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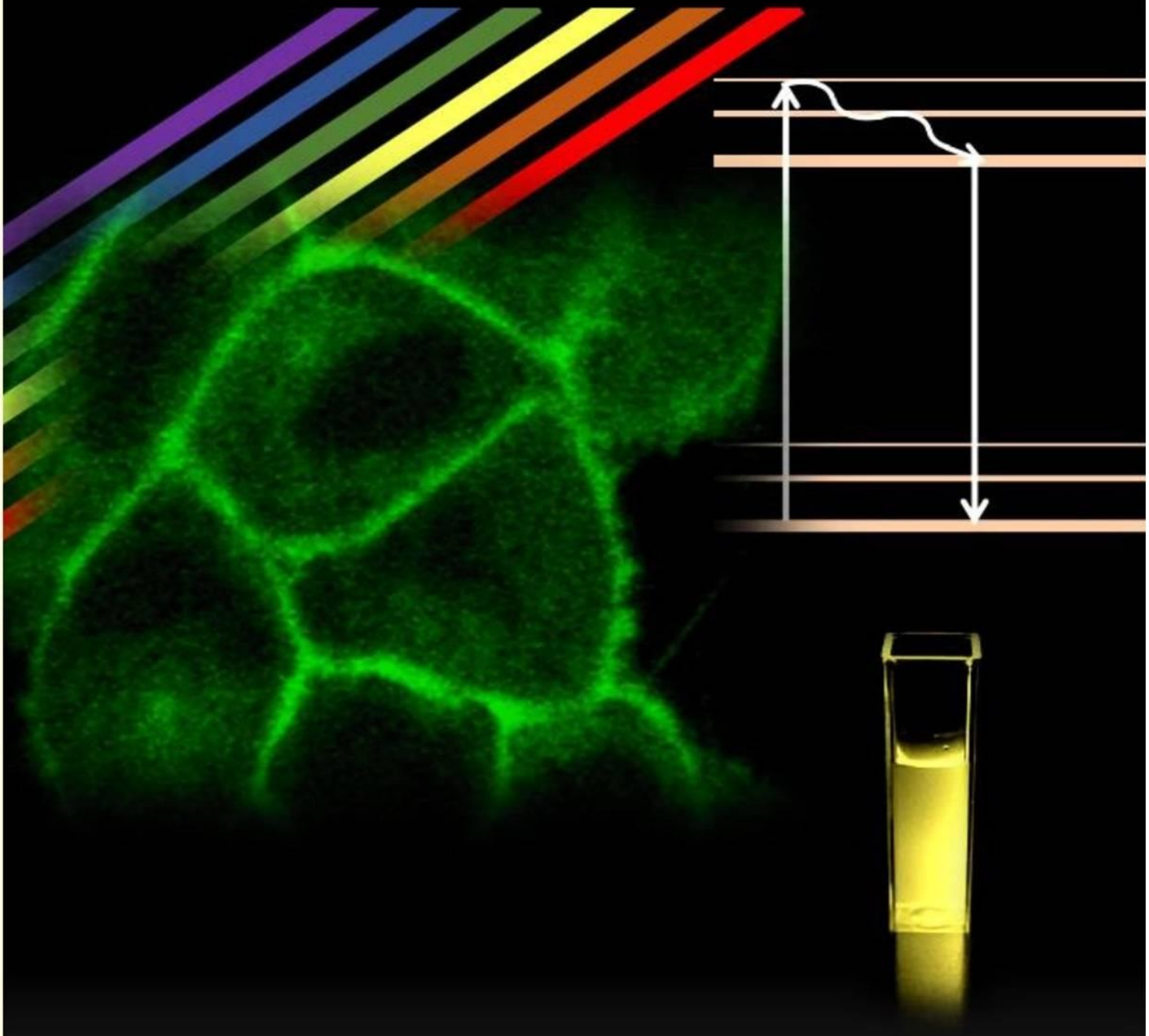
Fluorescence in Biology



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Commentary

GFP fluorescence: A few lesser-known nuggets that make it work

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Use of Green Fluorescent Protein (GFP) as a marker has revolutionized biological research in the last few decades. In this brief commentary, we reflect upon the success story of GFP and highlight a few lesser-known facets about GFP that add up to its usefulness.

