TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

KUMARAGURUBARAN KUMAR

pH Sensor Using Superfolder Green Fluorescent Protein for Intracellular Studies

MASTER OF SCIENCE THESIS

Subject approved by Department Council
9th Jan 2013

Examiners: Professor Matti Karp
Bobin George Abraham

ABSTRACT

The aim of this study is to create novel pH reporting probe for use in intracellular studies of thermophiles using superfolder green fluorescent protein. The strains were screened based on ratiometric spectral characteristics. For comparison of results, we have used deGFP mutant which is the best fluorescent protein based ratiometric probe currently available. The work was conducted in different steps including site directed mutagenesis, transformation and cloning, protein expression and spectral measurements. Mutations were created using overlap extension PCR method. We have demonstrated the influence of mutations T203C, H148G and C48S on both expression and spectral characteristics of "superfolder" GFP. sfGFPp1 with mutation T203C is selected as best strain from our study based on ratiometric spectral property. The linear emission response of sfGFPp1 at physiological pH range is very significant. To the best of our knowledge, except sfGFP, all the fluorescent proteins reported are unstable at high temperatures. This study demonstrated the stability of sfGFP and the newly developed variant sfGFPp1 at 70°C while retaining its pH sensitivity making it the only fluorescent protein based pH sensor for thermophilic conditions. . *In vitro* emission of sfGFPp1 respective to various pH at 70°C is matching emission calibration curve against pH at normal temperature. *In vivo* emission of sfGFPp1 shows better ratiometric characteristics compared to original superfolder GFP. Thus, T203C mutation along with superfolder mutations has unlocked GFP to become a successful probe in reporting intracellular pH of thermophiles.

PREFACE

This thesis work has been conducted by Department of Chemistry and Bioengineering of Tampere University of Technology, Tampere, Finland.

Past four years has been one of the best periods in my life. The calmness of Finnish atmosphere helped me greatly in my study. So, I cannot start without thanking Tampere University of Technology and Finnish education system for providing a place of study without any Tuition fees. I will always remember the high quality educational environment and flexibility offered in course structure. Secondly, I would like to thank my Professor Matti Karp for providing me a place for thesis work in his lab and for the flexibility in working hours and thesis duration. And finally I am very grateful to my co supervisor Bobin George Abraham for his kind guidance in experiments, result analysis and thesis writing.

I dedicate this work to my parents Mr. Kumar and Mrs. Tamilselvi, who have always been strong pillar of support in all my endeavors. As always, friends are best part at any situation of life, I would like to thank all my friends.

Tampere, April 28th 2014

Kumaragurubaran Kumar

TABLE OF CONTENTS

LIST OF TERMS AND ABBREVIATIONS

dNTP	deoxyribonucleotide
PCR	Polymerase chain reaction
MCS	Multiple cloning site
FRET	Fluorescence resonance energy transfer
GFP	Green Fluorescent Protein
sfGFP	Superfolder green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MES	2-(N-Morpholino) ethane sulfonic acid
PPI	Protein-protein interaction
pKa	Acid dissociation constant
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
IPTG	isopropylthio-β-galactoside
Tth	Thermus thermophilus
ESPT	Excited state proton transfer

1. INTRODUCTION

In 1962, Green fluorescent protein became the first fluorescent molecule identified in a living organism *Aequorea Victoria* (Heim and Tsien, 1996). Scientists Osama Shimomura, Martin Chalfie and Roger Tsien were awarded Nobel Prize in 2008 for the discovery and development of GFP (Nobel Media, 2013). Photoprotein aqueorin in *A. Victoria* emits blue luminescence which is converted into green emission by green fluorescent protein in the presence of molecular oxygen (Heim *et al.,* 1994; Ranieri *et al.,* 2009). But only thirty years after its discovery, the potential of GFP in molecular studies understood when GFP was independently cloned and expressed in another organism (Prasher *et al.,* 1992; Pedelacq *et al.,* 2005). The major advantage of GFP in expression of other organisms is its independent expression without need for any catalytic molecule from *A. Victoria* (Pedelacq *et al.,* 2005) and protected structure of its chromophore against many quenchers. The chromophore of GFP is made up of three amino acids - Ser65, Tyr66 and Gly66 which is present inside cylindrical β barrel structure of GFP and covalently attached to α-helix strand; three dimensional structure of GFP has studied using X ray crystallographic method (Yang *et al.,* 1996). Later with the aid of new molecular handling techniques, the structure and functionality of GFP have been studied for greater extent (Ranieri *et al.,* 2009). Many mutants of GFP were created which has modified spectral properties and chemical properties. Now there are GFP derived proteins available from spectral region blue to red (Pedelacq *et al.,* 2005; Matz *et al.,* 1999). Apart from genetic expression of GFP in various organisms, the application in bioengineering field became broader like monitoring protein-protein interaction (Cabantous *et al.,* 2005; Wilson *et al.,* 2004), intracellular ionic concentration (Stauffer *et al.,* 1998) and receptor interaction (Lippincott-Schwartz *et al.,* 2003).

Proton concentration inside the cell plays major role in controlling cellular metabolisms, detailed study on effects of pH is important in industrial and therapeutic applications (Kellokumpu *et al.,* 2002). Furthermore, the distribution of pH among different parts inside the cell is not uniform for example, mitochondria maintains little alkaline pH whereas vesicles maintains more acidic to digest peptides on exocytosis (Abad *et al.,* 2004; Jahn *et al.,* 2003). There are many organic dyes available for reporting intracellular studies but those dyes often cause cell damage through photo and chemo toxicity (Ranieri *et al.,* 2006). But GFP variants has been proved for better reporting and sensing probe which can be expressed in almost any part of the organism without causing any toxic effects (Zhang *et al.,* 2002). Moreover, GFP suits the ideal characteristics as intracellular reporter molecule such as accuracy in spatial and temporal dynamics

inside the cell (Ranieri et *al.,* 2009). Even though, more than two decades spent in intracellular pH studies, there are no significant probes available for studying thermophile metabolism and genetic expression. For many years, thermophiles has occupied major role in many industrial applications like enzyme production, downstream processing. But only few strains were subjected to laboratory studies due to less availability of detailed methods to study its proteomics and genomics (Zhang *et al.,* 1999). Thus developing any probe which promotes the study of molecular processes of thermophiles is highly inevitable.

The property of GFP has been changed by mutagenesis for different sensing and reporting properties. Notably, S65T (EGFP) mutation is one of the major amino acid changes which proved that the mutation can cause significant change in protein's physico-chemical properties like ionization state (Kneen *et al., 1998*), emission intensity, thermal property (Ranieri *et al.,* 2006) and change in *pKa* value (Elsliger *et al.,* 1999). EGFP were the first variant noted for pH responsive absorption and emission characteristics (Kneen *et al., 1998*). The acidic *pKa* range (5.8) of EGFP was further changed by mutations which yielded neutral (*pKa* 7.1) strains called as "Ecliptic" pHluorins (Sankaranarayanan *et al.,* 2000). First evolved variants of GFP through mutagenesis were non-ratiometric in absorption and emission properties. However, those firstly emerged variants had provided strong basis for understanding protein's protonation mechanism, spectral shift and ionic dependency (Ranieri *et al.,* 2006). There were models namely single-site model and two-site model developed which explains the protonation pathways of GFP's chromophore (Hess *et al.,* 2004).

In pH sensitive experiments non ratiometric strains had certain drawbacks like inaccuracy in fluorescence signal relative to proton concentration and unable to predict spatial resolution of molecular process in live cell. In 1998, Miesenbock et al., categorized a GFP variant called "Ratiometric pHluorin" with mutation S202H which showed increased emission 460 nm range when pH is below 5.5 and consecutive decrease in anionic emission range. Furthermore, experimenting GFP with different combinations of amino acid change yielded many ratiometric probes with specific characteristics. Interestingly, first major dual emission variant engineered was named as dual emission GFPs (deGFP) which showed higher emission at 460 nm range relative to low pH (George *et al.,* 2002). Crystal structure analysis of deGFPs suggested strong influence of mutations T203C, H148G and S65T on coupling between E222 and chromophore which affects the ionization state of protein (Ranieri *et al.,* 2009). Apart from the advantageous of deGFPs like clear pH calibration curve, pKa range 7.2 suitable for *in vivo* studies, there are drawbacks like poor emission, noisy background signal and low expression level (George *et al.,* 2002). To improve ratiometric probes, Ranieri *et al,* in 2006, engineered EGFP variant with red shifted mutation T203Y which also had good characteristics for ratiometric probe in live cell experiments. The motivation in engineering GFPs kept on increasing due to its scope for many new applications.

The ability of mutations were proved again through engineering "superfolder" GFP (sfGFP) which folds well when even fused to poorly folding peptides (Pedelacq *et al.,* 2006). Emerging new molecular techniques using GFP employs more number of applications for fusion proteins (Reid *et al.,* 1997; Waldo *et al.,* 1999). Superfolder GFP is the only variant of GFP which folds 3.5 times faster than folding reporter GFP (frGFP) (Felipe et al., 2009). There were series of mutations involved in creating sfGFP namely S30R, Y39N, Y145F, I171V and A206V along with cycle3 mutations F99S, (M153T, and V163A) and EGFP mutations (F64L and S65T). Furthermore, it is reported that sfGFP also has greater stability against denaturants and higher circular permutation (Pedelacq *et al.,* 2006). Apart from 100% refolding efficiency of sfGFP, its folding and stability at extreme temperature was also studied (Felipe *et al.,* 2009). Protein localization at thermophiles using GFP promotes new targeting and tracking techniques in thermophilic organisms (Felipe *et al.,* 2009). But the pH reporting characteristics of sfGFP has not been studies till now. In this study, we made an attempt to engineer dual emission characteristics in sfGFP in order to successful introduction of ratiometric pH probe for *in vivo* thermophilic studies.

2. THEORITICAL BACKGROUND

2.1 Overview of Fluorescence

2.1.1 A brief History and introduction

Sir John Frederick William Herschel in 1845 reported fluorescence from quinine solution for the first time, he stated that "Though quinine solution exhibits transparent and colorless when held between the eye and the normal light or a white object, it yet exhibits in certain aspects, and under certain incidences of light, an extremely vivid and beautiful celestial blue color" (Herschel, 1845). Interestingly, the first discovered substance quinine stands till date as one of the best examples for fluorescent subjects and used for creating first spectrofluorometer in 1950s (Undenfriend 1995).

The term Luminescence refers to emission of light from any substance which occurs in electronically excited state. The light produced by chemical and biological processes is called as chemiluminescence and bioluminescence respectively (Joseph, 2006). When the light incident on a substance involves absorption of some photon by its molecules, which is enough to transfer its electron from ground state to higher energy level is called excited state. Based on the nature of excited state, luminescence is divided into two categories namely: Fluorescence and phosphorescence. Depending on the nature of substance, molecule from excited state will restore back to ground state through losing absorbed energy by emission of heat or light or structural change.

Even though, fluorescence or phosphorescence is the radioactive decay process in which the amount of absorbed photon is released to reach ground state, both differ owing to its lifetime. Fluorescent lifetime of a substance is the average time taken by its molecule to restore from excited state to ground state (Ana luiza et $al.,$ 2009). Fluorescence has lifetime nearly 10 ns (10×10^{-9} s), whereas phosphorescence lifetime is rather slower and varies from milliseconds to seconds or even several months to years.

2.1.2   Jablonski Diagram

Professor Alexander Jablonski was a physicist, musician and soldier during world war and is regarded as father of fluorescence spectroscopy for his contribution in defining the processes between absorption and emission of light and defining the term anisotropy, which is used to describe polarized emission of a solution (Acta, 1978). Jabloski diagrams are referred as the basic steps towards understanding the absorption and emission spectra and there are many forms available depending on molecular processes in excited state (Jablonski, 1935).
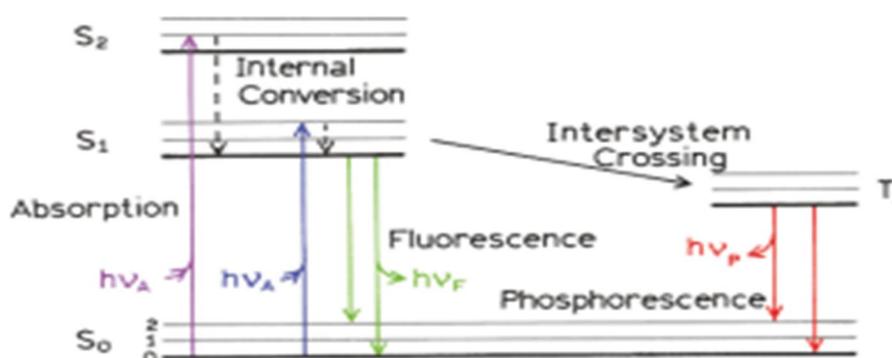
Figure 2.1 Example of jablonski diagram (picture adapted from chapter 1, Joseph, 2006)

Figure 2.1 explains the major parts of jablonski diagram like energy state, various energy levels, major events like fluorescence and phosphorescence etc. S_0, S_1 and S_2 are singlet ground state, first and second electronically excited states, respectively. Depending on fluorophore there exist different energy levels for each state, for example 0, 1 and 2 for ground state (S_0 in figure 2.1). Absorption occurs for the time period of 10^{-15} s; a time too short for significant displacement of nuclei called Franck-Condon principle (Joseph, 2006).

Following the absorption of light, fluorophore is excited to the higher energy levels. During the course of relaxation, substance follows fluorescence emission which occurs at 10^{-8} s. Internal conversion is a rare event which leads to fluorescence by rapidly relaxing to lowest energy levels of S_1 and happens closely till 10^{-12} s or less, since fluorescence starts from this point. It is proved that fluorescence occurs in thermally equilibrated excited state, which is lowest energy level of first excited state S_1.

Emission from T_1 or triplet state is called phosphorescence, which occurs for longer wavelength. Molecules in S_1 state spinning around into triplet state is called intersystem crossing, and it is the state which doesn't emit the absorbed light immediately like fluorescence, so immediate transformation into singlet ground state is forbidden (Joseph, 2006). Bromine and iodine are examples for frequent phosphorescent substance.

In excitation of substance, light source is considered as more ideal than heat energy. In figure 2.2, absorption and emission spectrums of perylene and quinine solution are presented. For example, in spectrum of perylene, emission and absorption maximum is 1500cm^{-1} wavenumber apart, and emission occurs at lower energy and in equilibrium between two states S1 and S_0, it is not possible to create enough excited molecules cloud at room temperature but increasing temperature cause larger energy difference between two excited states S1 and S_0 (Joseph, 2006; Berlman, 1977). Moreover, the spectroscopic event takes place when the singlet ground state and S_1 are in thermally equilibrium state. Hence, light creates smaller energy difference between these two states S_0 and S_1 than heat.
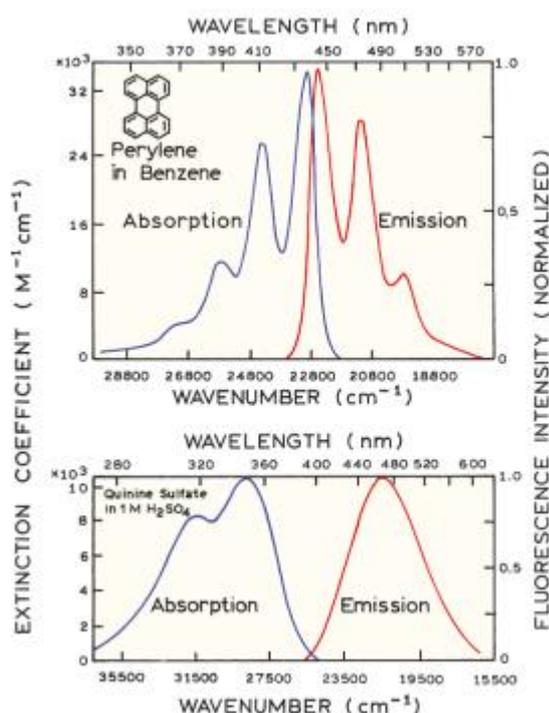
Figure 2.2 Absorption and emission of perylene and quinine presented against wavenumber (cm^{-1}) (picture adapted from chapter 3, Berlman, 1977)

2.1.3   Significant Characteristics of Fluorescence and its application

2.1.3.1   Stokes shift

Energy loss for the fluorescent molecule was first reported by Professor Stokes in 1852 from University of Cambridge. The phenomenon of fluorescence occurring only in lower energies of absorption spectra or at higher wavelength is called stokes shift and it is a common phenomenon for all fluorophores (Stokes, 1852). Energy difference between excitation and emission rays is due to energy decay of higher energy level to lower energy level and properties of solvent. Earliest demonstration of stokes shift by Professor Stokes was through a simple experimental setup, where a glass of quinine solution was

introduced to sunlight with a glass window as excitation filter and a glass of yellow wine as emission filter in between observer and quinine solution. Glass window limits the sunlight by only allowing the rays of shorter wavelength (400nm) to pass thorough, therefore quinine solution which has 450nm as excitation wavelength get excited to produce blue color (Berlman *et al.,* 1977). In his later works, he demonstrated many methods to identify many organic compounds through absorption, fluorescence and colored reflection.

Major application of stokes shift is its ability to interpret solvent-fluorophore interaction and predict the solvent binding site of macromolecule (Lakowicz *et al.,* 1995). This stokes shift property of a fluorophore is determined by its chemical structure, polarization nature and side-chain confirmation (Haugland *et al.,* 2002). By understanding this property, supports the specific selection of buffer based on its buffering capacity in bimolecular research (Joseph, 2006).

2.1.3.2 Mirror image rule between emission and excitation spectra

In 1926, Kasha's rule reported that the excitation wavelength doesn't alter the emission spectrum of fluorophores (Kasha, 1985). During excitation, the excess energy is dissipated; it might be due to the strong overlapping between the energy levels to maintain equilibrium and shorter relaxation time of about 10^{-12} s. Due to this reason, many fluorophores emission spectra of the range from S_1 to S_0 are mirror image of absorption spectra. This is also due to emission that occurs largely from the relaxation of lowest energy level of S_1 to singlet ground state S_0. Upon absorption and emission, the transition between energy levels follows reciprocal process, so it presents symmetric spectrum in nature. According to Frank codon principle all transitions occurs with no time to change in position of nuclei and it results in reciprocal transition between lowest vibrational level of 1st and 0th state (Lakowicz *et al.,* 1982). But there are some exceptions in mirror image rule, for example anthracene. Exception to mirror image rule is also due to some pH sensitive compounds exhibiting different spectral properties upon pH change, for example, pH-sensitive fluorophore 1-hydroxypyrene-3, 6, 8-trisulfonate (HPTS) gets ionized at low pH levels and due to decreased dissociation constant (*pKa*) it gives different emission spectrum rather than mirror image of absorption spectrum (Birks *et al.*, 1973). Some poly nuclear hydrocarbons form complex at excited state which gives unstructured emission, these complexes are also called as exciplexes. Thus, the relation between absorption and emission spectra is influenced by structural confirmation, ionization nature and excited state behavior of fluorophore (Lakowicz *et al.,* 1982; Joseph, 2006). Accordingly, linear graphical relation between excitation and emission spectra of pH sensitiveness of a fluorophore can be plotted.

2.1.3.3   Fluorescence Lifetime and quantum yield of a fluorophore

Quantum yield is number of emitted photons relative to number of photons absorbed (Joseph, 2006). It is a significant property of fluorophore, because substance which has high quantum yield has brighter fluorescence for example, rhodamines. There are many analytical methods devolved using quantum yield and lifetime calculation in sensing and other photochemical experiments.

Quantum yield is the ratio of the number of photons emitted to the number of photons absorbed. It is given in the below equation (Joseph, 2006).

$$Quantum\ yield, Q = \frac{\Gamma}{\Gamma + k_{nr}}$$

Where, Γ and k_{nr} is the rate constants of radioactive and non-radioactive decay respectively.

Fluorescence lifetime is the average time spent by molecules in the excited state before returning to ground state. It also depends on the time taken by fluorophore respective with its environment interaction to produce fluorescence. For many fluorophores, fluoresce lifetime is nearly 10 ns (Joseph, 2006).

$$Fluorescence\ life\ time,\ \tau = \frac{1}{\Gamma + k_{nr}}$$

Stokes rule suggests that the emitted energy is less than absorbed due to stokes shift of energy, so non-radioactive decay constant is mostly close to unity or less than Γ. Thus calculating emissive rate of fluorophore leads to calculation of quantum yield and lifetime (Birks *et al.,* 1973). Radioactive decay rate can be calculated by the following equation but the method is constantly upgraded for more accuracy (Strickler *et al.,* 1962).

$$\Gamma = 2.88 \times 10^{9} n^{2} \frac{\int F(\bar{v}) d(\bar{v}}{\int F(\bar{v}) d(\bar{v}/\bar{v}^{3}} \int \frac{\in \bar{v}}{\bar{v}} d\bar{v}$$

Where, F (v⁻) and ∈⁻v is the emission spectrum and absorption spectrum on wavenumber scale respectively, n is refractive index of the medium (Joseph, 2006).

2.1.3.4   Fluorescence Quenching and its timescale

Even though each fluorophore has unique photo-chemical properties of its own, upon experimental environment there are more external factors that influence fluorescence. Such factors decrease the fluorescence intensity and lifetime, which is called fluorescence quenching. The major quenching is due to molecular collision between fluoro-

phore and solvent elements, which is called collision quenching. Solvent elements which suppress fluorescence activity are called quenchers. Biological fluorophores like GFP which are present inside a cell or tissue has less substituted to these kinds of molecular collisions; hence fluorescence decrease due to quenching can be neglected. There can be anything acting as quenchers for example, oxygen, halogen, pH, amines etc. In collisional quenching there is no molecular alteration, but fluorophore returns to ground state due to diffusive gathering of quencher.

