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An overview of GFP and its application in biology

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Abstract

GFP is a naturally occurring fluorescent protein that absorbs a certain wave-length of light (absorption maxima= 395 and 475nm) and emits green fluorescence (emission maxima= 510nm). Later, similar red, yellow, orange, cyan, blue colored natural as well as laboratory-developed fluorescent proteins were discovered. Due to their fluorescent property these are widely used as bio-marker in diverse field of biological sciences. Green fluorescent protein is coded from a heritable gene known as *gfp*. GFP as well as Aequorin, a blue light emitting protein, were discovered from *Aequorea victoria*. Aequorin is used as calcium probe in modern researches. Activated chromophores which are located in the core of the fluorescent proteins, are responsible for their fluorescence emission. After accumulating several research-data, here, we discuss a precise synopsis on immense potential of GFPs and its variants in the modern sciences such as cancer biology, drug discovery etc. also including the historical background and their structural and chemical aspects.

Key words: GFP, *Aequorea Victoria*, chromophore, fluorescence, bioluminescence.

1. Introduction

Green fluorescent protein, discovered from *Aequorea Victoria*, is a barrel shaped protein containing a centrally-located, stable chromophore which is spontaneously synthesized without any aids from cofactors, accessory proteins or enzymes thus making it an excellent biological marker to study any living systems. GFP and its genetically-engineered enhanced blue, cyan, yellow variants as well as later-discovered other fluorescent proteins from different animals have become an essential tool in the studies of Molecular, medicinal, cellular and developmental biology.

Professor Yoshimasa Hirata, Osamu Shimomura became interested in the study of bioluminescence of *Cypridina*. After successfully separating the protein luciferin through crystallization from *Cypridina*, Shimomura gathered all the knowledge and experience to

proceed in the research on *Aequorea victoria* which led to his successful discovery of Green Fluorescent Protein (GFP) while working with other renowned scientists in America.

Osamu Shimomura, Roger Tsien and Marty Chalfie in 2008 won the Noble Prize in chemistry for their discovery and work on GFP (Kumar *et al*., 2016). In the scientific field of GFP studies the researches of Douglas Prasher and Sergey A. Lukyanov also provided an enormous contribution.

Osamu Shimomura (1962) first isolated GFP from the luminous organ of the jellyfish *Aequorea Victoria* and also identified the portion of GFP that produce fluorescence. Douglas Prasher (1987) invented the use of GFP as tracer molecule and reporter molecule. Gene for GFP was also cloned by him. After acquiring the GFP clone from Prasher, Marty Chalfie (1993, 1994) demonstrated the expression of the fluorescence of GFP in *Escherichia coli* and *Caenorhabditis elegans* without adding auxiliary heterologous factors, thus, pioneering the potential of GFP as a universal genetic marker. Sergey A. Lukyanov (1999, 2000) and his colleagues found a type red fluorescent protein named DsRed from *Discosoma* coral. Roger Tsien (1999, 2008) and his group was able to develop new variants and mutants of GFP with improved overall functionality.

Here, we have accumulated current information and data by studying progression of scientific works on GFP. Thus, finding the necessary details and gap areas, researchers can conduct future researches for new discoveries.

2. Phylogeny

According to the data recorded till 2008, fluorescent proteins are expressed in two different classes of phylum Cnidaria i.e., Anthozoa and Hydrozoa. Actiniaria, Scleractinia, Pennatulacea and Ceriantharia these four orders of class Anthozoa possess some forms of FP. Scleractinia comprises of clade B, C (C1, C2 and C3) and D. Actiniaria is denoted as clade A (Figure-1) (Alieva *et al.*, 2008).