Keywords. GFP; REES; FRAP; lateral diffusion

Abbreviations: AOT, sodium bis(2-ethylhexyl)sulfosuccinate; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; REES, red edge excitation shift

1. Introduction: The green revolution in biology

Ever since the discovery of fluorescence by Herschel (Herschel 1845; also see Jameson 2014 for a lucid description of the early history of fluorescence), very few discoveries in fluorescence have become as popular as the discovery and application of the Green Fluorescent Protein (GFP) in biology. The GFP story as a cellular fluorescent tool is fascinating, which resulted in the award of the Nobel Prize in Chemistry to Osamu Shimomura, Martin Chalfie and Roger Tsien in 2008 (reviewed in Chalfie 2009; Haldar and Chattopadhyay 2009a, b). Interestingly, GFP was initially discovered by Shimomura in the jellyfish *Aequorea victoria* as far back as the early 60's (Shimomura *et al.* 1962). In spite of this early lead, it took almost three decades to clone the gene that encodes GFP (Prasher *et al.* 1992). It was later shown by Martin Chalfie and co-workers that GFP could be expressed in heterologous systems (Chalfie *et al*. 1994). This served as the beginning of the 'green revolution in biology' (see figure 1). Subsequently, Roger Tsien and co-workers provided the structural and molecular details of GFP (Ormö *et al*. 1996).

The popularity of GFP as a reporter molecule in modern biological research is apparent from the ever-increasing number of publications associated with GFP (see figure 1). There are a number of reasons for the popularity enjoyed by GFP. These include intrinsic, cofactor-independent fluorescence which displays remarkable stability in the presence of denaturants and over a wide range of pH (Tsien 1998). As a result, GFP fluorescence is extensively used for monitoring gene expression, localization, mobility, traffic and interaction of various membrane and cytoplasmic proteins, protein folding, and protein-protein interactions. In addition to visualization by fluorescence microscopy, GFP-tagged molecules in a cellular milieu can be monitored by a variety of approaches such as Fluorescence Resonance Energy Transfer (FRET), Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Correlation Spectroscopy (FCS) to explore lateral dynamics and molecular interactions of membrane proteins and receptors.

In molecular terms, GFP represents a compact barrel-shaped protein containing 238 amino acids which folds to give rise to 11 β-strands with an α-helix running through the central axis of the cylindrical structure (see figure 2a). The fluorophore inside GFP (*p*-hydroxybenzylideneimidazolidinone) that provides its green fluorescence occupies the center of the β barrel structure. The fluorophore moiety is spontaneously generated upon folding of the polypeptide chain by internal cyclization, followed by oxidation of the vital residues Ser65-Tyr66-Gly67 (see figure 2b). The unique aspect of GFP fluorescence lies in the fact that its fluorescence is independent of any cofactor or specific enzyme of the jellyfish. All that is needed is molecular oxygen for GFP to fluoresce. In terms of stereochemistry, the fluorophore in GFP is localized in a constrained environment, and remains protected from the bulk solvent by the surrounding rigid β-strands (see later). Interestingly, it is possible to alter the photophysical and spectral properties of wild type GFP by mutations in the fluorophore region and also in the surrounding β barrel structure. Using this approach, Roger Tsien generated a number of variants of GFP (often termed as "cornucopia" of fluorescent proteins) with better spectral properties and photostability (Tsien 1998, 2009; Patterson 2004). A unique feature of GFP fluorescence is that secondary structure of the protein is necessary for GFP to fluoresce. As a result, GFP does not fluoresce when denatured (unlike tryptophans in proteins). The green fluorescence is restored only when the protein refolds

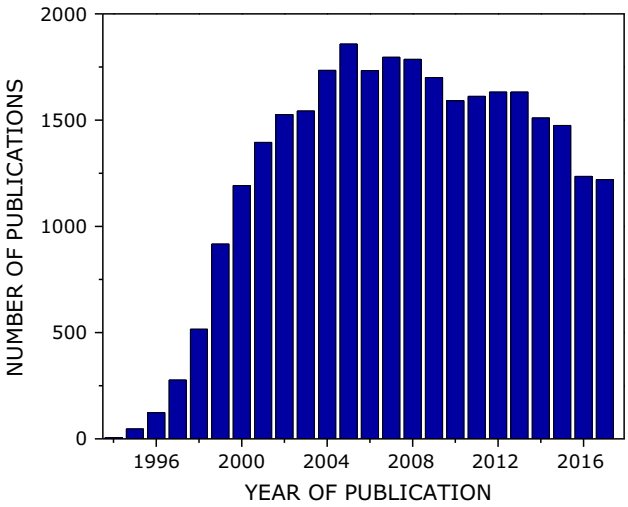

Figure 1. GFP popularity in biomedical literature. Number of publications (year-wise) in which the words green fluorescent protein appear either in the title or abstract (data taken from PubMed). This list is not exhaustive.

(Prendergast 1999; Ganim and Rief 2017). This aspect of GFP fluorescence has proved to be very useful in protein folding studies.

A number of informative and comprehensive reviews describing the usefulness of GFP fluorescence in biology are available in the literature along with an excellent monograph (Chalfie and Kain 2006). The aim of this brief commentary, therefore, is not to provide an exhaustive overview of applications of GFP in biology. Instead, we provide here a glimpse of lesser-known nuggets (some collated from our previous work) about GFP fluorescence, and elucidate how these features add up to make GFP such a useful probe. We believe that knowledge of these interesting, yet subtle features of GFP fluorescence, would empower future users of GFP fluorescence to efficiently optimize their experiments.

