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Review

Nanoscale gizmos – the novel fluorescent probes for monitoring protein activity

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ABSTRACT

Nanobiotechnology has emerged inherently as an interdisciplinary field, with collaborations from researchers belonging to diverse backgrounds like molecular biology, materials science and organic chemistry. Till the current times, researchers have been able to design numerous types of nanoscale fluorescent tool kits for monitoring protein–protein interactions through real time cellular imagery in a fluorescence microscope. It is apparent that supplementing any protein of interest with a fluorescence habit traces its function and regulation within a cell. Our review therefore highlights the application of several fluorescent probes such as molecular organic dyes, quantum dots (QD) and fluorescent proteins (FPs) to determine activity state, expression and localization of proteins in live and fixed cells. The focus is on Fluorescence Resonance Energy Transfer (FRET) based nanosensors that have been developed by researchers to visualize and monitor protein dynamics and quantify metabolites of diverse nature. FRET based toolkits permit the resolution of ambiguities that arise due to the rotation of sensor molecules and flexibility of the probe. Achievements of live cell imaging and efficient spatiotemporal resolution however have been possible only with the advent of fluorescence microscopic technology, equipped with precisely sensitive automated softwares.

Contents

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1. Introduction

Fluorescence is a luminescence process in which atoms and molecules absorb a specific lower wavelength light and after a brief gap of fluorescence lifetime emit a longer wavelength light. Over the past many years in the service of biology, fluorescence microscopy has emerged as the prime pillar of microscopy due to its inherent selectivity to expose only objects of interest against black background [1]. To investigate myriad of cellular processes, fluorescence imaging has been realistic for the visualization of molecules and whole organisms. It started with the attachment of organic dyes to proteins through antibodies and later sought the genetic tagging of target proteins with fluorescent proteins. However, the linking of antibodies has to be supplemented with the fixation and permeabilization of cells [2]. With the efficacy of fluorophores as direct recognition agents for various cellular molecules like nucleic acids, ions and other cell organelles, the technique of immuno-fluorescence became less valuable. Additionally, with the development of FRET based sensors, noninvasive behavior and live cell imaging in both prokaryotic and eukaryotic cells proved to be a novelty for researchers exploring fluorescent probes for real time cellular imaging. Originally, FRET stands for Forster Resonance Energy Transfer in the honour of Theodor Forster, physical chemist, who first discovered and understood it. The term "fluorescence resonance energy transfer" is often used, when both the chromophores are fluorescent. The latter enjoys common usage in scientific literature and has been in practice as such commonly.

However, It has been established that compared to other fluorophores, amplified brightness and photo-stability are the two critical parameters of semiconductor nanocrystals that makes them unique for fluorescent microscopic imaging (Fig. 1), although their targeting potential still endures many hindrances [3]. In recent years, using newly engineered photo-controllable fluorescent proteins; numerous super-resolution microscopic techniques became ready to use in order to get better visualization of objects having dimensions smaller than 500 nm and 200 nm in axial and lateral directions, respectively. In current times, researchers are now powerfully aided with more precisely optimized FPs. There are three groups of photo-controllable FPs- photo-convertible FPs (PCFPs), photoactivatable FPs (PAFPs), and reversibly photo-switchable FPs (rsFPs). PAFPs are activated from non-fluorescent (dark) to fluorescent state, whereas, rsFPs are reversibly photo-switched between inactive and active states, and PCFPs are made to convert from original color to another. The super-resolution microscopic imaging using these FPs is always carried out by controllably turning them on and off [4].

Moreover, proteases have emerged favorable enzymes for synthesizing their inhibitory compounds as potential drugs for some major human diseases such as cancer, AIDS and other neurodegenerative infections. Because of the latest coherent advancements between genomic, proteomic and biophysical techniques, peptide substrates for several proteases have been ascertained, that paved a way to trace and analyze the functioning of their equivalent proteases through a simplistic and speedy mode. By combining such peptide substrates with appropriate FPs, researchers develop a chimeric protein and then sequentially analyze many protease inhibitors through FRET disruption. Present review will focus on latest advances in major fluorophores and diverse fluorescence strategies that are in current use of fluorescence microscopy to visualize protein dynamics involved in their location and function within a cell.

2. Variety of fluorescent probes

Varieties of fluorophores that are beyond the scope of this review are now available for evaluating the protein activity. Regarding fluorescent probes, two critical considerations must be acknowledged first the fact that spectral properties of fluorescent probes determine the basic settings of time resolution and wavelength of instruments, and secondly certain fluorescent probes are used to monitor specific activities. For example, fluorophores that are sensitive to pH can only be explored to measure pH, and rotational diffusion can be tracked by only those probes having non-zero anisotropies. For histological studies, probes possessing long excitation and emission wavelengths are utilized to display auto-fluorescence at short excitation wavelengths. We are elaborating here some basic fluorophores which are being exploited through various bioengineering approaches to visualize the sea of protein dynamics, so as to come up with innovative nanoscale toolkits for monitoring molecular interactions operating within a living cell in real time.

2.1. Molecular organic dyes

In the synthesis of organic fluorophores, many molecular strategies have been adapted. For this purpose, addition of electrophiles or other charged substituents like sulfonates, conjugation of double bonds, extra ring rigidification by locking rotatable rings into parent ring structures, etc. are some of the most important assignments. Though the dyes made by such strategies are now available commercially (Haugland, 2005), however, such dyes are handicapped by being non-specific to all proteins. Therefore, their use in permeabilized and fixed cells has to be supplemented with antibodies (Fig. 2A). These organic fluorophores are usually of low magnitude (<1 kD) and their important etiquettes like reduction in self-quenching, good brightness and appropriate wavelength have made them industrially optimized.

For a successful delivery of fluorophore into a cell, broadly two types of cellular environments- intracellular and extracellular are available. The major confrontations to overcome lies in optimized intracellular delivery of fluorophores, the perfect labeling of target biomolecule and negligible cytotoxicity. Due to smaller size of organic fluorophores there is least spatial hindrance to impede with functioning of target biomolecule. Therefore, this opportunity has been explored by attaching many fluorescent probes with a single target biomolecule in order to gather maximum fluorescence signal. Because of high label densities the strong electrostatic repulsion between neighboring molecules, the dye structural conformation and hydrophilicity altogether can cause fluorescence quenching [5–7] and may also affect functioning of the biomolecule [8].

For the selective detection of enzymatic and non-enzymatic proteins, fluorescent chemical turn-on probes are synthesized by fusing an environment sensitive fluorophore with a protein-specific small molecule [9]. In this scenario, localized deposition of proteins in proteostasis has been elaborately exploited to develop sensors for understanding the mechanism of protein deposition in neurodegenerative disease progression [10]. The process of proteostatis involves biogenesis, traffic and protein degradation within and outside a cell, involving different integrated biological pathways. The basis of such a designed fluorescent probe is that most of the ligand binding sites in proteins are hydrophobic, that constitute the prime thermodynamic driving force for the binding of small molecule ligands to their respective proteins. This approach has been exploited to develop a precious turn on folding sensor for effective live cell monitoring of proteostatis [11]. This small fluorogenic molecule became fluorescent when it binds and reacts with folded and functional retroaldolase enzyme. Recently, in a similar approach, dual signal fluorescence-enhanced sensor, based on Cu^{2+} mediated fluorescence switchable strategy has been designed to detect Cysteine (Cys) in a simple and fast way. It was observed that two fluorescence emissions of ultrathin films (calcein@NFR/LDHs UTFs) are effec-

