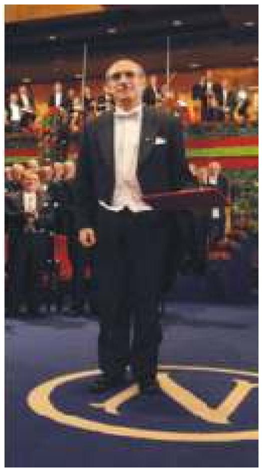
Green Fluorescent Proteins: Invisible to Visible

K. Sivakumar

Introduction

The Royal Swedish Academy of Sciences has awarded the Nobel Prize in Chemistry for 2008 jointly to Osamu Shimomura, of Marine Biological Laboratory, and Boston University Medical School along with Martin Chalfie, Columbia University, New York and Roger Y. Tsien, University of California, San Diego "for the discovery and development of the Green Fluorescent Protein (GFP)".



Osamu Shimomura, Martin Chalfie and Roger Y. Tsien with their Nobel Prize

Nobel medal

Nobel Prize in Chemistry for 2008 honors the discovery of GFP (by Osamu Shimomura in 1962) and developments (by Martin Chalfie and Roger Y. Tsien) that have led GFP's use as a tagging tool in bioscience to research and study the various invisible biological processes visibly.

Nobel diploma of Osamu Shimomura

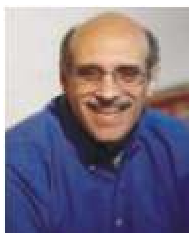
Nobel diploma of Martin Chalfie

Nobel Laureates in Chemistry for 2008

Osamu Shimomura first isolated GFP from the jellyfish Aequorea victoria, which drifts with the currents off the west coast of North America. He discovered that this protein glowed bright green under ultraviolet light.

Osamu Shimomura (Japan) was born in 1928 in Kyoto, Japan. He obtained his Ph.D. in organic chemistry in 1960 from Nagoya University, Japan. He is now professor emeritus at the Marine Biological Laboratory in Woods Hole, MA, and at Boston University Medical School.

Martin Chalfie demonstrated the value of GFP as a luminous genetic tag for various biological phenomena. In one of his first experiments, he coloured six individual cells in the transparent roundworm Caenorhabditis elegans with the aid of GFP.



Martin Chalfie (USA) was born in 1947 and grew up in Chicago. He obtained his Ph.D. in neurobiology in 1977 from Harvard University.

Roger Y. Tsien contributed to our general understanding of how GFP fluoresces. He also extended the colour palette beyond green allowing researchers to give various proteins and cells different colours. This enables scientists to follow several different biological processes at the same time.

Roger Y. Tsien (USA) was born in 1952 in New York. He obtained his Ph.D. in physiology in 1977 from Cambridge University. He has been Professor at University of California, San Diego since 1989.

Nobel diploma of Roger Y. Tsien

Green Fluorescent Protein

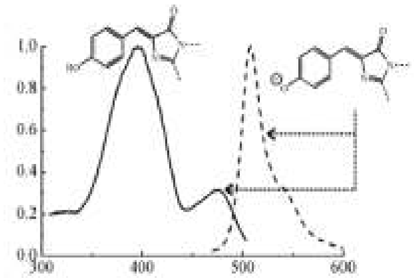
Green Fluorescent Protein is a protein produced by brightly luminescent jellyfish *Aequorea victoria*. The jellyfish is approximately 4 inches wide and shaped like

Jellyfish *Aequorea victoria*

an umbrella, which is characterized by glowing (bioluminescence) points around its margin. This bioluminescence is due to the light emission by ~ 6000 photogenic cells that consists of required components for emission in the cytoplasm. The component reasonable for emission is aequorin protein. Aequorin protein is a photoprotein with the molecular weight of 21.4kDa. In order to bioluminescence, *Aequorea* jellyfish releases Ca^{2+} ions and Aequorin protein emits blue light upon calcium binding. The blue light is absorbed by green fluorescent protein, which in turn gives off the green light. The green fluorescent protein accepts energy from Aequorin protein and reemits as green light.