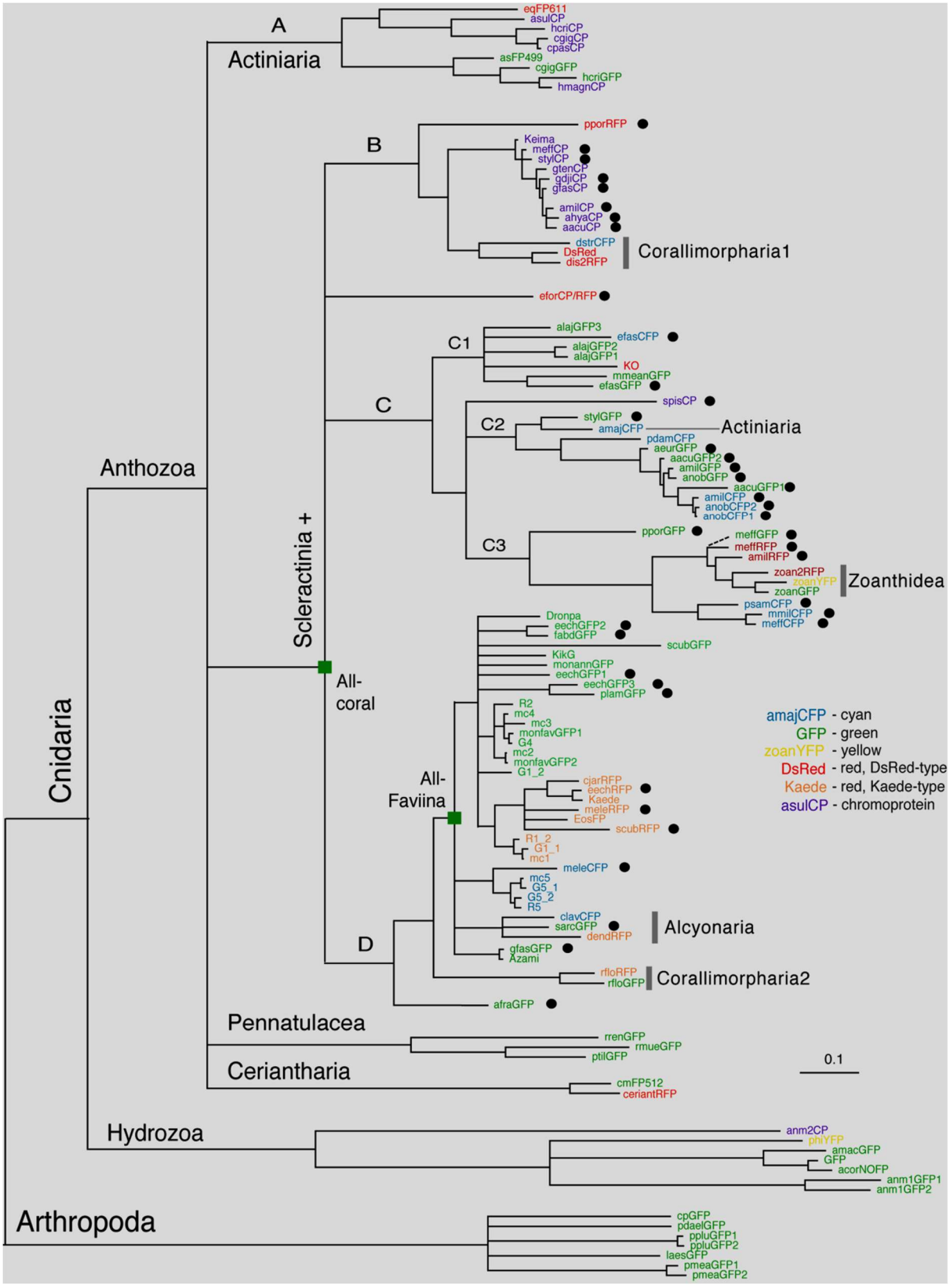

Fig. 1. Fluorescent proteins of the members of phylum Cnidaria are manifested in the form of Bayesian phylogenetic tree; FPs found in arthropod members are depicted as outgroup. Proteins that are first described by Alieva *et al.* are designated with black dots (Alieva *et al.*, 2008).

3. Natural bioluminescence through GFP in *Aequorea victoria*

According to Davenport and Nicol (1955), the bioluminescent jellyfish *Aequorea victoria* produces green fluorescence via chemical reactions which stimulate photon emission by producing enough energy. After completing his research on the bioluminescence of *Cypridina*, a small ostracod, Osamu Shimomura came to Princeton, USA to work on the molecular mechanism of the bioluminescence of *Aequorea victoria* jellyfish which were first collected from Friday Harbor of Washington State on the coast of the Pacific Ocean (Shimomura, 2005). Ca^{2+} dependent blue light emitting aequorin protein was discovered as the working component of the bioluminescence of *Aequorea* (Shimomura *et al.*, 1962). Photogenic cells of *Aequorea* consist of green fluorescent protein (GFP) and aequorin which act as acceptor and donor respectively in a FRET (Fluorescence resonance energy transfer) reaction. GFP's electronic dipole gets excited when the energy is transferred non-radiatively from chemically excited electronic dipole of aequorin. When returning to ground state GFP emit photons in the form of green light (Morin and Hastings, 1971). The peak of emission spectrum of GFP is about 510 nm (λ_{emmax} =508-515 nm). λ_{max} of the blue colored bioluminescence of aequorin is 470 nm which is almost similar to the peak value of wavelength (λ_{exmax}=460 nm) within GFP's excitation spectrum (Morise *et al.*, 1974 and Cormier *et al.*, 1973).

4. Structure

Wild type GFP is a 27 KDa molecular weight cylindrical protein with 30 Å diameters and 40 Å length containing 238 amino acids. With the application of 1.9 Å resolutions, the phase estimating multi-wavelength anomalous diffraction method (MAD method) was used to explain the crystal structure of recombinant wild-type green fluorescent protein by Yang *et al.* (1996) and Ormo *et al.* (1996). Crystal tertiary structure of wild type GFP consists of eleven-stranded β sheets forming a barrel shaped structure with α-helix occupying apex and bottom portion of the barrel. While continuing through the hollow of the barrel, α-helix threads also house centrally located chromophore (Kumar and Pal, 2016) (Figure-2). "Beta can" is a new protein class which has been substantiated by GFP (Hraška *et al.*, 2006).

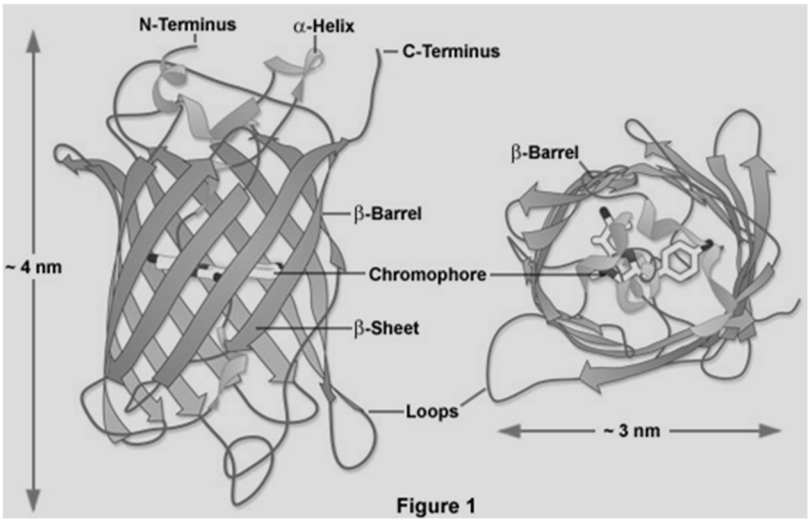

Figure 1

Fig. 2. 3D structure of GFP (image source: http://zeiss-campus.magnet.fsu.edu/articles/probes/jellyfishfps.html, accessed: 14 May 2021)

Functional chromophore formation

Ser65-Tyr66-Gly67 tripeptide forms the GFP chromophore i.e., p-hydroxybenzylideneimidazolinone (Figure-3) through folding, cyclization, dehydration and aerial oxidation reactions (Kumar and Pal, 2016; Chalfie, 1995).