2. Environmental insensitivity of GFP fluorescence: A boon or a bane?

Environmental sensitivity (solvatochromism) is a hallmark of many fluorophores and forms the basis of biological applications of many fluorophores. The fluorescence (intensity and wavelength of maximum emission) of environment-sensitive fluorophores display shift when the polarity of the environment exhibits a change. Popular fluorescent probes such as Laurdan (Parasassi and Gratton 1995) and NBD (Chattopadhyay 1990; Haldar and Chattopadhyay 2013) exhibit high degree of environmental sensitivity. In contrast to this, GFP fluorescence is rather insensitive to its environment. This is consistent with its small Stokes' shift and a high fluorescence quantum yield (Brejc *et al.* 1997).

The reason for environment insensitivity of GFP fluorescence could be due to the fact that the fluorophore in GFP is protected from the bulk solvent and lies within a highly constrained environment. If this is indeed the case, measurements of solvent dynamics (relaxation) around the fluorophore in GFP under various conditions could provide insight into this process. Keeping this in mind, we previously carried out red edge excitation shift (REES) measurements of enhanced green fluorescent protein (EGFP) under various conditions (Haldar and Chattopadhyay 2007). REES is a phenomenon often observed in case of fluorophores localized in a motionally restricted environment, such as the membrane interface or in dynamically constrained regions of proteins (Demchenko 2008; Haldar *et al.* 2011; Chattopadhyay and Haldar 2014). A major factor governing REES is that the fluorophore should experience an environment in which the rate of solvent relaxation in the excited state is comparable to or slow relative to its fluorescence lifetime (typically $\sim$ns). REES is expressed as the shift in the wavelength of maximum fluorescence emission toward higher wavelengths, induced by a shift in the excitation wavelength toward the red edge of absorption spectrum. REES has emerged as a powerful tool to probe the mobility parameters of the environment itself (which is represented by the relaxing "optically silent" water molecules) using the fluorophore *merely* as a reporter in case of membranes (Chattopadhyay and Mukherjee 1993, 1999), and for soluble and membrane proteins (Guha *et al.* 1996; Raja *et al.* 1999; Chattopadhyay *et al.* 2003; Rawat *et al.* 2004; Jain *et al.* 2013; Chakraborty and Chattopadhyay 2017).

However, photophysics of GFP is complex and is characterized by multiple absorption bands resulting in ground state heterogeneity (Tsien 1998). Since ground state heterogeneity could complicate interpretation of REES results, we chose to