Ster-Volmer equation is used to calculate the decreased effect in fluorescence. The equation is given below (Joseph, 2006).

$$\frac{F_0}{F} = 1 + k[Q] = 1 + k_q \tau_0 [Q]$$

Where K is Stern-Volmer quenching constant, k_q is biomolecular quenching constant, τ_0 is unquenched lifetime and Q is the concentration gradient of quencher.

Sometimes fluorophores form complex with quenchers, which restrict it to get excited and is called static quenching. The basic idea of studying quenching is to predict the molecular dynamics of solvent respective to photonic properties. Usually quenching through molecular interactions is less common in absorption spectrum, due to the fact that absorption occurs at no time to change the position of nuclei according to Franck codon principle. The timescale of absorption spectra is 10^{-15} ns; therefore the absorption spectrum reveals only information of molecules which are very adjacent to it and the average molecules that absorb the light at singlet ground state. Contrastingly, fluorescence occurs for longer period of time (about 10 ns), so there is enough time scale for molecular interactions. Thus collision quenching can be noted only in emission spectrum, oxygen, acrylamides and halogens are best examples for quenchers. Unlike ground state, fluorophores at excited state form dipole and interact through rotational diffusion; this kind of interaction is called solvent relaxation which occurs at 10^{-10} ns (Joseph, 2010).

Effects of quenching gathered large attention in scientific community, due its greater influence on emission intensity of fluorophores. Biological fluorophores has higher degree of dependency on the presence of molecular quenchers and this property also aids to detect the movement of biological fluorophore in complex environment (Haugland *et al*, 2002; Joseph, 2006). In creating mutation in protein, quenching study is necessary to predict the effect of mutation in porosity of the protein structure. For example, in GFP, the mutations like T203Y, H148G are making the protein's emission responsive to molecular quenchers (Romoser *et al.*, 1997; Piotr *et al.*, 2012).

2.1.3.5   **Fluorescence anisotropy**

Professor Jablonski first coined the term anisotropy in the study of polarization effects in fluorescence, which has become significant property of fluorescence in biochemical studies. Light is an electromagnetic wave, which oscillates through the field it travels. Light can be polarized by polarizers which has wide applications in photonic studies. When the polarized light used for excitation, emission occurs which is called photo selective emission, this polarized light provides screening of fluorophores molecules to be excited (Joseph, 2006). Fluorophores get excited when its dipole plane is parallel to the direction of polarized light. Fluorescence spectra of such emission reveal more molecular dynamic process. The term anisotropy (r) and polarization (P) can be explained through following equation.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

Where $I_{\parallel}$ and $I_{\perp}$ are vertical and horizontal fluorescence intensities emitted by polarized light.

Anisotropy values for more viscous solution are higher because of less displacement in the fluorophores. In contrast, fluorophores suspended in non-viscous solution tends to rotate during emission leading to low anisotropy. In recent protein studies, proteins are subjected to bind macromolecule or other surface to neglect rotational diffusion. In that case, anisotropy can be expressed by following equation (Berberan-Santos 2001).

$$r = \frac{r_0}{1 + (\frac{\tau}{\theta})}$$

Where r_0 is the anisotropy in absence of rotational diffusion, τ is fluorescence lifetime, θ is rotational correlation time for diffusion.

Anisotropy measurement has various applications in studying protein structure and shape. In recent years, there are many antibodies, which are studied for its binding properties through fluorescence anisotropy. It gives accurate information on molecular volume, forming complex with other molecules and biochemistry of molecular assays (Lakowicz *et al.*, 1994).

2.1.3.6   FRET

Resonance energy transfer (RET) or Fluorescence resonance energy transfer (FRET) is the overlapping region between absorption and emission spectrums of different fluorophores. In other words, FRET is the radiation-less transfer of energy which doesn't require intermediate proton and can also be non-fluorescent. Firstly, German scientist Theodor Förster described the phenomenon as RET; it is also called as Förster resonance energy transfer. The fluorescent fluorophore is called donor and fluorophore with overlapping absorption spectrum is called acceptor (Figure 2.3). Close proximity between fluorophores, parallel dipole orientation and overlapping spectrum between acceptor and donor are preliminary conditions for FRET. Donor and acceptor interact only through dipole coupling, and not through emission and absorption. The rate of energy transfer ($k_T(r)$) can be explained by following expression. FRET is dependent on distance between donor and acceptor, solvent nature and lifetime of fluorophores.

$$k_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$$

Where r is distance between donor and acceptor, R_0 is Förster distance and τ_D is lifetime of donor in absence of acceptor.
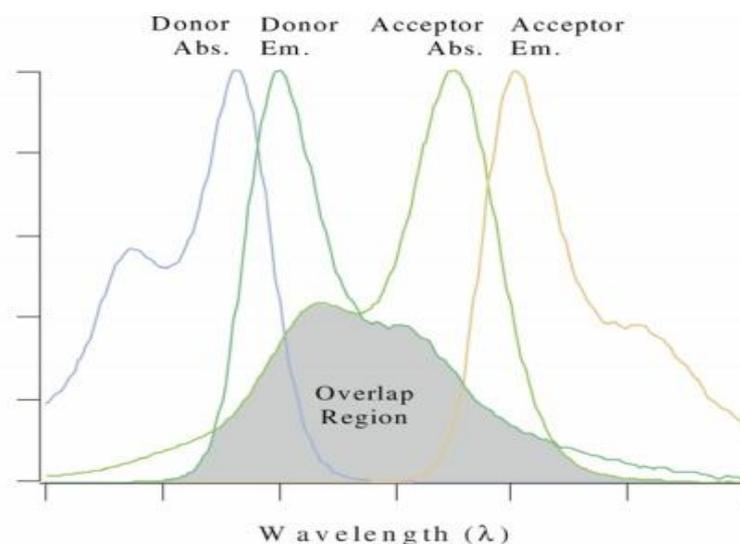
Figure 2.3 Schematic representation of FRET (image adapted from Time and Stefan, 2014).

FRET analysis of biomolecule provides precise information on distance between donor and acceptor. Efficiency of energy transfer is directly related to distance and orientation between them. So, the diameter of biomolecules can be calculated using FRET analysis. Moreover, interaction between two different fluorophores and its binding nature in solution can be predicted. Förster distance is the distance between donor and acceptor when energy transfer is 50%, and it is between 15-60Å for most macromolecules (Stryer *et al.,* 1978; Joseph, 2006).

2.1.4 Measurement systems

2.1.4.1   Steady state and time resolved measurements

The fluorescence can be measured by two different methods namely steady state measurement and time resolved measurement (Joseph, 2006). Steady state measurement is the earliest and simple measurement, which has conventional measurement principle and setup. In steady state measurement, from the excited sample the emission is monitored constantly and the average intensities of fluorescence by sample as whole is measured. In time resolved measurement, sample is monitored as a function of time after excitation by a short pulse of light; the measured value gives the decay of limited number of fluorophores. The basic difference between these two methods is represented in figure 2.4. Time resolved measurement can be used to measure sample size and shape. Due to complexity in nano scale measurement, time resolved measurement requires more sophisticated instruments but rather useful considering the accuracy on anisotropy experiments.
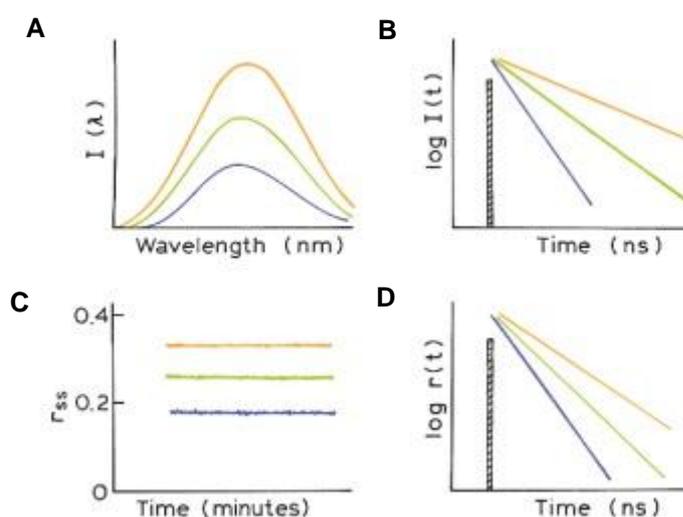
Figure 2.4 Schematic representation of steady state and time resolved measurement. A. Steady state measurement records with constant illumination and the steady state is maintained constantly throughout the observation. B. Time resolved experiments records intensity decays with small exposure time to illumination. C. Steady state maintained constantly with time in steady state measurement. D. Intensity decay or anisotropy decay lasts for few nano seconds (picture adapted from Joseph, 2010).

2.2 Overview of green fluorescent protein

It took centuries after introduction of fluorescence studies, to identify a fluorescent substance in a living organism. Scientists Osama Shimomura, Martin Chalfie and Roger Tsien were awarded Nobel Prize in 2008 for discovery and development of first living protein from a jelly fish *Aequorea Victoria* since 1962 (Nobel Media, 2013).. Bioluminescence of photoprotein aqueorin from *A. Victoria* is originally blue in color, but its associated protein named green fluorescent protein turns it to green color. Shimomura later identified the gene coding for GFP and characterized its crystal structure. Fluorescence is emitted from the chromophore of GFP which is covered by β-barrel sheet of peptides. The major property of GFP is the tendency of its polypeptide chain to spontaneously fold nature after its expression in the cell and it doesn't require any enzymatic activity (Chalfie *et al.,* 1994; Niwa *et al.,* 1996). Due to its protected chromophore structure, GFP had gained the name for good photostablity, high quantum yield and environment stable.

After thirty years of GFP study, in 1992, Prasher successfully cloned and expressed GFP in *E.Coli* (Prasher *et al.,* 1992). Subsequent experiments using GFP yielded new mutants of GFP with enhanced spectral properties (Inouye *et al.,* 1994; Tsien RY, 1998). It can be expressed alone or even as hybrid with other protein. Expression of GFP is proved in many species ranging from single cell organism to mammals. The independent expression and stability of GFP had promoted numerous research interests and became the most studied fluorescent protein in recent years (Wiedenmann *et al.,* 2009). Sometimes it is said that GFP is a biological torch to sense large or minute biological process (Patterson *et al.,* 1997). The continuous increase in fluorescent studies has resulted in many fluorescent proteins from other sea species like sea corals and sea anemones being identified (Matz *et al.,* 1999). However, some non-fluorescent proteins from sea when found, many proteins discovered from sea are photoactivable and are useful for new molecular studies (Nienhaus *et al.,* 2009). DsRed from Anthozoa corals has been identified as potential element to increase the diversity of spectral studies (Miyawaki *et al.,* 2002). Interestingly, the first identified GFP (sometimes called as avGFP) from *A. Victoria* has advantage over several photochemical proteins of other fluorescent proteins (Pakhomov *et al.,* 2008).

2.2.1 Chemical structure of GFP

The choromophore (p-hydroxybenzylidene-imidazolidone) of GFP is covalently linked to its β-sheet barrel and it consists of amino acid residues serine–tyrosine–glycine residues at 65-67 forming the chromophore of 238 amino acid protein (Cubitt *et al.*, 1995). The crystal structure of GFP is made up of 11 β-barrel strands of polypeptide arranged like a cylinder and α-helix strand running through its center (Brejc *et al.,* 1997). The choromophore is present inside the center of this cylinder which is shown in figure 2.5.
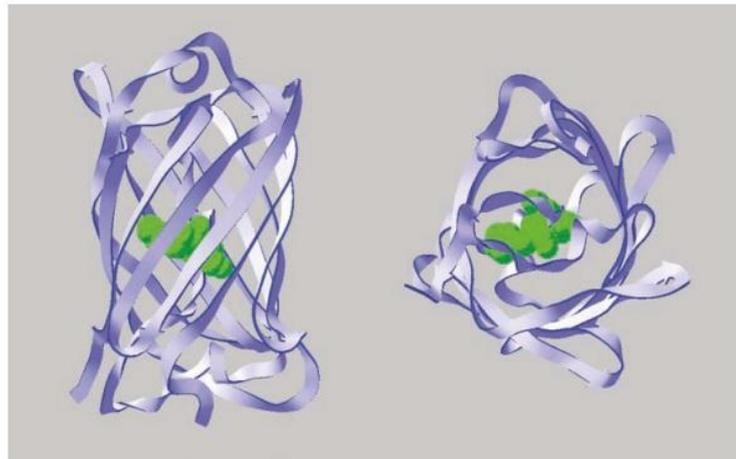
Figure 2.5 Structure of green fluorescent protein with its chromophore at center (side and top view) (picture adapted from chapter 3, Joseph R, 2010).

The formation of GFP as fluorescence protein doesn't require any enzyme from its origin organism *A.Victoria* (Ward at al., 1982). After the expression of polypeptide GFP inside the cell, it undergoes step by step folding process and cyclization to form its tripeptide chromophore (Reid *et al.*, 1997). The chromophore of GFP is responsible for flourescence of GFP by simple oxidation process; it is proved by expressing GFP anaerobically which is non fluorescent but structurally similar (Wachter, 2007).

The process of chromophore formation is also termed as maturation of GFP which requires only molecular oxygen. Backbone condensation of amino acid residues Ser65, Gly67 and oxidation of Tyr66 to didehydrotyrosine are the proceses involved in choromophore maturation (Figure 2.6). Arg96 is proved to be a promoting factor in fluorophore formation. Chromophore is covalently connected to its α-helix strand and R group of first 64 and last 170 residues. Imidazolinone core is common in all fluorescent protein.
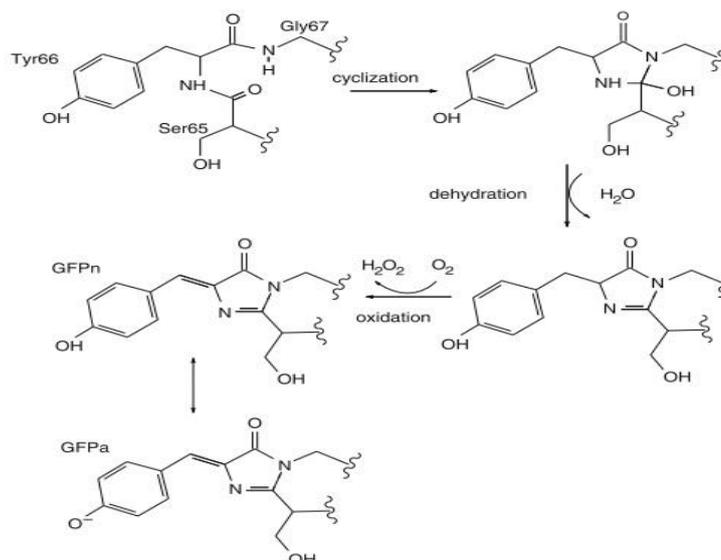
Figure 2.6 Spontaneous formation of fluorophore by cyclization, dehydration and oxidation process of residues Ser65, Tyr66 and Gly67 (picture adapted from Nifosi *et al.,* 2012).

2.2.2   Fluorescent properties of GFP

The spectral properties of GFP are unique and dynamic compared to other fluorescence substances. It is known for its high natural photo stability and quantum yield. GFP shows absorption peak at three different wavelengths 278 nm, 397 nm and 510 nm (Figure 2.7). Existence of aromatic amino acids in all molecules exhibits absorption at 278 nm, but the major peak at 397 nm and minor peak at 510 nm are the major unique characteristics of GFP. The pH concentration influences the height of peaks; at low pH the minor band is completely depleted and vice versa. Phenolic group of Tyr 66 residue of chromosphere gets protonated at 397 nm and deprotonated at 510 nm. Nitrogen and carbonyl oxygen group of imidazolinone gets deprotonated at both peaks (Bell *et al.,* 2000). Emission spectrum of GFP shows peak at 510 nm and quantum yield 0.79. Chromophore is responsible for excited state proton transfer (ESPT) and respective release of its anionic form (Chattoraj *et al.,* 1996). GFP exhibits two states in spectrum such as, protonated form with excitation maximum at 397 nm and unprotonated form with absorption maximum at 477 nm.

Even though, there are many fluorescent proteins identified from other species, GFP still maintains its significance in scientific research because of protected chromophore by its outer structure. The stability and orientation of chromophore affects physico-chemical and spectral properties. Precise change in amino acid near chromophore has shown significant results in fine tuning GFP for necessary sensing property (Mark *et al.,* 2005). It leads to introduction of new varieties of GFP mutants and its homologous proteins.
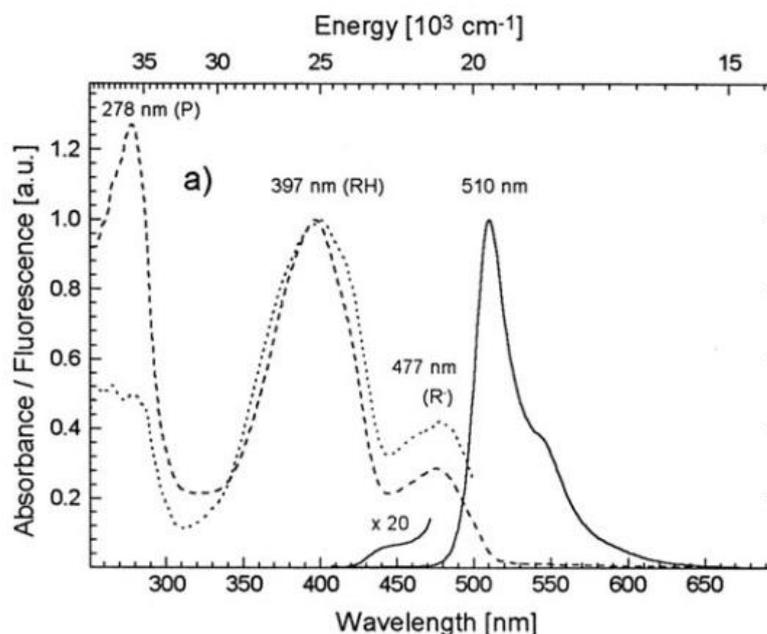
Figure 2.7 Absorption (dashed line), excitation (dotted line) and emission (solid line) of GFP at room temperature and pH 8 (Kummer *et al.,* 1998).

2.2.3 Mutants of GFP and other fluorescent proteins

Even though, wild type GFP gene cloned in other organisms gained significance in scientific research, the protein had certain downsides like poor folding at 37°C, less photo stability, excitation maximum near UV spectral range and quantum yield, dual emission peak etc (Pedelacq *et al.,* 2005; George *et al.,* 2002) . It was a spectacular approach for molecular biology, to identify the importance of mutation towards optimizing spectral properties of GFP. At first it was reported by Heim with co-researchers, when they introduced mutation S65T to GFP coding sequence in 1995 (Heim *et al.,* 1995). The mutation S65T increased the folding several times faster, provided stability at higher temperatures, shifting excitation peak from 395nm to 489nm and providing high quantum yield. Consecutively, incorporation of amino acid change F64L along with S65T gave a stable protein at higher temperatures which was named as enhanced GFP (EGFP) (Kneen *et al.,* 1998). In many mammals cell research, EGFP had been studied as reporter and delivery tool. The major advantage of GFP in microbial and mammalian cells research is its non-toxicity towards sub cellular process and its diverse reaction to molecular process (Kneen *et al.,* 1998).

In recent years, GFP mutants with modified spectral properties and chemical properties have been engineered. In modifying spectral properties, GFP ranging from spectrum blue to red is available now (Table 2.1). In FRET studies, multicolor fluorescent protein gained significance for its spectral divergence in reporting protein-protein interactions. Even though, many colored variants have less quantum yield and pH sensi-

tivity, they have specific useful properties, for example, BFP is prone to photo bleaching but useful in FRET analysis and multicolor studies.

Apart from spectral diversity of GFP, it has proved as significant sensing element in biomedical research. It is possible to incorporate GFP with any vector to deliver any part of the tissue or organ. At first, the thermal stability of GFP mutant increased from 28°C to 37°C through mutation F64L, and also had increased fluorescence intensity (Cormack *et al.,* 1996). As pH and temperature are major factors in living organism research, interest towards best behaving mutants of GFP in those conditions increased dramatically. Blue fluorescent protein (BFP) has been reported as novel strain in reporting intracellular pH 5 to 7, but it lacked photo stability and temperature stability (Wachter *et al.,* 1997).

Table 2.1 Availability of green fluorescent protein variants in spectral range between blue to red.

Color	Protein Name	Excitation (nm)	Emission (nm)	*pKa*	Reference
Blue	BFP	381	445	5.3	Yang *et al.,* 1997
	EBFP	383	448	5.3	Ai *et al.,* 2007
Cyan	ECFP	434 and 445	476 and 503	4.7	Kneen *et al.,* 1998
	mCerulean	433 and 445	475 and 503	4.7	Rizzo *et al.,* 2004
Green	EGFP	488	509	6.0	Kneen *et al.,* 1998
	sfGFP	485	510	5.5	Pedelacq *et al.,* 2006
	deGFP	485	512	7.2	George *et al.,* 2002
Yellow	EYFP	514	527	6.9	Bizzare *et al.,* 2008
	E^{2}GFP	408 and 473	508 and 523	7.0	Bizzare *et al.,* 2008
	GdFP	457	574		Prajna *et al.,* 2008
	Citrine	516	529	5.7	Patterson *et al.,* 2001
Red	mRFP	484	607	4.5	Campbell *et al.,* 2002
	R10-3	555	585		Mishin *et al.,* 2008
	mPlum	590	649	<4.5	Wang *et al.,* 2004

Halide sensitivity of GFP has been studied with certain mutants. Mutations T203Y/S65G/V68L/S72A displaced the GFP emission from green to yellow region of spectrum and was named as yellow fluorescent protein (YFP) (Piotr *et al.,* 2012). Apart from advantage of increased intensity of fluorescence, YFP has also showed better halide sensitivity with mutation H148Q (Cormack *et al.,* 1996). T203Y mutation in EGFP has also been studied for significant halide and chloride sensitivity inside the cells (Piotr *et al.,* 2012).