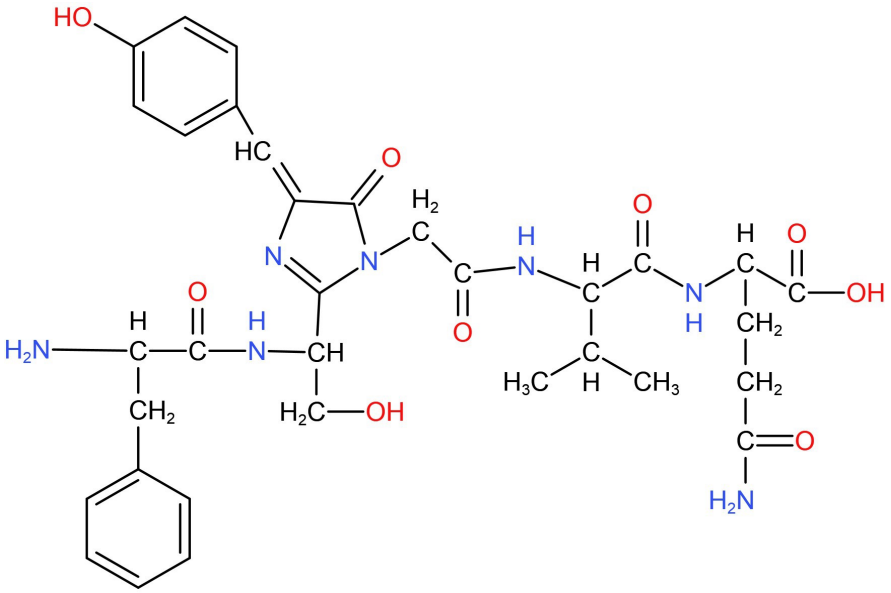

Fig. 3. Chemical structure of GFP chromophore.

When GFP retains the primary structure within its unfurled condition, the tripeptide chromophore content does not exhibit any natural fluorescence property (Figure-4, uppermost). When GFP starts to fold itself shaping up to its native form and those 3 amino acids experience a keen twist at the same time in the process, then the amide group of Gly67 is induced to initiate nucleophilic attack on the carbonyl group of Ser65 (Figure-4, middle-left). Through the process of cyclization (Figure-4, middle-right) and dehydration, the aforementioned changes and reactions ultimately result in the creation of imidazolinone (Figure-4, bottom-left). Although fluorescence still does not appear at this stage. But when molecular oxygen is available, α- β bond of Tyr66 gets dehydrogenated. This ultimately leads to its conjugation with previously formed imidazolinone. Thus, chromophore reaches its final form (Figure-4, bottom-right) that is capable of GFP fluorescence emission (Heim *et al.*, 1994).

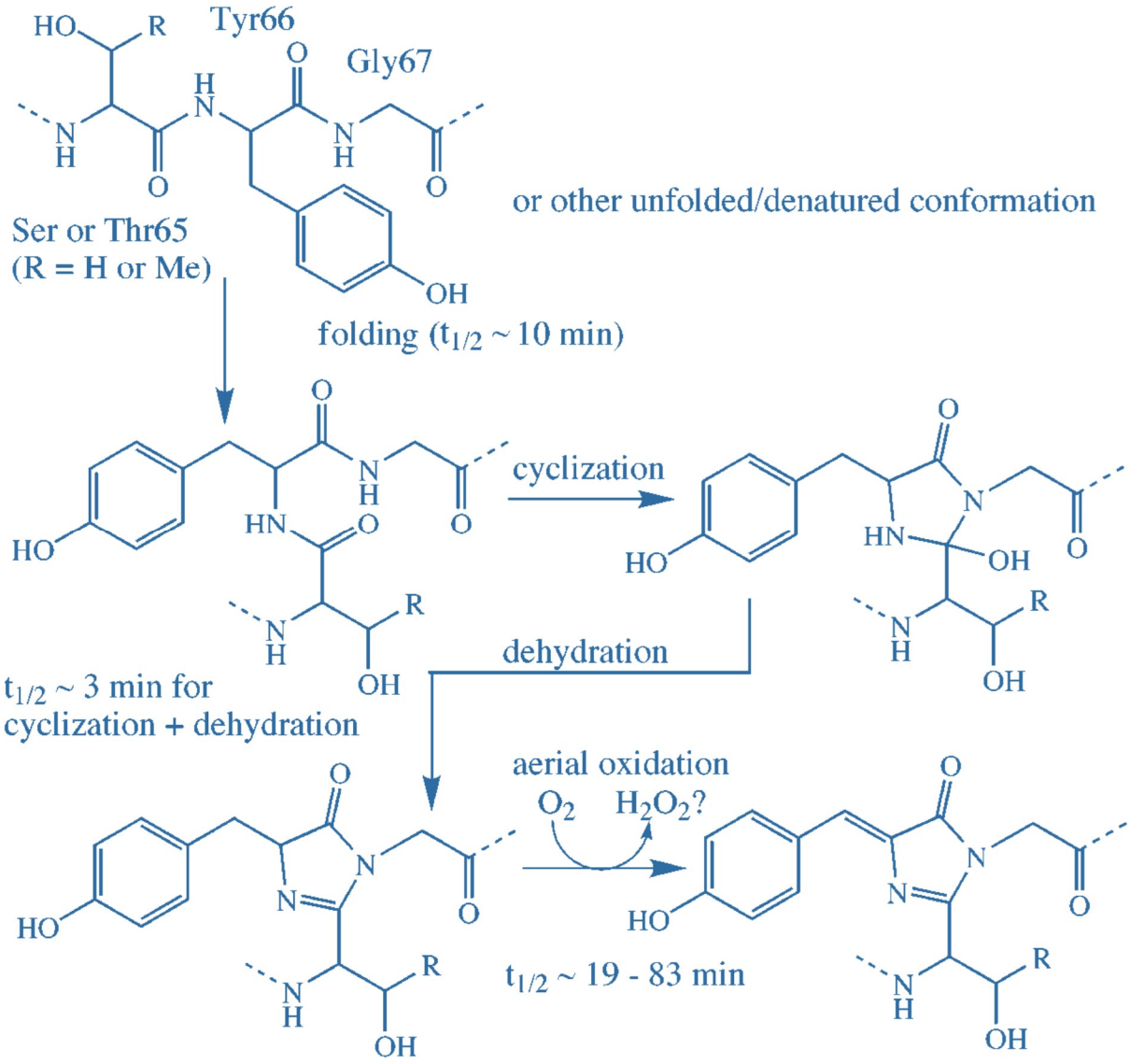

Fig. 4. Consecutive steps of active GFP chromophore formation (Tsien, 1998).