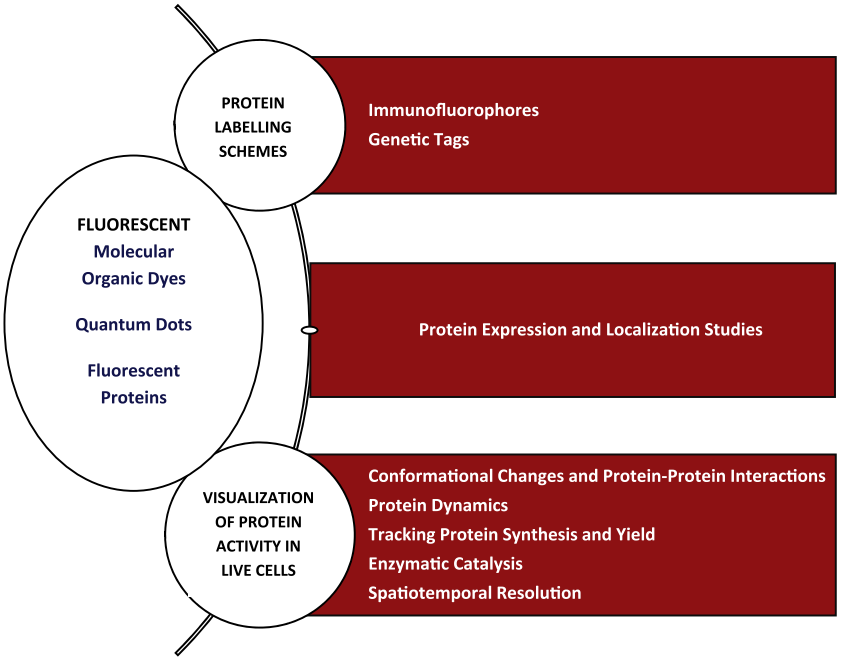

Fig. 1. Outline of fluorescent probes, their types, applications and different methods to study protein dynamics and localization within a cell or whole organism.

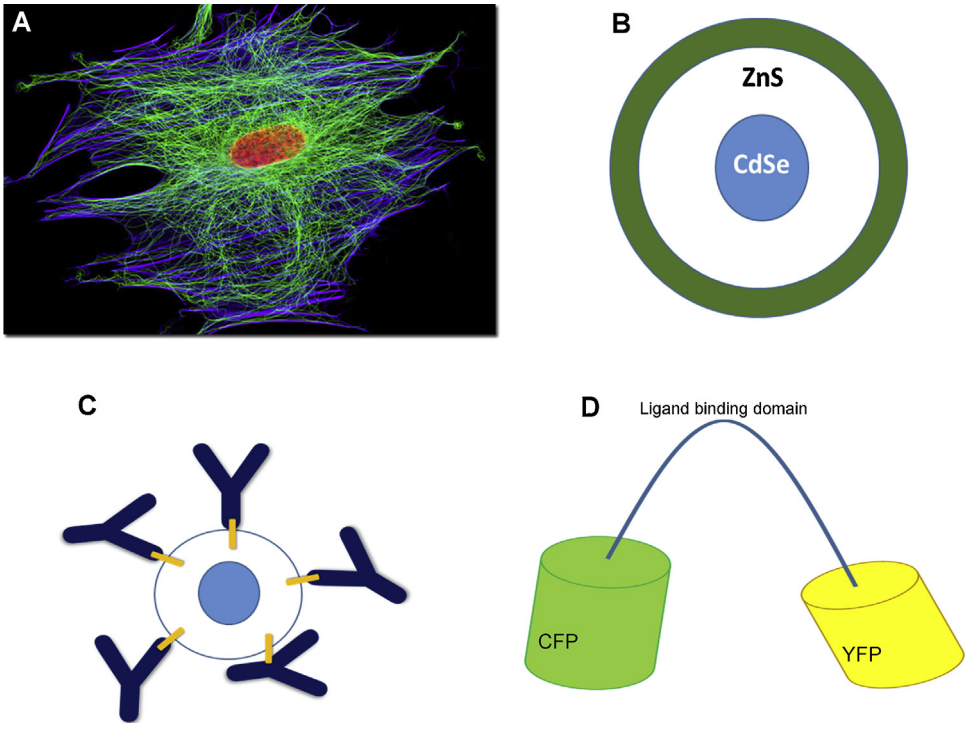

Fig. 2. Application of different types of fluorophores, exploited for labeling and detection of proteins. (**A**) A 3T3 cell from an adherent culture, immunolabelled with Alexa Fluor 488. (**B**) Quantum Dot with CdSe core and ZnS shell. (**C**) A QD conjugated with many antibodies, preventing its mobility. (**D**) A ligand sensing domain, inserted between CFP and YFP.

tively quenched by Cu^{2+} (off state), and then reversibly recovered by Cys (on state), leading to the specific coordination of Cys and Cu^{2+} [12]. Similarly, a novel, simple and rapid fluorescent probe based on excited-state intramolecular proton transfer (ESIPT) was the latest addition to such Turn On sensors, allowing easy way detection and quantification of biothiols in living cells [13].

For site specific protein labeling in live cells, Roger Y. Tsien and colleagues in 1998 pursued the use of biarsenical reagents. In real sense, it is the high affinity interaction of arsenic for thiols that forms the basis of biarsenical labeling technology. The fluorescent derivative FlAsH contains two arsenic atoms at a set distance from each other. Similarly, ReAsH is modified to contain resorufin. With the aid of fluorescence microscopy, FlAsH and ReAsH technology has been exploited to bind to tetracysteine (TC) sequences. The common TC sequence employed for this purpose is the six aminoacid sequence Cys-Cys-Pro-Gly-Cys-Cys. When bound to ethane dithiol (EDT), both FlAsH and ReAsH are non-fluorescent. Thus, upon binding with recombinant proteins containing such

a tetracysteine motif, both these biarsenical labeling reagents-FlAsH-EDT and ReAsH-EDT turn to become highly fluorescent with green or red color respectively, with subsequent displacement of EDT.

Another most important bacterial enzymatic protein *Haloalkane dehalogenase* a hydrolase is modified and designed to covalently bind to a synthetic ligand with subsequent fusion to the protein of interest. Therefore, this enzyme has been efficiently exploited for visualizing subcellular localization of protein of interest, to capture the binding partners of a protein or for protein immobilization [2]. The function of such a single protein tag is altered by attaching different chemical moieties of synthetic ligands called HaloTag ligands, through a chloroalkane linker, attached to useful molecules such as fluorescent dyes, affinity switches or solid surfaces [14]. The Halotag is a modified haloalkane dehalogenase designed to covalently bind to synthetic ligands (Halotag ligands). It needs to be understood that, covalent bond formation between FP tag and the chloroalkane linker is extremely specific, occurs swiftly under physiological settings, and is essentially irreversible. However, the choice of ligand is done in accordance with the type of experiments to be performed.

Because of the heavy load of metal pollutants, researchers have come up with some important electrochemical sensors to detect different metals in a specific, sensitive and selective way. For example, to detect attomolar (aM) concentrations of Hg^{2+} a precious electrochemical sensor was developed [15]. For detection of this target metal, three single-stranded DNA probes were rationally designed. These probes were developed due to combined thymine-Hg^{2+}-thymine (T-Hg^{2+}-T) based coordination chemistry principles. In a similar approach, a sensitive electrochemical lead ion sensor was developed for lead (Pb^{2+}) ion detection. It was observed that, it was due to Pb^{2+}-induced G-rich DNA conformation that upon Pb^{2+} addition, DNA duplex got unwound and formed a stabilized G-quadruplex (G4) [16]. Another approach for detecting Pb^{2+}, was based on an electrochemical DNA sensing strategy through modification of a glassy carbon electrode, with ordered mesoporous carbon nitride, gold nanoparticles and methylene blue [17].

2.2. Quantum dots

Quantum dots (QDs) contain some hundred to thousand atoms within a size of nanometer scale, distinctively made of an element of silicon or germanium or composed of a core of CdSe or CdTe and a ZnS shell (Fig. 2B). Such petite structures vary in their color. The sharp fluorescence of such nanocrystals at discrete wavelengths depends on their size. Compared to other fluorophores, QDs possess 10–100 times higher extinction coefficients and better quantum yields [18]. Here, a lone excitation wavelength readily excites the QDs of numerous emission spectra. QDs are preferably developed with coatings for biological investigations to facilitate their solubility in aqueous medium, help in conjugation with antibodies and prevents quenching by water [19,20]. However, QD conjugated biomolecules lack efficient migration potential through intact cellular membranes due to their larger size, and thus, their expenditure for endocytosed proteins or extracellular apartments, permeabilized cells is restricted. Remarkable about QDs is their tolerance to repetitive imaging of solitary molecules, by virtue of their photo-stability [21]. The portable electron-density and size permits analogous electron microscopy to be used for visualization of different objects of interest. However, a major issue with QDs is their toxicity in biological applications, and construction of conjugated polymer dots has helped a lot in this way.