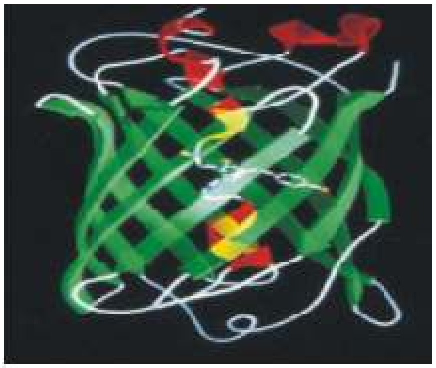
GFP from *Aequorea victoria* has a major absorption / excitation peak at a wavelength of 395nm with a minor peak at 475nm. GFP's emission peak is at 509nm which is in the lower green portion of the visible spectrum. The high molar extinction coefficients (30,000 and 7,000 M-1 cm-1) of GFPs bestow GFP highly fluorescent with a quantum efficiency of ~80%.

Fluorescence in GFP is due to the formation of fluorophoric moiety originates from an internal Ser65-Tyr66-Gly67 sequence. The

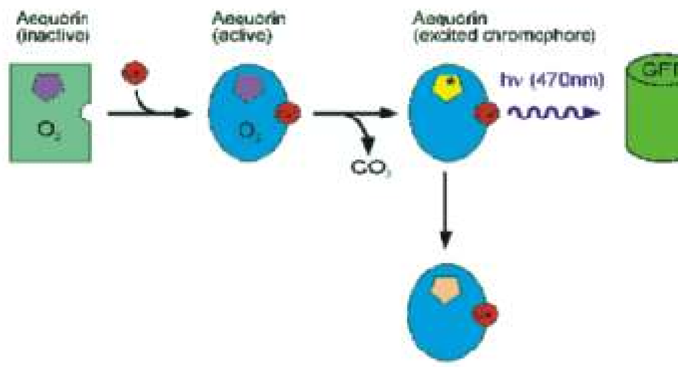


Absorption and Emission peaks of GFP

Tyr present in Ser65-Tyr66-Gly67 peptide gets oxidized during post-translational modification and cyclization reactions in between Ser65 - Gly67 in the tripeptide Ser65-Tyr66-Gly67 through a auto-catalytic process lead to the formation of fluorophoric moiety. When the protein folds this short segment (Ser65-Tyr66-Gly67) buried inside



GFP with Chromophore



Fluorescence in GFP

and own chromophore is formed after several transformations.

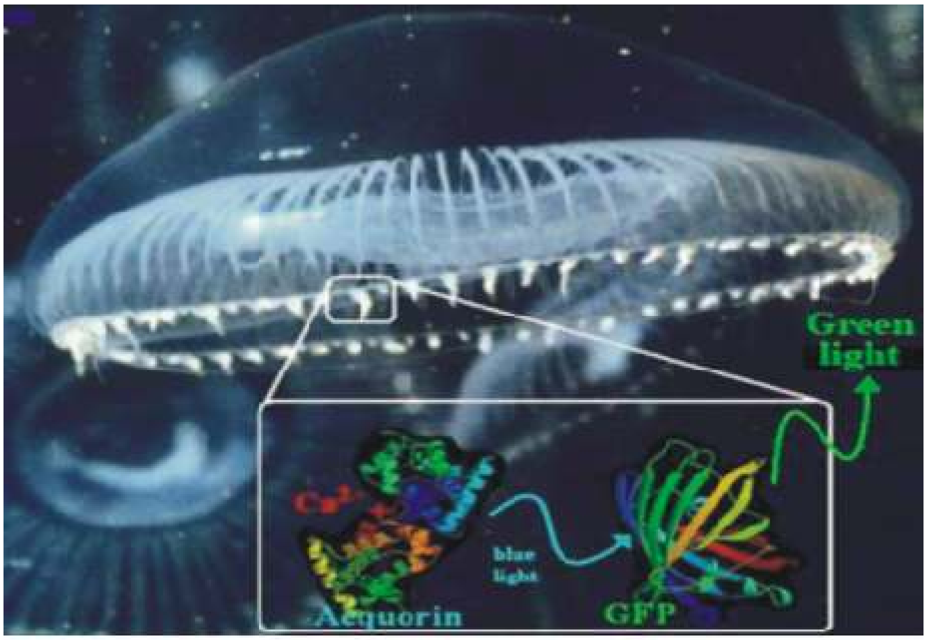
The green fluorescent protein is composed of 238 amino acids with the molecular weight of 26.9 kDa. The amino acid composition of GFP computed using BioEdit tool is given in the Table.1.