5. Characters

Two peaks at 395nm and 475nm are shown in the absorbance-excitation curve by wild-type GFP with extinction-coefficient of 30,000 and 7,000.m/cm respectively. GFP is denaturation resistant. It is well-stabilized against proteases, pH change (wide range of <4 or >12.0) and also remains intact up to 65ºC (Kumar and Pal, 2016). As the process of chromophore production and activation takes place via autocatalytic pathways in the presence of components that are abundant in cellular environment, neither any extra internal substrates and cofactors nor any external substances are needed for the functional fluorescence emission of GFP (Misteli and Spector 1997; Hraška *et al.*, 2006; Heim *et al.* 1994). Various powerful denaturing agents such as 6 M guanidine HCL, 1% sodium dodecyl sulphate, 8 M urea etc. are proved to be incapable of causing any effect against the active fluorescence of GFP (Yang *et al.* 1996; Hraška *et al.*, 2006).

6. Naturally occurring variations of GFP-like proteins

The chromophore of wild type GFP (from *Aequorea victoria*) generally exists in the equilibrium state between neutral/protonated and anionic/deprotonated conditions (Ward and Bokman, 1982) (Figure-5).

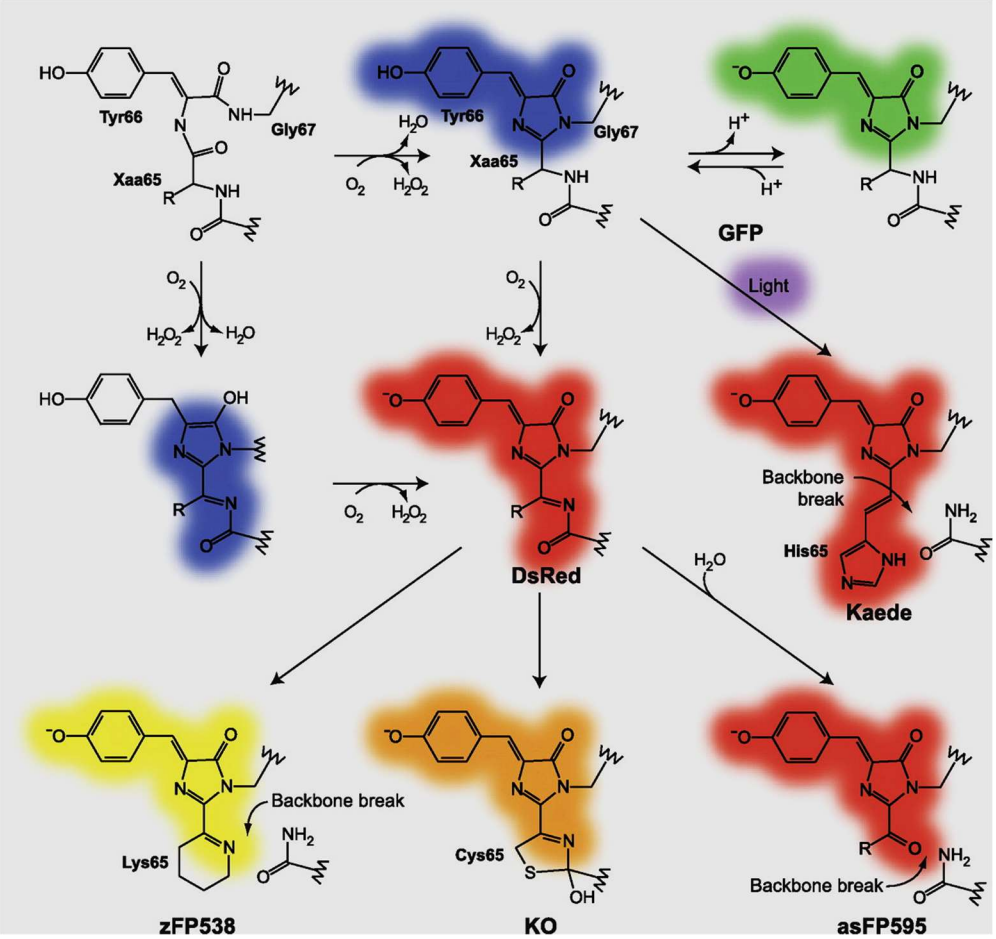

Fig. 5. Structures and maturation process of the chromophores of GFP and GFP-like proteins (Chudakov *et al.*, 2010**).**

PS-CFP, containing protonated GFP-like chromophore, shows absorption at ~400nm and blue-colored emission at about 460nm (Chudakov *et al.*, 2004).

Generally, a process known as excited state proton transfer (ESPT) occurs when GFP-like chromophores are in its protonated form. Green fluorescence emission is generally achieved through this process by being charged via the loss of single proton right after excitation and giving out the absorbed energy (Chudakov *et al.*, 2010).

The popular EGFP, containing anionic chromophore, shows absorption and green light emission at ~480nm and ~510nm respectively (Cormack *et al.*, 1996).

Molecular oxygen mediated additional oxidation of Cα-N bond of 65th residue leads to DsRed chromophore (anionic) formation. Conjugated π-system enlarging acylimine group's formation, as a result of the process of oxidation, causes bathochromic shift in

excitation and emission spectra (Strack *et al.*, 2010) (Figure-6). DsRed-like chromophores show peak of excitation at ~560-580nm and that of emission at ~570-610nm. Naturally occurring DsRed-like proteins sometimes show fluorescence color transition from green to red over time ("timer" phenotype) during chromophore biosynthesis (Labas *et al.*, 2002). Exposure to violet-light radiation seems to enhance the DsRed maturation process (Verkhusha *et al.*, 2004). DsRed chromophore exhibits cis planar conformation (Chudakov *et al.*, 2010).