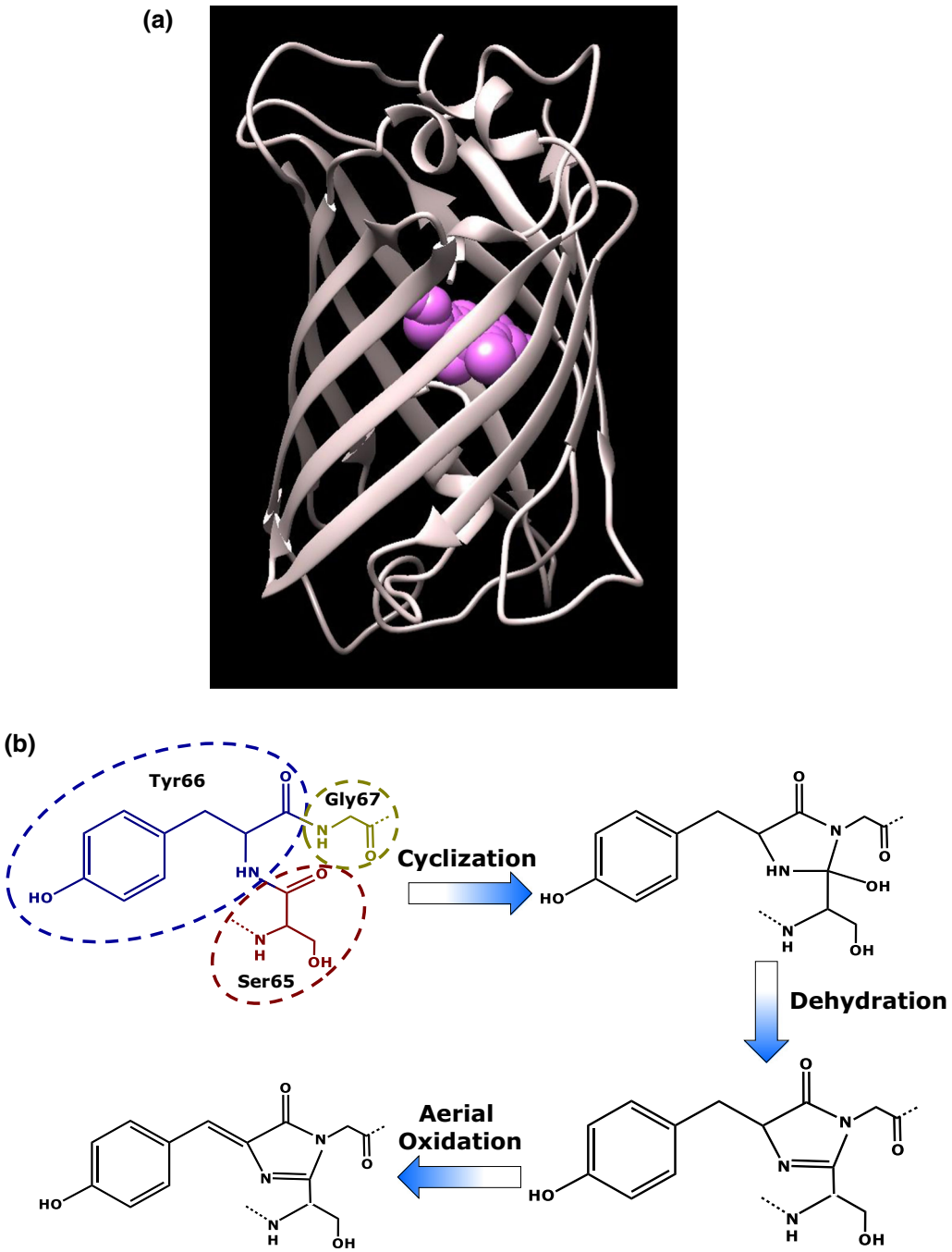

Figure 2. GFP crystal structure and molecular mechanism of fluorescence. (**a**) The crystal structure of GFP displaying a β-barrel structure with the chromophore (highlighted in purple) located in the core of the protein. Molecular graphics was generated using UCSF Chimera package (*https://www.cgl.ucsf.edu/chimera*) from the PDB entry 1EMB. (**b**) Molecular mechanism for the formation of the GFP fluorophore (*p*-hydroxybenzylideneimidazolidinone) by intramolecular cyclization involving the triad of amino acid Ser65-Tyr66-Gly67 residues, followed by aerial oxidation.

use a double mutant of GFP, termed EGFP, for REES experiments (Haldar and Chattopadhyay 2007). EGFP has two mutations (F64L and S65T), enjoys six-fold brighter fluorescence relative to wild type GFP, and displays a single absorption and emission peak (Patterson *et al*. 1997). This makes EGFP an ideal choice for use in REES experiments for monitoring the microenvironment around the fluorophore using dipolar relaxation.