GFP Amino Acid Sequence: Length: 238

MSKGEELFTGVVPVLVELDGDVNGQKFSVSGEGEGDATYGKLTLNFICT
TGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTI
FYKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKMEYNYNS
HNVYIMGDKPKNGIKVNFKIRHNIKDGSVQLADHYQQNTPIGDGPVLLP
DNHYLSTQSALSKDPNEKRDHMILLEFVTAARITHGMDELYK

Table 1: Amino acid composition of GFP

Amino acids	Number of Amino acids	% of Amino acids
Ala	7	2.94
Cys	2	0.84
Asp	18	7.56
Glu	15	6.30
Phe	12	5.04
Gly	22	9.24
His	9	3.78
Ile	12	5.04
Lys	20	8.40
Leu	18	7.56
Met	7	2.94
Asn	14	5.88
Pro	11	4.62
Gln	8	3.36
Arg	7	2.94
Ser	11	4.62
Thr	15	6.30
Val	17	7.14
Trp	1	0.42
Tyr	12	5.04



Bioluminesce in jellyfish *Aequorea victoria*

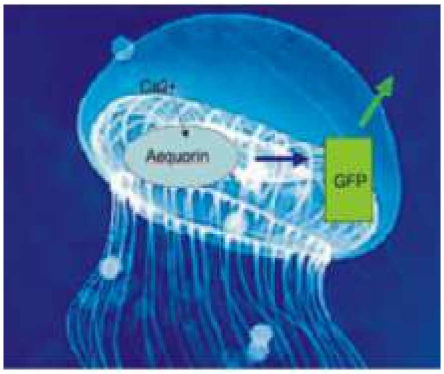
History of GFP

In 1955, Davenport and Nicol described that the jellyfish *Aequorea victoria* is bioluminescent and emits green light with the help of chemical reactions that provide the energy for photon emission.

- In 1962, Osamu Shimomura identified that the active component of the *Aequorea* bioluminescence was identified as a protein (extracted from 10,000 jellyfish), "a protein giving solutions that look slightly greenish in sunlight though only yellowish under tungsten lights, and exhibiting a very bright, greenish fluorescence in the ultraviolet of a Mineralite, has also been isolated from squeezates" Called "green protein".

Osamu Shimomura found that in order to bioluminesce, Aequorea releases calcium ions. These bind to a protein that he called **aequorin, which release blue light** upon calcium binding. The blue light is absorbed by **green fluorescent protein,** which in turn gives off the green light

- In 1969, J.W Hasting and J.G Morin named the "Green protein" as green fluorescent protein.