Concerning the use of QDs to label target biomolecules, there is no strict protocol. However to achieve an optimized labeling, QDs are first made to become water dispersible and are then attached to target biomolecules. Many interactions are exploited to achieve QD fluorophore labeling with target biomolecules. Some major linking methods include-*covalent linkages*, *biotin-avidin interactions* and poly-histidine tags. Compared to small organic fluorophores, many biomolecules are attached to a single QD [22] which leads to problematic orientation (Fig. 2C). On the other hand, because of the better cell and organelle permeability of *aryl fluorosulphates*, they have been employed for the development of protein-selective covalent probes. In this scenario, an environment sensitive fluorogenic probe, 1,3,4-oxadiazole has been designed to bind selectively to transthyretin (TTR). Addition of this fluorogenic probe to HEK293T cells allowed efficient binding and imaging of cellular organelles like mitochondria and endoplasmic reticulum, making it a new fluorescent tool for living cells [23]. Upon application of this fluorogenic probe in *Caenorhabditis elegans*, it successfully detected TTR in six macrophage like cells.

2.3. Fluorescent proteins

In real sense, the breakthrough of green fluorescent protein (GFP) from Jelly fish (*Aquorea victoria*) and then subsequent engineering of various other novel FPs from diverse organisms armed us with fluorophores, possessing extraordinary uniqueness for live cell imaging. Single GFP based sensors or chimeric FRET based nanosensors generate visible fluorescence for microscopic imagery. FRET involves the transfer of non-radiative energy from donor fluorophore to acceptor fluorophore, in the presence of a ligand. Such novel approach has been utilized to develop chimeric proteins that serve as nanosensors. These FRET based nanosensors consist of cyan fluorescent protein (CFP), a ligand binding domain and a yellow fluorescent protein (YFP) (Fig. 2D), with CFP and YFP as the two mutant forms of GFP. However, after GFP, large spectrums of FPs with varied colors were discovered from marine coelenterates. The increase in brightness and folding efficiencies, accompanied with a decrease in oligomerization is achieved by generation of affinity mutants. This leads to the diversification of spectral range of FPs, from one color to another, improving the overall protein monitoring system of the resultant biosensor. Mutagenesis increases both photo-stability and photo-switchability of FPs [24,25]. It is exactly the reversibility and irreversibility in photo-switching that makes FRET based biosensors useful to track protein trafficking. Quenching enforced by acidic pH is the major snag in the bio-sensing mechanism of FPs. However, recent developments have now better engineered the sensitivity of biosensors *vis a vis* ions, pH and redox potentials [26,27].

Prior to FRET based biosensors, phyco-bili proteins and cyanobacterial photosynthetic antenna pigments were used as prime tags for fluorescence. It is due to their bigger size that problems arise in their diffusion which renders them limited only for surface labeling purposes [28]. Therefore, such FPs are routinely utilized in conjugation with antibodies for enzyme-linked immunosorbent assays and flow cytometric measurements. For the FPs to offer their best character as fluorophores, some vital features include- varying spectral properties, maturation efficiency, photo stability, efficient brightness, and fidelity in fusions, monomeric character and their potential efficiency as FRET donors or acceptors. Furthermore, critical mutations have been exploited to generate different FP variants with enhanced spectral properties- mVenus [29] and mKO2 [30] are the two important examples of bright monomeric variants. To address spectral and structural snags of FPs, recently a family of GFP proteins in the cephalochordate *Branchiostoma floridae*, commonly known as amphioxus was discovered and named after it as bfloGFP [31]. So far, this animal is credited to be the only species representing the largest source of 16 FPs. A total of six clades of amphioxus possess these 16 GFPs, all emitting the green fluorescent light, and each clade owns discrete absorption spectra, extinction coefficients and fluorescence intensities.

Utilizing the x-ray crystallography derived three dimensional (3D) structures, biochemical and spectral characteristics of two FPs- a bright FP (bfloGFPa1) and a weak FP (bfloGFPc1), this group [31] deliberated about the role of structural differences in FPs vis a vis chromophore environments that modulate into their photonic properties. A latest approach [32] has been carried out to improve the brightness and photostability of green and red FPs for enhanced live cell imaging in FRET. The group reported an improved photostability of mClover3 a derivative of GFP and mRuby3 a derivative of Red Fluorescent Protein (RFP) by an extent of 60% and 200% respectively over the previous generation of fluorophores. Also mRuby3 was recorded to be 35% brighter than its previously engineered version- mRuby2. Till the present period so far, out of all Jelly fish GFP and coral RFP derivatives, expressed in various mammalian cell lines, mClover3 and mRuby3 offers the highest fluorescence signals.

3. Protein labeling schemes

In order to obtain the visualization of the protein of interest, two tagging schemes are generally followed- fluorescently labeled antibodies, in which antibodies are made to specifically bind with the protein of interest and the intrinsic fluorescent signal, where FP is genetically linked with the protein of interest. Though, in comparison to active antibodies, tagging through FPs is prolonged, yet when matched to QDs, genetic tags serve as the least cytotoxic fluorophores inside any cellular environment. The gene of interest is first cloned and then transformed into suitable cells. Once inside the cell, the bitterness due to FPs may be amplified, since they have their own unique proteinous feature which can lead to functional disorder of the attached protein of interest. In this direction, a striking effort [33] was made to genetically engineer one least cytotoxic red FP (FusionRed) for monitoring the protein dynamics in real time. In the chromophore surrounding region K69R and R203H mutations were transferred to mKate2.5, resulting in the dramatic pH stability of the protein. Earlier, mKate2.5 variant was yielded by inserting S132A, R164A, K182E and Y200N mutations along with the substituted C-terminus. In addition to pH stability, the final variant FusionRed exhibited photostability and maturation rate at par with other red-emitting fusions. Here we deliberate on the labeling schemes of immunofluorescent tags and genetic tags.

3.1. Labelling through immunofluorophores

There are two basic immunofluorescence techniques- *direct* and *indirect*. In former case, a single antibody conjugates directly with the fluorescent dye, whereas two different antibodies are exploited in latter- primary that recognizes target biomolecule and secondary that binds with fluorescent dye. However, for immunocytochemistry, direct labeling is carried out using both monoclonal and polyclonal antibodies. Background staining encountered with the use of secondary antibodies is also eliminated. In tissue sections, direct immunofluorescent technique (DIF) is principally applied in the identification of antibodies and other inflammatory proteins, to diagnose disease groups like pemphigus, lupus erythematosus, etc. that are histologically similar to separate under clinical investigations [34].

Additionally, labeled streptavidin binding (LSAB) and avidin-biotin complex (ABC) are the two common immuno-histochemical techniques that have been recently commercialized. In LSAB, primary antibodies are conjugated *directly* to fluorophores and detected by streptavidin [35] and in order to increase the spectral diversity for multiprotein analysis, antibodies are injected directly into live cells. Avidin-biotin labeling constitutes another *receptor-ligand* pair that works best in the secretory compartment. It is extremely useful for *in vitro* and histological investigations, however, for unknown reasons, this fluorophore toolbox is rarely explored in live cell imaging. pH measurements of various compartments in terms of different pK_a values has been carried out through chicken avidin-biotin fluorescein conjugates, recombinantly expressed in different secretory compartments [36,37]. This facilitates the study of mechanism of pH regulation in such secretory compartments. However, primarily the critical limitation includes the toxic nature of avidin-biotin fluorescein conjugate in cytosol and mitochondrial compartments or secondly avidin is saturated with biotin. G protein coupled receptors (GPCRs) regulate critical physiological functions through neurotransmitters, peptide hormones, etc. Therefore to visualize GPCRs in living or fixed cells, immunofluorescence is applied through two strategies. In one approach, through antibodies against the extracellular receptor regions, and in other case through an epitope tag [38]. Intracellularly sited receptors or receptor segments and epitope tags are recognized and visualized only after cell fixation and permeabilization (Fig. 3). The basic problem with immunolabelling is the larger

