From fixed to FRAP: measuring protein mobility and activity in living cells

Eric A.J. Reits and Jacques J. Neefjes*

Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
**e-mail: jneefjes@nki.nl*

Experiments with fluorescence recovery after photobleaching (FRAP) started 30 years ago to visualize the lateral mobility and dynamics of fluorescent proteins in living cells. Its popularity increased when non-invasive fluorescent tagging became possible with the green fluorescent protein (GFP). Many researchers use GFP to study the localization of fusion proteins in fixed or living cells, but the same fluorescent proteins can also be used to study protein mobility in living cells. Here we review the potential of FRAP to study protein dynamics and activity within a single living cell. These measurements can be made with most standard confocal laser-scanning microscopes equipped with photobleaching protocols.

In FRAP experiments, fluorescent molecules are irreversibly photobleached in a small area of the cell by a high-powered focused laser beam. Subsequent diffusion of surrounding non-bleached fluorescent molecules into the bleached area leads to a recovery of fluorescence, which is recorded at low laser power. FRAP experiments started in the 1970s using lipophilic or hydrophilic fluorophores, like fluorescein, coupled to proteins and lipids[1,2]. The cloning of GFP from the jellyfish *Aequorea victoria* introduced a new fluorescent reporter protein to study the localization of chimaeric proteins in living cells. This development made it possible to perform FRAP on living cells without disruption by microinjection. In addition, GFP-tagged molecules can be targeted to various sites in the cell and can be observed for long periods because the GFP molecule is very photostable. The attachment of GFP rarely affects the function and localization of the fusion protein. These properties of GFP thus considerably enhanced the biological applications of photobleaching studies.

Quantitative photobleaching

FRAP experiments provide information about the mobility of a fluorescent molecule in a defined compartment. Two parameters can be deduced from FRAP: the mobile fraction of fluorescent molecules and the rate of mobility, which is related to the characteristic diffusion time, τ_D. Figure 1 shows a typical fluorescence recovery curve, allowing the determination of the two parameters. The mobile fraction can be determined by comparing the fluorescence in the bleached region after full recovery (F_∞) with the fluorescence before bleaching (F_i) and just after bleaching (F_0). The mobile fraction R is defined as

$$R = (F_\infty - F_0)/(F_i - F_0)$$

The mobile fraction can change in different circumstances, for example when the fluorescent protein interacts with other molecules or membranes. The mobile fraction can also be affected by membrane barriers and microdomains in the membrane. These discontinuities can prevent, or temporarily restrict, the free diffusion of membrane molecules.

When motion due to active transport or unidirectional flow can be discounted, protein mobility in a cell is due to brownian motion. The mobility is expressed as the diffusion coefficient D, which is related to the diffusion time τ_D. Most formulas describing this relationship are based on the two-dimensional diffusion equation described by Axelrod *et al.*[3]:

$$\tau_D = \omega^2\gamma/4D$$

where ω is defined as the radius of the focused circular laser beam at the e^{-2} intensity and γ is a correction factor for the amount of bleaching. This equation assumes unrestricted two-dimensional diffusion in a circular bleached area, with no recovery from above and below the focal plane. Because this is valid for diffusion in membranes and thin films of liquid only, other formulas have evolved as well[4,5]. It is beyond the scope of this review to discuss all mathematical models in detail, but we shall mention some biologically important parameters determining diffusion of soluble and membrane-bound molecules.

Unrestricted diffusion of a particle in a free-volume model is described by the Stokes–Einstein formula[6]:

$$D = kT/6\pi\eta R_h$$

which correlates the hydrodynamic behaviour of a sphere with the absolute temperature T, the viscosity of the solution η, the Boltzmann constant k and the hydrodynamic radius of the particle R_h. Because the local absolute temperature is hardly affected by bleaching[7], and the viscosity of water and cytosol are relatively constant under experimental conditions, D is determined mainly by R_h. When a molecule is assumed to be a sphere with a volume proportional to its molecular mass, the diffusion coefficient is proportional to the inverse of the cube root of molecular mass ($D \sim M^{-1/3}$). The relationship between D and the hydrodynamic radius was confirmed with a range of macromolecules *in vitro*[8,9]. There is no clear size limit for free diffusion *in vivo* because even large protein complexes such as the proteasome[10] can diffuse freely through the cytoplasm and the nucleus. In addition to temperature, viscosity and radius, other factors affect diffusion in living cells, including specific interactions but also hindrance by mobile and immobile obstacles. Collision with other proteins and barriers such as cytoskeletal filaments affects mobility, as shown by experiments with swollen and shrunken cells[11,12].