2.2.4   Development of Superfolder GFP

A group of researchers made sequence of six mutations in EGFP, which yielded a new variety of GFP with higher folding efficiency when even fused to poorly folded polypeptides and this modified fluorescent protein is named superfolder GFP (Pédelacq *et al.,* 2006). It has also showed higher stability to environmental changes and improved tolerance of circular permutation. Major motivation in developing folding efficacy was due to the misfolding variants of GFPs when fused with other peptides (Pédelacq *et al.,* 2006, Waldo et al 1999). Even though, wtGFP had been improved for better sensing and reporting studies from its time of discovery, all the available mutants are better reporting probe only when expressed alone (crameri *et al.,* 1996). Recent studies are more interdisciplinary which leads to more hybrid studies of GFP linking with other proteins, chemical moiety, or nano particles (Nakayama *et al.,* 2003). But many sensing molecules are incapable of reporting and expressing alone. Expressing those molecules with available strains of GFP often expressed reduced fluorescence, aggregation, and wrong folding, which are very much prone to its environmental changes (Peeelle *et al.,* 2001).

It was a successful effort taken by Jean-Denis and his colleagues to select best fluorescent strain from incorporating different mutation combinations along with poorly folding N-terminus polypeptide addition. X-ray crystallographic studies were used to combine and prove results from already available variants. Mutation was created by random DNA shuffling and screening of bright strains in each cycle. Final superfolder GFP had the mutations S30R, Y39N, Y145F, I171V and A206V along with cycle3 mutations F99S, (M153T, and V163A) and EGFP mutations (F64L and S65T) (Figure 2.8). Different variants like BFP, CFP and YFP were also tested consecutively for results comparison (Pédelacq *et al.,* 2006). X-ray crystallographic evidences shows that mutation S30R plays major role in increasing folding efficiency. Superfolder showed quantum efficiency 0.65 and molar extinction coefficients 8.33×10^4 M^{-1} cm^{-1}.
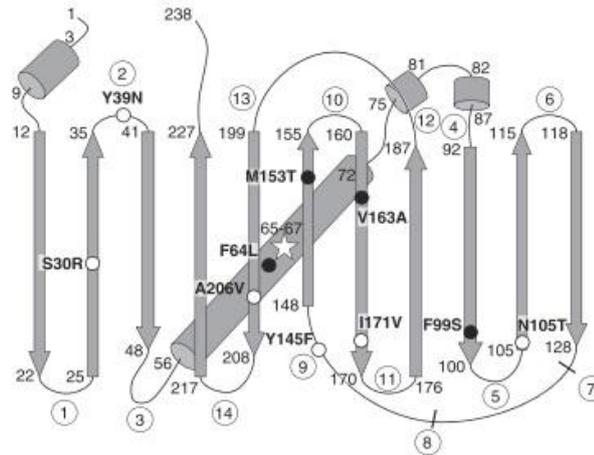
Figure 2.8 Schematic representation Superfolder GFP mutations (white circles) along with cycle3 mutations (dark circles) and EGFP mutations. (Picture adapted from Pédelacq *et al.*, 2006).

In recent studies, superfolder GFP has shown significant results in periplasmic protein localization (Thuy *et al.,* 2011). Other variants of GFP have failed previously in these studies because of misfolding during protein transport to periplasmic region. As perisplasmic region of *E.coli* and other cells are highly oxidative environments, even stable variants like EGFP failed to transport reporting molecules due to less folding efficiency. Superfolder has been shown as a stable delivery tool in oxidative environments (Aronson *et al.*, 2011). In another study, superfolder exhibited tenfold increase in folding efficiency and 100% folding back after denaturation (Benjamin *et al.*, 2007). Split protein studies were considerably increased using GFP variants, where protein can be engineered in such a way that it can be photoactivable at specific events (Kaddoum *et al.,* 2010). Some mutations in superfolder GFP have showed ideal characteristics in reporting protein-protein interaction (PPI) (Jun *et al.*, 2011). Eventually, increased number of studies on GFP variants provide basis to explore significance of special amino acids in 238 amino acid sequence of GFP. Except few important amino acids like Arg96 which play an important role in auto-cyclic formation of three dimensional structure of GFP, all other amino acids have the possibility to be changed and cause significant effect in changing protein spectral and physical characteristics (Miyawaki *et al.,* 2003).

2.3   Significance of pH in living environments

The basic definition of pH is negative logarithm of hydrogen ion activity in water, but understanding pH in biological environments is influenced by other aspects like proton to buffer component ratio, weakly acidic and basic cell components, amino acid side chain contents etc (for review, Rick *et al.,* 2011). In biological environment, biomolecules donate or take protons from its environment, so depending on the local molecular events pH inside the cell vary from region of a single cell to whole organ. Let us con-

sider cytosol as a buffer which constitutes proteins and other substituents and, almost all proteins are either weakly acidic or basic in nature (Pick *et al.,* 1990). Predicting the pH behavior of cytosol depends on buffer capacity (β) of each ingredient. Buffer capacity is the amount of strong base or acid needed to change pH by 1 unit (Srivastava *et al.,*, 2007). Majority of living cells employ exchange of CO_2 for maintaining this buffer activity, due to free diffusive nature of CO_2 (Rick *et al.,* 2011).

Proteins play various functions inside the living cell like signaling, transporting, reporting etc. (Wang *et al.,* 1997). pH influences all the interactions of protein such as movement of substances between cell surface to cell organelles, ionic current flow (Hunte *et al.,* 2007) and cellular contractibility (Fliegel, 2005). Thus, pH becomes a basic factor influencing the entire metabolism throughout the cell cycle (Srivastava *et al.,* 2007). In eukaryotes, mainly NHE proteins play major role in maintaining pH by transferring H^+, OH^- or HCO_3^- in and out across the membrane (Wakabayashi, 2006). Living cells control cytoskeletal movement through organizing pH along with some receptor activation and enzymes which supplies energy molecules (Kapus *et al.,* 1999).

Exocytosis is one of the major events influenced by pH, because interior lumen of synaptic vesicles exhibits acidic pH during the process (Jahn *et al.,* 2003). Exocytosis is the movement of metabolites or other cellular substance through vesicles to the outer surface. Endocytosis is the transportation of metabolites into the cell for subcellular pathways. Both endocytosis and exocytosis have pH influence for association and dissociation of molecules or ligands (Mellman and Warren, 2000). The study of sodium-potassium ATPase and chloride channels explains effects of pH during different stages (Rybak *et al.,* 1997). The movement of metabolites across the cell membrane is controlled by phosphorylation or carboxylation, because most of the biological environments maintain neutral pH, in which all water soluble metabolites entering into the cell gets ionized by phosphorylation or carboxylation in order to prevent role back to its environment (Davis, 1958). For example, glucose molecule turned charged by phosphorylation in glycolysis pathway (Rick *et al.,* 2011). Determination of *pKa* values for each metabolites and buffer components could define the pH behavior of particular cell (Milletti *et al.,* 2009).

Distribution of pH is not uniform in all region of cell; the subcellular organelles have specific pH. For instance, mitochondrial region has little alkaline pH which affects the life cycle of cell. Mitochondrial research has become mainstream focus in studying cell's life cycle and programmed cell death called apoptosis (Abad *et al.,* 2004). Proteins are transporting and reporting molecules in many cellular metabolisms including mitochondrial expression. These protein interactions are deeply controlled by pH. Studies on tumor cells have revealed change in pH respective to normal pH range (Kellokumpu *et al.,* 2002). Even protein folding pathways are deeply influenced by pH, because amino acid side chain affinity towards substrates is pH dependent (Pace *et al.,*

1990). Moreover, enzymes are made up of peptide sequence which influence the entire cellular metabolism and influence of pH in enzymatic activity have been studied (Akke, 1990). For example, in glycolytic pathway, effects of pH have been studied to improve enzyme efficiency (Van Hoek, 1998).

Lipids contribute to the major role in cellular metabolism; it acts as membrane and energy storage molecule. It is basically hydrophobic and amphiphilic nature in order to form cell wall, vesicles etc. Ketoacyle and isoprene are the two building blocks of lipids (Rajakumari *et al.,* 2008). In mammals, excess lipids are stored in specific muscular compartments which will be converted into glucose when necessary. Lipid metabolism is controlled through transcriptional regulation of enzymes and polarization. Proton concentration affects the affinity between lipids and enzymes. For example, pH change of 0.5 units determines the affinity between transcriptional regulator Opi1p and Phosphatidic acid (PA) near endoplasmic reticulum of yeast cells (Young *et al.,* 2010). So lipid is one of the major biological molecules influenced by minor change in pH (Rick *et al.,* 2011).

2.3.1 pH compartments inside the cell

In eukaryotic cell, each organelle is involved in different metabolic activities. Maintaining pH homeostasis along the cell is necessary for cellular metabolism (Figure 2.9). For example, mitochondria generate ATPase and acts as main power generator and signaling molecule generator of the cell, moreover, it maintains slightly alkaline pH. To maintain homeostasis, each organelle releases protons to its environment through its lumen receptors and vice versa. It is primarily regulated by Na^+/A^TPase along plasma membrane and provides room for Na^+/H^+ exchangers to maintain homeostasis in cytosol (Casey *et al.,* 2010). In compartmentalization, protein also plays a major role to maintain ionic homeostasis through side chain reactions (Chan *et al.,* 2006). Vesicles play a major role in endocytosis and exocytosis pathways and the processing of peptides in vesicles reportedly requires acidic pH, thus even a small change in vesicular pH leads to protein misfolding (Carnell *et al.,* 1994).
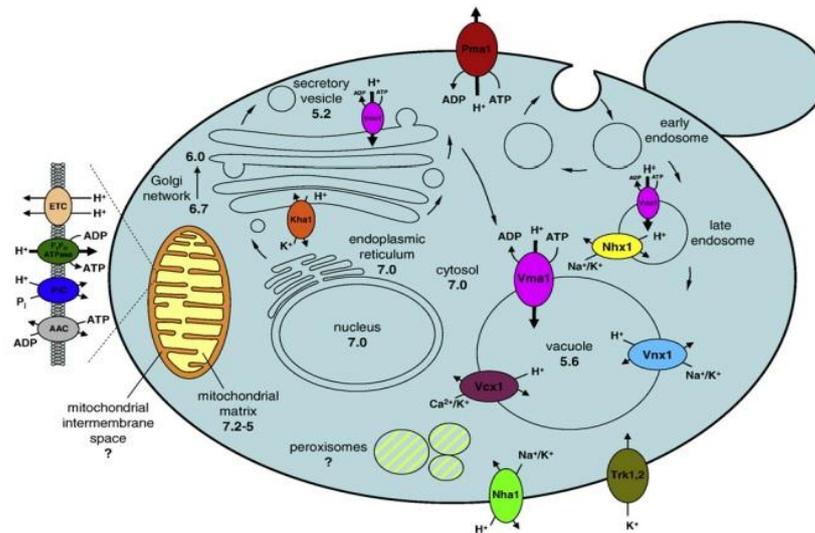
Figure 2.9 pH regulators and different pH compartments in eukaryotic cells (for reviews see Rick *et al.,* 2011).

2.3.2 Behavior of *E.coli* and other bacteria with pH homeostasis

Suspended medium pH is highly important in all bio-molecular and live cell studies. Maintaining pH is one of the strategies of *E.coli* against environmental stress, e.g. some halophiles produce hydrogen to maintain metabolism in saline environment (Anniina *et al.*, 2013). *E.Coli* maintains neutral pH range inside the cell throughout its lifecycle, but in order to maintain pH homeostasis, it generates large proton gradients respective to outside pH (Padan *et al.,* 1981). However, some acid tolerant fermentative bacteria pro-portionally decrease internal pH along when outside pH decreases, because maintaining pH gradient consumes more energy which will affect fermentation process (Kobayashi *et al.,* 1986). Different species employs specific mechanism against pH, but even strains within same species evolve itself differently against its own environment. Gram-positive and gram-negative bacteria has been extensively studied for its pH behavior and some neutrophils exhibit same growth in little alkaline medium also (Rius *et al.,* 1994). Maintaining large pH gradients increases organic acid anions inside the cell (Russell *et al.,* 1992). Lactic acid bacterial strains are tolerant to intracellular acidic environments which also facilitate increased tolerance to pathogens. Furthermore, these lactic acid bacteria are reported to be dynamically changing its intracellular pH relative to its environment (Henrik *et al.,* 2000). Each bacterial strain involves different mechanisms in maintaining pH gradient such as proton-trans locating ATPase (Hong *et al.,* 1999), Cytoplasmic buffering capacity (Hutkins *et al.,* 19993), cell membrane permeability (Henrik *et al.,* 2000).

2.4   Development of pH sensitivity of GFP variants and superfolder GFP

Self-catalytic property of wild type GFP to form three dimensional structures without the need of enzyme promoted it to clone and express in different cells. GFP was proved to be a successful reporter and sensing molecule in non-invasive experiments without toxic effects to host. With the aid of modern spectral and structural studies, it was able to track down characteristics of amino acid to photo-chemical property of GFP. There are many mutations found to be associated with spectral shift, temperature, pH, ligand interactions, ionic concentration, transporting property etc. As a result, GFP variants such as EGFP, YFP, BFP, and superfolder GFP are commercially successful in different applications (Yang *et al.,* 1996). These successful mutants are also sensitive to pH to certain extent, so the interest towards creating GFP mutants with different pH reporting property increased rapidly in both *in vitro* and *in vivo* experiments.

The chemical structure of GFP is largely dependent on amino acid residues and its side chain confirmations. These amino acids employ free proton release from its chromophore, which is responsible for fluorescence. Therefore, any change in amino acid sequence results in fluorescence and pH sensitivity (Palm *et al.,* 1997). Chromophore contains one protonation site, which is the basis for developing different pH variants (Kneen *et al.,* 1998). Change in genetic sequence changes some protonated amino acid to deprotonated amino acid and vice versa. It all started when James Rothman with his group, made S202H mutation in wild type GFP in order to find pH specific amino acid and successfully reported varying fluorescence between pH 5.5-7.5 (Miesenbock *et al.,* 1998). S202H strain was then popularly called as ratiometric pHluorins and extensively used in studying intracellular pH of bacteria, yeast and mould (Bagar *et al.,* 2009). As a result, there are hundreds of mutation combinations, which are made in GFP to improve its sensitivity to pH change. EGFP, which has mutations S65T and L64F is reported as better reporting molecule in acidic regions of the cell like Golgi apparatus (Llopis *et al.,* 1998). Yellow spectrum mutation T203Y in EGFP is better suitable for base regions like mitochondria (Kneen *et al,* 1998; Dang *et al.*, 2012; Michael *et al.,* 2004; Malea *et al.*, 1998).

The GFP mutant S65T with enhanced intensity of fluorescence was popular in late 90s (Heim *et al.,* 1995), it was also attempted to check the pH sensitivity *in vivo*. H148G and E222Q mutations are characterized as influential in polarity of the chromophore (Marc-Andre *et al.*, 1999). YFPs had T203Y substitution along with other mutations such as S65G/V68L/S72A. In a study in 1999, YFP along with H148G mutation combination was studied for its variability in *pKa* value; even though, *in vivo* results was not accurate enough on that time, there were significant change in *pKa* value of each mutant (Marc-Andre *et al.*, 1999). For example, YFP had pKa value 7.1 which is

bright at alkaline pH regions like Golgi apparatus, mitochondria etc. (Wachter *et al.,* 2007).

Another GFP variant (E^2GFP) with mutations F64L/S65T/T203Y/L231H was successfully cloned and expressed in both prokaryotic and eukaryotic cells for pH studies. Moreover E^2GFP was also reported for optical switching between bright and dark state even in single molecule level. GFP variants have also proved to be better reporter and signaling molecule, when fused with other peptides. For example, E^2GFP has been studied as fusion molecule with vesicle specific receptor protein in order to report pH behavior during exocytosis, (Miesenbock *et al.,* 1998).

GFP variants also proved to be better stable when fused with non-fluorescent peptides. There are well known studies for fusion site and size of fusion peptide, because abundant peptide addition to fusion can cause fluorescence reduction. In an attempt to create pH selective mutant, S202H mutation yielded a better ratiometric GFP probe also called as ecliptic synapto-pHluorin (Michael *et al.,* 2004). Interestingly, synapto-pHluorin had several advantages such as accuracy in ratiometric analysis, good fluorescence variation towards pH, higher sensitivity etc. Furthermore, two-point mutation in chromophore gave 20 fold increases in fluorescence, which is named as superecliptic pHluorin (Sankaranarayanan *et al.*, 2000). These fusion protein studies with GFP mutants have extended further to other cellular compartments like Golgi apparatus (Machen *et al.*, 2003), peroxisomes (Jankowski *et al.*, 2001), mitochondria and cytoplasm (Karagiannis *et al.*, 2001). These proteins provided a new approach to study plasma membrane receptor protein and its pH behavior using photo bleaching method (Michael *et al.*, 2004).

2.4.1   Behavior of deGFP and sfGFP with pH

The unprecedented importance of GFP in pH studies is due to less number of alternative systems developed for intracellular pH studies. Chemical or synthetic fluorescent dyes are major substituent for cellular pH studies (Lippincott-Schwartz *et al.*, 2003), but the major drawbacks of synthetic dyes are cytotoxicity (Llopis *et al.*, 1998), varying penetration nature and less intracellular targeting efficiency (Awaji *et al.*, 2001). Fortunately, there are some remarkable properties of GFP like chromophore dynamism (Reid *et al.*, 1997), nano size, suitability for improving analytical procedures, fusion with other molecules (Tozzini *et al.*, 2004), folding and stability and so on. Likewise, recently improved variant of GFP for intracellular pH studies are dual emission (deGFP) (George *et al.*, 2002) and superfolder GFP (sfGFP) (Felipe *et al.*, 2008).

It is known that wild type GFP absorption spectrum is bound to two bands A and B connected to neutral and anionic state of the chromophore respectively. Neutral state emission spectrum peaks at blue range and anionic state peaks at green spectral range,

but only green spectrum is visible due to anions produced by internal transfer of neutral state. There are many studies proposed for the study of atomic structure and proton transfer network of GFP variants (Palm *et al.*, 1997) (Brejc *et al.*, 1997). It is believed that Tyr66 has significant effect in changing protein spectral range which is further related to widening applications of GFP (Heim *et al.*, 1994). In principle, the emission of blue range can be possible by slowing down excited state proton transfer (ESPT) by neutral state of chromophore in excited state (George *et al.*, 2002).

In a recent study, GFP mutant called dual emission GFP (deGFP) was created. deGFP has two emission peaks which are essential for ratiometric fluorescence analysis (George *et al.*, 2002). In different rounds of mutations, the results were proposed for two ideally behaving mutants. deGFP strain with mutations S65T, C48S, H148C, and T203C was identified as novel pH dependent variant and proposed for *in vivo* mammal cells. Fluorescence of deGFP shifted from 515nm (green) to 460nm (blue) and *pKa* value shifted from 6.8 to 8.0. The study of its crystal structure, *pKa* value, and emission, compared to fluorescent dyes suggested deGFP to be better strain for *in vivo* pH measurements of mammalian cells (George *et al.*, 2002). Even though deGFP is suggested for better response and accuracy for change in pH, the properties like unsuitable *pKa* value for physiological conditions, noisy fluorescence signal and possible cellular damage due to UV spectral range, are its drawbacks (Ranieri *et al.*, 2006).

2.5   Thermophilic stability and a new approach on super-folder GFP

In a novel study of sfGFP in thermophiles, it has been proved to be better molecule for assays in intracellular metabolism and distribution (Felipe *et al.,* 2008). Before that, potential ability of superfolder in thermophiles was not understood completely. In that research, Felipe and his group cloned sfGFP with fusion protein and expressed in cytoplasm, membrane and periplasmic region of *Thermus thermophilus* (Tth) (Felipe *et al.,* 2008). It is also stated that promoters can control protein expression and temperature can be used to activate fluorescence of GFP (Felipe *et al.,* 2008). In principle, superfolder can also promote immunoisolation of protein from thermophiles, which provide more interests towards thermophilic application of sfGFP (Cristea *et al.*, 2005; Felipe *et al.*, 2008). Moreover, wild type GFP was often missfolded in physiological temperatures, but superfolder GFP had shown better stability even at higher temperatures suitable for thermophiles. Except superfolder GFP none of other variants or molecules is better suitable for *in vivo* studies in thermophiles.

In this research work, we used selective mutations for creating dual emission and pH sensitivity while maintaining the superfolder and thermostable properties of superfolder GFP. With the aid of multidisciplinary studies, advances in protein engineering, purification, delivery and reporting methods would probably increase more applications and understanding of GFP. GFP variant for pH sensitivity in thermophiles would probably become one of the significant technological advancements and may provide commercial applications. As hundreds of GFP mutations have been characterized and associated to specific properties, it is possible to select pH selective mutations in thermostable superfolder GFP.