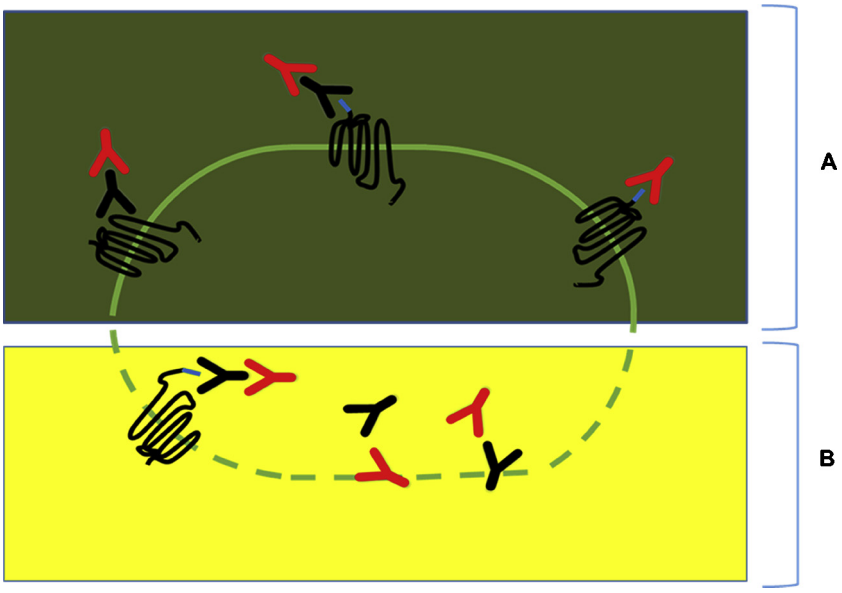

Fig. 3. In order to visualize GPCRs, how in non-permeabilized cells: (A) antibodies are used against extracellular receptor regions or N-terminal epitope tags. (B) In contrast, it's only after cell permeabilization, that the visualization of intracellularly located receptors/segments or C-terminal epitope tags takes place.

size of immunofluorophores, interfering with multiprotein recognition. Furthermore, immunofluorescent techniques are limited to permeabilized cells, extracellular proteins, and intracellular proteins. Whenever a problem arises due to availability of low-grade antibodies, an epitope tag is applied to the target protein to express it in a recombinant manner.

3.2. Labelling through genetic tags

The major tagging hindrances have been overcome through genetically encoded FPs, which ensure perfect covalent tagging of FPs with the protein of interest. Genetic engineering followed by transformation procedure ensures an easier and perfect delivery of exogenous DNA into cells as compared to chemical dyes. By successfully developing different fusion constructs, FPs describes temporal dynamics of different metabolic processes in real time. For example, substrates binding to fusion constructs containing GFP undergo ubiquitin-proteasome dependent proteolysis [39]. FRET disruption by proteases emerged as a classical biophysical parameter for investigators searching for novel drugs against major lethal diseases such as AIDS and cancer. FRET based biosensors possess a protease-sensitive linker fused between a blue fluorescent protein (BFP) and green fluorescent protein (GFP). The disruption of FRET by proteolysis separate the donor and acceptor fluorophores and individual domains of metallothionein (MT) were generated by proteolysis of CdMT with substilin [40]. In exact sense the cleavage occurred between two lysine residues- 30 and 31 in the hinge region, ultimately cleaving the polypeptide chain between two fluorophores and thus in comparison to the control sample, energy transfer of dual-labeled MT decreased more significantly.

Cellular metabolite quantification in live cells constitutes the most important application of fluorescent proteins. For example, by inserting the peptide linker CaM and M13 between CFP and YFP, cameleons- the genetically encoded Ca^{2+} sensors were constructed [26]. By increasing the intracellular levels of Ca^{2+} the affinity of CaM for the adjacent M13 sequence gets switched on. This causes a change in distance between two FPs that ultimately results into a large FRET. By replacing glutamate with glutamine residues in the pockets of Ca^{2+} −binding sites, the effective affinity of $Ca^{2+}$for cameleons is regulated. Recently, well optimized FRET based nanosensors have been developed for measuring *Ras* and *Rap1* activity [41] and imaging glutamate levels in brain [42]. We have successfully constructed special FRET based nanosensors for quantification of leucine [43] and methionine [44], for *in vivo* monitoring of zinc concentrations [45], lysine flux [46] and glycine betaine levels [47] and vitamin B12 levels [48].

4. Protein expression and localization studies

Fluorescent flow cytometry facilitates the visualization of endogenous proteins in their active state, very important for single cell profiling. However, immunofluorescent tagging is most suitable for endogenous proteins. To visualize the expression of particular proteins in situ within different cell lineages or individual cells, mutant phenotypes can be analyzed by immunofluorescence staining [49]. Although due to ample brightness of QDs, the detection limit advances much more and hence multi-label separation is achieved by varying spectral features. Though at light microscopic (LM) and electron microscopic levels (EM), QDs serve as appropriate tools for detection of proteins, however, it is only with the aid of electron microscopy that protein localization is effectively accomplished in subcellular structures. QDs are promising in electron microscopic observations, due to their size and electron dense core, and additionally, visualization is enhanced by silver staining. Recently, QDs have been utilized directly for concurrent immunolabelling of several endogenous proteins to acquire a correlated LM-EM imagery of cells [50]. Before proceeding for EM examination studies, we can swiftly get a precise analysis in LM by utilizing diverse range of QD labels. The fluorescent QDs at EM level overcome the need of antibody labeling, for example streptavidin labeled QDs detect surface proteins by using enzyme biotin ligase [51]. Correlated microscopy using tetracysteine ensures the preservation of ultrastructure against the immunolabelled microscopic imagery that needs permeabilization and makes inflexible fixation. Both FPs and immunofluorescent tags are extensively applied for subcellular allotment of proteins and there is a high correlation between live-cell and fixed localization investigations. Researchers utilize finest validated antibodies and suitable fixation procedures, critical to obtain better epitope accessibility and an accurate protein distribution *in vivo*. In terms of cellular and subcellular localization however, FRET based nanosensors are comparatively more efficient than all other labeling methods. Compared to adding chemical dyes exogenously, such nanosensors can be expressed within stable cell lines and transgenic animals. The strategic approach is combination of FRET with metabolite recognition capability of a bacterial periplasmic binding protein (PBP) [52], followed by successful transfection of resultant nanosensor into any cell type. This expands the possibilities for high-throughput screening, rigorous cell, developmental and physiological studies. In case of plants, most cells contain a large vacuole in their cytosol making it difficult to quantify plant metabolite concentrations in a given compartment in the little available cytoplasmic space. The FRET based nanosensors are comfortably targeted to sub-cellular locations, facilitating the high-resolution mapping of signals within plant cells. Since there is an enormous range of metabolites in plants with highly complicated transport pathways that are quite contrasting against the animal systems.

5. Visualization of protein activity in live cells

5.1. Monitoring protein conformational changes and protein–protein interactions

A healthier spatiotemporal resolution of protein conformational changes is usually achieved by fixing a protein domain in between two FPs- Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) respectively. This is followed by FRET between two FPs, as the ligand binds with ligand sensing domain. Protein that connects the two FPs is engineered to undergo conformational changes in reaction to very important signals. With the aid of an appropriate localization signal, the resultant biosensors are targeted to precise subcellular compartments. A wide range of FRET based nanosensors have been constructed for measuring various metabolites, to monitor the activity of many proteases, check the balance between kinases and phosphatases, for sensing neurotransmitters and other metabolites [53]. FRET efficiency varies with distance and orientation between donor and acceptor FPs. However, circular permutation of any of the two FPs [54] or slight adjustments in the length of linker region greatly alters the FRET, as a result the frequent crop up of FRET responses are more due to reorientation and less by means of change in their inter fluorophore distance.