FRAP has also been used extensively to study the lateral diffusion of membrane-associated proteins. This diffusion is considerably slower than that of soluble proteins because membranes have a much higher viscosity. The aqueous phase of a transmembrane molecule hardly affects diffusion because the viscosity of the mem-

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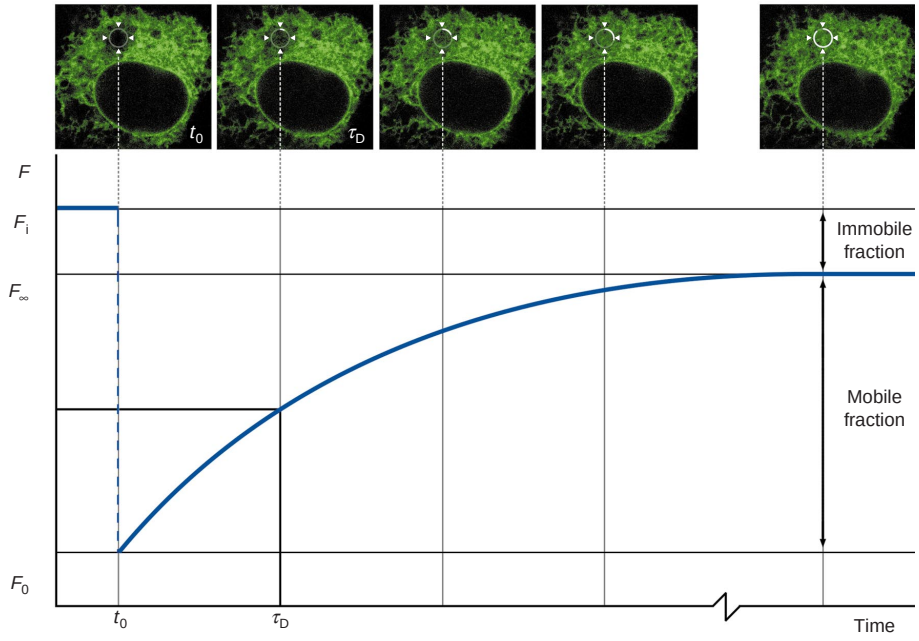

Figure 1 **Fluorescence recovery after photobleaching (FRAP).** When a region in the fluorescent area (here the endoplasmic reticulum) is bleached at time t_0 the fluorescence decreases from the initial fluorescence F_i to F_0. The fluorescence recovers over time by diffusion until it has fully recovered (F_∞). The characteristic diffusion time τ_D indicates the time at which half of the fluorescence has recovered. The mobile fraction can be calculated by comparing the fluorescence in the bleached region after full recovery (F_∞) with that before bleaching (F_i) and just after bleaching (F_0).

brane is much higher. For instance, large deletions in the cytoplasmic domain of the epidermal growth factor (EGF) receptor do not affect its lateral mobility[13]. Nevertheless, similar mathematics apply to the diffusion of membrane-associated molecules, although the main variable is now the radius of the protein segment located in the membrane phase. The Saffman–Delbrück equation[14] thus correlates the diffusion mainly with the radius of the transmembrane segment but again also with viscosity and the absolute temperature:

$$D = cT\ln[(k/ha) - 0.5772]$$

where D is the diffusion coefficient, c and k are constants incorporating the viscosity of the aqueous phase and the membrane bilayer thickness, T is the absolute temperature, η is the viscosity of the membrane and a is the radius of the transmembrane segment. When different large membrane proteins were compared, a dependence on radius and temperature was indeed observed[15].

The Saffman–Delbrück equation considers a theoretical protein with a cylindrical transmembrane segment without interactions with the surrounding lipid bilayer. However, most membrane molecules diffuse more slowly than expected for random brownian motion in a lipid layer. This might be due to interactions, tilted transmembrane segments, obstacles, and temporary binding sites in cell membranes[16]. The Saffman–Delbrück equation should therefore be considered an approximation from which the actual radius cannot be calculated. Few transmembrane proteins have a cylindrical transmembrane domain and most are subjected to conformational changes, aggregate formation and lipid interactions that can affect the mobility of the protein–lipid complex without changing the radius.

