Chapter 3
Novel Insights in Membrane Biology Utilizing Fluorescence Recovery After Photobleaching

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Abbreviations

25-NBD-cholesterol	25-[*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)methyl]amino]-27-norcholesterol
5-HT_{1A} receptor	5-Hydroxytryptamine-1A receptor
5-HT_{1A}R-EYFP	5-Hydroxytryptamine-1A receptor tagged to enhanced yellow fluorescent protein
$DiIC_{18}(3)$	1,1′-Dioctadecyl-3,3,3′,3′,-tetramethylindocarbocyanine perchlorate
EYFP	Enhanced yellow fluorescent protein
FAST DiI	1,1′-Dilinoleyl-3,3,3′,3′,-tetramethylindocarbocyanine 4-chlorobenzenesulfonate
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
NBD-PE	1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)
p-MPPI	4-(2′-Methoxy)phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido]ethylpiperazine

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The Dynamic Membrane

Biological membranes are complex two-dimensional, non-covalent assemblies of a diverse variety of lipids and proteins. They impart an identity to the cell and its organelles and represent an ideal milieu for the proper function of a diverse set of membrane proteins. A unique feature of biological membranes is their characteristic dynamics that gets manifested as lateral and rotational dynamics of the constituent lipids and proteins (Marguet et al. 2006; Baker et al. 2007a). It is becoming increasingly clear that membrane dynamics holds the key to membrane function. For example, the conformational dynamics of membrane receptors (such as G protein-coupled receptors (GPCRs)) is beginning to be appreciated in relation to their function (Nygaard et al. 2013; Schmidt et al. 2014). Understanding cellular signaling by membrane receptors in terms of their lateral dynamics represents a challenging area in contemporary biology (Calvert et al. 2001; Ganguly et al. 2008).

Fluorescence Recovery After Photobleaching

Fluorescence recovery after photobleaching (FRAP) represents a convenient approach to measure lateral (translational) diffusion and is widely used for measuring lateral diffusion of lipids and proteins in membranes (Edidin 1994; Lippincott-Schwartz et al. 2001; Klonis et al. 2002; Hagen et al. 2005). FRAP involves generation of a concentration gradient of fluorescent molecules by irreversibly photobleaching a fraction of fluorophores in the observation region (region of interest). The dissipation of this gradient with time owing to diffusion of fluorophores into the bleached region from the unbleached regions of the membrane is an indicator of the mobility of the fluorophores in the membrane. The recovery of fluorescence into the bleached area in FRAP experiments is described by two parameters, an apparent diffusion coefficient (D) and mobile fraction (M_f). The rate of fluorescence recovery provides an estimate of the lateral diffusion coefficient of diffusing molecules, whereas the extent of fluorescence recovery provides an estimate of the mobile fraction (in FRAP time scale). Figure 3.1 illustrates the basic principles of FRAP measurements. In this review, we will provide an overview of the range of research problems that could be addressed in membrane and receptor biology using FRAP, taking representative examples mostly from work carried out in our laboratory. This review is by no means an exhaustive review of FRAP methodology and its application in membrane biology.