In living cells, FRET visualizes the dynamic protein–protein interaction, by means of two FP tagged proteins, whence a ligand binds with the ligand binding domain (Fig. 4). FRET processing occurs as the two FPs are within the intermolecular distance of 6–8 nm. However, recent approaches extended to the three FP tagged FRET based biosensors. It was carried out by valuable addition of monomeric red fluorescent protein (mRFP) to the pair of CFP/YFP. Such trimeric FRET based nanosensors again endow with deeper resolutions. In such FRET experiments, CFP acts as a donor

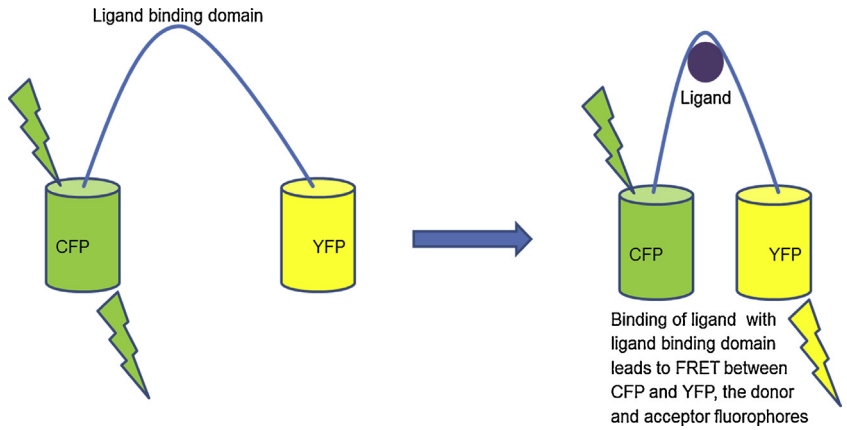

Fig. 4. FRET phenomenon: FRET takes place when ligand binds with the ligand sensing domain, through which CFP (donor) and YFP (acceptor) are in proximity.

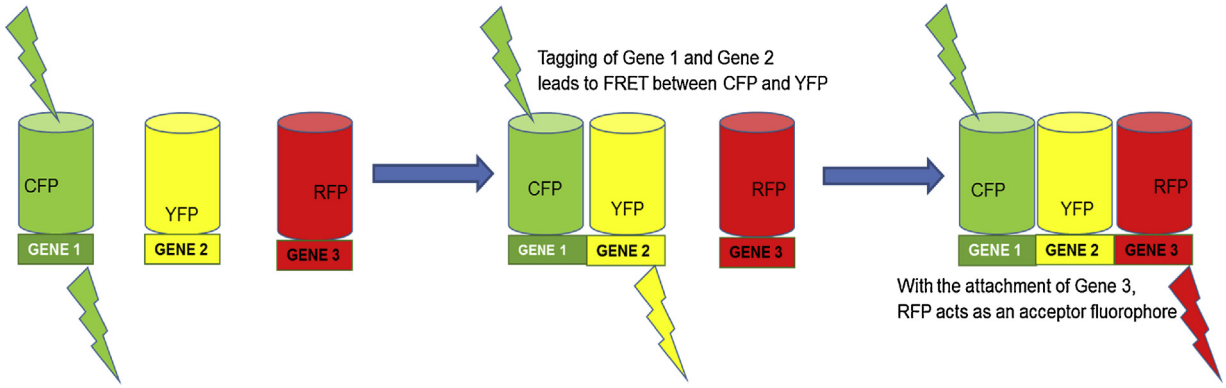

Fig. 5. Three fluorophore based FRET process: by the attachment of gene 1 with gene 2, intermolecular FRET occurs first between CFP and YFP. Afterwards, gene 3 encoding RFP also gets attached, which acts as new acceptor fluorophore for another FRET.

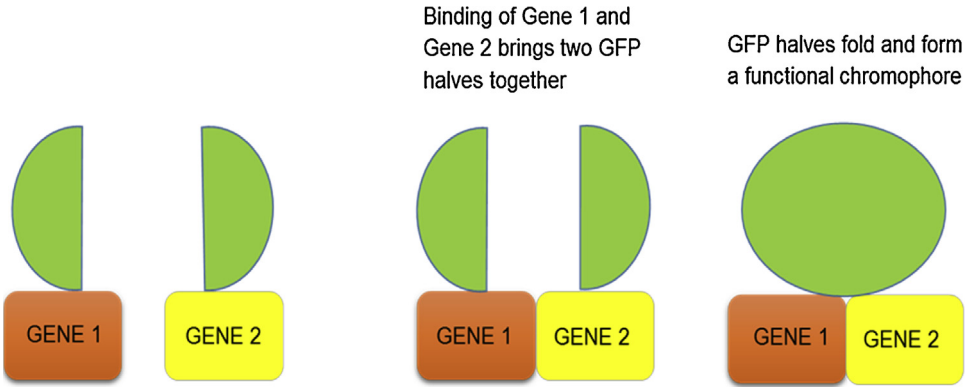

Fig. 6. BiFC leading to the formation of a functional chromophore. Before this process, the two GFP halves are separate and BiFC leading to their proper folding resulting into the formation of a functional chromophore.

for YFP and in turn, YFP as the next donor for mRFP (Fig. 5). On the basis of same principle, bimolecular fluorescence complementation (BiFC) is low pace chromophoric interaction between two split fragments of a FP (Fig. 6) fused with two interacting proteins. Fluorescence correlation spectroscopy works in a novel way- using the two different tags aided with two different colors that interact and diffuse as a pair [55]. There are simple colocalization events that indicate substantial protein–protein interactions. As seen in case of protein kinase A (Fig. 7), its regulatory subunit and catalytic subunit are co-expressed. The latter is fluorescently tagged and destined to plasma membrane, and whence the adenosine 3′, 5′ –monophosphate (cAMP) levels are increased, the two partners get dissociated, and therefore FRET visualization takes place.

Halotags have been used for FRET technique and it has been validated that compared to other bio-conjugated dyes, utilizing the Halotag protein linked with enhanced green fluorescent protein (GFP) displayed superior fluorescence stability [56]. Halotags have been applied to monitor protein–protein and protein-DNA interactions. They have been used to monitor the interaction of bromodomain protein (BRD4) and histone deacetylase (HDAC1) along with other additional proteins [57]. Halotag has been adapted for the investigation of epidermal growth factor receptor Ras-extracellular signal regulated kinase (ERK) mitogen activated

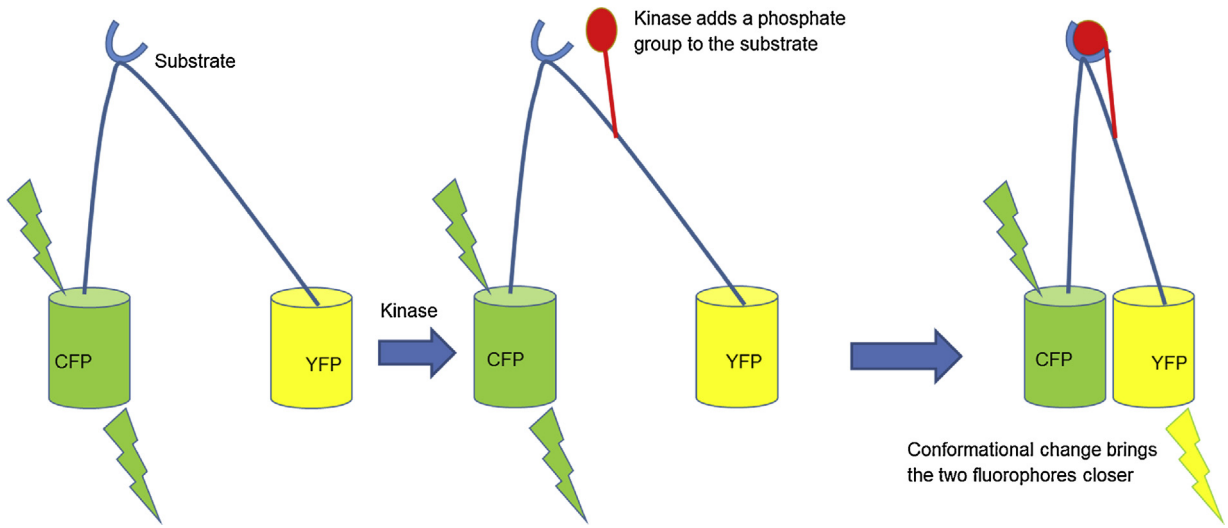

Fig. 7. Indirect FRET: Addition of a phosphate group to the substrate by Kinase enzyme leads to a conformational change that brings CFP and YFP close together, which ultimately results into FRET.

protein (MAP) kinase pathway in living cells. FRET has been further adapted for Halotag mediated conjugation and site specific decoration of molecular beacons, utilizing two different FP fusions, thus enabling easy detection of target nucleotide sequences [58]. In live cells, the protein–protein interactions and alternative protein conformations have been detected through bipartite tetracysteine display. Such profluorescent biarsenical reagents- FlAsH-EDT2 and ReAsH-EDT2 have led to detection of early protein misfolding events associated with Alzheimer's and Parkinson's diseases and for high-throughput screening of compounds that stabilize discrete protein folds [59].