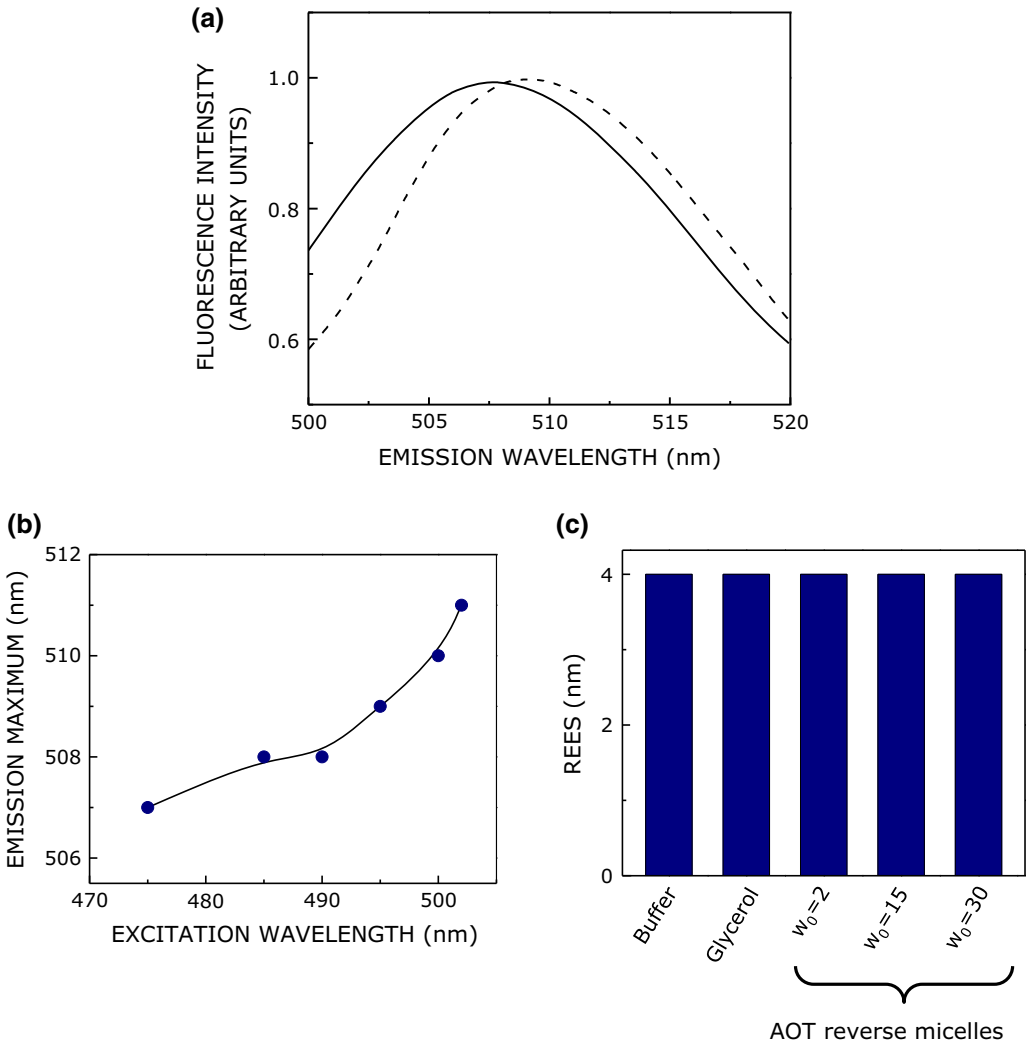

Figure 3. Environmental insensitivity of GFP fluorescence. (**a**) Representative intensity-normalized fluorescence emission spectra of EGFP at low concentration with varying excitation wavelengths (475 (—) and 495 (- - -) nm) in aqueous solution at pH 7.5. (**b**) REES of GFP fluorophore: effect of changing excitation wavelength on the wavelength of maximum emission of EGFP. The line joining the data points offers only a viewing guide. (**c**) *Invariance of GFP REES with environmental viscosity:* REES of EGFP remains unaltered in aqueous medium, 90% glycerol, and AOT reverse micelles of varying hydration. Adapted and modified with permission from Haldar and Chattopadhyay (2007).

Figure 3a shows the representative fluorescence emission spectra of EGFP in aqueous buffer with varying excitation wavelength. It is apparent from the figure that the emission maximum of EGFP depends on the choice of excitation wavelength. For example, the emission maximum exhibited a shift from 507 to 509 nm upon increasing the excitation wavelength to the red edge (from 475 to 495 nm). REES of EGFP under these conditions is shown in figure 3b. The figure shows that as the excitation wavelength is changed from 475 to 502 nm, the emission maximum exhibited a corresponding shift from 507 to 511 nm, giving rise to REES of 4 nm. As mentioned above, REES is observed predominantly with polar fluorophores in motionally restricted environments such as viscous solutions. In this context, our observation of 4 nm REES of EGFP in aqueous buffer (a nonviscous solvent) is surprising. It should be remembered here that EGFP is a compact barrel-shaped protein where the fluorophore is localized at the center and remains protected from the bulk solvent. We therefore attributed the observed REES of 4 nm *to the constrained protein matrix rather than the dynamics of the bulk solvent* (buffer). This claim was further supported by the observation that EGFP in 90% glycerol solution (a bulk viscous solvent) exhibits same extent of REES (4 nm, see figure 3c) as in buffer. In addition, we observed that REES of EGFP in sodium bis(2-ethylhexyl) sulfosuccinate (AOT) reverse micelles is completely independent of the reverse micellar hydration

state (defined as [water]/[surfactant] molar ratio or w_o, see figure 3c). This implies that neither entrapment in a reverse micelle, nor the hydration state of the reverse micelle, appears to influence the magnitude of REES of EGFP. This reinforces the fact that the extent of REES of EGFP is independent of the viscosity and hydration of the surrounding medium, implying that the dynamics of the protein matrix, rather than the dynamics of the surrounding medium, plays an important role.

The lack of environmental sensitivity of GFP fluorescence turns out to be a useful feature in many applications. In fact, the quantum yield of GFP has been shown to be by and large independent of its immediate environment (solvent) (Suhling *et al*. 2002; Uskova *et al.* 2000). This allows quantitation of fluorophore (GFP) concentrations under varying conditions *without worrying about intensity changes due to quantum yield variations* (Kalipatnapu and Chattopadhyay 2004, 2005; Soboleski *et al.* 2005; Lo *et al.* 2015).