2.6 Characteristics of mutations T203C, H148G, C48S, T203Y

The GFP variant superfolder has series of mutations which aid its properties like rapid folding, higher circular permutation, stability etc (Jean-Denis *et al.*, 2006). In addition to sfGFP mutations, we had selected few mutations which have pH specific and dual emission properties for this study. The mutations T203C, H148G and C48S were taken from dual emission variant deGFP (George *et al.*, 2002). However, Thr203 and His148 amino acid residues were proved to have influence in spectral property of GFP (Torsten *et al.*, 1995) and promote protein to become photoactivable (Patterson *et al.*, 2002). S65T is a well-known mutation for pH dependency, which is present in most of the GFP variants including superfolder. The presence of cysteine at 203rd position favors neutral chromophore emission and stability in acidic pH environment. Most of these mutation combinations have potential ability to construct GFP as pH sensitive and ratiometric probe. T203Y is a mutation which takes the protein to yellow spectral range (Jayaraman *et al.*, 2000) and creates an affinity site towards ions like chloride, bromide, halide etc (Wachter *et al.*, 2000; Ranieri *et al.*, 2006). Moreover, His148 and Tyr203 were two of the amino acid residues among the list characterized (Gln69, Gln94, Arg96, His148, Thr203, Ser205 and Glu222) as amino acids, which acts as proton donor and acceptor of hydrogen bonds network in GFP (Brejc *et al.*, 1997; Ranieri *et al.*, 2009).

2.7 Site directed mutagenesis

Natural properties of molecules like proteins, enzymes, antibodies etc. can be altered to get a desired change in thermo stability, pH stability, nutrition consumption etc. For example, there are many enzymes which are naturally unstable at high temperatures and are modified for thermo stability by inserting gene of interest into host gene; moreover it is possible to regulate the specific property of gene by altering amino acid sequence. It is said that many naturally available strains were not suitable for industrial applications, so they are mainly used to scale up industrial production (Bernard *et al.,* 2008).

Engineering new protein with desired property is not a simple method; the major obstacle is to know the role of each amino acid in its position in genetic sequence. However, it is feasible to engineer known proteins with defined physical and chemical properties like x-ray crystallography, because it is possible to predict which amino acid plays major role in three dimensional folding or other structural and physic-chemical properties of protein. The process of changing desired amino acid in genetic sequence is called site directed mutagenesis (Bernard *et al.,* 2008). In practice, site directed muta-genesis is a trial and error method, mainly focused on the best behaving strain out of random combinations of amino acid change. Even though, theory supports that change can be carried by both protein sequence and genetic sequence, changing through DNA sequence is quite suitable for large scale production.

There are different methods employed to create directed mutations such as clon-ing with M13 DNA, plasmid DNA, PCR amplification etc. Cloning with M13 DNA was an earlier method adopted for successful creation of point mutation. In this method, the cloned sequence is inserted into double stranded DNA of M13 bacteriophage. First-ly, the amino acid sequence to be changed and the mRNA codon for changing site should be known precisely. Single stranded vector DNA of M13 is mixed with synthetic DNA sequence which is perfectly complimentary with one difference in nucleotide which is to be changed. The synthetic oligonucleotide sequence binding is facilitated by low temperature, increased concentration and higher salinity. The difference in oligonu-cleotide creates a mismatch loop region in segment. The 3′ region of oligonucleotide acts as primer site for double strand synthesis in presence of DNA polymerase, four deoxyribonucleoside triphosphates and T4 DNA ligase. Then, mutant bacteriophage virus particle transformed into *E. coli* bacterial cells and the expression of viral parcel will eventually kill the cells. Moreover, the replication of plasmid DNA is semicon-servative in nature; accordingly replicating population should have 50 percentage of both mutant and native plasmid vector. The mutant cell line was screened by stringent screening process to isolate the mutant plasmid to clone in *E. coli* expression plasmid to produce mutant protein. Theoretical factor failed in practice to produce 50 percentage of expressed mutant cell line and this method has more time consuming steps (Sambrook *et al.,* 1990).

Next major method developed was to create mutation through plasmid DNA which simplifies the process by excluding extra cloning steps. The plasmids which have antibiotic resistance genes were selected for better screening. Target DNA which is to be mutated is inserted into multiple cloning site of plasmid DNA (Piechocki *et al.,* 1994). Then, double stranded plasmid were denatured to single stranded and annealed by synthetic primers with defined amino acid change. In three synthetic primers, one which anneals to multiple-cloning site has the property to change an amino acid in tar-get sequence; other two are used to control antibiotic resistance gene expression for

better screening (figure 2.10). In the presence of dNTPs and T4 DNA polymerase enzyme, 3´ end of primers synthesizes complementary strand for whole plasmid, then T4 DNA ligase confirms closing of 3` end to 5` end. Mutant plasmid finally cloned into *E. coli* cells, can be screened using antibiotic sensitivity property. Oligonucleotide site specific mutation with plasmid DNA has five times screening efficiency than with M13 vector.
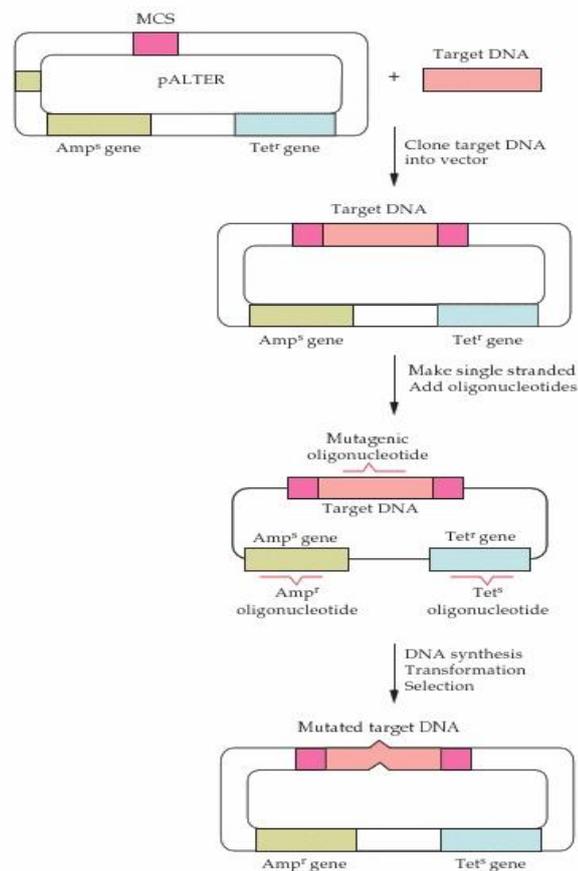

Figure 2.10 Site directed mutagenesis with plasmid DNA. Target DNA is inserted into multiple cloning site of plasmid DNA which then purifies and denatured into single stranded. Synthetic primers with desired property to change an amino acid annealed and synthesize mutant circular plasmid in the presence of DNA polymerase, T4 DNA ligase and dNTPs (picture adapted from Bernard *et al.,* 2008).

Even though many protocols of creating point mutation with plasmid DNA is in good practice in research, researchers keep pushing forward to more simplified and efficient techniques. Creating mutations with PCR amplification has recently gained large scale application in molecular biology research. In PCR amplification method, there is no need for plasmid vector, rather synthetic primer binds to single stranded target DNA. The target DNA sequence should be known and 5´ end of primers should be phosphorylated. For point mutation, the amino acid change should be inserted into middle of primer. To create deletion in DNA, the region of deletion should be the border for forward and reverse primer (figure 2.11). And to insert a sequence, the inserting sequence must be added to 5´ end of one or both primers. Synthesized single stranded DNA can be

annealed to its complementary sequence and circularized using T4 DNA ligase (Herlitz *et al.,* 1990).
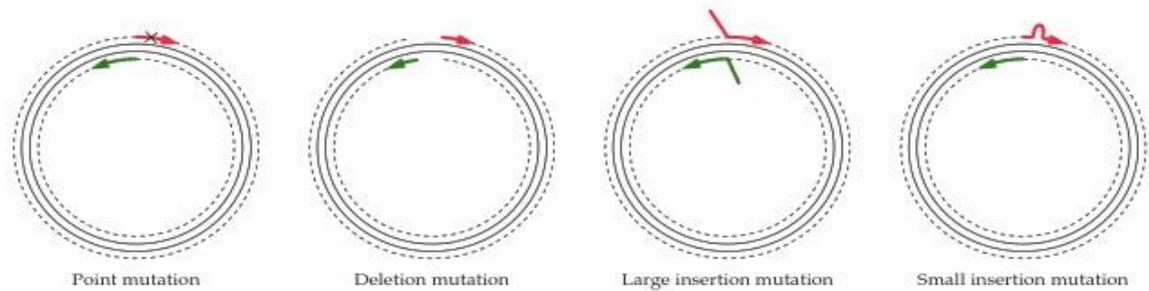

Figure 2.11 Schematic representation of PCR amplified method of creating point mutation, deletion mutation, and mutation by small or large insertion (Bernard *et al.,* 2008).

2.8   His-Tag protein purification

Protein is purified as homogenous mixture with other proteins expressed inside the cell. Even though, homogenous mixture of protein is applicable for several studies, isolation of specific protein is in high demand in modern day research. As studies to automate, simplify and optimize protein isolation emerges, there are many secondary methods available now commercially. Affinity chromatography is one of such secondary procedures, which is used in the isolation of targeting protein from homogenous mixture or protein extract. Affinity chromatography purifies protein based on polar nature of protein, ion exchange, enzyme affinity etc. Isolation based on polarity or ionic nature often leads to affinity towards other protein of same nature as well. His-tag affinity chromatography overcomes these drawbacks by combining recombinant protein technology and affinity chromatography (Michael, 2006).

Polyhistidine tag or 6xHis tag is added to the protein expressing gene in N- or C-terminal region. This kind of recombinant protein is often expressed in selective organism for optimized expression rather than origin organism. Expressed protein is isolated through normal procedure of protein expression using IPTG and destructing cells by lysosome. Final mixture contains all proteins along with His tagged protein; it is subjected for further purification with batch or column chromatography. Major principle of 6xHis tag purification is the characteristic of Histine to get pronated at low pH (4.8) and binding nature towards cobalt or Nickel ionic resin at high pH (7.9) (Paul, 1995). Crude protein mixture is allowed to drip through column filled with chelating resin which is charged by cobalt or Nickel buffer. There are different columns and buffers available commercially with specific protocols. After the specific protein binds to the column, elution buffer with respective pH promotes His tags to get protonated and to unbind the resin. Purity and concentration of protein in elution buffer can be determined using Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis or Western blotting. In this

study, 6xHis tag is added at the end of protein coding sequence as stop codon and it is expressed as extended tag in three dimensional structure of GFP. Compared to other proteins GFP purified using polyhistidine tag has higher purity (Scopes *et al.,* 1994).

2.9   Development of fluorescence experimental methods in pH studies of both *In vitro* and *In vivo* experiments

Even though there are better measuring methods for *in vitro* pH measurements, GFP variants have been extensively studied through *in vitro* experiments for pH sensitivity. Advanced analytical procedures and equipment in fluorescence studies facilitates engineering specific characteristics of GFP with more precision. With the aid of *in vitro* results, non-invasive applications of GFP increased dramatically. So far, all the mutants can be characterized as ratiometric or non-ratiometric based on their spectral property.

2.9.1   Non-ratiometric fluorescence method

The absorption spectrum of GFP is characterized by band A and band B: the equilibrium between these two states is influenced also by pH along with other factors. There is a mathematical expression which relates the concentration of chromophore population in two states with pH and spectral values. Through the given equation, sigmoidal dependence of fluorescence to pH can be seen in pH sensitive probes. First emerged GFP variants are mostly non ratiometric in absorption and emission characteristics (Kneen *et al.,* 1998). In the linear sigmoidal representation, pKa value is the pH where the emission variation reaches half to its overall change (Raneri *et al.,* 2009).

$$F(\lambda_x \lambda_e) = C_0 \left(\frac{F_B(\lambda_x \lambda_e) + F_A(\lambda_x \lambda_e) 10^{(pK-pH)}}{1 + 10^{(pK-pH)}} \right)$$

Where C_0 is the concentration of protein, F_A and F_B are molar fluorescence emission of two states A and B (Raneri *et al.,* 2009).

2.9.2   Ratiometric fluorescence method

Even though, there are many successful fluorescence analytical methods available to promote intracellular studies, the major drawback of these methods are its narrow dependency on fluorescence intensity which is further related to analyte concentration and fluorophore availability (George *et al.,* 2008). For example, in GFP, the intracellular distribution is often related to cellular permeability, homeostasis metabolism of cell, protein fusion molecule etc; thus the fluorescence is not just related to only analyte concentration. Rather, ratiometric probes report opposite changes in fluorescence, respective to analyte concentration thereby reducing the drawbacks in intracellular fluores-

cence studies such as photo bleaching, concentration of analyte independent, reliability and dynamic reporting from various cell compartments etc (Grynkiewicz *et al.*, 1985). Below equation explains the independency of emission or absorption ratio to concentration of protein which replaces F_A and F_B notations in non ratiometric equation.

$$\frac{F(\lambda_{x1}\lambda_{e1})}{F(\lambda_{x2}\lambda_{e2})} = \left(\frac{F_\infty(\lambda_{x1}\lambda_{e1}) + F_0(\lambda_{x1}\lambda_{e1})10^{(pK-pH)}}{F_\infty(\lambda_{x2}\lambda_{e2}) + F_0(\lambda_{x2}\lambda_{e2})10^{(pK-pH)}}\right)$$

Where F_∞ is higher molar fluorescence asymptote and F_0 is lower molar fluorescence asymptote (Ranieri *et al.,* 2009).

2.9.2.1   Basic characteristics of ratiometric pH probe

Chromophore of GFP lies at center of the protein structure protected by its β-barrel sheets. Arg96 and Glu222 are major amino acids in formation of chromophore (Sniegowski *et al,* 2005). There are some other amino acids and few water molecules surround the chromophore and create a hydrogen network, which acts as donor and acceptor or proton or polar groups to the chromophore. Those amino acids include residues Gln69, Gln94, His148, Thr203 and Ser205 (Brejc *et al.,* 1997). The spectral and photochemical property of GFP can be modulated largely by changing these amino acids. The excitation spectrum of GFP is characterized by its two bands namely band A and band B which are related to ionization characteristics of chromophore. Phenol and imidazolinone ring of Arg96 exhibits ionization reaction through hydrogen network. In solution, the equilibrium between these states is largely influenced by factors such as ionic concentration, protein concentration, enzyme activity, temperature and pH (Ranieri *et al.,* 2009).

The major mechanism behind creating ratiometric pH-GFP probe is due to the nature of ionization mechanism between two forms of chromophore respective to proton concentration. Interestingly, the intensity of GFP emission below pH 5.5 (460nm) is inversely converted to the emission above pH-7.5 (500nm) in ratiometric probes. It is further characterized that emission below pH 5.5 favored by protonated chromophore and emission above pH 7.5 favored by deprotonated or anionic chromophore (Ranieri et al., 2009). Mostly, emission of protonated chromophore is very less in GFP spectrum due to the mechanism called excited state proton transfer (ESPT) (Voityuk et al., 1998; Bonsma *et al.,* 2005), which results in lower intensity of emission at 460 nm of spectral range. Upon inter conversion of bands A and B at different pH, GFP exhibits a common point in the spectrum where the spectrum from all pH range coincides is called isobestic point. Even though, inter converting nature of band is the only essential feature in ratiometric analysis, presence of isobestic point creates an ideal ratiometric probe (Srivastava et al., 2007; Wang et al., 1997). Non ratiometric analysis of spectrum depends on the intensity of emission relative to pH and protein concentra-

tion, whereas, ratiometric analysis depends only on the ratio of emission peak value between protonated and deprotonated choromophore.

The mechanism behind pH dependent behavior of GFP were first explained by considering only single protonation site which is situated at Arg96, but later proposed two-site model fits most of the GFP mutants available now (Bizzarri *et al.,* 2007). Instead of emission dependency on single protonation site of Arg96, two-site model proposes greater suitability to spectral behavior of many mutants, which states that the presence of one more ionization site favors neutral state population of chromophore and also influences the equilibrium of GFP along with anionic and cationic state. Structural analysis shows influence of His148 and Glu222 in equilibrium between neutral state and anionic state of chromophore (Bizzarri *et al.,* 2007). One way or another, emission of GFP shows considerable difference in response between low and high pH, which elaborates the potentiality of GFP variants in live cell pH signaling.

2.10   Future applications

Fluorescence has been employed as spectroscopic, imaging and molecular probing techniques in last few decades (Alexander *et al.,* 2010). Due to its nano time scale, it demands the sophisticated methods for measurement and data processing. Unlike last century, scientists from various backgrounds combining and making way to find solutions for complex problems. Due to the result, it has gained importance in biotechnology, molecular biology, biophysics and biochemistry research fields. Consecutively, GFP become the first biomolecule which has larger variety of applications in fluorescence studies. GFP variants have been evolving dynamically along with new molecular techniques and experimental procedures.

The spectral diversity of GFP variants is useful in FRET studies which explain the interaction of GFP with other molecules (Sekay *et al.,* 2003). Even though the spectral range of some GFP variant is not suitable for intracellular studies due to photo toxicity, they can be useful in FRET experiments (Sekay *et al.,* 2003). Some of the latest applications of GFP have been reported that GFP can be used as fluorescent timers which changes emission from green to red after some time of expression: this color change can be used to study expression and degradation rate (Terskikh *et al.,* 2000). Photoactivable GFPs can be tracked its movement inside the cell thorough photo bleaching which is highly important in cancer studies (Lippincott-Schwartz *et al.,* 2003; Chudakov *et al.* 2003). Furthermore, fusion studies of GFP variants with other molecules are useful in studying ionic interactions, movement in cellular environment and apoptosis (Xu, X. *et al,.* 1998). Likewise, apart from the aim of this study, sfGFP variant created through this study could have significant property suitable for these applications.

3. MATERIALS AND METHODS

3.1 Materials Used

E. Coli XL1 blue strain from Stratagene was used throughout the study for cloning and expression of recombinant protein. The sequence of sfGFP (Pédelacq *et al.,* 2006) was obtained from the spring 2011 distribution of biobrick biological parts registry (iGEM) (number: BBa_I746907, http://parts.igem.org/partsdb/get_part.cgi?part=BBa_I746907). Agarose, Yeast extract, Nacl, glucose and tryptophan used for LB medium are from Sigma-Aldrich. For protein purification: Sigma 4K15 centrifuge was used, protease inhibitor cocktail tablets were from Roche, Germany, and Soniprep plus150, from MSE, USA was used for sonication. Lysozyme enzyme was from Sigma, Canada. Antibiotics Chloramphenicol and Ampicillin were from Sigma-Aldrich. The enzymes Hind III, XbaI, T4 DNA ligase, Taq DNA polymerase and buffers 1X tango buffer and 10X ligation buffer were from Fermentas. Primers were ordered from Thermo Fisher scientific, Germany. Plasmid and gel extraction kit was from Fermentas.

Protein purification His-bind resin was from Novagen and columns from Amersham biosciences. Electrophoresis setup used was from Bio-Rad laboratories. For fluorescence measurements Fluorolog from Jobin-Yvon-spex was used. Absorbance was measured with Schimadzu UV-Vis, NIR spectrophotometer. Results of fluorescence and absorbance were analyzed in Orogin8 software. All the reagents used for experiments were analytical grade.

3.2 Site Directed Mutagenesis

The major aim was to create different combinations of mutations to identify the best behaving strain. The amino acid changes selected was T203C, H148G, C48S and T203Y. Mutation was created using overlap extension method using polymerase chain reaction. The primers are listed in the table 3.1. The mutations are carried out by PCR in thermocycler. The PCR parameters are as follows: initial denaturation at 94°C for 2 minutes, 30 cycles of 1 minute denaturation at 94°C, 1minute annealing at 53°C, 1 minute elongation at 72°C, 10 minute extension at 72°C and final sample cooling temperature 4°C. For protein screening Histidine tag was introduced in Hind III restriction site. 5µM primer stocks were prepared from 100µM stocks of ordered primers and stored at -

20°C. The primers sfb12c is forward primer and Reb11k is reverse primer for complete coding sequence of 27kDa protein.

Primer name	Sequence	Description
sfb12c	5'gagttctagagaaggagatatacatatgcgtaaaggcgaagagctgttc3'	Sense primer, Xba I site
Reb11k	5'tctactcgagatccgtgacgcagtagcggt 3'	Antisense primer, HindIII site
sfb12h	5'-tcactatctgagctgccaaagcgttctg-3'	Antisense primer to create T203C
sfb12i	5'-cagaacgctttggcagctcagatagtga-3'	Overlaps sfb12h
sfb12j	5'-aacagcggcaatgtttacatcaccgccgat-3'	Antisense primer to create H148G
sfb12k	5'-atcggcggtgatgtaaacattgccgctgtt-3'	Overlaps sfb12j
sfb12l	5'-acgctgaagttcatcagcactactggtaaac-3'	Antisense primer to create H148G
sfb12m	5'-gtttaccagtagtgctgatgaacttcagcgt-3'	Overlaps sfb12l
sfb12p	5'-tcactatctgagctatcaaagcgttctg-3'	Antisense primer to create T203Y
sfb12q	5'-cagaacgctttgatagctcagatagtga-3'	Overlaps sfb12p

   Plasmid from superfolder GFP strain was isolated and purified using Fermentas plasmid extraction kit. Isolated plasmid (conc.221ng/µl) was used as template for initial PCR which is to create T203C using primers sfb12c, sfb12h, sfb12i and Reb11k. The PCR reaction mixture concentration as follows: template 0.5µl, 5x buffer 10µl, forward and reverse primers 5µl, dNTPs 1.25 µl, DNA polymerase 0.5µl and Millipore water 27.75µl.

3.3 Cloning and sequencing

The plasmid vector used for cloning was SpT5.10/pAK400cB. PCR products were confirmed for its size using 1% agarose gel electrophoresis at 150 volts for 35 minutes and bands were purified using Fermentas gel extraction kit. Both plasmid and DNA was restricted using restriction enzymes Xba I and Hind III. The restriction mixture was purified DNA 20µl or plasmid 13µl, XbaI 3µl, Hind III 1.5 µl and milli-Q water for total volume of 30µl. Restriction mixture was kept at 37 °C for 3-4 hours. Then, restriction mixture was purified from right band of electrophoresis and purified. Overnight ligation was carried out with 8µl plasmid, 16µl PCR product, 3µl 10X ligation buffer and Milli Q water for total volume 30µl at room temperature.