5.2. Visualizing protein dynamics

Protein labeling enables us to track its redistribution within a cell by using various imaging techniques which facilitate visuals of protein dynamics. Imaging readouts are therefore sought for such translocation processes that includes- monitoring buildup of polyphosphoinositides through FP-tagging of pleckistrin homology domains, proteins diffusing at steady state rates or involved in inter-compartmental exchange. Intensity fluctuations are statistically interpreted in fluorescence correlation spectroscopy. These fluctuations are because of fluorescent object mobilities, focused in front of a laser. Correlation of such images gained through spectroscopy efficiently measures these fluorescent variations. This facilitates the easy way illustration of mobility and interfacings of labeled molecules in real time. The critical factor for photomarking method is photochemical sensitivity. It involves dequenching of fluorophores, either through photoactivation (Fig. 8) [60] or FP destruction. These two photo-marking modifications in fluorophores lead to the imaging of participant proteins. However, it needs to be emphasized, that the role of large fluorescent tag sizes is critical for passive mode of translocation.

This technique sees the tracking down of unimolecular protein translocations. It harvests the dual only fluorescent behavior (both poor and bright fluorescence in molecules or organelles) and uses automated software to track visualizations in the form of single videos. In the macromolecular structures of filamentous protein aggregates like actins and microtubules, upon fluorescent tagging the whole protein turnover processing is echoed by dynamic translocation of fluorescent patches. QDs were conjugated with EGF that resulted in specific co-localization with ErbB1-GFP chimeric receptor [61]. It was revealed that against activated ErbB1, Cy5-conjugated antibodies determined the activation of the receptor. Colocalization of transferrin labeled with Alexa fluor revealed that the complex internalization was a clathrin dependent phe-

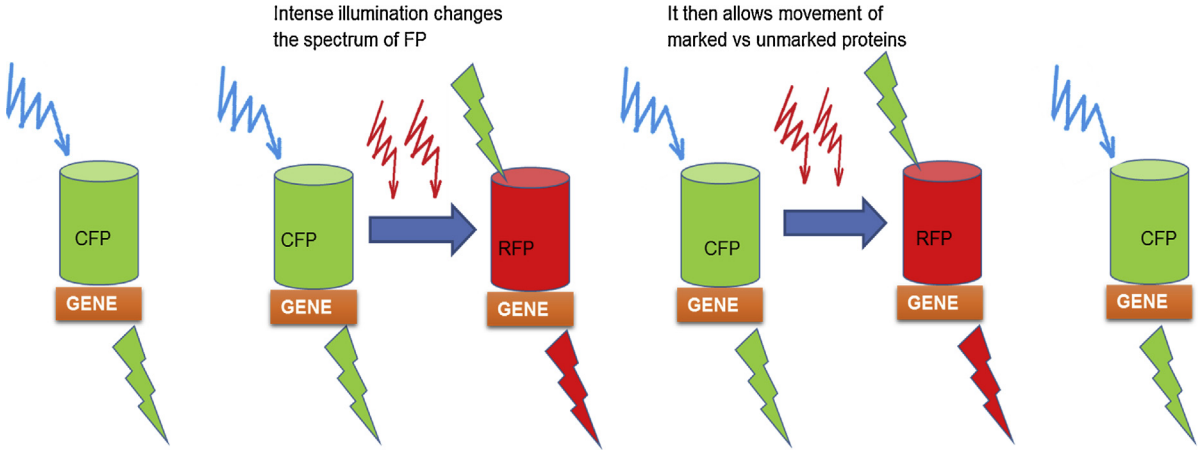

Fig. 8. Showing the process of photoactivation, in which intense illumination causes the quenching of a fluorescent protein, changing its spectrum. This is followed by movement of fluorophores towards each other.

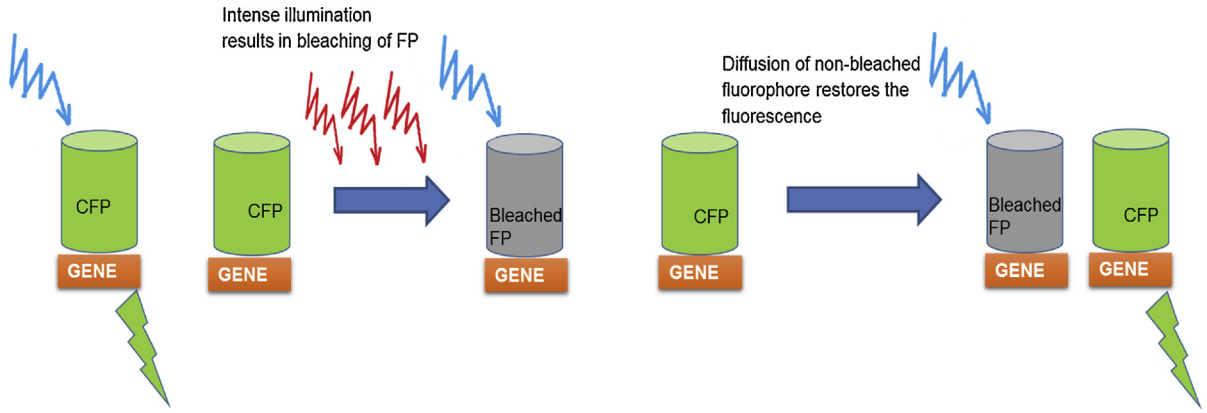

Fig. 9. Depicting the intense illumination causes bleaching of the fluorescent protein in case of FRAP. The fluorescence is however restored back by the diffusion of non-bleached fluorophore CFP.

nomenon. By utilizing, distinctly synthesized EGF-QD, it was found that receptor oligomerization lead to retrograde transportation of receptor bounded single EGF-QD. It was furthermore revealed by the novel phenomenon of fluorescent recovery after photo bleaching (FRAP) that both actin flow and retrograde receptor transport occurred simultaneously (Fig. 9). With the use of biotin Alexa, the specific quenching of EGF-QDs revealed that receptor internalization occurred at cell body and not on filopodia. Henceforth, the involvement of filopodia to transport the activated receptors to cell body is brightly persuasive.

In the latest approaches an RFP based cAMP indicator "Pink Flamindo" applicable in optogenetics and *in vivo* imaging was developed. It was observed that, in presence of a saturating cAMP dose, fluorescence intensity of Pink Flamindo increased 4.2-fold at 567 nm and 590 nm excitation and emission peaks respectively. Recently, a FRET based system for detection of oversulfated chondroitin sulfate was constructed using the energy donor supercharged green fluorescent protein and dye labeled Hep (Hep-RF1) as the energy acceptor. Upon giving heparinase treatment to this sensor system, Hep-RF1 got hydrolysed into minute fragments, thus quenching the FRET signal [62].

5.3. Tracking protein synthesis and yield

It is imperative to gather the important information about protein synthesis and yield, involved in diverse physiological and developmental processes of a cell. However, in case of living cells or tissues, in no way we can adopt the methodologies that incorporate radioactive amino acids into nascent polypeptides. To solve this problem, only genetic tagging offers a great scope. The molecular strategy for tracking down the protein synthesis and yield involves the labeling of proteins with fluorescent probe of one color at initial stage of process up to a time limit. A new fluorescent probe of different color is used for labeling after an adequate time delay. For chimeric proteins, the phenomenon of bleaching and photo activation is explored to make the synthesized proteins get visualized. Varying temperature gradients lead to irreversible misfolding of FP mutants. Some FPs mature and change color spontaneously from green FP to red FP over a period of hours. Commonly, flexible linkers without hydrophobic residues are applied for monitoring the protein yield. Though in some cases, compared to flexible linkers, helix-forming hydrophobic linkers have been found to be better [63], GFP tags are however removed through an enzymatic cleavage site (e.g., enterokinase) already engineered into the linker. Splicing of GFP or linker to N terminus of the target protein is usually preferred if it does not impairs its function and stability. In a novel investigation [64] protein synthesis has been monitored by exploiting fluorescent labeled tRNA FRET pairs in live cells through protein monitoring system (PSM). The synthesis of a viral protein during infectious stage was monitored through PSM using isoleucine tRNA. In a similar fashion, in mouse, synthesis of collagen in fibroblasts was monitored using tRNA-Gly and tRNA-Pro.