Anemonia sulcata has asFP595, a purple-colored chromoprotein that contains a similar core as GFP's but carries an extension having keto-group. The backbone of the protein is also cleaved just before the chromophore's structure. These structural modifications are most likely to occur due to hydrolysis of acylimine resulting in a DsRed-like intermediate formation. This non-fluorescent protein's chromophore shows trans non-planar conformation. It shows the peak of absorption at 560-570nm and then exhibits a "kindling" transition to a form which has lasting fluorescence with emission maximum at 595nm (Quillin *et al.*, 2005; Chudakov *et al.*, 2010).

The Lys65 side chain takes part in the formation of a third heterocyclic ring containing six members in the chromophore of yellow-colored fluorescent protein zFP538, found in *Zoanthus* sp. (button polyp; "z" comes from first letter of *Zoanthus* sp.), thus resulting an extension of the GFP-like core via an extra conjugated C=N bonding. Protein backbone breakage occurs when terminal amino group of Lys65 attacks a temporary acylimine as a form of transimination reaction resulting in the aforementioned structural peculiarity. Fluorescence-emitting zFP538 chromophore shows cis planar conformation and excitation-emission maxima at 527nm and 538nm, respectively (Gurskaya *et al.*, 2001; Pletneva *et al.*, 2007; Remington *et al.*, 2005; Chudakov *et al.*, 2010).

Cyclic thioamide ring, containing five members, formed from Cys65, found in *Fungia concinna* (stony coral), involves in the extension of GFP-like core portion of Kusabira-Orange's chromophore which emits orange fluorescence and shows cis planar configuration. Kusabira-Orange (KO) chromophore exhibits excitation peak at 548nm and emission peak at 559nm (Karasawa *et al.*, 2004; Kikuchi *et al.*, 2008; Chudakov *et al.*, 2010).

Cis planar chromophore of Kaede protein, containing His65, matures to obtain GFP-like conformation that maintains equilibrium between neutral and anionic states in the dark. But during exposure to ultra-violet radiation, in the structure of His65, it has been observed that the backbone of protein breaks betwixt the amide N and the alpha carbon, and also a double bond forms within alpha and beta carbon. These steps ultimately lead to protonated chromophore's conversion and red fluorescence emission (Mizuno *et al.*, 2003; Chudakov *et al.*, 2010).

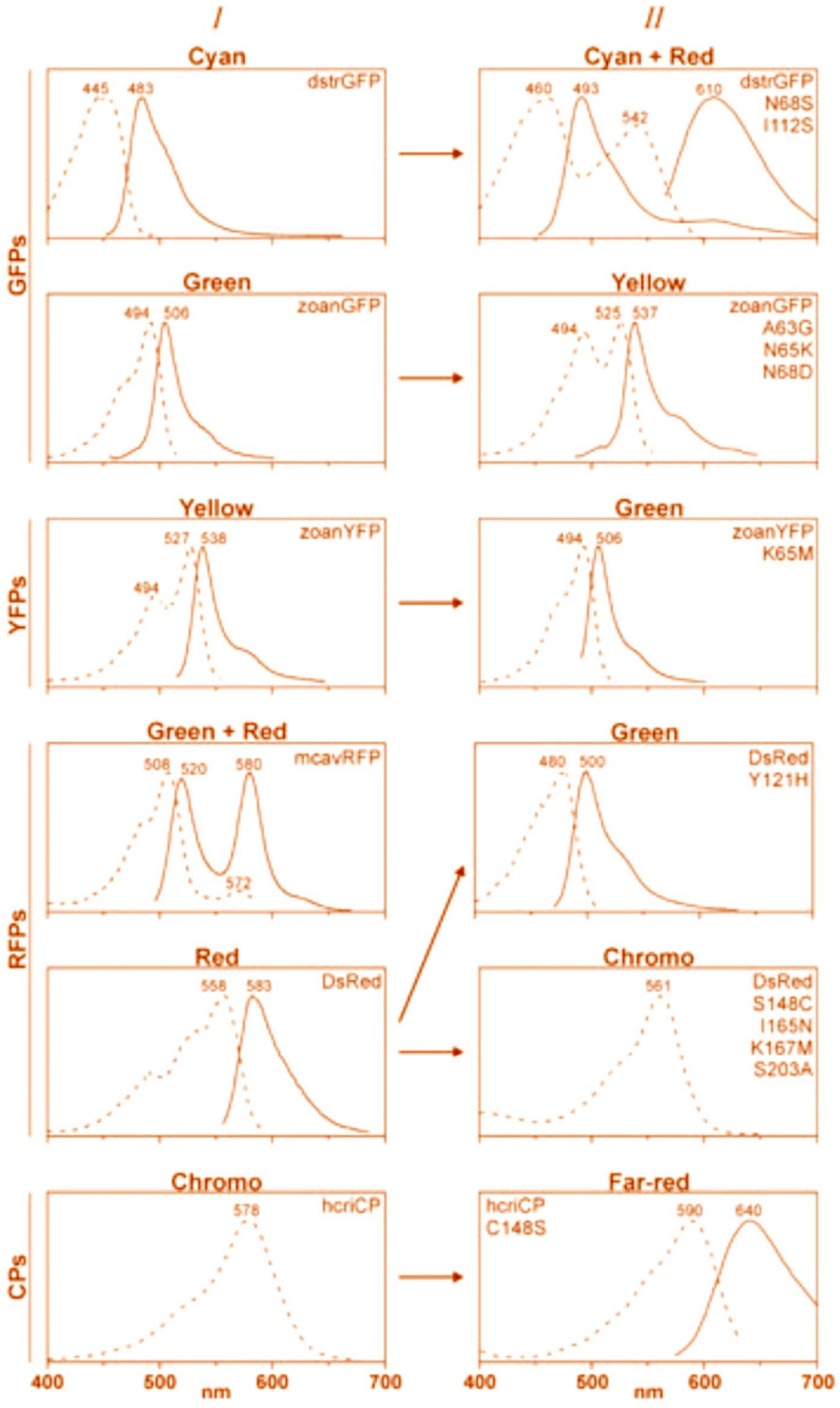

Fig. 6. Absorption and emission spectrum of GFPs, RFPs, YFPs, CPs. Column-I shows the spectra of original/wild-types and column-II shows the spectra of mutants. Solid lines indicate emission spectra whereas dotted lines indicate absorption spectra (Stepanenko *et al.*, 2008)**.**