Electro-competent *E.Coli* XL1-Blue cells were prepared using standard procedure (Sambrook *et al*, 1990) and stored at -40°C. Ligation mixture was kept at 65°C in order to terminate. Electroporation cuvettes were pre cooled. Transformation was carried out with 2µl of ligation mixture in BioRad Micropulser electroporater (BioRad, USA). 1ml of LB medium with nutrients and chloramphenicol added to transformation cuvettes and incubated at 37°C for 1 hour. Then cultures were plated in LA agar plates with antibiotic chloramphenicol, colonies were allow to grow overnight at 37°C. Colonies were screened based on antibiotic resistance and restriction reaction of plasmid DNA. Strains with correct restriction band size were selected and confirmed thorough sequencing. All strains were sequenced from Macrogen, Korea.

3.4 Protein Production and Screening

The strains which were confirmed from sequencing were used for protein production. Cells were cultured in 1000ml LB medium with 25µg/ml chloramphenicol, 0.4% glucose at 300 rpm. Along with sfGFPp1, sfGFPp2, and sfGFP p3, deGFP and original sf GFP strain were also used throughout studies for comparison. When optical density reached 0.6, 1mM of isopropyl β-D-thiogalactoside (IPTG) added to the culture and temperature was reduced to 30°C. After 4 hours of protein production induction temperature was reduced to 20°C and grown overnight.

Cultures were kept on ice immediately after taken from incubator. Cells were pelleted in 250ml centrifuge bottles by centrifuging at 4°C for 6000 rpm and 20 minutes. Resuspended pellet in 3 times its weight of 1X binding buffer (40mM imidazole, 4M NaCl, 160mM Tris-HCL, pH 7.9). Added lysozyme (100mg/1 liter culture) and protease inhibitor cocktail (500µl from 1ml of dissolved tablet) directly to the suspended cells and incubated for 30 minutes. Samples were subjected to sonication for 3 times

with each 1 minute and cooled in between. Finally, centrifuged for 30 minutes at 10000 rpm and collected supernatant in fresh sample tubes. In order to filter debris further incubated with 1µl of bezonase nuclease for 30 minutes and filtered through 0.2 mm membrane filters.

3.4.1   Column purification

Proteins were purified by His-bind resin from Novagen. All the column purification steps were given by manufacturer. Firstly, column was prepared by adding 4 ml of novagen, which was washed with 6 ml of de ionized water, 10ml of 1X charge buffer (400mM $NiSO_4$) and 6 ml of 1X binding buffer (40mM imidazole, pH 7.9). Samples were applied to the column and allowed to drip by gravity. After samples drips off completely, column was washed with 20 ml of wash buffer (390mM imidazole, 4M NaCl, 160mM Tris-HCL, pH 7.9). Samples were collected in each step for PAGE analysis. Elution buffer (800mM imidazole, 2M NaCl, 80mM Tris-HCL, pH 7.9) which elutes only His-tagged protein added to column and collected until colored protein elutes. Column is stored in strip buffer for reuse. Purified protein was collected in glass sample tubes and stored at 4°C.

Concentrations of purified protein for *in vitro* analysis were measured through absorbance spectrum in Nano drop instrument from Thermo Scientific, USA. Protocol for measurement was given by manufacturer. 1µl of purified sample was used to measure with elution buffer as blank. In nano drop software, measurements were taken using pre-programed option 'proteins and labels' at absorbance wavelength 488nm.

3.5   Buffer preparation for *In vitro* measurements

Buffers were selected based on their less toxic characteristics with biomolecules in desired pH level. The buffers are MES anhydrate (pH 5.5 to 6.5), HEPES (pH 6.6 to 7.5) and TRIZMA base (pH 8.5 to 9.0). All buffer substances were supplied from SIGMA and concentration was 75mM. Different pH points ranging from 6.0 to 9 were prepared by adjusting with 3M NaOH and 37% HCL.

3.6   Steady-state spectroscopy

3.6.1   *In vitro* measurements

Concentration of protein samples (sfGFPp1, sfGFPp2, sfGFP p3, deGFP and original sfGFP) were measured using nanodrop machine and adjusted each sample to approximately near 0.800ng/ml. Fluorescence and absorbance measurements were made with mixture of 1400µl of buffer and 300µl of protein. For *in vitro* measurement protein was

measured with pH 6, 6.25, 6.5, 6.75, 7, 7.25, 7.5, 8, 8.5 and 9. UV-VIS spectrophotometer (shimadzu) was used to measure absorbance. Consecutively, fluorescence measurements were made with same samples in Fluorometer Fluorolog-3-111 (ISA-Jobin Yvon, France). Emission was recorded between 400nm to 650nm with excitation wavelength 400nm and slit with 1:1. Measurement results were saved in both excel and origin8 file format.

In the stability experiment of sfGFP at 70°C, each protein sfGFP, sfGFPp1 and deGFP suspended in pH 7.25 were used. Sample prepared with 1400µl of buffer and 300µl of protein. Emission was recorded for every 3 minutes up to 30 minutes. Temperature was controlled using hot water circulation which maintains the sample holder at elevated temperature.

3.6.2 *In vivo* measurements

The strains sfGFPp1, sfGFPp2, sfGFP p3, deGFP and original sfGFP were culture with respective antibodies in 6 ml culture tubes at 37°C for 6-8 hours. Then added 1mM of IPTG and allowed to grow overnight at 20°C at 300 rpm. Cells were pelleted by centrifuging at 12000 rpm for 10 minutes. In order to make pH change inside the cell equivalent to pH of medium, cells were resuspended in pH buffer 7.5 with 10µM cccp (Carbonyl cyanide 3-chlorophenylhydrazone) for 30 minutes and then suspended in respective pH buffers. cccp disrupts the protonosphere and polarity of cell wall and facilitates passive diffusion of H^+ ions inwards the cell. Finally, cells were suspended in 3 ml of respective buffer with final optical density of 0.6. Fluorescence measurements were made with same samples in Fluorometer Fluorolog-3-111 (ISA-Jobin Yvon, France). Emission was recorded between 425nm to 625nm with excitation wavelength 400nm and slit width 2:2.

4.   RESULTS

Results were presented as different parts in this section which include construction of mutants, protein production, *in vitro* fluorescence measurements, *in vivo* fluorescence measurements, stability at 70°C and finally fluorescence at 70°C. The best strain with pH sensitive characteristics was selected from *in vitro* fluorescence measurements to continue *in vivo* and higher temperature experiments. Totally three mutants were constructed, they are sfGFPp1, sfGFPp2 and sfGFPp3 out of that sfGFPp1 had shown pH sensitive characteristics.

4.1   Construction of mutants

The mutations were created by site directed mutagenesis (SOE-PCR method). Transformed colonies were screened based on antibiotic resistance and restriction digestion conformation by agarose gel electrophoresis. While plating all strains in agar plates, sfGFPp1 showed higher brightness when viewed on blue light (Not shown in results). Transformed colonies are sent for sequencing in Macrogen (Korea). Sequencing results were analyzed using vector NTI suit. Results are shown in figure 4.1 and the amino acid change is shown compared to original superfolder GFP. For convenience, mutants were named as follows, sfGFPp1 (T203C), sfGFPp2 (T203C, H148G, C48S) and sfGFPp3 (C203Y, H148G, C48S).

Figure 4.1 Amino acid change of each mutant compared to superfolder GFP. sfGFPp1 (T203C), sfGFPp2 (C48S, H148G, T203C), sfGFPp3 (C48S, H148G, T203Y)

4.2   Protein production and concentration

All the strains were grown at 500 ml conical flask except deGFP which was cultivated using 1000 ml bioreactor. Proteins were isolated using 6×His tag and Sephadex resin (Novagen) for affinity column purification. Purified samples were quantified using SDS-PAGE and purity was identified as >95%. Concentration values are given in Table

4.1 and 4.2, given stock solution was directly used for measurement with respective pH buffer.

Table 4.1 Concentration of proteins for in vitro measurements at room temperature (23°C).

Protein	Stock concentration for measurement (ng/µl)
sfGFPp1	0.683
sfGFPp2	0.399
sfGFPp3	0.092
sfGFP	1.817
deGFP	0.155

Table 4.2 Concentration of proteins for in vitro measurements at 70°C.

Protein	Total Concentration of purified sample (ng/µl)	Stock concentration for measurement (ng/µl)
sfGFPp1	1.771	0.719
sfGFP	1.767	0.709

4.3   *In vitro* Fluorescence and Absorbance measurments

Absorbance measurements were taken from UV-VIS spectrophotometer (shimadzu) and fluorescence measurements from Fluorometer Fluorolog-3-111 (ISA-Jobin Yvon, France). Both measurements were taken with the same sample. For *in vitro* measurements at room temperature (23°C), the sample volume of 300 µl from stock of each mutant purified protein was added to 1200 µl of respective pH buffer. Therefore the concentrations of each protein differ between each strain which is given in table 4.1. Absorbance was measured at 400 nm and emission was recorded between 410 to 650 nm for all strains with slit width (1, 1) except for sfGFPp3 (slit 2, 1); integration time 0.1 seconds. *In vitro* experiments were repeated for five times for sfGFPp1, four times for deGFP and sfGFP, three times for sfGFPp2 and sfGFPp3.

Absorbance spectrum of all strains is characterized by two bands with peaks at 400 nm and above 500 nm. But at pH below 4.8, absorption from deprotonated chromophore diminished completely. Absorbance and emission spectrum of all strains retained interconverting nature between two states of chromophore.

Spectroscopic measurements of sfGFPp1 displayed emission maximum at 510 to 512 nm for low pH and between 514 to515nm for high pH.; absorption peaks at 400 nm and 500 nm. Figure 4.2 shows fluorescence and emission spectrum of mutant sfGFPp1. Emission of neutral chromophore at 400 nm is inversely converted to the emission at 515nm. Interestingly, the common isobestic point is observed near 490 nm wavelength of absorption spectra of sfGFPp1 is an added value for ratiometric fluorescence studies. *pKa* value of sfGFP1 had calculated from pH titration curve as respective pH to 50% fluorescence. sfGFPp1 showed *pKa* as 6.45±0.15, experiments were repeated for five times for *in vitro* measurements of sfGFPp1, *pKa* value is the average of all the experiments. Most of the spectral characteristics are similar to sfGFP original strain, but due to mutation Thr203 to Cysteine it shows higher dependency of fluorescence between pH 6 to pH 7.5. Compared to other strains sfGFP1 shows higher intensity of fluorescence.
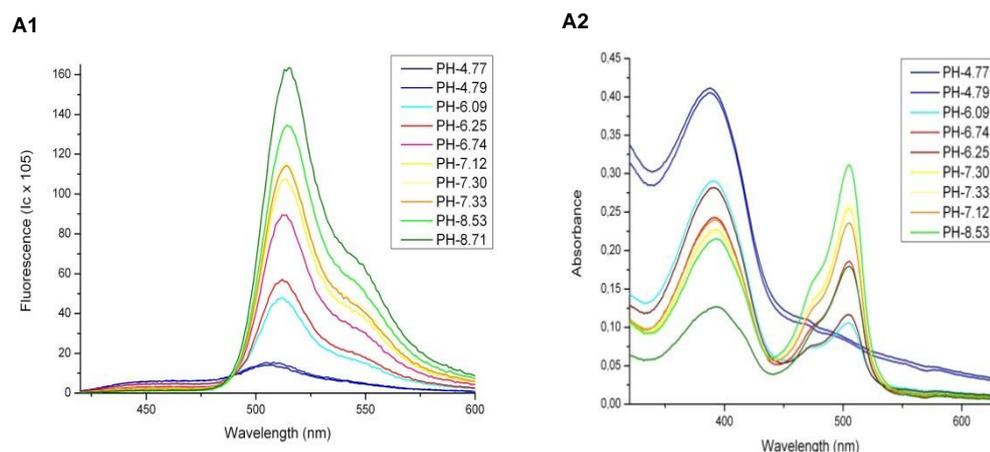

Figure 4.2 Emission (A1) and absorption (A2) spectrum of sfGFPp1-Mutations T203C: Emission maximum between 510 to 515 nm, Absorption maximum at 400 nm and 500 nm.

sfGFPp2 shows emission maximum between 508 to 512 at low pH and 512 to 516 at pH >8. Figure 4.3 shows the emission and absorption spectrum of sfGFPp2. In contrast with sfGFPp1, it does not have a common isobestic point between anionic and neutral chromophore; also the second absorption band is weak above 500nm. In other words, absorption of anionic chromophore is notable only at higher pH with low intensity. It gives confirmation that the presence of H148G and C48S mutations strongly alters the spectral properties of superfolder GFP both in absorption and emission.
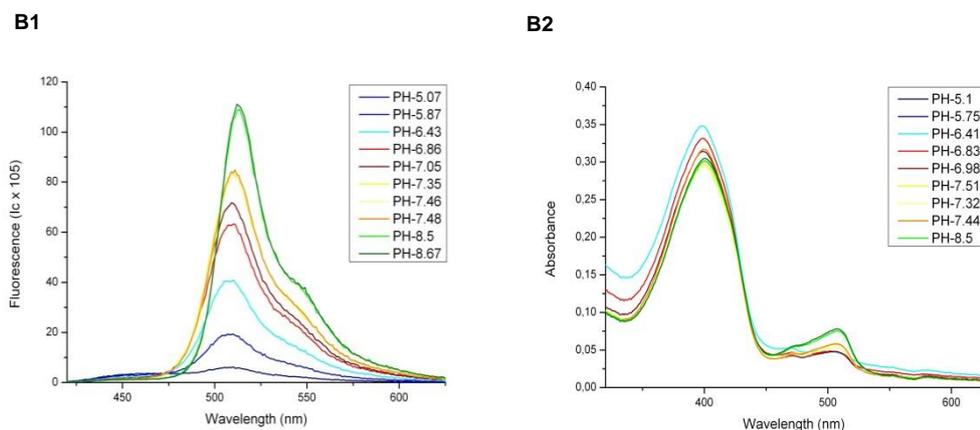
Figure 4.3 Emission (B1) and absorption (B2) of sfGFPp2-Mutations T203C, H148G, and C48S: Emission maximum between 508 to 516 nm, Absorption maximum at 400 nm and 500 nm.

sfGFPp3 has created with mutation which shifts the spectral range of GFP from green to yellow region. Figure 4.4 shows the emission peak is at 523 nm at the yellow region of the spectrum. Like sfGFPp2, absorbance of sfGFP3 also diminished at anionic absorbance region (>500 nm). Fluorescence of sfGFPp3 has emission maximum between 510-514 for pH less than 7.5 and at 523 nm for pH above 8. This result confirms the previous research conclusions about the amino acid change T203Y (Chan *et al.,* 2001; Ranieri *et al.,* 2006). Despite the change in spectral emission range, sfGFP3 doesn't have any significant property of pH sensitivity.

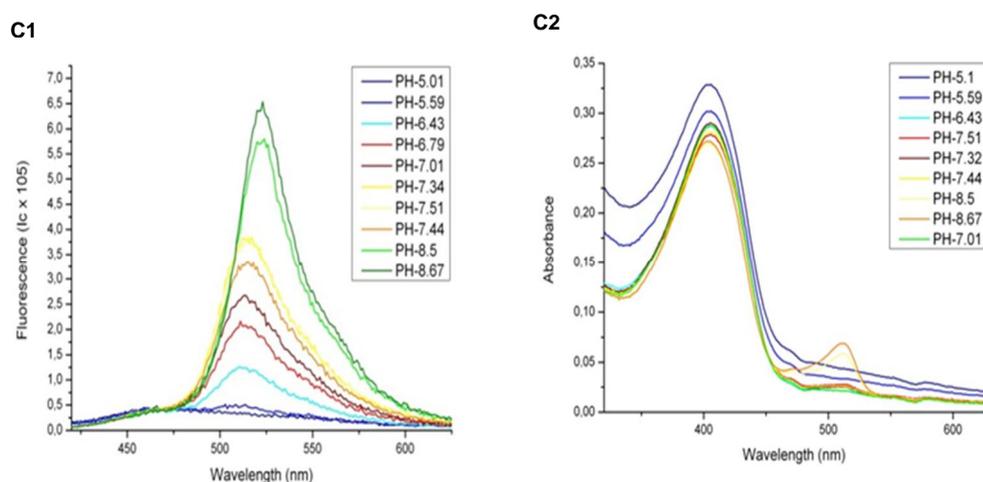**Figure 4.4** Emission (C1) and absorption (C2) of sfGFPp3-Mutations C203Y, H148G, C48S: Emission maximum between 510 to 523 nm, Absorption maximum at 400 nm and 500 nm.

Apart from stability and folding properties, spectral characteristics of sfGFP have significant pH relative changes. Emission peaks were observed between 508 and 512 at low pH and between 513 and 515 at pH above 7.5 (Figure 4.5). sfGFP reported to

have *pKa* 5.5 (Nathan *et al.,* 2011). The difference between sfGFP1 and sfGFP is the fluorescence intensity change in the physiological range between pH 6.5 to 7.5.
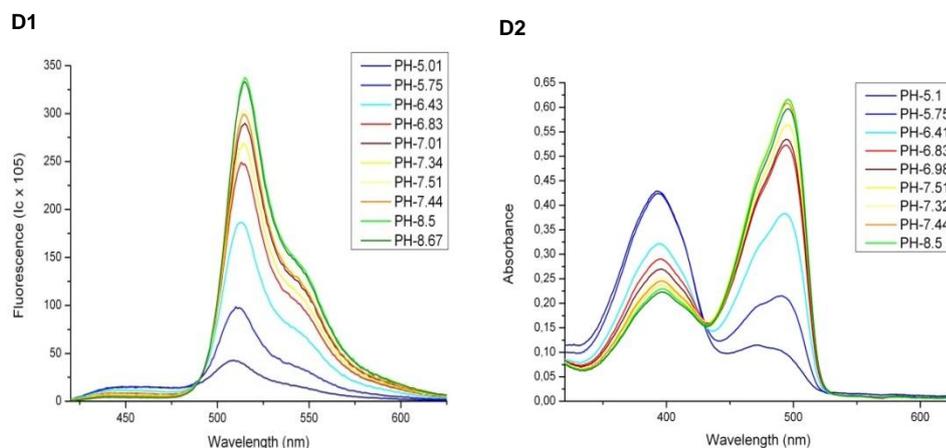

Figure 4.5 Emission (D1) and absorption (D2) of sfGFP: Mutations compared to wtGFP (S30R, Y39N, Y145F, I171V, A206V along with cycle3 mutations F99S, M153T, V163A and EGFP mutations SF64L and S65T): Emission maximum is between 508 nm to 515 nm.

For comparison of results, we have used deGFP strain as control throughout the study. Dual emission spectrum characteristics of deGFP are well-matched with results presented by George and his group (George *et al.,* 2002). The presence of S65T along with deGFP mutation combinations strongly favors neutral chromophore. As a result, dual emission peak is observed at 460nm and 515nm (figure 4.6); pKa of deGFP is close to neutral (7.2±0.1) (George *et al.,* 2002). Even the absorbance of neutral chromophore is higher compared to absorbance by anionic choromophore. Reportedly, deGFP with two emission peaks is a better ratiometric probe at 37°C, but the fluorescence and expression of protein is observed to be very low both in *in vitro* and *in vivo,* respectively.
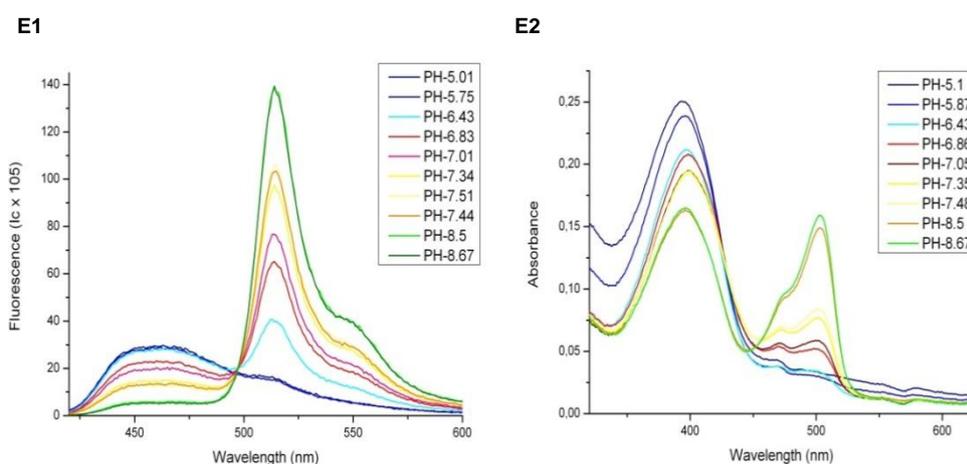

Figure 4.6 Emission (E1) and absorption (E2) of deGFP: Mutations compared to wtGFP (S65T, C48S, H148C, and T203C): Emission maximum at 460 nm and 515 nm, Absorption maximum at 400 nm and 500 nm.

Even though, the presence of S65T mutation favors deprotonated state of chromophore in GFP, the combination of H148G and C48S mutations suppress the absorbance properties of GFP above 500 nm. From the above fluorescence and absorbance results, it is concluded that sfGFPp1 is better strain by considering common isobestic point, higher fluorescence intensity, and absorption above 500 nm. sfGFPp1 was selected as best strain among three strains for further analysis and experiments along with deGFP and original superfolder.