In chromophore activated light inactivation (CALI), protein activity is manipulated by immuno-targetting the protein of interest with organic dye (Fig. 10), as observed in malachite green [65]. Different fluorophore targeting has been investigated to overcome

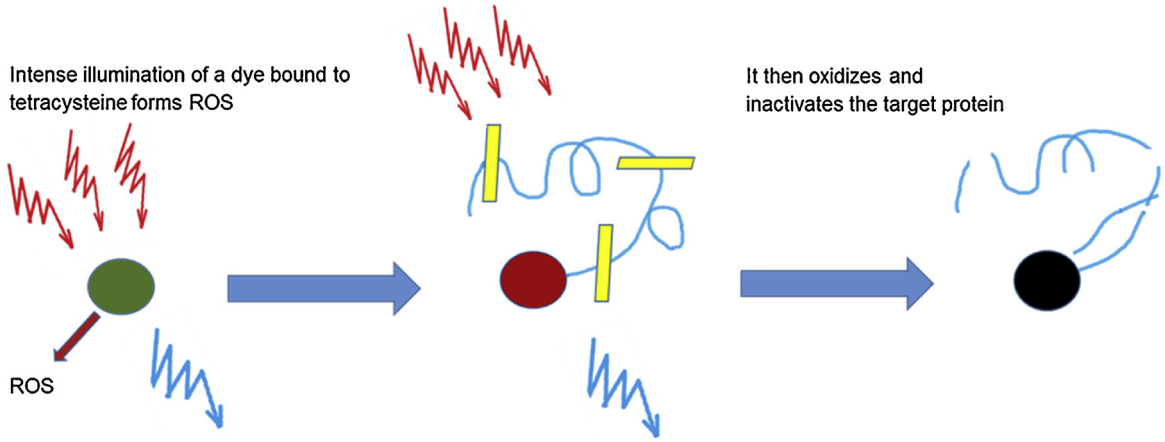

Fig. 10. Chromophore activated light inactivation: As a result of intense illumination of fluorescent dye, the target protein gets oxidized and inactivated due to ROS generation. This results in the loss of its fluorescent property.

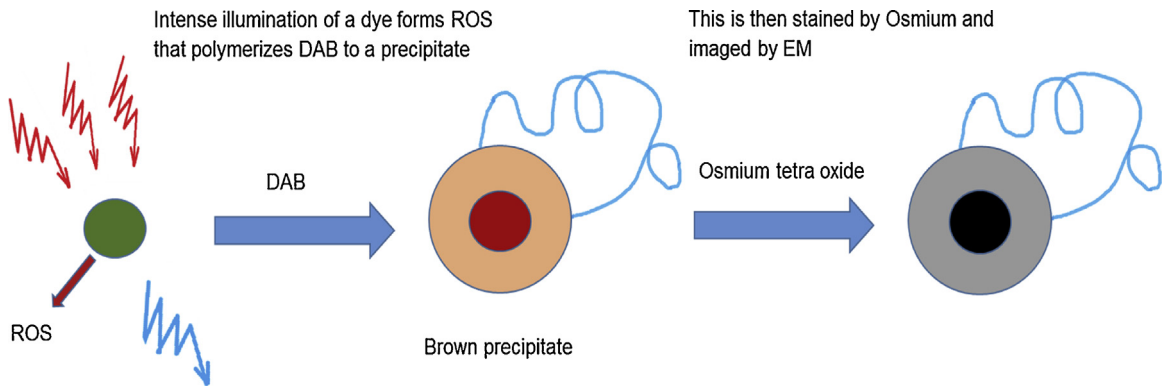

Fig. 11. Photo-oxidation is carried out for electron microscopy. The generation of ROS due to intense illumination causes the precipitation. Staining is carried out by osmium tetraoxide for imaging it on an electron microscope.

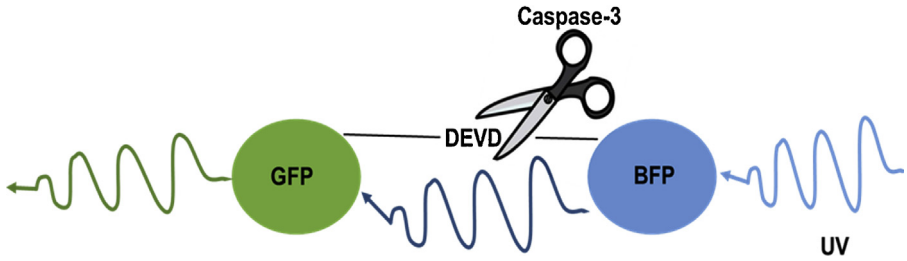

Fig. 12. Illustration of the design of fluorescence resonance energy transfer probe for caspase-3. Caspase cleavage site DEVD is positioned between GFP and BFP.

the post delivery problems of dye conjugated antibodies into living cells. Mostly, the tetracycline bound biarsenical dyes ReAsH [66] and FlAsH [67], genetic tags GFP [68] and other fluorescein conjugates are employed in molecular works to achieve the obliteration of proteins expressed ectopically. Another novel approach to minimize non-specific toxic effects involves shifting towards longer wavelengths, that are least damaging. The obligate dimer is the genetically encoded prototype for CALI known as KillerRed. Though being less efficient than ReAsH, it operates as an effective toolkit against photo-oxidation (Fig. 11) of FPs [69]. FRET process has been further exploited in tune with FlAsH and ReAsH based biarsenical dyes. In this scenario, fluorescence of a donor fluorophore attached to a protein is measured. This is followed by reaction of biarsenical acceptor dye to tetracysteine motif and the quantity of donor quenching is measured [70], providing a direct measurement of FRET efficiency. In membrane receptors, this technique has been applied to draw structural transitions in membrane receptors.

5.4. Visualizing enzymatic catalysis

Many fluorescent imaging techniques have been explored to monitor and visualize activity of proteases, considering their biochemical importance in tumorigenesis, apoptosis, metastasis and inflammatory responses. Usually, the FRET disruption has been done with the use of proteases like subtilisin (Hong and Maret [40]). A comparative analysis of subtilisin treated metallothionein and those of free FPs remarkably abolished transfer of energy from donor FP to acceptor FP. In order to get a valuable understanding of proteolysis w.r.t. different inhibitors, good advances have been made to develop a range of fluorescence based assays. This facilitates us to unravel protease activities working in cells. For example, inhibitors of unrelated serine proteases influence the ruining of GFP based substrates in ubiquitin-protease system [71]. Recently, it has been seen that GFP-reporter mice allows the *in vivo* tracking of proteasome activities, along with the outcome of proteasome inhibitors [72]. Numerous works are also focusing to develop protease inhibiting compounds of life threatening viruses, e.g., hepatitis C virus [73] and human corona virus, responsible for acute respiratory syndrome [74]. In this direction, development of HIV-1 protease inhibitors was a major breakthrough to target viral proteases. Halotag technique has been tailored to study several disease models of bacteriology and virology. For example, Liu et al. studied the membrane topology of glycoprotein-41 of HIV in mammalian cells using two distinct Halotag ligands [75]. In another study [76] involving relative intensity of fluorescence from the Halotag and GFP system it was revealed that Halotag showed superior fluorescence intensity and also functioned better than GFP under acidic conditions.

Similarly, for caspases, which are cysteine proteases involved in apoptosis, numerous FRET based probes are available for monitoring their activity [77]. Because, involvement of apoptosis within disease processing of neurodegenerative disorders [77] and cancer [78], caspases are potential drug targets, considering their critical role as inducers and executors of cell death [79].The first functional caspase FRET (Fig. 12) was developed by fusing BFP, a peptide target sequence for caspase-3 effector and GFP [80]. Due to the caspase-3 activation, as the apoptosis got induced, transfected cells expressing this chimeric protein lost their FRET signal between donor (BFP) and acceptor (GFP). However, as the BFP donor in BFP-GFP FRET pair is excited by ultraviolet light, it resulted in enough cellular damage, preventing its application as a fluorescent toolkit in living cells. This problem has been overcome by generating new spectral variants of FPs.