7. Functions

Generally, *gfp* gene is incorporated with the gene of target protein of the experimented organism which can keep up the normal protein-activity unchanged while also keeping the fluorescence of GFP simultaneously. Thus, monitoring the GFP-fluorescence, several activities of the desired protein can be visualized. GFP has diverse applications in modern biological sciences which include:

1. detection of gene manifestation in different types of cells when it is used as reporter gene, 2. detection of localization and observing the destiny of a protein when GFP is applied genetically as a fusion tag with that protein which is working as host, 3. tracking and examining different organelles and any environment that surrounds GFP when GFP acts as an active indicator, 4. use in quantum-mechanical phenomenon known as Fluorescence Resonance Energy Transfer (FRET), 5. use in high throughput screening (HTS) where GFP functions as a reporter, 6. observing the expression of gene in antimycobacterial agents such as *Mycobacterium smegmatis* and *M. bovis* BCG when GFP acts as a marker, 7. use in high-content screening (HCS) technique that utilizes the full potential of GFP in the field of drug discovery etc. (Kumar and Pal, 2016; Cubitt *et al.*,1995) (Figure- 7).

By using green fluorescent protein-based biosensors we can keep track of several significant biological events such as incorporation of the receptors that are present on cell surface, trafficking of transcription factors (TF), protein kinase translocation and subsequent progressive changes and arrangements of organelles as well as cytoskeletons during cell division (Kumar and Pal, 2016). GFP has been applied in the modern-day researches regarding the study within a vast array of organisms such as plants, yeasts, bacteria, nematodes, insects, fish and mammals (Hraška *et al.*, 2006). Developing embryo and fetus cells can also be studied with the help of GFP.

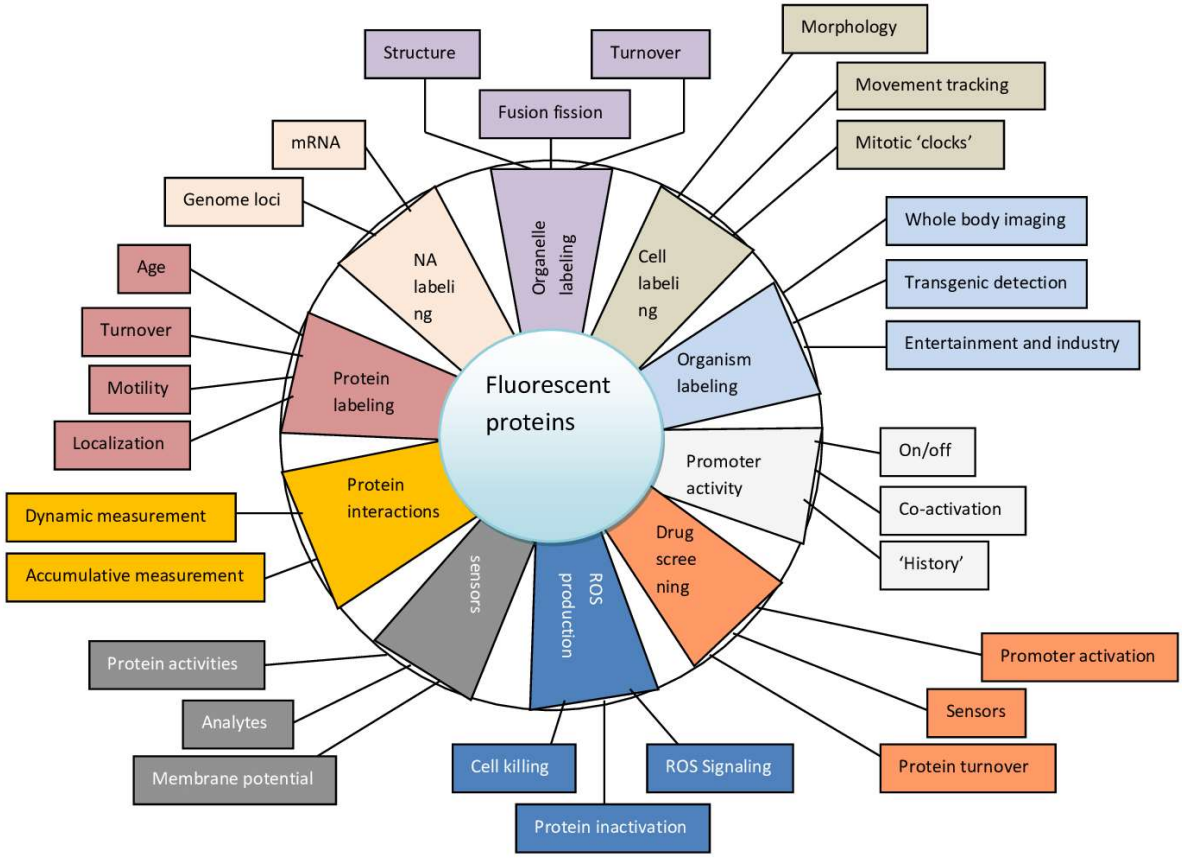

Fig. 7. Schematic representation of multifarious functions of GFP.