In ratiometric experiments, *pKa* and quantum yield of substance is not depends on its concentration in the solvent, rather it depends on the difference in excitation and emission of two states of chromophores. An ideal ratiometric indicator can be identified from graphical representation of those ratios against respective pH. Calibration curve of emission ratio against pH is given in Figure 4.6. As stated, only sfGFP1 mutant is taken from this step onwards for further analysis. There was no significant change in fluorescence ratio below pH 5.5 and above pH 8.0. *pKa* (50% maximum) for sfGFPp1 and deGFP is 6.45±0.15 and 7.2±0.1 respectively (figure 4.7). Presence of Cysteine at 203rd position in sfGFPp1 makes the protein to display more variation in fluorescence ratio at physiological pH range (pH 6 to 7.5).

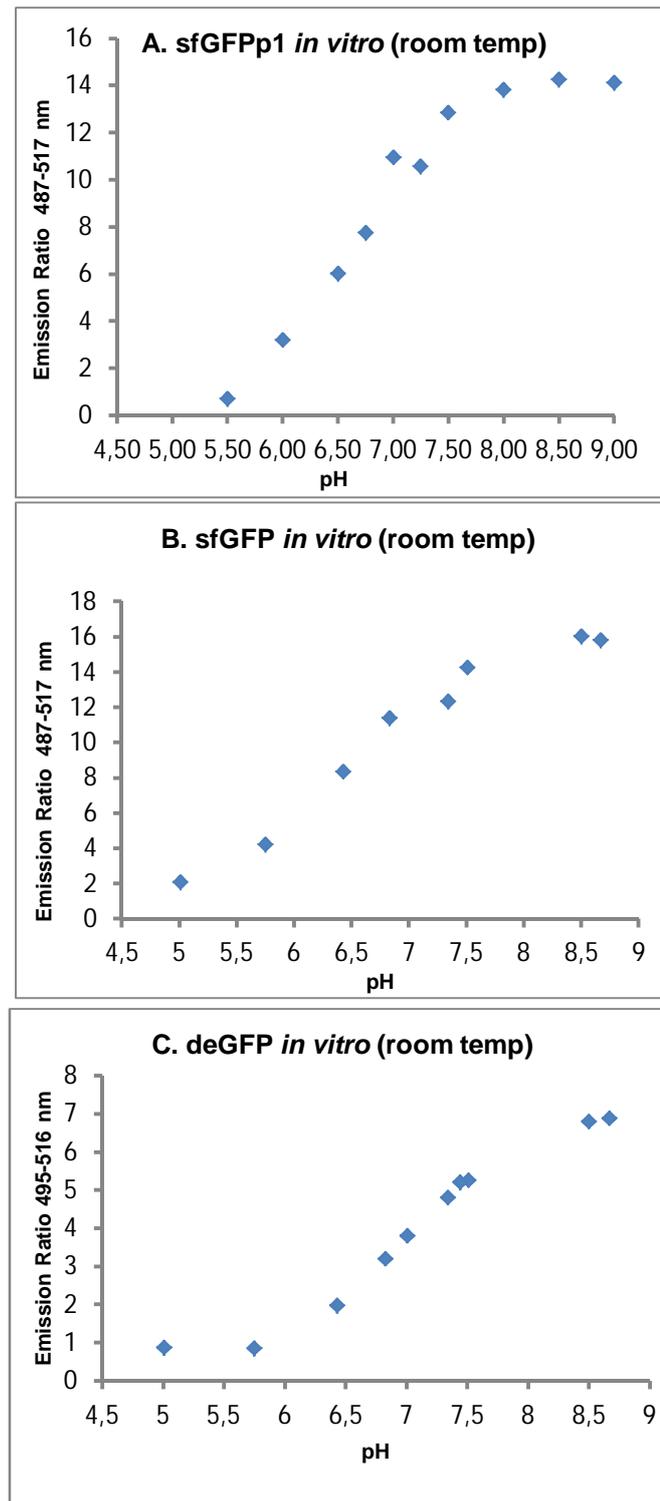**Figure 4.7** Calibration curve of emission ratio against pH: Calculated from the ratio of fluorescence maximum of band B to fluorescence at isobestic point (wavelength is given in vertical axis title). A. sfGFP1 shows difference in fluorescence from pH 4.79 to 8.53 with pKa 6.45±0.15. B. sfGFP original strain emission ratio curve versus different pH. C. deGFP shows better response between pH range 5.75 and 8.0 with *pKa* 7.2±0.1.

4.4 *In vivo* fluorescence

Fluorescence of cells was measured between 400 nm to 650 nm with excitation wavelength at 400 nm; Slit width was 2, 2 and integration time 0.1 s. Fluorescence by control *E coli* XL1 blue strain also measured in order to neglect emission by cells rather emission from protein inside it. Fluorescence spectra of *in vivo* measurements shows normalized emission by subtracting emission values of control *E coli* XL1 strain in respective pH.

As reported earlier (George *et al.,* 2002), expression of deGFP is low inside the cell, which leads to weak emission compared to sfGFP. As a result, emission above 500 nm gives noisy signal; moreover, opposing behavior of anionic to neutral chromophore at 515 nm and 460 nm is not seen (Figure 4.8). Contradictory behavior of two states of chromophore is essential for ratiometric analysis, therefore, it is not possible to make emission ratio graph relative to pH for deGFP. But emission peaks were observed at both states of chromophore.
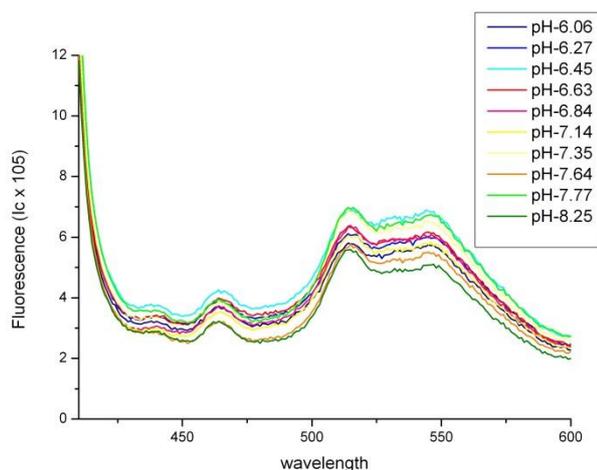
Figure 4.8 Emission of deGFP by excitation at 460 nm and emission between 375 nm and 600 nm. Emission peaks at 460 nm and 515 nm with noisy signal. Instead of interchanging behavior of anionic and neutral chromophore emission, emission spectrum gives linear response at both spectral range.

Unlike deGFP, sfGFPp1 expressed in higher concentration inside the cell, as a result, intensity of fluorescence is high and reliable degree of response relative to pH. Even though, there is very less deviation of fluorescence among different pH, the presence of isobestic point between two states makes protein as *in vivo* ratiometeric probe. Emission patterns of *in vivo* results correlate with the results of *in vitro* experiments with emission maximum at 515 nm for both sfGFP (Figure 8) and sfGFPp1 (Figure 4.9) . Even though, emission patterns of sfGFP and sfGFPp1 are same, sfGFPp1 mutant shows increased degree of fluorescence variation respective to pH at protonated chromophore emission (460 nm).

Figure 4.9 Emission spectra of sfGFPp1(A) and sfGFP(B) *in vivo* at excitation wavelength 460 nm. PCR9-Mutations T203C.Emission of protonated chromophore has hig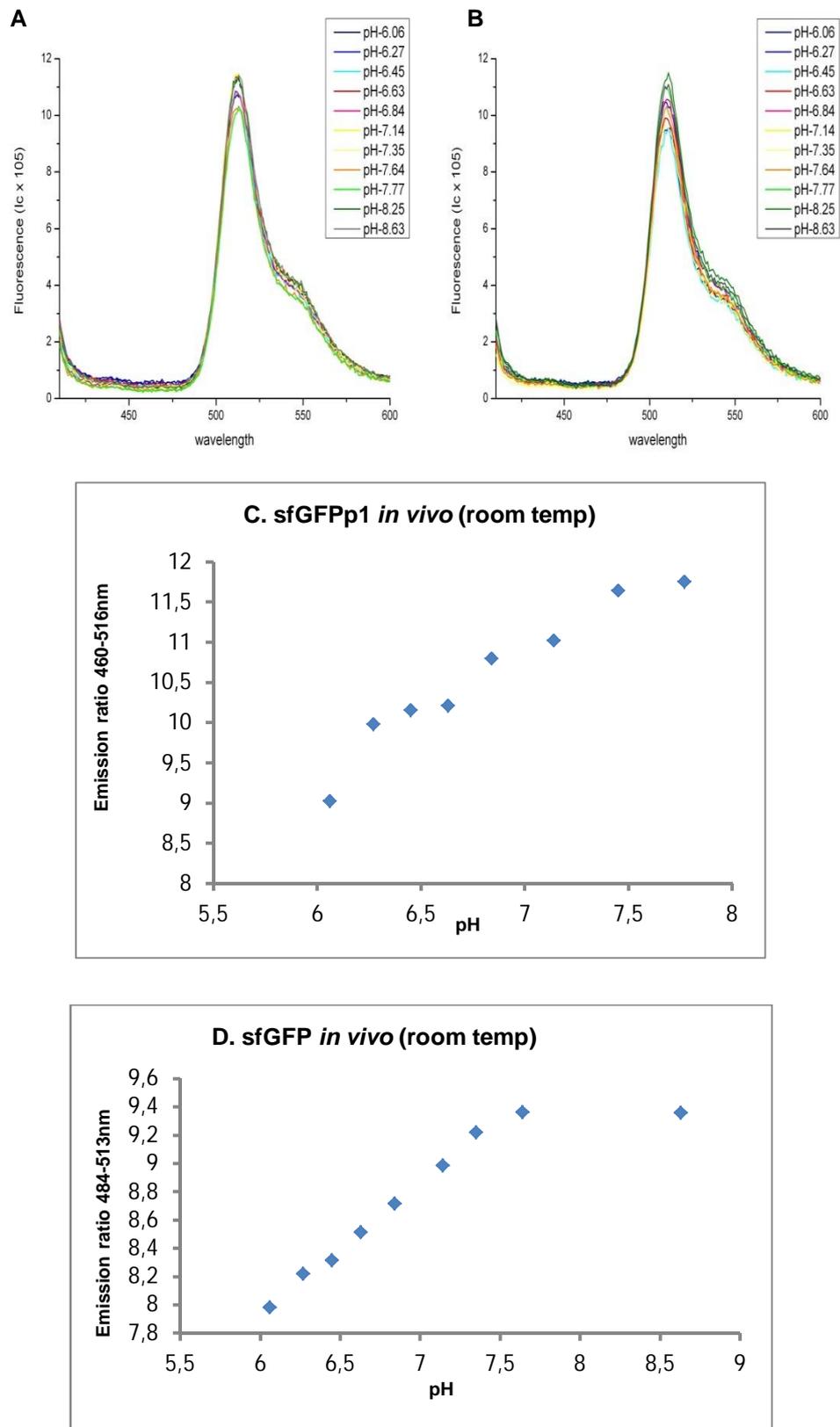her varies respective to pH in sfGFPp1 than sfGFP. Emission ratio of sfGFPp1(C) and sfGFP(D) respective to pH. Room temperature was 23°C.

4.5   Stability of sfGFP, deGFP and sfGFPp1 at 70°C *in vitro*

Emission of all proteins was measured at pH 7.25 and temperature 70°C. Measurement was taken for every 3 minutes using same sample for each protein sfGFPp1, sfGFP and deGFP in pH 7.25 by maintaining at 70°C for 35 minutes. In spite of equal concentration, superfolder GFPp1 had higher intensity than sfGFP and deGFP, ratio of intensity change is normalized to fit in common graph (figure 4.10). After approximately 6 minutes sample of deGFP precipitated and emission became very weak, however, both superfolder GFP showed good stability over 30 minutes.
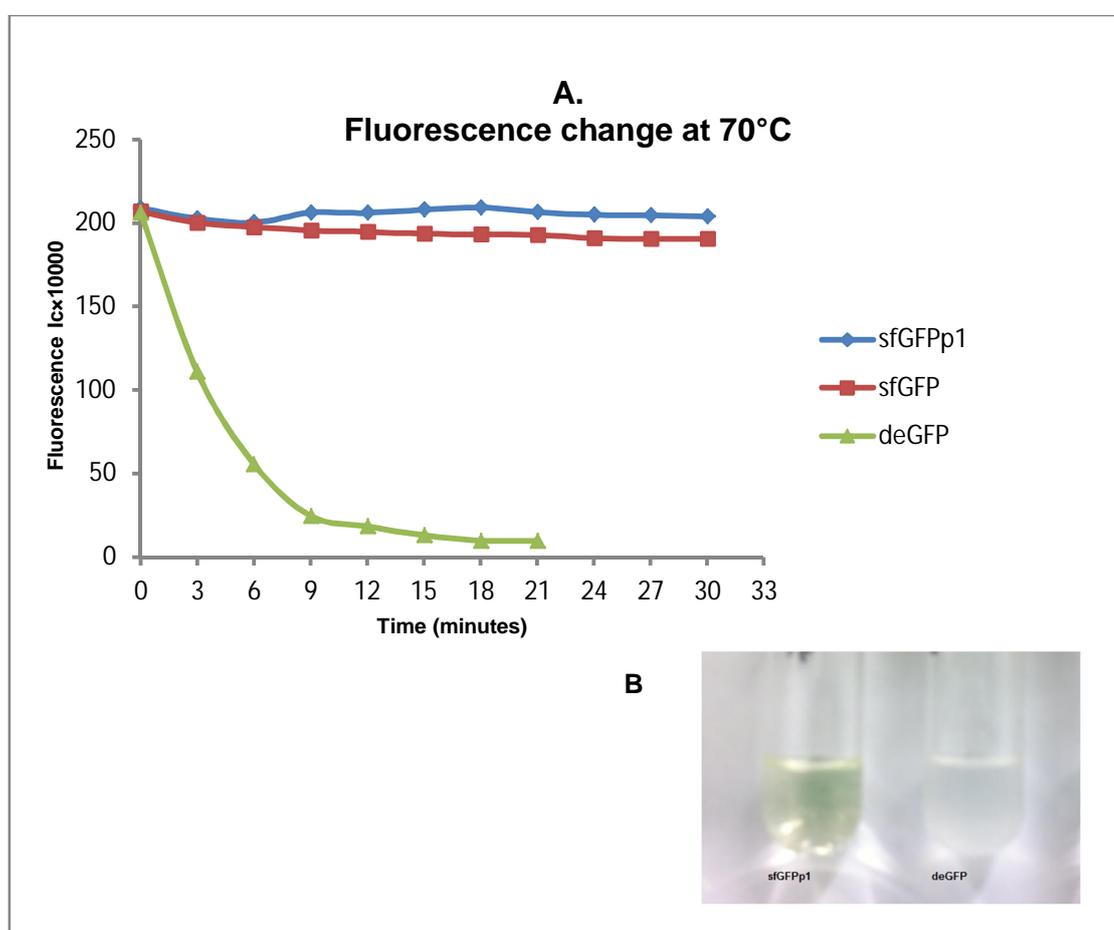
Figure 4.10 A. Stability of sfGFP, deGFP, sfGFPp1 at 70 degree in vitro. Normalized emission value for every 3 minutes of sfGFPp1 (circles), sfGFP (square) and deGFP (triangle). Intensity of sfGFPp1 has higher value than other proteins; emission of deGFP has dropped completely after 6 minutes and got precipitated.   B. deGFP solution precipitates and becomes white after 6 minutes at 70°C, whereas sfGFPp1 maintains the solution same after 30 minutes.

4.6   *In vitro* emission at 70°C

Concentration of protein in elution buffer was made equal based on nano drop UV-Vis Spectrophotometer measurement for both sfGFP and sfGFPp1. 400 µl of protein is added to 1300 µl of respective buffers. During measurement, each sample is heated up to 70°C by flowing hot water around cuvette holder and the temperature of sample is monitored. When the temperature reaches 70°C emission was recorded with excitation wavelength 400 nm. Even though, concentration of protein in each measurement is same, sfGFPp1 shows higher intensity of fluorescence by anionic chromophore than superfolder GFP, emission maximum was shown at 515 nm (Figure 4.11 A and B). Ratiometric calibration curve of sfGFPp1 (Figure 4.11D) and sfGFP (Figure 4.11C) at 70°C in *in vitro*. Emission of anionic chromophore has more dependency towards pH range 6 to 7.0. Emission of sfGFPp1 shows higher variation between pH 6.0 to 7.5 compared to sfGFP. Moreover, emission ratio maintains about two-unit change linearly between pH 6.0 to 7.15.
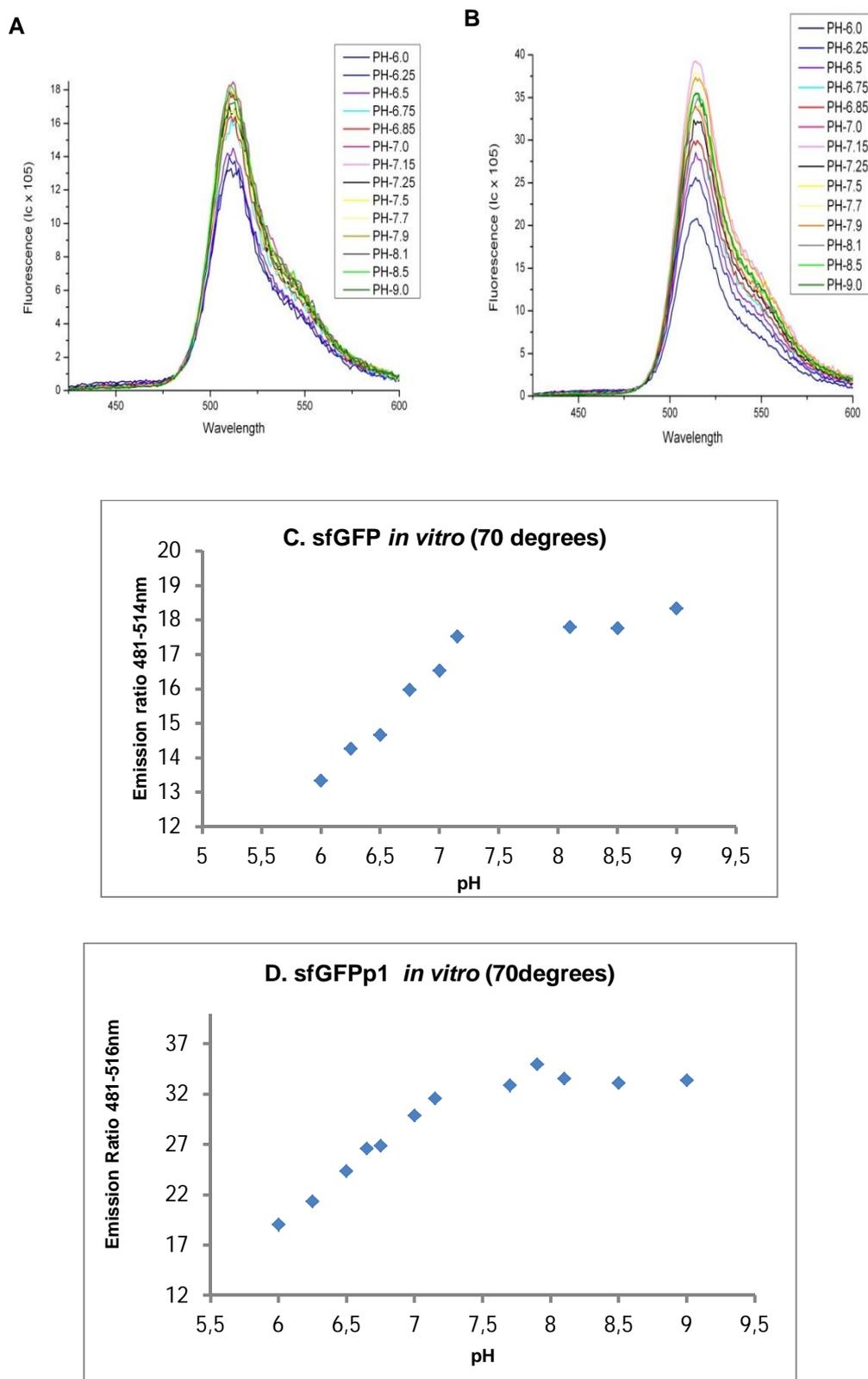
Figure 4.11 Emission of sfGFP (A) and sfGFPp1(B) at 70°C in vitro. Both (A and B) shows emission maximum at 515 nm with excitation wavelength 400 nm. Calibration curve against pH of sfGFP (C) and sfGFPp1 (D) relative to pH change.

Table 4.3 Spectral characteristics of all mutants

	Emission peak above pH 7.5 (nm)	Emission peak below pH 5.5 (nm)	Isobestic point (nm)	*pKa* (50% emission maximum)
In vitro at room temperature				
sfGFPp1	512-515	510-512	487-490	6.45±0.15
sfGFPp2	512-516	508-512	-	-
sfGFPp3	520-523	510-515	-	-
sfGFP	512-515	510-512	487-490	5.5 (Nathan *et al.,* 2011)
deGFP	515	460	492-495	7.2±0.1 (George *et al.,* 2002)
In vivo				
sfGFPp1	515	-	485-490	-
sfGFP	515	-	485-490	-
deGFP	515	460	-	-
In vitro at 70°C				
sfGFPp1	515	515	480	-
sfGFP	515	515	480	-
deGFP	-	-	-	-

5.   DISCUSSION

5.1   Significance of Mutation

The major advantage of GFP in intracellular pH studies is due to its non-toxic, good sensitivity, accuracy of signal, spectral range suitable for live cells and targeting towards any part of cell or tissue (Kneen *et al.,* 1998). Moreover, the structure of GFP has protected environment for its chromophore makes its fluorescence stable against quenchers and other chemical agents (Swaminathan *et al.,* 1997). Even though, most mutants of GFP have *pKa* range towards more acidic, it is possible to change *pKa* towards physiological range by mutations. It is promising that even ratiometric probes of GFP are emerging through mutagenesis (Ranieri *et al,* 2006). This study explores again the prospects of mutagenesis on its spectral characteristics and physico-chemical properties. Other than pH relative studies of GFP, there are considerable number of works relative to GFP's calcium sensitivity (Romoser *et al.,* 1997), protease activity (Heim and Tsien, 1996), halide detection (Piotr *et al,* 2012) etc.. Mutagenesis plays major role even in ionic detecting studies to change the *pKa* less than to the detecting environment (Kneen *et al.,* 1998). Therefore, pH sensitive variant of GFP also changes the interaction of GFP with other molecules or ions which influence Förster distance of the protein (Kneen *et al.,* 1998).