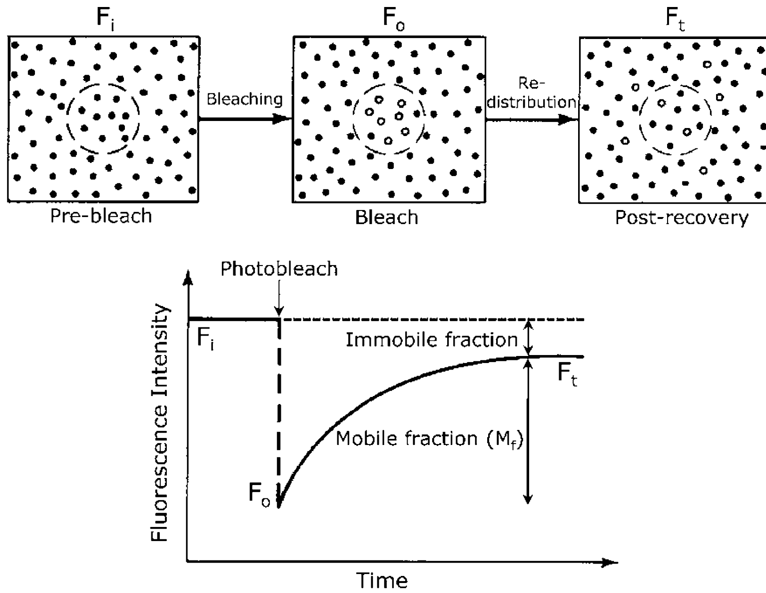

Fig. 3.1 Basic design of FRAP measurements. F_i represents the initial total fluorescence intensity in the region of interest (ROI) prior to photobleaching. A concentration gradient of fluorescent molecules is generated by photobleaching a population of fluorophores in the ROI (shown as a *dashed circle*) using a strong laser beam. F_o represents the total fluorescence intensity in the ROI immediately after photobleaching. The concentration gradient of fluorophores created this way gets dissipated with progress of time due to lateral diffusion of unbleached fluorophores (outside ROI) into the bleached region. F_t represents the total fluorescence intensity in the region at a given time (t) after photobleaching. Careful analysis of the rate of recovery of fluorescence (from F_o to F_t) yields lateral diffusion coefficient (D). Note that the diffusion coefficient obtained in case of membranes represents two-dimensional diffusion (since the membrane is considered to be two-dimensional). The extent of fluorescence recovery provides information on the fraction of molecules that are mobile in this time scale (termed mobile fraction, M_f)

Lipid Dynamics by FRAP

Fluorescently labeled lipid probes are widely used for measuring lipid dynamics in model and natural membranes. The DiI series of lipid analogues are commonly used probes for such measurements. The DiI analogues are composed of a polar indocarbocyanine headgroup and two hydrophobic alkyl chains (see Fig. 3.2) which impart an overall amphiphilic character. They have earlier been shown to preferentially partition into gel (ordered) or fluid (disordered) phases depending on the degree of matching between their acyl chain length and those of lipids that comprise the host membrane (Klausner and Wolf 1980; Spink et al. 1990; Kalipatnapu and Chattopadhyay 2004). $DiIC_{18}(3)$ and *FAST* DiI (Fig. 3.2) represent two such probes