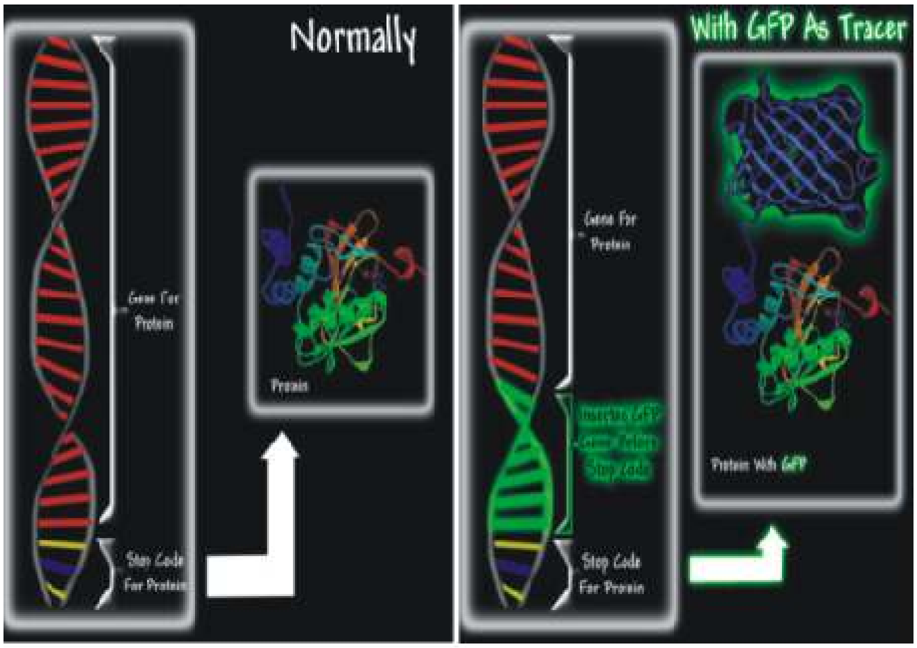


Intermolecular energy transfer between aequorin and GFPin jellyfish

- In 1974, Intermolecular energy transfer between aequorin and GFP in jellyfish was reported by J.G Morin *et.al.*, and Osamu Shimomura *et al.*,
- In 1979, Shimomura characterized the structure of chromophore.
- In 1985, Douglas Prasher clones and expresses aequorin.

Douglas Prasher

Manufacture of proteins using the instructions from the gene is called protein expression. Douglas Prasher was the first person to realize the potential of GFP as a tracer molecule. Douglas Prasher envisioned that it would be possible to use biomolecular techniques to insert the GFP gene at the end of the hemoglobin gene, right before the stop codon. When the cell needed to make hemoglobin, it would go to the hemoglobin gene, use the information encoded in the gene to make it, but instead of stopping when the hemoglobin was made, this cell would carry on making GFP until it reached the stop codon at the end of the GFP gene. As a result, the cell would produce a hemoglobin molecule with a GFP attached to it.



Expression of GFP tagged proteins

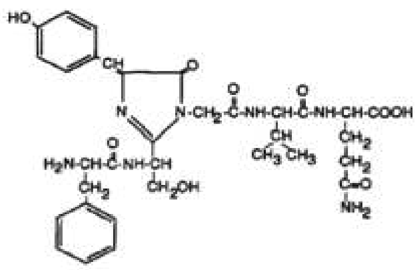
- In 1992, Prasher reported the (GFP cloned) primary structure of the Aequorea victorea green fluorescent protein.

- In 1994, GFP expressed in E. coli was reported by Martin Chalfie

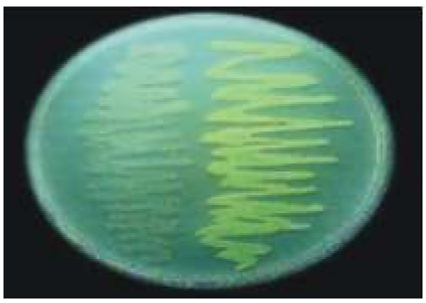
GFP Amino Acid Sequence: Length: 238

MSKGEELFTGVVPVLVELDGDVNGQKFSVSGEGEGDATYGKLTLNFICT
TGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTI
FYKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKMEYNYNS
HNVYIMGDKPKNGIKVNFKIRHNIKDGSVQLADHYQQNTPIGDGPVLLP
DNHYLSTQSALSKDPNEKRDHMILLEFVTAARITHGMDELYK

- In 1993, C.W Cody and D. C Prasher reported the Chemical Structure of the hexapeptide chromophore of the Aequorea Green fluorescent protein



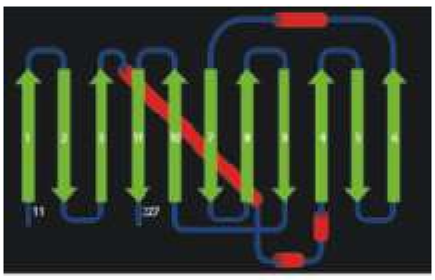
Fluorescence chromophore formed by amino acid residues 65-67 (Ser-Tyr-Gly) in the primary structure of GFP



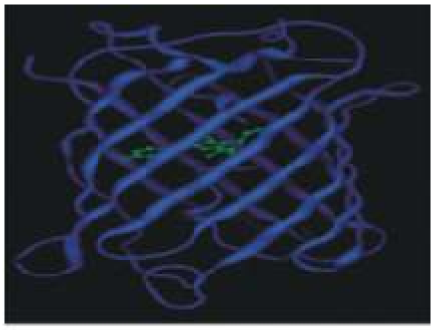
Expression of GFP in *E. coli.*

- In 1995, R.Y Tsien et.al, reported the Enhanced GFP (EGFP) i.e., S65T mutant (Ser65 mutated to Thr) and found that posttranslational oxidation of S65T GFP was four times faster than wild type GFP.