Applications of FRAP in living cells

FRAP can be used to address a number of questions about protein localization, dynamics and interactions with other components in living cells. The mobility of molecules within specific cell compartments has been visualized, as has membrane continuity. Experiments with GFP-tagged molecules targeted to specific organelles and compartments such as mitochondria[17] and the nucleus[18] show that most proteins can move freely within these compartments. Because their mobility is independent of ATP, diffusion is the primary means of movement. FRAP showed that protein immobilization is not responsible for the retention of either misfolded proteins in the endoplasmic reticulum (ER) membrane[19] or Golgi molecules[20], as these membrane molecules diffuse rapidly as well. Proteins in the ER lumen and membrane can diffuse with a high mobility through the ER and the perinuclear envelope, which forms a continuous membrane system. In comparison with organelles, some cell-surface proteins have a much lower mobility[21] and an altered mobile fraction. This is possibly due to interactions with cytoskeleton elements or the extracellular matrix.

Whereas these studies visualize the dynamics of proteins in a cell organelle, FRAP can also be used to follow events during cell division and signalling. For example, the nuclear envelope fuses with the ER during mitosis, as was observed using the GFP-tagged nuclear membrane molecule lamin-B receptor[22]. Although immobile in the nuclear envelope of interphase cells, the lamin-B receptor diffuses freely and rapidly when redistributed to the ER. Similarly, the disassembly and reassociation of nucleoli during mitosis was monitored with fibrillarin–GFP[23], showing a large mobile fraction of nucleolar components during reassembly. The import of fluorescent molecules can be followed by the bleaching of compartments in the cell. When the nuclear pool of proteasomes is bleached, a slow recovery of fluorescence is observed that is due to the unidirectional transport of cytosolic proteasomes into the nucleus[10].

FRAP can be used to measure protein interactions and conformational changes as well as protein dynamics. The association of fluorescent proteins with other molecules can be determined *in vivo* by lateral mobility, as shown for GFP-tagged MHC class I molecules in the ER[24]. Empty MHC class I molecules are bound to the TAP peptide transporter complex in the ER, decreasing its lateral mobility. The diffusion coefficient increases after peptide loading,

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as the class I molecule is released from the antigen loading complex. By measuring mobility in living cells, protein activity can be studied at the single-cell level. The activities of GTPases such as ARF1–GFP[25] and K-ras[26] have been quantified by FRAP. Because the GDP-bound form resides in the cytosol and the GTP-bound form is membrane-associated, photobleaching the membrane pool reveals the kinetics of the GTP cycle *in vivo*. Finally, conformational changes associated with activity can be visualized by FRAP, as shown for GFP–TAP[27]. The lateral mobility of the peptide pump decreases during peptide translocation and increases when inactive. The diffusion rate can be used to show the relative quantities of cytosolic peptides present in living cells. These powerful observations demonstrate the value of FRAP in single-cell biology.

Considerations

Although diffusion coefficients can be deduced from FRAP experiments, one should be careful to compare diffusion coefficients and mobile fractions between different cell lines or compartments. Diffusion coefficients of the same molecule in different membranes vary not only because of specific interactions but also as a result of membrane topology and viscosity. For example, the ER forms a complex three-dimensional structure that differs from the plasma membrane in both architecture and membrane viscosity. Similarly, membrane factors such as cholesterol, phospholipids and proteins as well as cytoplasmic viscosity can result in cell-type-specific diffusion coefficients for the same molecule of interest.

Temperature affects the mobility of both soluble and membrane-associated molecules. Because viscosity is highly dependent on temperature, the effect of temperature on diffusion can be striking. FRAP experiments performed at 37 °C with soluble GFP targeted to the ER result in a 1.4-fold faster recovery rate than at 23 °C, owing to differences in viscosity[28]. The diffusion coefficient of TAP complexes in the ER doubles when the temperature is raised from 25 to 37 °C (E.A.J.R. and J.J.N., unpublished results). The diffusion of phospholipid probes in liposomes was also strongly dependent on temperature over the range 15–37 °C. The temperature effect was biphasic owing to a sharp phase transition in the membrane lipid, affecting lateral diffusion when solid-phase lipid regions turned into fluid-phase lipid regions[29]. This implies that FRAP experiments should be performed in a carefully controlled temperature stage, ideally at a physiological temperature.

Future roads and perspectives

FRAP is a powerful and continuously improving tool, available on most commercial confocal laser-scanning microscope systems, that can be used to address a number of questions regarding protein localization, activity, interactions and dynamics within a living cell. FRAP has been used to measure the continuity of membrane compartments and the behaviour of proteins during mitosis. However, the diffusion rate can also be interpreted in biochemical terms, being a readout for protein interactions and activity. FRAP will become a rapid and non-invasive technique to study biochemical processes not with isolated proteins but in the most complicated but relevant biological system: the living cell. □

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