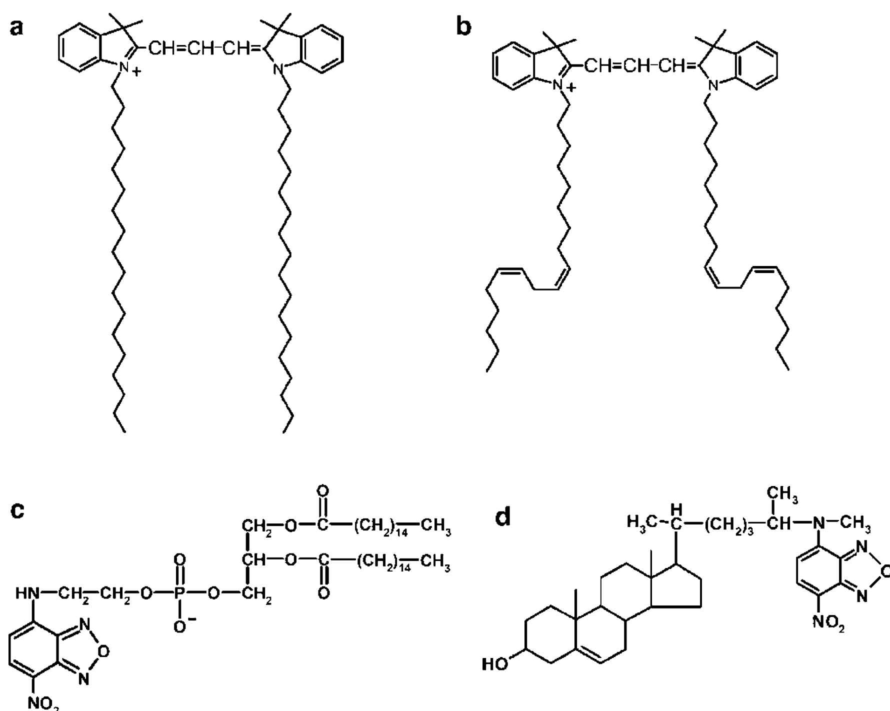

Fig. 3.2 Chemical structures of common fluorescent probes used for measuring lipid dynamics in membranes using FRAP: (**a**) DiIC_{18}(3), (**b**) *FAST* DiI, (**c**) NBD-PE and (**d**) 25-NBD-cholesterol

that are similar in their intrinsic fluorescence properties but differ in their phase partitioning preference. Lateral diffusion characteristics of these probes in native hippocampal membranes have been analyzed in detail using FRAP (Pucadyil and Chattopadhyay 2006). The results show that mobility of these probes in hippocampal membranes varies with membrane cholesterol content. Lateral mobility was found to be higher in cholesterol-depleted membranes. These results could provide insight in the function of neuronal receptors present in these membranes. In another study, *FAST* DiI was used to monitor lateral diffusion in membranes of the wild type and *erg* mutants of the pathogenic yeast, *Candida albicans* (Mukhopadhyay et al. 2004). Interestingly, lipid diffusion in membranes of the wild type and *erg* mutants of *C. albicans* (mutants for ergosterol; *erg2* and *erg16*) correlate well with their drug resistance characteristics. These results represent the first report of analysis of lipid dynamics in *C. albicans* using FRAP. Another interesting application of FRAP to study lipid dynamics is the demonstration of the presence of cholesterol monomers and transbilayer dimers in membranes at low concentration (Pucadyil et al. 2007).

The fluorescent probes used in this case were NBD-PE and 25-NBD-cholesterol (see Fig. 3.2). The NBD group is a commonly used fluorescent lipid probe for studies with model and natural membranes (for a recent review, see Haldar and Chattopadhyay 2013).

GPCR Activation: Manifestations in Receptor Dynamics

The G protein-coupled receptor (GPCR) superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes (Pierce et al. 2002; Rosenbaum et al. 2009). GPCRs are seven transmembrane domain proteins and include >800 members which are encoded by ~5 % of human genes (Zhang et al. 2006). Since GPCRs regulate multiple cellular processes, they have emerged as major targets for the development of novel drug candidates in all clinical areas (Heilker et al. 2009). It is estimated that ~50 % of clinically prescribed drugs act as ligands of GPCRs (Schlyer and Horuk 2006). The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is a representative member of the GPCR family and is implicated in the generation and modulation of various cognitive, behavioral and developmental functions (Pucadyil et al. 2005; Kalipatnapu and Chattopadhyay 2007; Müller et al. 2007). Ligands that bind to the serotonin$_{1A}$ receptor are reported to possess potential therapeutic effects in anxiety or stress-related disorders (Pucadyil et al. 2005). As a consequence, the serotonin$_{1A}$ receptor serves as an important target in the development of therapeutic agents for neuropsychiatric disorders such as anxiety and depression (Celada et al. 2013).

Signaling by GPCRs provides an efficient way for cells to communicate with each other and with their environment. This is achieved through the activation of GPCRs upon binding of ligands present in the extracellular environment that leads to transduction of signals to the interior of the cell through concerted changes in the transmembrane helices (Nygaard et al. 2013). Ligand stimulation of GPCRs generally leads to the recruitment and activation of the heterotrimeric G-proteins. The activation process stimulates the GDP-GTP exchange leading to the dissociation of the GTP-bound α-subunit and the βγ-dimer of the G-protein from the GPCR. This activation could lead to dissociation of G-proteins from the receptors, increasing receptor diffusion. This was validated by FRAP measurements of the serotonin$_{1A}$ receptor tagged to enhanced yellow fluorescent protein (5-HT$_{1A}$R-EYFP) upon activation of the receptor (Pucadyil et al. 2004; Pucadyil and Chattopadhyay 2007a). Figure 3.3 shows a representative FRAP experiment with 5-HT$_{1A}$R-EYFP in CHO cells. The results show that activation with the natural agonist serotonin resulted in a significant increase in the diffusion coefficient of the serotonin$_{1A}$ receptor, while treatment with the antagonist *p*-MPPI did not exhibit any significant difference (see Fig. 3.4). Interestingly, the increase in the diffusion