3. Use of GFP-tagged membrane receptors for FRAP measurements: Advantage of the GFP tag

The G protein-coupled receptor (GPCR) superfamily is the largest seven transmembrane domain protein family in mammals (Pierce *et al*. 2002; Venkatakrishnan *et al*. 2013; Chattopadhyay 2014). Cells communicate and respond to their external environment *via* signal transduction events mediated by GPCRs. This is achieved through the activation of GPCRs upon binding to ligands present in the extracellular environment, which subsequently results in transduction of signal to cellular interior through concerted changes in their transmembrane domain structure. These initial events, which are fundamental to all types of GPCR signaling, occur at the plasma membrane *via* protein-protein interactions. An important consequence of this is that dynamics (lateral diffusion) of the receptor at the cell surface represents an important determinant of the overall efficiency of the signal transduction process. In this context, GFP and its variants have become popular reporter molecules for monitoring expression, localization and mobility of various membrane proteins (Tsien 1998; White and Stelzer 1999, Carayon *et al.* 2014). More specifically, tagging of GPCRs with GFP has allowed direct visualization of signaling and real-time trafficking in living cells (Barak *et al*. 1999; Milligan 1999; Kallal and Benovic 2000). The use of GFP-tagged GPCRs to visualize the receptor has a number of advantages: (i) the stoichiometry of the receptor and fluorescent protein is well defined as the latter is covalently attached to the receptor at the DNA level, (ii) complications encountered while using fluorescent ligands such as ligand dissociation are avoided, (iii) this approach allows analysis of un-liganded states of the receptor which is otherwise not possible with fluorescently labeled ligand, (iv) the possibility of perturbations induced by bulky fluorescent groups attached to small endogenous ligands such as biogenic amines limits their use, and (v) cellular biosynthesis ensures the presence of receptors attached to fluorescent proteins in cells and eliminate the necessity of labeling receptors with fluorescent ligands before each experiment.

In our previous work, we expressed the human serotonin$_{1A}$ receptor tagged at its C-terminus to the red-shifted emission variant of GFP, the enhanced yellow fluorescent protein (EYFP, previously known as GFP-10C, Ormö *et al.* 1996) stably expressed in CHO cells (figure 4a, Pucadyil *et al*. 2004; Pucadyil and Chattopadhyay 2007a). The serotonin$_{1A}$ receptor is a neurotransmitter receptor belonging to the GPCR superfamily, implicated in cognition and behavioral functions (Pucadyil *et al*. 2005), and serve as an important target in drug discovery (Lacivita *et al.* 2008; Fiorino *et al.* 2014). The EYFP variant is advantageous since it avoids cellular autofluorescence, it is relatively photostable and has a higher quantum yield (Ormö *et al.* 1996). A remarkable aspect of GFP is that in spite of its large size (27 kDa), most (but not all, see Fucile *et al*. 2002) proteins maintain their biochemical characteristics upon fusion with GFP (or its variants). We demonstrated that tagging of the receptor with EYFP does not alter the function of the serotonin$_{1A}$ receptor and this tagged receptor could be used to faithfully mimic the native receptor based on pharmacological and cell signaling criteria (Pucadyil *et al.* 2004). The site of the EYFP tag in the serotonin$_{1A}$ receptor and its typical fluorescence distribution when stably expressed in CHO cells are shown in figures 4a and 4b, respectively. We analyzed the cell surface dynamics and diffusion characteristics of the EYFP-tagged serotonin$_{1A}$ receptor, using fluorescence recovery after photobleaching (FRAP).

FRAP is a widely used approach to quantitatively measure lateral (translational) diffusion of lipids and proteins in membranes (Edidin 1994; Lippincott-Schwartz *et al*. 2001; Chattopadhyay and Jafurulla 2015; see figure 4). In FRAP, a concentration gradient of fluorescently labeled molecules is generated by irreversibly photobleaching (using a high power laser) a fraction of fluorophores in a region of interest of $\sim$µm dimension (see figure 4b). The time-dependent loss of this concentration gradient due to lateral (transverse) diffusion of fluorescently tagged molecules into the bleached region from the unbleached region is a faithful indicator of the lateral mobility of receptors in the membrane (figure 4c). Figure 4b shows a representative FRAP experiment in CHO cells expressing the serotonin$_{1A}$-EYFP receptor. The bright fluorescent periphery of cells representing the plasma membrane was selected for bleaching and monitoring recovery of fluorescence. Confocal images of the same group of cells before bleach (pre-bleach), immediately after bleach (bleach) and after recovery (post-recovery) from a representative FRAP experiment are shown in figure 4b. Region 1 was monitored to measure fluorescence