In this study we have modified sfGFP with mutations T203C or Y, H148G and C48S. T203C mutation has previously shown to be favoring protonated population in deGFP, which resulted deGFP to be better ratiometric variant with higher intensity of emission at 460 nm (George *et al.,* 2002). Other mutations which play a role in pH are S65T and F64L which are already present in sfGFP making it suitable for further small modifications to create a pH sensor while maintaining its superfolder properties (Kneen *et al.,* 1998). S65T mutation was known to favor anionic chromophore population by promoting excited state proton transfer (ESPT) (Marc-Andre *et al.,* 1999). The deGFP mutations made the protein to have clear dual emission characteristic, but it has less intensity of emission in *in vivo* experiments. Therefore we used these mutations in sfGFP to modify its pH sensing property with higher intensity of emission in intracellular studies. This study showed better pH sensitivity for the construct with mutation T203C (sfGFPp1). This indicated that sfGFP+ T203C could make the protein a pH sensor.

5.1.1   sfGFP variants as in vivo pH sensor and thermo stability

Superfolder GFP was reported as a robustly folding protein when fused to poorly folded peptides. But apart from the folding and stability (Pédelacq *et al.,* 2006), its application with pH sensitivity has not explored even though it has few mutations (S65T, F64L) (Kneen *et al.,* 1998) which is present in the pH sensitive variants of GFP. Many pH sensitive mutants available already have some disadvantages like noisy back ground signal, less stability, unsuitable *pKa* value, non ratiometric and less intensity of fluorescence (Ranieri *et al.,* 2009). Additionally, sfGFP also reported to have higher circular permutation, stability to oscillating environmental factors like temperature, ionic concentration etc., and these characteristics promotes it to be studied as novel *in vivo* pH reporter for thermophiles. However, the expression of sfGFP in thermophiles up to 70°C was already reported in a study (Felipe *et al*., 2008) and denaturation occurs at temperature over 95°C (Thuy and Thomas, 2011). From the results of this study, thermal stability of superfolder complies very much with previous study (Felipe *et al*., 2008); among the developed variants, the construct sfGFPp1 showed higher intensity at 70°C. This intensity was observed to be higher even on comparison to original superfolder GFP (figure 4.10).

The mutations of original superfolder GFP are S30R, Y39N, Y145F, I171V and A206V along with cycle3 mutations (F99S, M153T, and V163A) and EGFP mutations (F64L and S65T) (Pédelacq *et al.,* 2006). The presence of S65T in EGFP had already reported for its influence on anionic chromophore emission (Ranieri *et al.,* 2006). Mutations like T203CorY and H148G had showed change in emission pattern of neutral chromophore respective to pH (George *et al.,* 2002; Ranieri *et al.,* 2006). Reportedly, dual emission GFP (deGFP) is an ideal ratiometric probe, but it is well suited for the temperature up to 36°C and often gives noisy background signal at low pH and in live cell experiments (Ranieri *et al.,* 2009). Considering all these characteristics, this study gives a new approach to include dual emission mutations in superfolder to make a novel ratiometric pH probe which can be even used for high temperature experiments and *in vivo* thermophilic studies.

5.2   Analysis of Ratiometric property from *in vitro* spectral results

According to *in vitro* experimental results of this study, sfGFPp1 shows greater degree of pH sensitivity than other strains**.** Moreover sfGFP1 showed and *pKa* value of 6.45±0.15 which is in the physiological range on comparison to sfGFP which has *pKa* of 5.5 (Nathan *et al.,* 2011). This was clear from the emission between pH 6.5 to 7.5 which displayed a higher degree of variation for sfGFP1 on comparison with sfGFP.

Presence of cysteine at position 203 reduces the absorption of protein at 500 nm. The mutations C48S and H148G further reduce the absorption at 500 nm among all other strains. One reason could be the loss of hydrogen network due to amino acid change H148G, because, as stated in two-site model, H148G also influences ionization state of the chromophore (Bizzarri *et al.,* 2007).

The replacement of Thr203 to aromatic amino acid residues shifts GFP into yellow spectral region (Marc-Andre *et al.,* 1999). In this study, amino acid Thr203 is changed with Tyr residue which shifts the emission of sfGFPp3 from 512 to 523 nm displaying yellow emission. Moreover, it was thought that the mutation T303Y favors protonated chromophore emission at 460 nm; because in previous studies this mutation reported to have influence on protonated chromophore emission (Bizzarri *et al.* 2006). EGFP mutant which have S65T mutation is well known for promoting anionic chromophore emission (Kneen *et al.,* 1988; Chattoraj *et al.,* 1996). But the variants sfGFPp2 and sfGFPp3 doesn't have any significant emission at 460 nm. Furthermore, the absorption of state B for those two strains is very low which indicates the presence of phenol form of chromophores in the solution than phenolate form for all pH range; because B state (phenolate form) excitation is favored in wtGFP at low pH (Brjec *et al.,* 1997). The major cause of reduction in phenolate form of choromophore may be due to mutation H148G in combination with "superfolder" mutation, because this mutation was characterized to break hydrogen bond between His148 and Arg168 (Battistutta *et al.,* 2000; Seifert *et al.,* 2002), which further influence solvent diffusion towards chromophore (Barondaeu *et al.,* 2002) and protein flexibility (Seifert *et al.,* 2003; Barbara *et al.,* 2013). But in the emission spectrum of sfGFPp2 and sfGFPp3, anionic form emission is favored which shows that those strains follow excited state proton transfer (ESPT).

Considering the absorption and emission spectrum of all three mutant sfGFPp1, sfGFPp2 and sfGFPp3, sfGFPp1 with mutation T203C shows superior characteristics towards pH sensitivity. It correlates with the aim of creating better ratiometric probe for intracellular pH studies. In ratiometric graph of sfGFPp1 (Figure 4.7A), emission ratio shows change of more than 1 unit at physiological pH range and this is highly advantageous in live cell experiments. Its *pKa* 6.45±0.15 is well suitable for both acidic and neutral cellular compartments like mitochondria, Golgi apparatus, vesicles and endoplasmic reticulum. According to previous studies the mutation combinations in deGFP4 have greater dual emission characteristics on GFP but in the same study another mutant called deGFP3 with mutations S65T0 and T203C didn't had ratiometric absorption or emission (George *et al.,* 2002). It clearly shows the combinations of "superfolder" mutation with T203C have significant influence on ratiometric spectral property of GFP. Compared to emission and absorption of sfGFPp1 *in vitro* at room temperature with deGFP4, deGFP4 is better ratiometric probe with clear dual emission at 460 nm and 515 nm, but considering stability and fluorescence properties sfGFPp1 could be considerable.

5.3   *In vivo* pH sensitive spectral results of sfGFP, sfGFPp1 and deGFP

The major aim of this study is to create better ratiometric pH probe for *in vivo* studies and possibly for the application in thermophiles. There are significant numbers of mutant GFP's created for both *in vitro* and *in vivo* studies. Suitability of *pKa* value for *in vivo* experiments also changed by mutagenesis which yielded successful variants like YFPs (Llopis et al., 1998), pHluorins (Dang *et al.,* 2012), EcGFP (ecliptic GFP), sEcGFP (super ecliptic GFP), E^{2}GFP (Ranieri *et al.,* 2006), CFPs (Urra *et al.,* 2008) and deGFPs (George *et al,* 2002).

Available ratiometric probes for *in vivo* experiments were characterized mainly based on its spectral property, spatial resolution, *pKa* value, stability in cytoplasm, intensity of emission, spectral range suitability for live cell and interaction with ions. First engineered RaGFP was used to study intracellular pH of cytoplasm (Karagiannis *et al.,* 2001), peroxisomes (Jankowski *et al.,* 2001) and trans-Golgi network (Machen *et al.,* 2003), and moreover, RaGFP had *pKa* range close to neutral.

Later emerged deGFP and E^{2}GFP showed that the coupling between 222rd amino acid and chromophore affects excited state proton transfer (ESPT) in order to promote emission or neutral chromophore (Bizzare *et al.,* 2009). The *in vitro* emission results of deGFP correlated with this thesis results, but *in vivo* emission had very low intensity with noisy signal and it is also not suitable for ratiometric analysis. Experimental method difference should be also considered for reasoning the difference in live cell experiments, because deGFP *in vivo* spectral experiments were carried out using confocal and two-photon spectroscopy (George *et al.,* 2002). The *in vivo* emission of deGFP in that study using those methods was just above auto fluorescence of the cell. So there is greater chance that the reason for noisy *in vivo* fluorescence deGFP in this study is due to interruption of cell's auto fluorescence (George *et al.,* 2002). But in this study sfGFPp1 shows higher intensity far away from cell's auto fluorescence and also clear difference in emission of neutral chromophore respective to pH. Unlike *deGFP in vivo* emission has isobestic point near 480 nm widens the property of GFP as better intracellular pH reporter protein. Considering ratiometric property and intensity, E^{2}GFP (Bizzare *et al.,* 2006) and E^{1}GFP (Arosio *et al.,* 2007) is also has better pH sensing property for alkaline and acidic environments.

This experiment includes the activity of Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for perfusion which affects the polarization of ion exchange channel and ion exchange pathway of cells. Thus the spectral behavior or pro-

tein expressed in cell also influenced by activity of CCCP. More study to fine-tune the adjustment of equilibrium of intracellular pH to its environment could modify the emission results of this study. Spatial resolution is the significant kinetic property of a GFP variant to report shift in pH along different region in the cell (Bizzare *et al.,* 2009). Spatial resolution of GFP can be engineered using fusion experiments, in which superfolder GFP already has an advantage for being better fusion carrier. Furthermore, the evolution of new instruments in fluorescence measurements with higher resolution, low detector noise, different excitation principle and higher spatial detection is promising for future live cell studies.

5.4   Spectral property of sfGFPp1 at 70° C and its potentiality in molecular dynamics research of Thermophiles.

Thermophiles are characterized as those organisms living at extreme temperatures. The major significance of extremophiles is its suitability for harsh industrial application. Enzymes are the major substance produced by these extremophiles which has already proved its commercial application in many industrial processes includes bio fuel production (Zhaco *et al.,* 2013), bio leaching (Vilcáez *et al.,* 2008), fermentation (Olle *et al.,* 1997) and substrate degradation (Petros *et al.,* 2006). It is highly important that development of new techniques to increase understanding of each molecular processes of thermophiles.

Unfortunately, except sfGFP no other fluorescent proteins can be expressed in thermophilic organisms as fluorescent protein precipitates in extreme temperatures (Felipe *et al.,* 2008). In another study, sfGFP proved to be carrier molecule of periplasmic peptides of thermopiles and able to express it in periplasmic region of *Thermus thermophiles (Tth)* (Felipe *et al.,* 2008). It made us to check the thermophilic property of newly constructed variant sfGFPp1 along with its pH sensing property, which is again confirmed in this study as deGFP precipitated in 6 minutes at 70°C (Figure 4.10).

From this study, we suggest that sfGFPp1 has novel property to be used as pH reporting probe in thermophiles. Compared to *in vitro* fluorescence of superfolder, sfGFPp1 showed linear variation in emission ratio between pH 6 to 7.5 (Figure 4.11). Considering the importance of pH in thermophile metabolism, there is no intracellular probe available to study its pH homeostasis. As confocal microscopy method is already proved to detect signal from superfolder expressed intracellular in *Thermus thermophiles (Tth)* (Felipe *et al.,* 2008), it is promising to detect pH sensitive emission from sfGFPp1. Furthermore, the stability of sfGFPp1 over 30 minutes at 70°C is appropriate for many experimental procedures. In future, we recommend further studies using sfGFPp1 by expressing in thermophiles, because of potential ability to report intracellular pH behavior.

6.   CONCLUSION

This work demonstrates that "superfolder" GFP with mutation T203C is better ratiometric probe for studying pH in environments of extreme temperatures. Moreover, we have also reported the influence of mutations H148G, T203Y and C48S on spectral behavior of "superfolder" which elaborates the available information about these residues. By comparing the *in vitro* spectral results of all mutants, sfGFPp1 were selected for further experiments as better variant based on pH responsiveness. Emission Intensity of sfGFPp1 was higher than all other strains throughout the studies. There is significant emission of neutral chromophore region in *in vivo* spectral measurements, which makes the protein as intracellular ratiometric pH probe, but further evolving of new molecular techniques to regulate activity of cccp or other perfusion mechanism could give more detail signal from neutral choromophore emission. The emission result at 70°C shows that sfGFPp1 maintains same spectral characteristics as in the room temperature. The stability of sfGFP, sfGFPp1 and deGFP at 70°C for 30 minutes were also reported, in which deGFP fails to hold its emission after 6 minutes.

REFERENCES

Ana Luiza P, N., Rodrigo M, L., & Carmen, F. 2009. Fluorescence and Chemiluminescence: Teaching Basic Principles by Simple Demonstration Experiments. *Chemical education journal* 13, 2, 27.

Anniina, K., Ville S., Matti, K. 2013. Non-sterile process for biohydrogen and 1,3-propanediol production from raw glycerol, *International Journal of Hydrogen Energy.* 38, 27, 11749–11755.

Alexander, P, D,. 2010. Advanced fluorescence reporter in chemistry and biology I: fundamentals and molecular design. Springer series on fluorescence. 8, 9, 1-14.

Abad, M, F, C., Di Benedetto, G., Magalhaes, P,J., Filippin, L., Pozzan, T. 2004. *J Biol Chem* 279:11521 – 11529

Acta, P, P. 1978. Polska Akademia Nauk Instytut Fizyki. *Europhys J*, Vol. A65, 6.

Ai, H., Shaner, N. C., Cheng, Z., Tsien, R. Y. and Campbell, R. E. 2007. Exploration of new chromophore structures leads to the identification of improved blue fluorescent proteins. *Biochemistry* 46, 5904-5910.

Akke, M. Forsen S. 1990. Protein stability and electrostatic interactions between solvent exposed charged side chains, *Proteins*. 8, 23–29

Aronson, D, E., Costantini. L, M., Snapp E, L. 2011. Superfolder GFP is fluorescent in oxidizing environments when targeted via the Sec translocon. *Pub med, NCBI*. 5, 543.

Arosio, D., Garau, G., Ricci F., Marchetti L., Bizzarri R., Nifosi R., Beltram, F. 2007. *Biophys J* 93:232 – 244

Awaji, T., Hirasawa, A,. Shirakawa, H., Tsujimoto, G., and Miyazaki, S. 2001. Novel green fluorescent protein-based ratiometric indicators for monitoring pH in defined intracellular microdomains. *Biochem. Biophys. Res. Commun.* 289:457–462.

Bagar, T., Altenbach, K., Read, N.D., Bencina, M., 2009. Live-cell imaging and measurement of intracellular pH in filamentous fungi using a genetically encoded ratiometric probe. *Eukaryotic Cell* 8, 703–712.

Barbara, C., Barbara, P., Samanta, R., Paolo, F., Immacolata, G., Laura, D., Maddalena C., Giuseppe, C., Stefano, B. 2013. Role of histidine 148 in stability and dynamics of a

highly fluorescent GFP variant. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics.* Volume 1834, 4, 770-779.'

Battistutta, R., Negro, A., Zanotti, G. 2000. Crystal structure and refolding properties of the mutant F99S/M153T/V163A of the green fluorescent protein. *Proteins Struct. Funct. Genet.* 41, 429-437.

Berlman, I,B. 1971. Handbook of fluorescence spectra of aromatic molecules. *Academic Press, New York.* 2

Berlemont. R., Gerday, C. 2011. Extremophiles, In Comprehensive Biotechnology edited by Murray Moo-Young, *Academic Press, Burlington.* edition 2, 229-242.

Benjamin, T, A., Andrea, R, S., Melinda, R., Geoffrey, W., Patricia, A. J.2007. The Rough Energy Landscape of Superfolder GFP Is Linked to the Chromophore. *Journal of Molecular Biology.* Volume 373, Issue 2, 19 476-490.

Birks J,B. 1973. Organic molecular photophysics. *John Wiley & Sons, New York.*

Brejc K, Sixma T.K, Kitts P.A. Kain S.R, Tsien R.Y, Orm M, Remington S.J. 1997. Structural basis for dual excitation and photoisomerization of the Aequorea victoria green fluorescent protein. *Proc Natl Acad Sci* USA 94:2306–2311

Bell A.F, He X, Wachter R.M, Tonge P.J. 2000. Probing the ground state structure of the green fluorescent protein chromophore using Raman spectroscopy. *Biochemistry.* 39: 4423–4431

Bernard R. G. Jack J. P. Cheryl L. P. 2008. Molecular biology: principles and applications of recombinant DNA. *American Society for Microbiology. 3*

Berberan-Santos M. N. 2001. Pioneering contributions of Jean and Francis Perrin to molecular luminescence. In New trends in fluorescence spectroscopy: applications to chemical and life sciences. *Ed B Valeur, J-C Brochon. Springer, New York.* Vol. 18, pp. 7–33.

Bizzarri R, Nifosi R, Abbruzzetti S, Rocchia W, Guidi S, Arosio D, Garau G, Campanini B, Grandi E, Ricci F, Viappiani C, Beltram F. 2007.Green fluorescent protein ground states: the influence of a second protonation site near chromophore. *Biochemistry.* 46:5494 – 5504

Barondeau D.P., Kassmann C.J., Tainer J.A., Getzoff E.D. 2002. Structural chemistry of a green fluorescent protein Zn biosensor. *J. Am. Chem. Soc.,* 124, 3522–3524.

Bonsma S, Purchase R, Jezowski S, Gallus J, Konz F, Volker S. 2005. Green and red fluorescent proteins: photo and thermally induced dynamics probed by site selective spectroscopy and hole burning. *Chemphyschem.* 6:838 – 849

Cabantous, S., Pedelacq, J. D., Mark, B. L., Naranjo, C., Terwilliger, T. C. & Waldo, G. S. 2005. Recent advances in GFP folding reporter and split-GFP solubility reporter technologies; Application to improving the folding and solubility of recalcitrant proteins from Mycobacterium tuberculosis. *J. Struct. Funct. Genom.* 6, 113–119.

Cabantous, S., Terwilliger, T. C. & Waldo, G. S. 2005. Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nature Biotechnol.* 23, 102–107.

Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A. and Tsien, R. Y. 2002. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* 99, 7877-7882.

Carnell L, Moore HP. 1994. *J Cell Biol.* 127:693 – 705

Chalfie M, Tu Y, Euskirchen. G. Ward W. W. Prasher D. C. 1994. Green fluorescent protein as a marker for gene expression. *Science.* 263:802–805.

Casey, J.R. Grinstein, S. Orlowski, J. 2010. Sensors and regulators of intracellular pH, *Nat. Rev. Mol. Cell Biol.* 11, 50–61

Chan, P. Lovric, J. Warwicker, J. 2006. Subcellular pH and predicted pH-dependent features of proteins, *Proteomics.* 6, 3494–3501.

Chattoraj M, King B,A, Bublitz G,U, Boxer S,G. 1996. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer. Proc Natl Acad Sci USA 93:8362–8367

Chudakov, D.M. et al. 2003. Kindling fluorescent proteins for precise in vivo photolabeling. *Nat. Biotechnol.* 21, 191–194

Cormack, B. P., Valdivia, R. H., and Falkow, S. 1996. FACS-optimized mutants of green fluorescent protein gene. *Gene.* 173, 33 – 38.

Crameri, A. Whitehorn, E. A. Tate, E. Stemmer, W.P. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat Biotechnol.* 14315-319.

Cristea, I.M., Williams, R., Chait, B.T., and Rout, M.P. 2005. Fluorescent proteins as proteomic probes. *Mol CellProteomics* 4: 1933–1941.

Cubitt A.B, Heim R, Adams S.R, Boyd A.E, Gross L.A, Tsien R.Y. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem Sci* 20:448–455

Davis. B. D. 1958. On the importance of being ionized. *Arch. Biochem. Biophys.* 78, 497–509.

Dang, T.D.T. De Maeseneire S.L. Zhang B.Y. De Vos, W.H. Rajkovic. A. Vermeulen, A. Van Impe J.F. Devlieghere. F. 2012. Monitoring the intracellular pH of Zygosaccharomyces bailii by green fluorescent protein. *International Journal of Food Microbiology.* 156, 290-295.

Digman M.A, Brown C.M, Sengupta P, Wiseman P.W, Horwitz A.R, Gratton E. 2005. *Biophys J* 89:1317 – 1327

Elsliger, M.-A., Wachter, R. M., Hanson, G. T., Kallio, K., and Remington, S. J. 1999 *Biochemistry* 38, 5296 - 5301.

Fliegel L. 2005. *Int J Biochem Cell Biol* 37:33 – 37

Felipe C, Miguel A P, Emilio B, Geoffrey S. W, Lars F. W. José B.2008. Expression and use of superfolder green fluorescent protein at high temperatures *in vivo*: a tool to study extreme thermophile biology. *Environmental microbiology.* 10(3), 605–613.