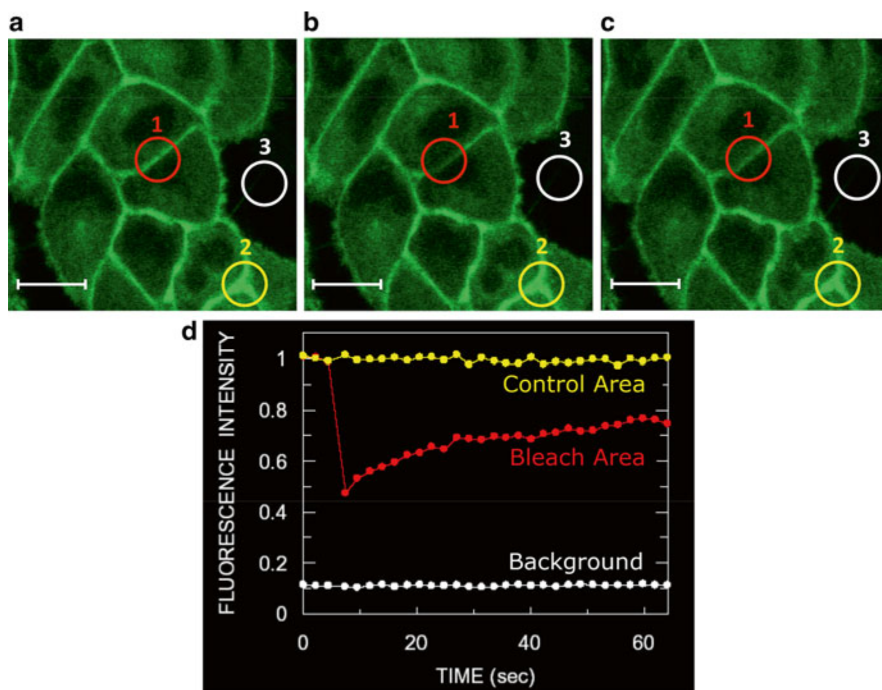

Fig. 3.3 Lateral dynamics of the serotonin$_{1A}$ receptor tagged to enhanced yellow fluorescent protein (5-HT$_{1A}$R-EYFP) stably expressed in CHO cells. The cellular periphery with distinct plasma membrane localization of 5-HT$_{1A}$R-EYFP was chosen for FRAP measurements. Typical images corresponding to (**a**) pre-bleach, (**b**) bleach and (**c**) post-bleach are shown. Regions 1, 2 and 3 represent bleach area, control area and background, respectively. The scale bar represents 10 μm. The plot in (**d**) shows a representative set of normalized fluorescence intensity of 5-HT$_{1A}$R-EYFP corresponding to regions 1 and 2, and normalized background intensity in region 3. The normalized fluorescence intensity in control area (2) was monitored for same duration of time, and shows no significant photobleaching

coefficient with serotonin could be reversed upon addition of *p*-MPPI. The observed increase in receptor diffusion coefficient upon stimulation with the agonist (but not with the antagonist) clearly suggested that activation of G-proteins resulted in an increase in mobility of the receptor. This was further supported by an increase in diffusion coefficient of the receptor in presence of mastoparan and AlF_4^- (see Fig. 3.4), both of which activate G-proteins in a receptor-independent manner. In addition, treatment of cells with pertussis toxin (PTX), that abolishes receptor and G-protein interaction, resulted in an increase in diffusion coefficient of the receptor. Taken together, these results show that receptor diffusion is dependent on its interaction with G-proteins.