5.5. Spatiotemporal resolution

In the myriad of cellular and molecular processes, operating over a wide range of distances (1–100 nm), efforts are being done to overcome the microscopic limits (>200 nm) and achieve a definite spatiotemporal resolution. The major advantage of this approach facilitates the dimensional interpretation of motor proteins and other downstream enzymes [81]. In recent approaches, to get a high

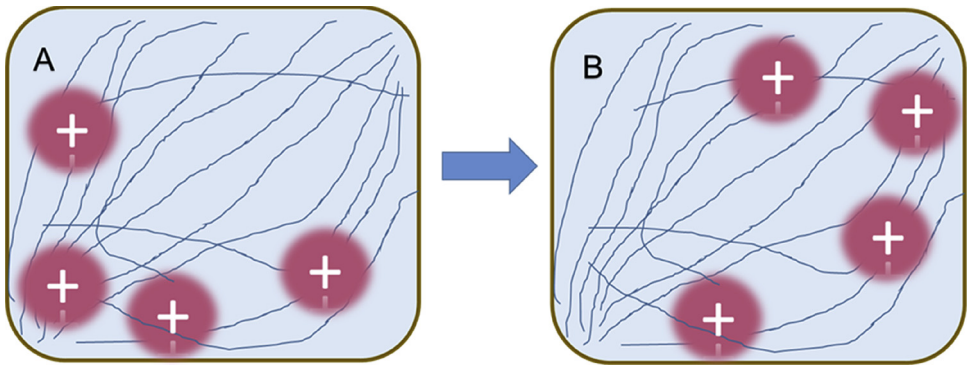

Fig. 13. High precision localization of photo-switchable fluorophores in super-resolution imaging. (**A**) Set of fluorophores is activated to fluorescent state, such that their images (red dots) do not overlap. (**B**) Fluorophores are activated and their positions are determined, and after multiple activation sites, a high-resolution image is constructed (red dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resolution imaging up to 1 mm depths, infrared multiphoton excitation is very advantageous, however, this technique must fulfill the critical condition of collection and harnessing emission photons that have originated from illumination focus. Though tomography is favored for imaging of live tissues yet its major bottleneck is seen in reduced resolution and this problem is solved by serial reconstruction technique utilized to visualize samples through fluorescence imaging at greater depths [82].

Latest developments for better resolutions explain FPs have evolved the photo-activated localization microscopy (PALM). Point spread function (PSF) of a microscope represents the intensity outline of focused emission from a lone protein molecule producing a diffracted spot and a blurred spot. In case visible light and high numerical aperture (NA) lenses are used, this spot size is approximately 200 × 500 nm along lateral and axial dimensions. Since the individual images are merged, there is no distinction of fluorescing protein molecules in the distances smaller than the dimensions of this spot. When there is simultaneous fluorescing of FPs, PALM ensures their separation much greater than this diffraction-limited distance (Fig. 13), thus individually localizing each FP. Applying a low intensity light, in a densely labeled structure there occurs stochastic activation of photo-controllable FPs. Therefore, an appropriate separation distance is achieved between fluorophores [83,84]. These activated molecules are then imaged and localized before being deactivated either through perpetual photo-bleaching or by turning off the fluorescence. However in order to collect such molecular positions in millions before deactivation, this process is repeated many thousand times. This microscopic technique of super-resolution imaging based on single molecule can work with all shades of photo-controllable FPs, whether rsFPs, PAFPs or PSFPs. Practically, in single molecule super-resolution microscopy, two critical factors- the fineness of localization of individual molecules and their density of localization regulate the spatial resolution. The microscopic technique involves photo-manipulation of such protein molecules. This type of imaging technology uses both synthetic dyes [71] as well as photo-controllable FPs and has been applied in total internal reflection [83] and epifluorescence [71,84].

6. Conclusion and future outlook

Fluorescent microscopic imagery is an exquisitely efficient technology that has come to the aid of researchers due to coordinated expansion of targeting strategies, fluorescent probes, highly optimized microscopes and precise data analyzing softwares, which synchronously facilitated live cell and multiprotein imaging, single molecule detection and high throughput screening. Mutagenesis on the other hand expanded the spectral diversity of natural FPs, generating a valuable range of phenotypes, which are very much desirable as non-cytotoxic fluorescent tags. A library of reporters has been developed for different metabolites, biochemical processes and regulatory enzymes, and it marks certainly boundless achievement in systems biology. For visualizing protein of interest through live cell imaging, although genetic tags provide a remarkable advantage, attempts should be made by genetic engineers to explore other fluorescent probes that cause no irritation to the functioning of endogenous proteins. For example, in the technique of immunofluorescence the detection limit is decreased with the application of QDs that are stable and possess increased brightness and this advantage amplifies its prospects for multiprotein detection, correlated EM examinations and other ELISA and Western blot based in vitro assays. However, while designing such prized QDs efficient targeting and better penetration is very crucial to achieve flawless fluorescence visualizations. In recent advancements, because of their efficient photo-physical and biochemical properties photo-controllable FPs has emerged as the key tools for super-resolution microscopy.

Each of three discussed fluorescent probe is equipped with their unique advantages and limitations. With organic dyes, the pulse-labeling procedures are much easier. Secondly, targeting motifs are small and, therefore, a very less opportunity remains for introduced sequences to disrupt gross folding and function of a labeled protein. However, with biarsenical dyes, there are three fundamental problems during labeling; the effect of tag causes disturbed protein localization and function. The background fluorescence level and the cellular toxicity due to ligands further deteriorate the fluorescence intensity measurements and imaging in eukaryotic living cells. The largest problem associated with quantum dots is their toxicity, since these QD nanomaterials are composed of heavy metals, which are potentially toxic during *in vitro* and *in vivo* imaging. QD toxicity depends on different parameters such as charge, size, shape, composition, redox reactivity, surface coating, solubility, photostability and exposure time. On the other hand, whether GFP or its variants are non-toxic to cells but some limitations does exist. First, it is the aggregation of FPs and secondly, exciting GFP for an extended time generates free radicals. It has been further established that GFP variants induce apoptosis, that indicates the possible reason of difficulty in establishing the stable cell lines expressing GFP [85]. Therefore, FRET based nanosensors undergo a healthy expression under *in vivo* environments of prokaryotic model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae,* and animal cell lines. In practice, several fluorescent probe based sensors have been patented for the specific detection of target ligands, drugs or metabolites. From the time of the first report of colorimetric detection of influenza virus through a polydiacetylene (PDA) film, researchers developed a great interest in their application as efficient sensor chips [86]. PDA-based fluorescent

chemo-sensor systems are compatible with microarray technologies. In response to a range of environmental perturbations such as temperature, pH and ligand-receptor interactions, these chemosensors undergo a blue to red visible color change. The important FRET based sensors for apoptosis detection [87] to detect caspase-3, and proteolytic activity of matrix metalloproteinase 9 (MMP-9) with high spatiotemporal resolution [88] are some of the novel sensing systems available to us.

Fluorescent probe based measurements and imaging technology has a vast role to play ahead. In medicine and clinical research, application of fluorescent probes for biopsies and biochemical tests such as assays to analyze profiles of protein activity in patient cells is emerging very fast. Furthermore, fluorescence detection will be applicable to precisely investigate in individual patients the special modulatory effects of drugs on cellular signaling. Thus, fluorescence assays ensure a high-throughput drug screening by means of both functional assays in animal models or live cells and through protein microarrays. To achieve a better spatiotemporal resolution, photo-activated localization microscopy guarantees the individual localization of each FP by means of a low intensity light in a densely labeled structure attaining a suitable separation between photo-controllable FPs, much greater than the diffraction- limited distance. Therefore, in the inquiries of metabolomics, proteomics and systems biology, the sui-generis alliance of high molecular specificity, harmonized and nondestructive compatibility with prokaryotic or eukaryotic organisms and living cells, and certain spatiotemporal resolution based microscopic technology will prevail as pivotal.

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