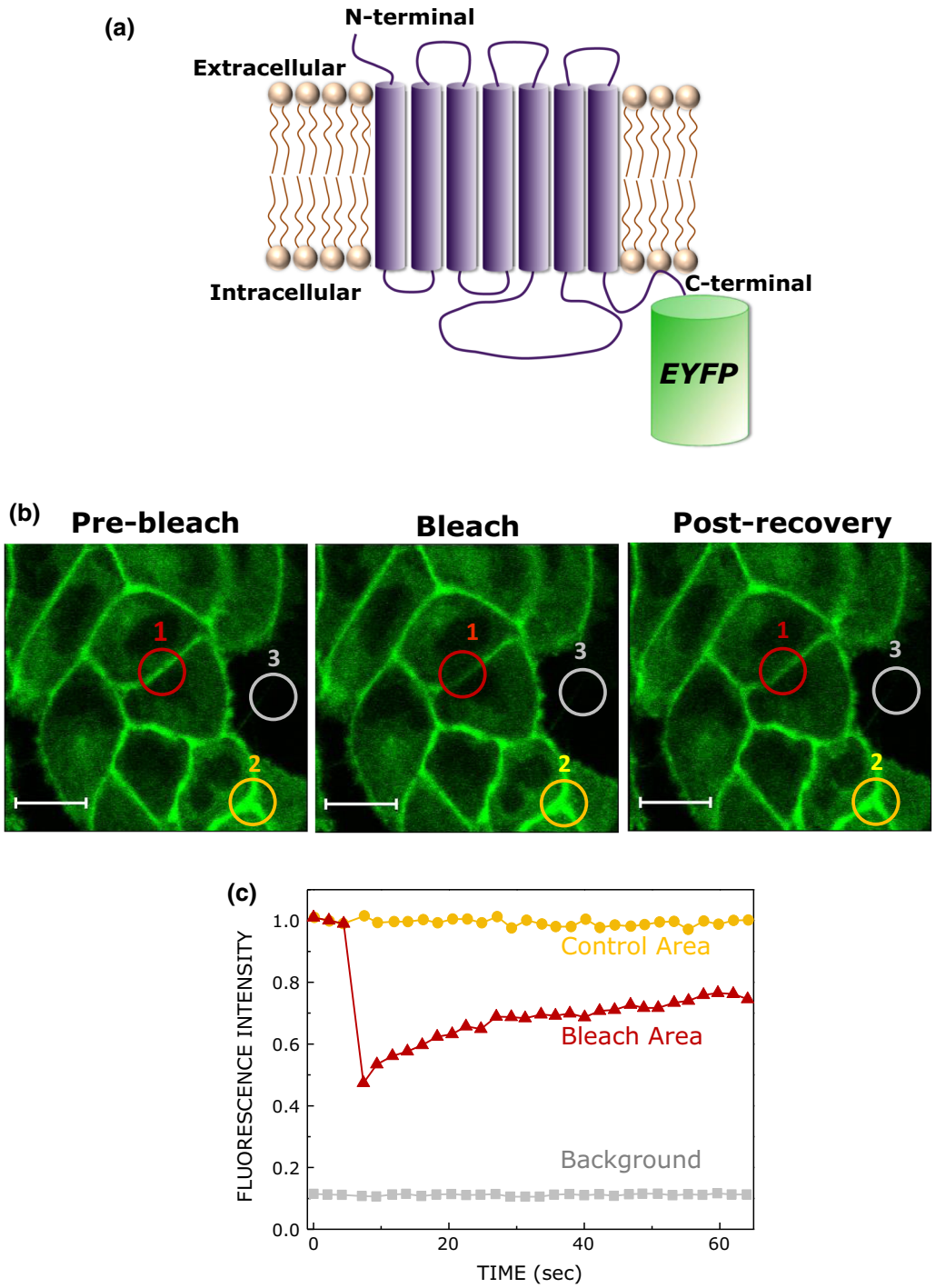

Figure 4. FRAP measurements in membranes using GFP-tagged receptor. Lateral dynamics of the serotonin$_{1A}$ receptor tagged to enhanced yellow fluorescent protein (5-HT$_{1A}$R-EYFP) stably expressed in CHO cells utilizing FRAP. (**a**) A schematic representation of the topological features of the serotonin$_{1A}$ receptor fused with EYFP at its C-terminus. (**b**) The cellular periphery shows distinct plasma membrane localization of 5-HT$_{1A}$R-EYFP and was chosen for FRAP measurements. Typical confocal images corresponding to prior to bleach (pre-bleach), immediately after bleach (bleach) and after recovery (post-recovery) are shown. Regions 1, 2 and 3 represent bleach area, control area (to detect any possible bleaching during scanning) and background, respectively. The scale bar represents 10 µm. (**c**) A representative set of normalized fluorescence intensity of 5-HT$_{1A}$R-EYFP corresponding to regions 1–3 for the entire duration of the FRAP measurement. The normalized fluorescence intensity in control area (region 2) shows no significant photobleaching. Adapted and modified with permission from Pucadyil and Chattopadhyay (2007b).