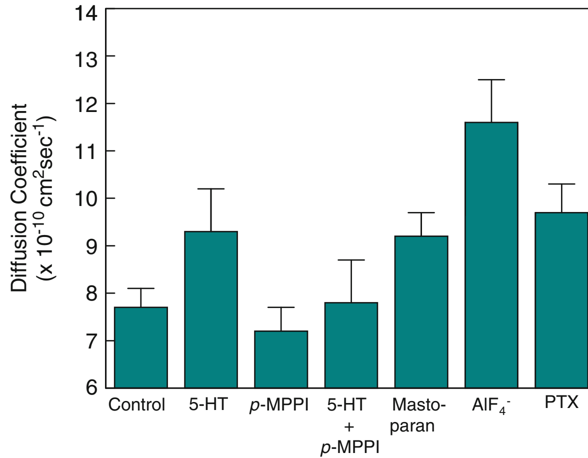

Fig. 3.4 Lateral diffusion coefficients of 5-HT$_{1A}$R-EYFP under various conditions. Serotonin (5-HT) and *p*-MPPI act as agonist and antagonist of the serotonin$_{1A}$ receptor. Both mastoparan and AlF$_4^-$ activate G-proteins in a receptor-independent manner, whereas pertussis toxin (PTX) inactivates G-proteins of G$_{i/o}$ subtype, thereby abrogating interaction of G-proteins with the receptor. Note that the diffusion coefficient of the receptor exhibits an increase upon activation of G-proteins, irrespective of whether G-proteins are activated in receptor-dependent or independent manner. Adapted and modified from Pucadyil et al. (2004)

Dynamic Confinement of GPCRs Upon Cholesterol Depletion: Insight from Bleach Area-Dependent FRAP

An interesting source of cell membrane heterogeneity (domain) is the relative confinement of membrane components. From this perspective, cellular signaling could be viewed as a consequence of differential mobility of the various interacting partners (Peters 1988). The fluorescence recovery kinetics in FRAP measurements contains information on the area being monitored. This provides a handle to explore spatial organization of molecules in the cell membrane by systematically varying the area monitored in FRAP measurements (Edidin 1992). Differences in diffusion properties obtained from FRAP measurements performed with bleach areas of different sizes can be correlated to the presence of domains on the cell membrane, with dimensions that fall in the same range as the area monitored in these measurements (Yechiel and Edidin 1987; Edidin and Stroynowski 1991; Salomé et al. 1998; Cézanne et al. 2004; Baker et al. 2007b; Saulière-Nzeh Ndong et al. 2010). This interpretation is based on the following model (see below), and was earlier validated by simulations and FRAP experiments performed on physically domainized model membrane systems (Salomé et al. 1998).

The rate of fluorescence recovery provides an estimate of the apparent diffusion coefficient of molecules, while the extent to which fluorescence recovers provides an estimate of mobile fraction of molecules. In general, for molecules diffusing in