Friedrich, A., Prust, C., Hartsch, T., Henne, A., and Averhoff, B. 2002. Molecular analyses of the natural transformation machinery and identification of pilus structures in the extremely thermophilic bacterium Thermus thermophilus strain HB27. *Appl Environ Microbiol* 68: 745–755.

Fukuda, H., Arai, M. & Kuwajima, K. 2000. Folding of green fluorescent protein and the cycle3 mutant. *Biochemistry,* 39, 12025–12032.

George T. H, Tim B. M, Eun Sun P, Marla E. P. R, Daniel K. Y, Shaoyou C Lixuan Xi, Steven G. B, Marshall H. MJames R. 2002. Green Fluorescent Protein Variants as Ratiometric Dual Emission pH Sensors. 1.Structural Characterization and Preliminary Application. *Biochemistry*. 41, 15477 – 15488.

Grynkiewicz, G., Martin, P., and Tsien, R. Y. 1985. J. Biol. Chem. 260, 3440 - 3450

Herlitz S., Koenen M. 1990. A general and rapid mutagenesis method using polymerase chain reaction. *Gene*. 91, 143-147.

Herschel, Sir JFW. 1845. On a case of superficial colour presented by a homogeneous liquid internally colourless. *Phil Trans Roy Soc (London).* 135:143–145.

Heim R, Cubitt A.B, Tsien R.Y. 1995. Improved green fluorescence. *Nature.* 373:663–664

Heim R, Prasher D.C, Tsien R.Y 1994 Wavelength mutations and posttranslational auto oxidation of green fluorescent protein. *Proc Natl Acad Sci USA*. 91:12501–12504

Henrik S., Bjorn R., Mogens J. 2000. Dynamic Changes of Intracellular pH in Individual Lactic Acid Bacterium Cells in Response to a Rapid Drop in Extracellular pH. *Applied and environmental microbiology*. 66, 6: 2330–2335.

Hess S, T., Heikal A, A., Webb W, W. 2004. *J Phys Chem B* 108:10138 – 10148

Hong, S.-I., Y.-J. Kim, and Y.-R. Pyun. 1999. Acid tolerance of Lactobacillus plantarum from Kimchi. *Food Sci. Technol.- Lebensm.- Wiss. Technol.* 32:142–148.

Haugland R.P. 2002. LIVE/DEAD BacLight bacterial viability kits. In Handbook of fluorescent probes and research products,. *Ed J Gregory. Molecular Probes, Eugene.* 9, 626–628

Hutkins, R. W., and N. L. Nannen. 1993. pH homeostasis in lactic acid bacteria. *J. Dairy Sci.* 76:2354–2365.

Hunte C, Screpanti E, Venturi M, Rimon A, Padan E, Michel H. 2005. *Nature* 435:1197 – 1202

Inouye S. Tsuji F.I. 1994. Aequorea green fluorescent protein: expression of the gene and fluorescent characteristics of the recombinant protein. *FEBS Lett.* 341:277–280

Jahn R, Lang T, Sudhof TC. 2003. *Cell* 112:519 – 533

Jablonski A. 1935. Über den Mechanisms des Photolumineszenz von Farbstoffphosphoren, *Z Phys.* 94:38–46.

Jankowski, A. *et al.* 2001 In situ measurements of the pH of mammalian peroxisomes using the fluorescent protein pHluorin. *J. Biol. Chem.* 276, 48748–48753

Jayaraman, S., P. Haggie, R. M. Wachter, S. J. Remington, and A. S. Verkman. 2000. Mechanism and cellular applications of a green fluorescent protein-based halide sensor. *J. Biol. Chem.* 275:6047–6050.

Jean-Denis. P. Stephanie, C. Timothy, T. Thomas, C. T. Geoffrey, s. W. 2006. Engineering and characterization of superfolder green fluorescent protein. *Nature Biotechnol.* 24: 1 79-88

Joseph R. L. 2010. Principles of fluorescence spectroscopy. *Springer science.* 3.

Jun Z, Jian L, Cuihong Z, Xiaoyan D, and Bin X. 2011. An improved bimolecular fluorescence complementation tool based on superfolder green fluorescent protein. *Acta Biochim Biophys Sin.* 1, 128-128.

Kasha M. 1950. Characterization of electronic transitions in complex molecules. *Disc Faraday Soc* 9:14–19.

Kaddoum L, Magdeleine E, Waldo G. S, Joly E, Cabantous S. 2010. One-step split GFP staining for sensitive protein detection and localization in mammalian cells. *Biotechniques.* 49:727-736.

Kapus A, Szaszi K, Sun J, Rizoli S, Rotstein O.D. 1999. *J Biol Chem.* 274:8093 – 8102

Karagiannis, J. and Young, P.G. 2001 Intracellular pH homeostasis during cell-cycle progression and growth state transition in Schizo-saccharomyces pombe. *J. Cell Sci.* 114, 2929–2941

Kellokumpu S, Sormunen R, Kellokumpu I. 2002. *FEBS Lett* 516:217 – 224

Kneen, M. *et al.* 1998. Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys. J.* 74, 1591–1599

Kobayashi, H., T. Suzuki, and T. Unemoto. 1986. Streptococcal cytoplasmic pH is regulated by changes in amount and activity of a proton-translocating ATPase. *J. Biol. Chem.* 261:627–630.

Kummer A.D, Kompa C, Lossau H, Pollinger-Dammer F, Michel-Beyerle M.E, Silva C.M, Bylina E.J, Coleman W.J, Yang M.M, Youvan D.C. 1998. Dramatic reduction in fluorescence quantum yield in mutants of green fluorescent protein due to fast internal conversion. *Chem Phys.* 237:183–193

Lakowicz J. R, Balter A. 1982. Analysis of excited state processes by phase-modulation fluorescence spectroscopy. *Biophys Chem.* 16:117–132.

Lakowicz J. R. 1995. Fluorescence spectroscopy of biomolecules. In Encyclopedia of molecular biology and molecular medicine, pp. 294–306. *Ed RA Meyers.* VCH Publishers, New York.

Lakowicz J. R, Gryczynski I, Laczko G, Wiczk W, Johnson ML. 1994. Distribution of distances between the tryptophan and the N-terminal residue of melittin in its complex with calmodulin, troponin, C, and phospholipids. *Protein Sci* 3:628–637.

Llopis, J., J. M. McCaffery, A. Miyawaki, M. G. Farquhar, and R. Y. Tsien. 1998. Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad.Sci. USA*. 95:6803–6808.

Lippincott-Schwartz, J., and G. H. Patterson. 2003. Development and use of fluorescent protein markers in living cells. *Science.* 300:87–91.

Lippincott-Schwartz, J. et al. 2003. Photobleaching and photoactiva-tion: following protein dynamics in living cells. *Nat. Cell Biol. (Suppl).* S7–14

Oriel Corporation, 250 Long Beach Blvd., PO Box 872, Stratford,CT 06497: Light Sources, Monochromators and Spectrographs, Detectors and Detection Systems, Fiber Optics.

Matz M., Fradkov A,F., Labas Y., Savitsky A, Zaraisky A,G., Markelov A,Z., Luky-anov SA. 1999. Fluorescent proteins from non-bioluminescent Anthozoa species. *Nat Biotechnol.* 17:969–973

Mark K, R. David, W, P. 2005. Fluorescent protein tracking and detection. *live cell imaging lab manual, Cold spring harbor laboratory press, Nat Biotechnol.* Chapter 1, pp 3-24.

Marc-Andre E, Rebekka M. W, George T. H, Karen K, James R. 1999. Structural and Spectral Response of Green Fluorescent Protein Variants to Changes in pH. *Biochemistry.* 38, 5296 - 5301

Machen, T.E. *et al.* 2003. pH of TGN and recycling endosomes of H^+/K^+-ATPase-transfected HEK-293 cells: implications for pHregulation in the secretory pathway. *Am. J. Physiol. Cell Physiol.*285, C205–C214

Magliery, T. J., Wilson, C. G., Pan, W., Mishler, D., Ghosh, I., Hamilton, A. D. & Regan, L. 2005. Detecting protein–protein interactions with a green fluorescent protein fragment reassembly trap: scope and mechanism. *J. Am. Chem. Soc.* 127, 146–157.

Mellman I, Warren G. 2000. *Cell* 100:99 – 112

Michael C. A. Kyoko I. Jeremy M. H. 2004. It's green outside: tracking cell surface proteins with pH-sensitive GFP. *TRENDS in Neurosciences.* Vol.27, 5, 258-263.

Michael. W. 2006. An introduction to molecular biology. *Wiley-VCH, Germany.* 131-149.

Miyawaki, A. *et al.* 2003. Lighting up cells: labelling proteins with fluorophores. *Nat. Cell Biol.* (Suppl), S1–S7

Miyawaki, A. 2002. Green fluorescent protein-like proteins in reef Anthozoa animals. *Cell Struct. Funct.* 27, 343–347

Miesenbock, G. *et al.* 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature.* 394, 192–195

Milletti. F, Storchi. L, Cruciani. G. 2009. Predicting protein *pKa* by environment similarity. *Proteins.* 76, 484–495.

Nakayama, M. Ohara. o. 2003. A system using convertible vectors for screening soluble recombinant proteins produced in *Escherichia coli* from randomly fragmented cDNAs. *Biochem. Biophys. Res. Commun.* 312. 825-830.

Nathan. C. S, George. H. P, Michael. W, D. 2011. Advances in fluorescent protein technology. *J cell Sci.* 120, 4247-4260.

Niwa H. Inouye S. Hirano T. Matsuno T. Kojima S. Kubota M. Ohashi M. Tsuji F. I. 1996. Chemical nature of the light emitter of the Aequorea green fluorescent protein. *Proc Natl Acad Sci USA* 93:13617–13622.

Nienhaus G, U. Wiedenmann J. 2009. Structure, dynamics and optical properties of fluores-cent proteins: perspectives for marker development. *Chemphyschem* 10:1369–1379

Nifosi. R. Tozzini. V. 2012. Fluorescent Proteins I. *G. Jung (ed.), Springer Ser Fluoresc.* 11: 3-40.

"The Nobel Prize in Chemistry 2008". *Nobelprize.org. Nobel Media AB 2013.* Web. 24 Mar 2014. <http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2008/>

Olle. H, Åsa. M, Martin. K, Herbert. M, Neil. R, Richard. S. 1997. Thermophiles and fermentation technology, Comparative Biochemistry and Physiology Part A: *Physiology.* Volume 118, 3, 415-422.

Padan, E., D. Zilberstein, and S. Schuldiner. 1981. pH homeostasis in bac-teria. *Biochim. Biophys. Acta* 650:151–166.

Palm, G.J. *et al.* 1997. The structural basis for spectral variations in green fluorescent protein. *Nat. Struct. Biol.* 4, 361–365

Patterson G.H, Knobel S.M, Sharif WD, Kain S.R, Piston D.W. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys J.* 73(5):2782–2790

Patterson, G. H., and Lippincott-Schwartz, J. 2002. A Photoactivatable GFP for Selective Photolabeling of Proteins and Cells. *Science* 297,1873 – 1877.

Pakhomov A.A. Martynov V.I. 2008. GFP family: structural insights into spectral tuning. *Chem Biol* 15(8):755–764

Pace, C. N. Laurents, D.V. Thomson, J.A. 1990. pH dependence of the urea and guan-idine hydrochloride denaturation of ribonuclease A and ribonuclease T1. *Biochemistry.* 29, 2564–2572.

Paul N. H. 1995 Purification of His-Tag fusion proteins from Escherichia coli. *Trends in Biochemical Sciences.* 20, 7, 285-286.

Peelle, B et al 2001. Intracellular protein scaffold-mediated display of random peptide libraries for phenotypic screens for mammalian cells. *Chem Biol.* 8, 521-534.

Pédelacq J, Cabantous S, Tran T, Terwilliger T, Waldo G. 2006. "Engineering and characterization of a superfolder green fluorescent protein". *Nat Biotechnol* 24 (1): 79–88.

Petros. K, Wim. N, Marc. C, Paul. C. 2006. Purification and characterization of a thermostable intracellular β-xylosidase from the thermophilic fungus Sporotrichum thermophile. *Process Biochemistry*. 41, 2402–2409.

Piotr B. Daniele A. 2012 . Green Fluorescent Protein-Based Chloride Ion Sensors for *In vivo* Imaging. *Fluorescent Proteins II, Springer Ser Fluoresc.* 12: 99–124.

Pick, U. Bental, M. Chitlaru, E. Weiss, M. 1990. Polyphosphate-hydrolysis—a protective mechanism against alkaline stress? FEBS Lett. 274 15–18.

Piechocki M., Hiens, R. N. 1994. Oligonucleotide design and optimized protocol for site directed mutagenesis. *Biotechniques.*16 702-707.

Prasher D., C. Eckenrode. V.,K. Ward. W., Pendergast F. G., Cormier M. J. 1992. Primary structure of the Aequorea Victoria green fluorescent protein. *Gene.* 111 229-33.

Prajna. P. P, Nediljko, B. 2008. Engineering green fluorescent protein using expanded genetic code. *springer science, reviews in fluorescence.*359-375

Rajakumari, S. Grillitsch, K. Daum, G. 2008. Synthesis and turnover of non-polar lipids in yeast, Prog. *Lipid Res.* 47, 157–171.

Ranieri B, Caterina A, Daniele A, Fernanda R, Paolo F, Francesco C. Fabio B. 2006. Development of a Novel GFP-based Ratiometric Excitation and Emission pH Indicator for Intracellular Studies. *Biophysical Journal.* Volume 90, 3300–3314

Rius, N., M. Sole´, A. Francia, and J. G. Lore´n. (1994). Buffering capacity and membrane H 1 conductance of lactic acid bacteria. *FEMS Microbiol. Lett.* 120:291–296.

Rizzo M.A, Springer G.H, Granada B, Piston D.W. 2004. An improved cyan fluorescent protein variant useful for fret. *Nat Biotechnol.* 22(4):445–449

Reid, B.G. and Flynn, G.C. 1997. Chromophore formation in green fluorescent protein. *Biochemistry.* 36, 6786–6791.

Rick O, Stanley B, Gertien J. S. 2011. Intracellular pH is a tightly controlled signal in yeast. *Biochimica et Biophysica Acta.* 1810, 933–944

Romoser, V. A., P. M. Hinkle, and A. Persechini. 1997. Detection in living cells of Ca^{2+}-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. J. Biol. Chem. 272:13270–13274.

Russell, J. B. 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *J. Appl. Bacteriol.*73:363–370.

Rybak S.L, Lanni F, Murphy R.F. 1997. *Biophys J* 73:674 – 687

Sambrook. J. Fritsch. E.F . maniatis. T. 1990. Molecular cloning. A laboratory manual. *Cold Spring Harbor Laboratory press, New York.*

Scopes, R. K. 1994. Protein purification, principles and practice. *Springer, New York.*

Srivastava, J. Barber, D.L. Jacobson, M.P. 2007. Intracellular pH sensors: design principles and functional significance. *Physiology (Bethesda)* 22. 30–39.

Sankaranarayanan, S. *et al.* 2000 The use of pHluorins for optical measurements of presynaptic activity. *Biophys.* J. 79, 2199–2208

Sekar, R.B. and Periasamy, A. 2003. Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *J. Cell Biol.* 160, 629–633

Seifert M.H., Ksiazek D., Azim M.K., Smialowski P., Budisa N., Holak T.A. 2002. Slow exchange in the chromophore of a green fluorescent protein variant. *J. Am. Chem. Soc.* 124, 7932–7942

Seifert M.H.J., Georgescu J., Ksiazek D., Smialowski P., Rehm T., Steipe B., Holak T.A. 2003. Backbone dynamics of green fluorescent protein and the effect of histidine 148 substitution. *Biochemistry,* 42, 2500–2512.

Szudy J . 1998. Born 100 years ago: Aleksander Jablonski (1898–1980), Uniwersytet Mikolaja Kopernika, Torun, Poland

Stokes G.G. 1852. On the change of refrangibility of light. *Phil Trans R Soc (London).* 142:463–562.

Strickler S., J. Berg R., A. 1962. Relationship between absorption intensity and fluorescence lifetime of molecules. *J Chem Phys*. 37(4):814–822.

Stryer L. 1978. Fluorescence energy transfer as a spectroscopic ruler. *Annu Rev Biochem*. 47:819–846.

Stauffer, T.P. et al. 1998. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. *Curr. Biol.* 8, 343–346

Shimomura. Johnson F. H. Saiga. Y. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. J. *Cell. Comp. Physiol.* 59 223-29.

Shimomura. 1979. Structure of the chromophore of Aequorea green fluorescent protein. *FEBS Letters 104,* 220-22

Shaner N.C. Steinbach P.A. Tsien R.Y. 2005. A guide to choosing fluorescent proteins. *Nat Methods.* 2(12):905–909

Sniegowski J. A, Lappe J. W, Patel H. N, Huffman H. A, Wachter R. M. 2005. Base catalysis of chromophore formation in Arg96 and Glu222 variants of green fluorescent protein. *J Biol Chem* 280:26248 – 26255

Swaminathan, R., C. P. Hoang, and A. S. Verkman. 1997. Photobleaching recovery and anisotropy decay of green fluorescent protein GFP-S65T in solution and cells: cytoplasmic viscosity probed by green fluorescent protein translational and rotational diffusion. *Biophys. J.* 72:1900–1907

Terskikh, A. et al. 2000. Fluorescent timer: protein that changes color with time. *Science* 290, 1585–1588

Tozzini, V., V. Pellegrini, and F. Beltram. 2004. Green fluorescent proteins and their applications to cell biology and bioelectronics. In CRC Handbook of Organic Photochemistry and Photobiology. *W. Horspool and F. Lenci, editors. CRC Press, Boca Raton,* FL. 1–15.

Thuy D. Thomas G. B. 2011. Using Superfolder Green Fluorescent Protein for Periplasmic Protein Localization Studies. *American society for microbiology, Journal of bacteriology.* 193, 18, 4984-4987.

Timo.Z and Stefan.T. 2014. FRET Basics. Retrieved 3 April 2014 from http://www.embl.de/eamnet/downloads/modules/FRET_teaching_module.pdf

Tsien R. Y. 1998. The green fluorescent protein. *Annu Rev Biochem.* 67:509–544

Torsten E, Dennis J. O, Franklyn G. P. 1995 Green-fluorescent protein mutants with altered fluorescence excitation spectra, *FEBS Letters.* 367, 2, 163-166.

Undenfriend S. 1995. Development of the spectrophotofluorometer and its commercialization. Protein Sci 4:542–551.

Urra J, Sandoval M, Cornejo I, Barros LF, Sepúlveda FV, Cid LP. 2008. *Pflugers Arch* 457:233 – 242

Van Hoek, P. Van Dijken, J.P. Pronk, J.T. 1998. Effect of specific growth rate on fermentative capacity of baker's yeast, *Appl. Environ. Microbiol.* 64, 4226–4233.

Vilcáez. J, Suto. K, Inoue. C. 2008. Bioleaching of chalcopyrite with thermophiles: Temperature–pH–ORP dependence. *International Journal of Mineral Processing.* Volume 88, 1–2, 37-44.

Voityuk A. A, Michel-Beyerle M. E, Rosch N. 1998. Quantum chemical modelling of structure and absorption spectra of the chromophore in green fluorescent proteins. *Chem Phys.* 231:13 – 25

Wang H, Singh D, Fliegel L. 1997. *J Biol Chem.* 272:26545–26549

Ward W,W., Bokman S,H. 1982. Reversible denaturation of Aequorea green fluorescent protein: physical separation and characterization of the renatured protein. *Biochemistry.* 21:4535–4540

Wachter R,M. 2007. Chromogenic cross-link formation in green fluorescent protein. *Acc Chem Res* 40(2):120–127

Wachter, R, M., Yarbrough, D., Kallio, K., and Remington, S, J. 2000. Crystallographic and energetic analysis of binding of selected anionsto the yellow variants of green fluorescent protein. *J. Mol. Biol.* 301:157–171

Wakabayashi I, Poteser M, Groschner K. 2006. *J Vasc Res* 43:238 – 250

Waldo G. S. Standish. B. M. Berendzen. J. Terwilliger. T. C. 1999. Rapid protein-folding assay using green fluorescent protein. *Nat. Biotechnol.* 17, 691-695.

Wiedenmann J, Oswald F, Nienhaus GU. 2009. Fluorescent proteins for live cell imaging: opportunities, limitations, and challenges. *Iubmb Life* 61:1029–1042

Wilson, C. G., Magliery, T. J. & Regan, L. 2004. Detecting protein–protein interactions with GFP-fragment reassembly. *Nature Methods,* 1, 255–262. 7

Xu, X. et al. 1998. Detection of programmed cell death using fluorescence energy transfer. *Nucleic Acids Res.* 26, 2034–2035

Young, B.P., Shin, J.J., Orij, R., Chao, J.T., Guan, S.C., Li, X.L., Khong, A., Jan, E., Wenk, M.R,. Prinz, W.A., Smits, G.J., Loewen, C.J. 2010. Phosphatidic acid is a pH biosensor that links membrane biogenesis to metabolism, *Science*. 329, 1085–1088.

Yang, T.T. *et al.* 1996. Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucleic Acids Res.* 24, 4592–4593

Zhang, G., Campbell, E.A., Minakhin, L., Richter, C., Severinov, K., and Darst, S.A. 1999. Crystal structure of Thermus aquaticus core RNA polymerase at 3.3 A resolution. *Cell* 98: 811–824.

Zhaco, C, Lu, W., Wang. H., Pan, X. 2013. Simultaneous hydrogen and ethanol production from a mixture of glucose and xylose using extreme thermophiles I: Effect of substrate and pH. 38, 9701-9706.