- In 1996, First crystal structures of wild-type GFP (and enhanced GFP) was reported by Tsien et.al. GFP has a unique soda can shape. Eleven beta-strands make up the beta-barrel and an alpha-helix runs through the center. The chromophore is located in the middle of the beta-barrel, it is occasionally referred to as the “light in the can.”

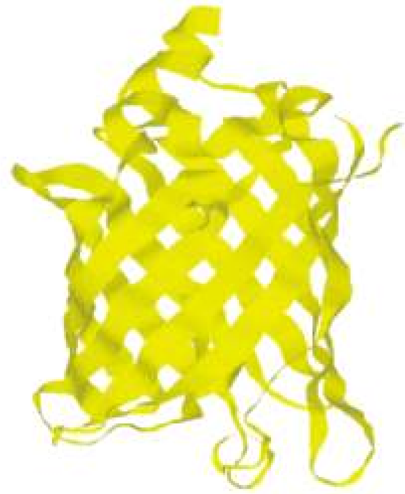


Structure of GFP reported by Tsien *et.al*



GFP beta barrel (Chromophore shown in green)

- Tsien et.al, reported a yellow fluorescent protein T203Y; mutant based on S65T GFP.



Structure of yellow fluorescent protein

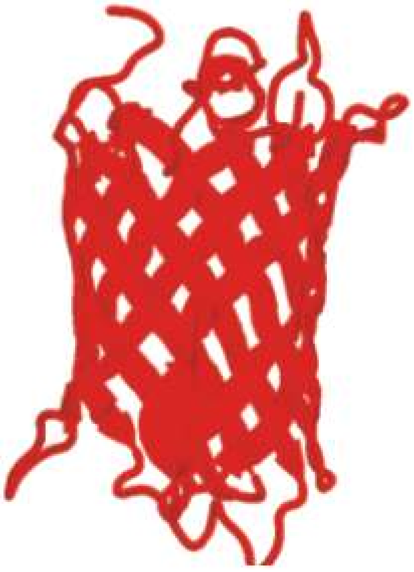
- In 1997, Tsien *et.al*, reported fluorescence resonance energy transfer (FRET) between two fluorescent proteins linked by the calmodulin (a protein that changes its shape in the presence of Ca^{2+}. Photoisomerization between A(neutral) and B(anionic) forms of wild-type GFP elucidated.

- In 1999, Sergey A. Lukyanov *et.al*, reported Red fluorescent proteins (DsRed) in anthozoan corals. Discovery of DsRed leads to discovery of many new fluorescent proteins and chromoproteins.

- In 2000, Sergey A. Lukyanov *et.al*, reported Fluorescent timer protein (mutant of red fluorescent protein which changes its

S. A. Lukyanov

fluorescence from green to red over time). The rate of color conversion is independent of protein concentration and therefore "fluorescent timer" was used to monitor activation and down-regulation of target promoters on the whole-organism scale.



Structure of
Red fluorescent protein

- In 2002, Monomeric DsRed (Tsien *et.al,)* photoconverible (Miyawakil *et.al,*) and photoactivatable (Lippincott-Schwartz *et.al,*) fluorescence proteins was created.

- In 2003, Sergey A. Lukyanov *et.al,* reported kindling protein (KFP) that can undergo irreversible photoconversion from non-fluorescent to stable red fluorescent form upon photoactivation.

- In 2004, Tsien *et.al,* reported a new "fruit" fluorescence protein generated by in vitro and in vivo directed evolution.

- In 2007, Molecular thermometer; small-scale temperature measurements based on the detection of fluorescent protein blinking was reported by C. Fradin *et.al.*

- In 2008, the first mutant of the *Aequorea victoria* GFP that forms a red chromophore was reported by Sergey A. Lukyanov *et.al*.

Green Fluorescent Protein: A Tagging Tool

Since the discovery of GFP, researchers have developed various methods to study the biological processes (that were previously invisible, such as development of nerve cells in the brain and cancer cell spreading etc.,) by connecting GFP with the proteins under study (i.e., invisible proteins). Connecting GFP, with other invisible protein helps to observe and study the positions, movements, properties and interactions of the tagged proteins. Researchers have succeeded in tagging different nerve cells in the brain of a mouse with a kaleidoscope of colours.