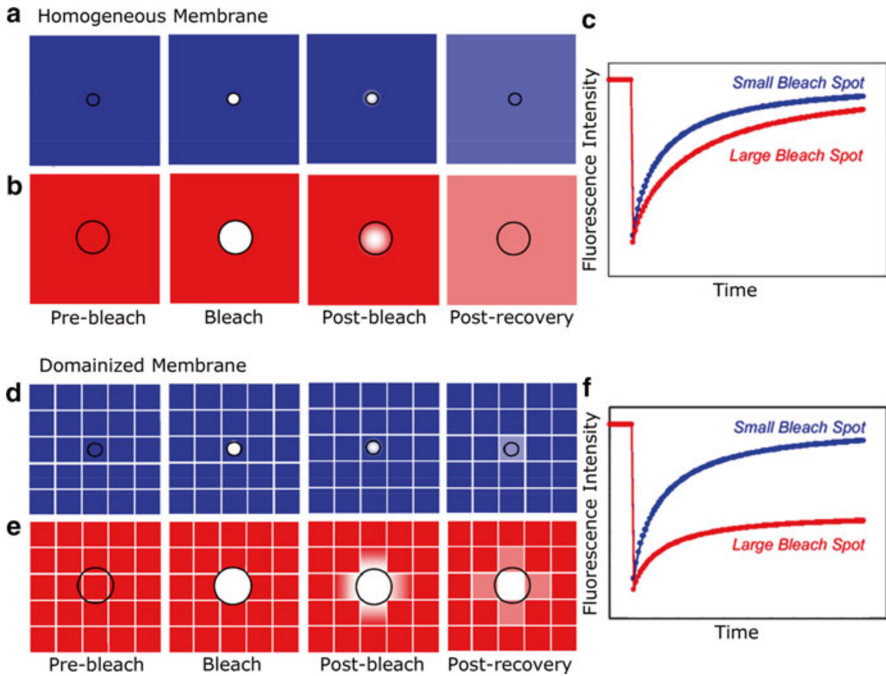

Fig. 3.5 Fluorescence recovery plots with a small or large bleach area performed on homogeneous or domainized membranes. The region of interest for FRAP is represented by a *circle*. The homogeneous membrane is characterized by random diffusion throughout the total area of the membrane in the experimental time scale. In contrast, diffusion on the domainized membrane is confined to closed areas (of comparable dimension as that of the bleached area) termed as 'domains'. The diffusion coefficient and mobile fraction in homogeneous membranes (panels (**a**) and (**b**)) would be independent of the size of the bleach area (see panel (**c**)). In contrast, these parameters would depend on the bleach area size in case of a domainized membrane (panel (**d**) and (**e**)). FRAP measurements on such a domainized membrane therefore would show an increase in diffusion coefficient and reduction in mobile fraction with increasing bleach area size (panel (**f**)). See text for details. Adapted and modified from Pucadyil and Chattopadhyay (2007b)

a homogeneous membrane, the diffusion coefficient is independent of the dimensions of the bleach area in FRAP measurements. A small bleach area (see Fig. 3.5a) would result in faster recovery of fluorescence while a large bleach area (Fig. 3.5b) would produce a slower fluorescence recovery. Yet, the rate of fluorescence recovery would be same in both cases, irrespective of the size of the bleach area. This means that the diffusion coefficient would remain same in both cases. In addition, if the bleached area is significantly smaller than the total area of the membrane, the extent of fluorescence recovery is the same in both cases resulting in a constant mobile fraction (Fig. 3.5c).

On the other hand, if diffusion was confined to closed domains of dimensions of the same scale as that of the bleach area, and static in FRAP time scale, diffusion coefficient would no longer be constant. A small bleach area (Fig. 3.5d) would tend

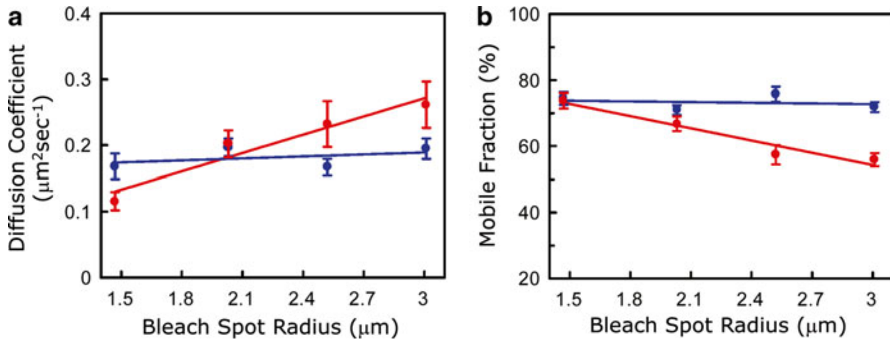

Fig. 3.6 Diffusion parameters from FRAP measurements with varying bleach area size. The (**a**) diffusion coefficient and (**b**) mobile fraction of 5-HT$_{1A}$R-EYFP obtained are shown for normal (*blue line*) and cholesterol-depleted (*red line*) cells. Adapted and modified from Pucadyil and Chattopadhyay (2007b)