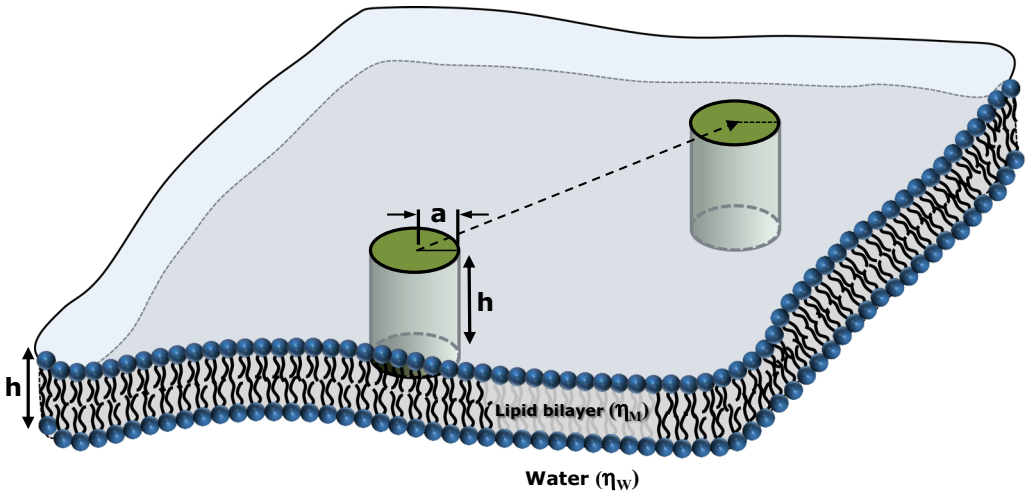

Figure 5. Lateral diffusion in biological membranes: The Saffman and Delbrück model. A membrane protein, shown as a cylinder of radius a and height h, undergoing lateral diffusion in a lipid bilayer membrane surrounded by water on both sides. The viscosity of the lipid bilayer and surrounding water are denoted as η_M and η_W, respectively. The membrane protein is permitted to move laterally in the x-y plane, and to rotate around the z-axis. An interesting consequence of the model is the rather weak (logarithmic) dependence of lateral diffusion coefficient on the mass of the diffusing membrane protein. Interestingly, this has acted as a boon for FRAP measurements of GFP-tagged membrane proteins and receptors, in the context of the considerable size of the GFP tag relative to typical size of membrane proteins. See text for more details.

recovery after photobleaching, region 2 to detect any possible bleaching during scanning, and region 3 to monitor background fluorescence. The relatively constant fluorescence intensity in control region (figure 4c) shows that the imaging conditions were optimized and no significant photobleaching of fluorescence during time-lapse imaging took place. Diffusion parameters were then extracted from a typical fluorescence recovery kinetics data shown in figure 4c (bleach area). Based on FRAP performed with the serotonin$_{1A}$ receptor tagged to EYFP, we demonstrated that cell surface dynamics of the receptor is altered upon activation of G-proteins (Pucadyil *et al.* 2004; Pucadyil and Chattopadhyay 2007a).

Diffusion of transmembrane proteins is a fundamental biophysical process that controls the dynamics of lipid-protein and protein-protein interactions in the membrane. However, the physics of diffusion of molecules immersed in membranes is different than that of molecules diffusing in a solvent. Therefore, unlike soluble proteins, understanding the size dependence of the diffusivity of membrane proteins is rather subtle. It is well known that the mobility of a rigid, spherical particle in a solvent is given by the Stokes-Einstein equation, which has an inverse dependence on the particle radius (or mass) (Dix and Verkman 2008). Interestingly, the mobility (diffusion) of the same particle when embedded in a membrane bilayer, is more complex. The classical framework of diffusion in membranes is provided by the Saffman and Delbrück model (Saffman and Delbrück 1975). According to the Saffman and Delbrück model (see figure 5), lateral (translational) diffusion is relatively insensitive to the size of the diffusing molecule, since the diffusion coefficient in this model is proportional to the logarithm of the reciprocal of the hydrodynamic radius of the diffusant. *This unique aspect of lateral diffusion in membranes has turned out to be advantageous for FRAP measurements of GFP-tagged membrane proteins and receptors, since the diffusion coefficients obtained are not influenced by the rather large size of the GFP tag (typically ∼27 kDa) !*

4. The road ahead

Clearly, GFP technology has been a real "game changer" in modern biology. Future applications of GFP in post-genomic biology could be exciting. For example, experiments using a combination of GFP-tagged proteins and super-resolution microscopy offer the possibility of high-throughput super-resolution imaging (Ries *et al.* 2012). GFPs that undergo repeated cycles of on/off state (thereby exhibiting blinking) appear well suited for monitoring time-dependent cellular processes and can serve as molecular photonic switches (Dickson *et al.* 1997). Other promising applications include quantitation of protein levels in single living cells (Lo *et al.* 2015), development of flow cytometry based assays using GFP-labeled parasites for

quantitation of infection and high-throughput usage (Wilson *et al.* 2010), and protein folding at a single molecule level (Ganim and Rief 2017).

About ten years back, we wrote a review for this journal in which we described GFP as "*the molecular lantern that illuminates the cellular interior*" (Haldar and Chattopadhyay 2009b). It appears now that the lantern has worked well and the cellular interior is indeed well lit!

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PARIJAT SARKAR and AMITABHA CHATTOPADHYAY*

CSIR-Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

**Corresponding author (Email, amit@ccmb.res.in)*