Reasons to use GFP as a Tracer / Reporter molecule

1. If enough protein with attached GFP were made, it would be easy to detect and to trace it when it moves through the cell. Because irradiating the cell with ultra violet light would cause the GFP attached to the protein to fluoresce.

2. GFP is a small protein (of 238 amino acids). A small protein attached to the protein of interest will not affect its property or function. GFPs small size would also allow it to follow the fused protein.

3. It had been shown by the researchers that once GFP was made in the jellyfish, it was fluorescent. Most other bioluminescent molecules require the addition of other substances before they glow. For example, aequorin will glow only if calcium ions and coelenterazine have been added, and firefly luciferase requires ATP, magnesium, and luciferin before it luminesces. This would make GFP a much more versatile tracer than either aequorin or firefly luciferase, which were being used as tracers.

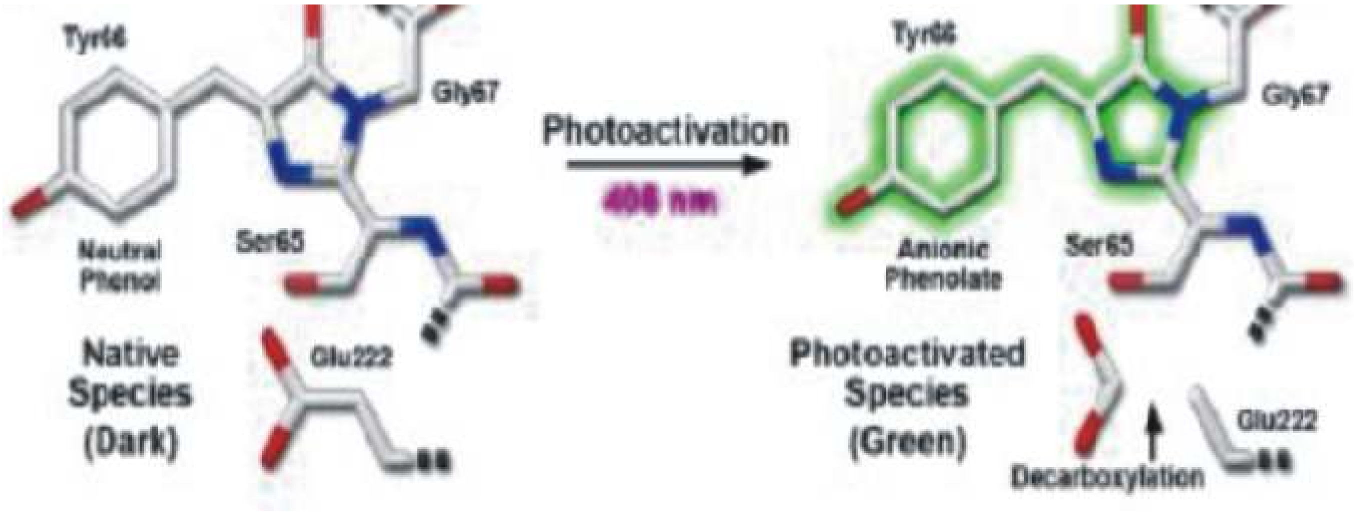
Concluding remarks: Tagging studies using GFP

The most common use of GFP has been to monitor the location, movement and chemical reactions involving proteins expressed as fusion partners with GFP. Some of the successful tagging research studies using GFPs are listed here.

- Dynamics of protein populations to chemical inhibitors, mutations and gene knock outs (Ellenberg *et al.*, 1998).

- Temporal expression of genes (Kalir *et al.*, 2001).

- Monitoring the intracellular location and concentration spectrum of whole proteasome of different organisms (Ghaemmaghami *et al.*, 2003).



- Kinetic phenomena in cells by single-cell, single-molecule microscopy based on GFP fluorescence (Xie *et al.*, 2008).

The SwissProt database and Protein Data Bank (PDB) consists of many sequences and structures of various fluorescent proteins. Any researcher from all over the world can download these protein sequences and structures for computational studies.

Brain cells of a laboratory mouse are shown glowing with multicolor fluorescent proteins (Harvard University in Cambridge)

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He has published many research papers, book chapters and review articles in International and National journals, books and magazines. He is a recipient of Summer Research Fellowship in the year 2008 offered jointly by theIndian Academy of Sciences, Indian National Science Academy and The National Academy of Sciences, India.