to monitor diffusion properties of molecules within domains. Fluorescence recovery kinetics with a small bleach area on a domainized (heterogeneous) membrane therefore would be similar to that observed in a homogeneous membrane. On the other hand, a large bleach area (overlapping different domains to varying extents, shown in Fig. 3.5e) would result in non-uniform bleaching of domains since the bleached area would be partial for a few and complete for others. As a consequence, fluorescence recovery kinetics in the entire region of observation would not be proportional to the actual size of the bleach area. While kinetics of fluorescence recovery within domains would be proportional to the area bleached in these domains, the apparent diffusion coefficient would show an increase (since diffusion coefficient is calculated taking into account the actual size of the bleach area). Importantly, a large bleach area would reduce mobile fraction since it could bleach an entire domain resulting in total loss of fluorescence in such a domain (Fig. 3.5e, f).

Analysis of fluorescence recovery kinetics of 5-HT$_{1A}$R-EYFP in CHO cells with bleach areas of different sizes exhibited relatively constant diffusion coefficient and mobile fraction (Pucadyil and Chattopadhyay 2007b; see Fig. 3.6). This suggests that serotonin$_{1A}$ receptors experience a homogeneous membrane environment. Interestingly, FRAP experiments performed on cholesterol-depleted cells with an identical range of bleach area size showed a marked dependence of diffusion coefficient and mobile fraction of the receptor on the dimension of the bleach area (see Fig. 3.6). This characteristic dependence of diffusion coefficient and mobile fraction in cholesterol-depleted membranes is consistent with a model describing confined diffusion in a domainized membrane (see Fig. 3.5c, d) (Yechiel and Edidin 1987; Edidin and Stroynowski 1991; Salomé et al. 1998; Cézanne et al. 2004; Baker et al. 2007b; Saulière-Nzeh Ndong et al. 2010). The dependence of the lateral diffusion parameters on the bleach area size in cholesterol-depleted cells indicates that cholesterol depletion induces dynamic confinement of the receptor resulting in confined diffusion into domains.

Are Signaling and Dynamics Correlated?

Cellular signaling has been hypothesized to be a consequence of differential mobility of various interacting components. This forms the basis of the 'mobile receptor' hypothesis, which proposes that receptor-effector interactions at the plasma membrane are controlled by lateral mobility of the interacting components (Kahn 1976; Peters 1988). Although conceptually elegant, this hypothesis has been difficult to validate experimentally. This was addressed by monitoring lateral mobility of 5-HT_{1A}R-EYFP utilizing FRAP and measuring downstream signaling by the reduction in cellular cAMP level upon activation of the receptor under the same condition (Ganguly et al. 2008). Lateral diffusion of membrane lipids and proteins is known to be influenced by cytoskeletal proteins. Upon destabilization of the actin cytoskeleton by increasing concentrations of cytochalasin D, the mobile fraction of the receptor showed a significant increase, whereas diffusion coefficient remained constant (see Fig. 3.7a, b). This was accompanied by an increase in signaling by the receptor, as measured by reduction in cAMP (Fig. 3.7c). The fact that the change in