When examined in the presence of simple UV light source, gene expression of an entire living plant body can be visualized via luminous characteristics of GFPs. By applying GFP in the plant cells, fluorescence microscopy is capable of revealing numerous accurate and detailed living cell activities that we can easily observe without affecting these cells and their components (Haseloff, 1999). Several researches and reports till date suggest that application of GFP in the plant body generally does not have any harmful effect on growth related physiological changes, developmental phases and their fertility (Hraška *et al.*, 2006). However floral pigments can sometime inhibit us from visualizing the green fluorescence emission as shown by Mercuri *et al.* (2001) in the case of transgenic *Limonium* flower which has various floral pigments blocking the sight of macroscopic fluorescence emission of GFP. Mature leaf-cell vacuoles without any GFP content take up the maximum space in them; thus, alteration in cytoplasmic density leads to worse expression of GFP in case of older leaves and their cells than the younger ones (Hraška *et al.*, 2006; Maximova *et al.*, 1998; Cho and Widholm, 2002; Molinier *et al.*, 2000). Although seldom fluorescence takes place in specific regions of mature leaves such as trichomes and stomatal guard cells (Hraška *et al.*, 2006). Different kinds of spare unwanted signal emissions along with the GFP signal emission also create a negative impact on GFP fluorescence (Hraška *et al.*, 2006). Expression of GFP mostly takes place in the tissues of plant like roots where albinism occurs in the absence of chlorophyll as green fluorescence expressed by GFP is hampered by the red autofluorescence of chlorophyll (Cho *et al.*, 2000; Hraška *et al.*, 2006; Huber *et al.*, 2002; Carlson *et al.*, 2001).

35S promoter manifestation in developmental or cell specific situations, gene silencing and occurrence of diluted GFP during cell growth and division are other three plausible causes of obstruction against proper GFP expression (Zhou *et al*., 2004; Ponappa *et al.*, 1999; Hraška *et al.*, 2006; Voinnet and Baulcombe, 1997).

Application of different molecular weight GFPs that are soluble in *Arabidopsis* reveal the stages from embryogenesis to postgermination, the gradual increase in density of plasmodesmata tissues from young to more mature stages, the intercellular macromolecular transport pattern through plasmodesmata in undifferentiated tissues, the symplastic pathway in different areas of maturing leaves (Kim *et al*., 2005).

Successful introduction of wild type GFP into the protoplasts of *Citrus sinensis* was first accomplished by Niedz *et al.* (1995). Thus they pioneered the application of GFP in the field of plant transformation science. In case of development of transgenic plants GFP fluorescence helps in differentiation of transformed and non-transformed cells without invading them, thus helping in the removal of non-transformed cells and sprouts before advancing in further cultivation. Various parameters of transformation can be estimated via GFP fluorescence, thus letting us understand how and what to modify in successive procedures of transformation. It assists in the detection and minimization of negative incidents of plant transformation in a more efficient way. The quantitative analysis of the green fluorescence leads to detection of homozygotes in advance and evaluation of the amount of recombinant protein. Successful GFP applied experiment on Carrot transformation mediated by *Agrobacterium rhizogenes* and recognition of different intensity of GFP allowed Baranski *et al.* (2006) to sort out *Agrobacterium* strain that is the most virulent, accurately potent concentration of acetosyringone and the most capable and competent genotype of carrot that is to be transformed. The tardy introduction of these bacterial strains in the plant-body creates effect on the adventitious roots production quantity as also evaluated by them. Along with wild type there are several variants of GFPs that serve several purposes in the study and researches regarding plant transformation technology. Some of the significant examples of these variants are mGFP4, mGFP5, sGFP, SGFP S65T, EGFP, mut3GFP, smGFP, smRS-GFP, smBFP, synGFP, BFP, mYFP, mCFP etc (Hraška *et al.*, 2006).

GFP helps in distinguishing living embryos and larvae with balancer chromosomes from the ones without them as well as revealing the gradual structural changes and positioning of yolks during embryogenesis of *Drosophila melanogaster* (Casso *et al*., 1999).

Japanese flounder tumor necrosis factor (TNF) and GFP containing Promoter region of 2381bp resided within a recombinant plasmid was microinjected into fertilized eggs of zebrafish by Yazawa *et al*. Transgenic zebrafish lines represented as TNF-GFP15 and TNF-GFP4 showed TNF promoter induced GFP expression in heart and surrounding regions of the pharynx during unstressed state and after 72 hours of fertilization. On the other hand, GFP was expressed throughout the surface during initial developmental stages of zebrafish embryo and also when lipopolysaccharide (LPS) was introduced to the embryo. Combined effect of concanavalin A and phorbol myristate acetate triggered GFP expression in transgenic embryos similarly as LPS. On the other hand, it was observed that Concanavalin A was not

capable of GFP expression induction at any concentration but only elevated level of phorbol myristate acetate concentration (300 µg/µl) was able to induce a small amount of GFP expression. Bacteria infected disease detection and study in live fish can be made possible via using GFP and Japanese flounder tumor necrosis factor (TNF) together. These scientific studies suggest that the influence of Japanese flounder tumor necrosis factor (TNF) is possibly capable of producing disease-resistant transgenic variants which may express antiviral or antibacterial peptides during pathogen-infection (Yazawa *et al*., 2005).

The dependency of the strength of spindle assembly checkpoint (SAC) during mitotic cell division has been successfully experimented and determined using GFP with Tubulin, $Mad1^{MDF-1}$, $Mad2^{MDF-2}$, $Apc1^{MAT-2}$ and PH (GFP-PH marker) at several embryonic developmental stages of *Caenorhabditis elegans* (Galli and Morgan, 2016).

Mouse embryonic germ (EG) cells containing Enhanced GFP marker genes, unveil differentiation and "developmental fate" of the EG4-GFP cells in chimeric embryos. EG4-GFP cells also participate in development of host embryo (Xin *et al*., 1999).

In the host embryo of *Sander lucioperca*, Gonad facing movement of donor-embryo's primordial germ cells can be detected via injecting synthesized GFP within 3'UTR of m-RNA of *nanos3*. Thus, the results of Güralp *et al*. explain the effective use of blastomere transplantation and potential creation of "germline chimeras" in percidae (Güralp *et al*., 2017).

Scientists have made it possible to develop a special type of detectable fluorescence emitting chimeric protein with combination of GFP and spike (S) protein of corona virus by recombining enhanced S-GFP gene, in the place of wild-type S gene into the mouse hepatitis corona virus (MHV) genome. Similarly, a type of easily detectable fluorescence emitting recombinant vesicular stomatitis virus (VSV) has been developed by using both the wild type VSV G (glycoprotein) gene and G/GFP gene which produce trimer mixtures comprising of G/GFP fusion protein and wild type G protein complexes in the virus. With the help of Time-lapsed imaging fluorescence microscopy technique we can detect the pathway of viral infection by investigating the fluorescence producing variants of that virus. Incorporation of external foreign proteins with the structural proteins has given rise to recombinant viruses which can specifically perform certain biological activities at a time. These activities may include bringing enzymatic activities into cells or having the ability to target certain type of cells (targeting function) (Bosch *et al.*, 2004).