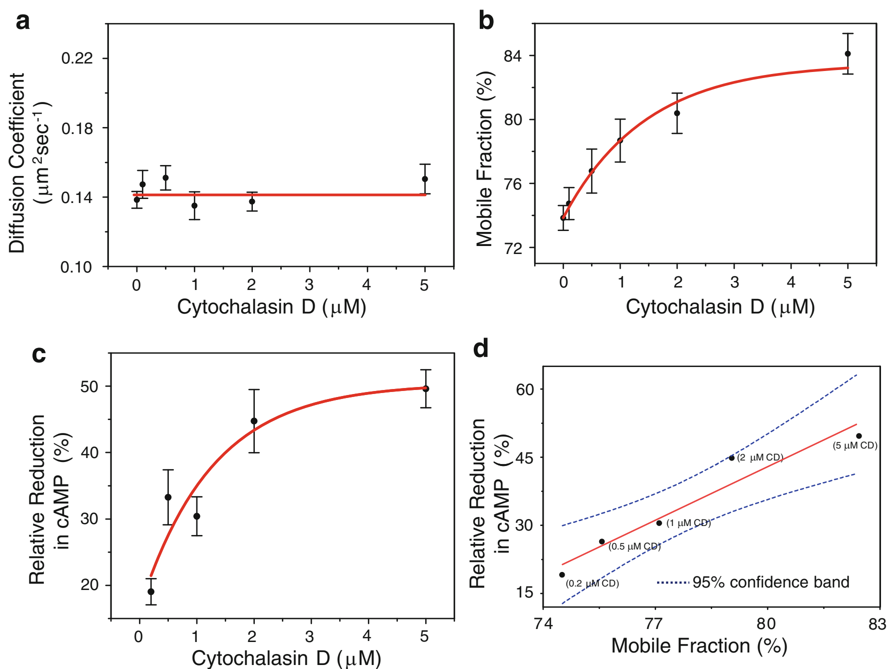

Fig. 3.7 A tight correlation between receptor dynamics and signaling. Effect of increasing cytoskeletal destabilization on (**a**) diffusion coefficient, (**b**) mobile fraction and (**c**) agonist-mediated signaling of the serotonin$_{1A}$ receptor. Cytoskeletal destabilization was achieved by treatment with cytochalasin D. Panel (**d**) shows that signaling of the receptor is strongly correlated with its dynamics (mobile fraction), with a correlation coefficient (r) ~ 0.95. Adapted and modified from Ganguly et al. (2008)

signaling was correlated with the change in receptor dynamics was supported by a positive correlation of ~0.95 obtained from a plot of these two parameters (see Fig. 3.7d). Such a tight correlation between the mobile fraction of the receptor and its signaling is supportive of the mobile receptor hypothesis.

Lateral Dynamics as Readout of Infection

The above example shows that lateral dynamics could be correlated with cellular signaling (Ganguly et al. 2008). Interestingly, a few studies have highlighted the correlation of lateral dynamics of host cell membrane proteins to infection by obligate intracellular parasites. For example, lateral dynamics has been related to the stage of infection of intracellular obligate parasites such as *Plasmodium falciparum*. In an elegant study, Parker et al. (2004) showed that the lateral diffusion coefficient and mobile fraction of host erythrocyte proteins (such as band 3 and glycophorin) depend on the stage of the infection. The diffusion coefficient and mobile fraction of these proteins were reported to be lower for mature stage-infected cells compared to ring stage-infected cells. The corresponding values of diffusion parameters were found to be the highest in case of uninfected cells. This observation points out the potential of lateral dynamics as an indicator of progress of infection. In another study, HIV-1 fusion and entry into target cells have been shown to be dependent on the lateral mobility of CD4 receptors (which serve as one of the receptors for viral entry) in host cell membranes (Rawat et al. 2008).

Conclusion and Future Perspectives

Although we have discussed only representative examples of the application of FRAP in membrane and receptor biology, it is clear that this approach is capable of providing a variety of information depending on experimental design and question asked. With the advent of confocal microscopy and our ability to optically section the cellular interior, FRAP is being increasingly used to explore dynamics of intracellular organelles (Lippincott-Schwartz et al. 2001; Aguila et al. 2011; Staras et al. 2013) using reporters such as GFP (Haldar and Chattopadhyay 2009). This is an exciting area of research and was not possible a few years back. A particularly exciting application is dynamics of nuclear proteins using FRAP (Dundr and Misteli 2003; Mariappan and Parnaik 2005). We envision that future applications of FRAP will involve generating a dynamic map of intracellular components and their modulation with differentiation and development, thereby enabling a novel dynamic view of cellular signaling and function in healthy and diseased states.

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