Green, red, orange, yellow and blue colored fluorescence emitting variants of fluorescent proteins have proven its immense significance in the field of cancer biology and drug discovery. In the cancer biology of living organism Distinction of tumor cells, live monitoring of the process of mitosis, apoptosis, metastasis, angiogenesis, observation of different abnormal stages of cell cycle all have been made possible by GFP and its variants (Hoffman, 2015).

Behavioral comparison can be examined at the same time between GFP labeled cancerous stem cells and RFP labeled non-stem cells (Hoffman, 2015). Application of GFP

helped in better understanding of extravasational and intravasational activities in addition to chemokine activity related polarity of cancerous cells (Farina *et al.*, 1998; Condeelis and Segall, 2003). By applying dual-color fluorescence imaging technique, angiogenesis was observed through examining the expression of NDGFP (nestin drives GFP) in nascent blood vessels and endothelial cells in RFP labeled growing tumor. FUCCI (fluorescence ubiquitious cell cycle indicator) 3D confocal imaging unveiled the cessation of S-, G2- and M-phase cancer cells having green FP expressing nuclei and migration initiation of G0-, G1-phase cancer cells containing red FP labeled nuclei (Sakaue-Sawano *et al.*, 2008; Yano *et al.*, 2014). Migration and trafficking of cancerous cells through blood-vessel as well as mammary and lung cancer cells' interactions with blood-vessel wall were observed by studying GFP expression via a technique known as skin window chambers in rodents (Chishima *et al.,* 1997; Huang *et al.*, 2002; Li *et al.*, 2000). Contact of colon cancer cells with vessel-lumen was also observed via GFP-labeling (Hoffman, 2015). Route of metastasis, starting from orthotopic position towards liver and lung, along with the proliferation process happening there were clearly identified by studying fluorescence emission of GFP-infected PC-3 human prostate adenocarcinoma cells (Yang *et al.*, 1999).

Studying GFP's expression from GFP-applied MDA-MB-435 human breast cancer (BC) cells provided insight into osteolytic metastasis of those cells in bone (Harms and Welch, 2003). Brain metastasis of 4T1-GFP or MDA231BRGFP BC cells was also examined in BALB/c or SCID rodents. Role of interactions of ALCAM/ALCAM and VLA-4/VCAM-1 during the initial metastasis and the beginning of tumor formation within brain has proven important. Reduction of this tumor seeding was done by applying antibody neutralization on ALCAM or VLA-4 (Soto *et al.*, 2014). Prevention of brain metastases of GFP expressed CN34-BrM2 and MDA-MB231BR breast cancer cell lines were made successful via the inhibition of mTOR by rapamycin and Temsirolimus-CCI-779 (Zhao *et al.*, 2012). Spantide III, an SP (substance P) inhibitor, proved its crucial role in the inhibition of BC cell mediated brain tumor formation and blood-brain-barrier breaching (Rodriguez *et al.*, 2014). Multi-kinase inhibitors such as sunitinib and dasatinib have potentiality to inhibit brain metastasis, as shown in the case of human 231BR cancer cells via GFP fluorescence (Zhao *et al.*, 2013). SU (sunitinib) enhanced METG (metronomic dose schedule) based GEM (Gemcitabine) treatment brought more success in metastasis suppression of MiaPaCa-2 RFP pancreatic cancer cells than SU-absent MTDG (maximum tolerated dose) schedule-based treatment (Katz *et al.*, 2003). Entire-body GFP imaging helped to recognize the effectiveness of angiostatin (angiogenesis inhibitor) in the inhibition of osteoclastic activity and ultimately in the prevention of tumor-growth in bone (Peyruchaud *et al.*, 2003). Temozolomide treated cell-apoptosis was examined in real-time by using craniotomy open window imaging which revealed expression of GFP from apoptotic bodies and RFP-expression from destroyed cytoplasm (Momiyama *et al.*, 2013). Use of GFP-imaging and radiography techniques showed the immense potentiality of bisphosphonate, 3, 3-dimethylaminopropane-1-hydroxy-1, 1-diphosphonic acid (olpadronate) in tumor reduction (Hoffman, 2015). Careful observation of GFP's expression in the MTLn3 and MTC cells obtained from the rat 13762NF mammary cancer cell line, revealed that metastatic MTLn3 cells shows better affinity towards blood vessels, whereas, while making contact with blood-vessel, non-

metastatic MTC cells lose its cytoplasmic content due to shearing of pseudo-pod which interacts with blood-flow (Condeelis and Segall, 2003).

8. Conclusion

GFP has proven its necessity as reporter molecule in the field of biology of plants and animals. Several aspects such as the invasion pathways and receptor-cellular interactions of infectious disease-causing pathogens, retaining the capability to create a pandemic, as we all have recently gone thorough in the case of coronavirus, create extensive possibilities for the usage of GFP. Cancer treatment and diagnosis can be more efficient, effective and simplified through applying GFP in cellular level and observing its expression in nuclear-cytoplasmic dynamics, cell cycle, mitosis, apoptosis, metastasis, angiogenesis, motility and invasion. Although, recent researches have shown that in vivo application of GFP can lead to immunogenicity and cytotoxicity. It has been demonstrated that life span of a cell decreases when it is tagged with GFP. Thus, GFP expression cannot be traced in a consistent manner over a certain period of time. In spite of having such aforementioned limitations GFP's modern application in complex brain study, intracellular signaling and cellular responses have widened a greater horizon in the field of research. A clear idea is established here unveiling the marvelous progression in the scientific-studies and new discoveries of different aspects related to GFPs and its diverse applications. In the field of developmental biology, GFP's immense potential has been proven by several researchers although vast areas are yet to be discovered.

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