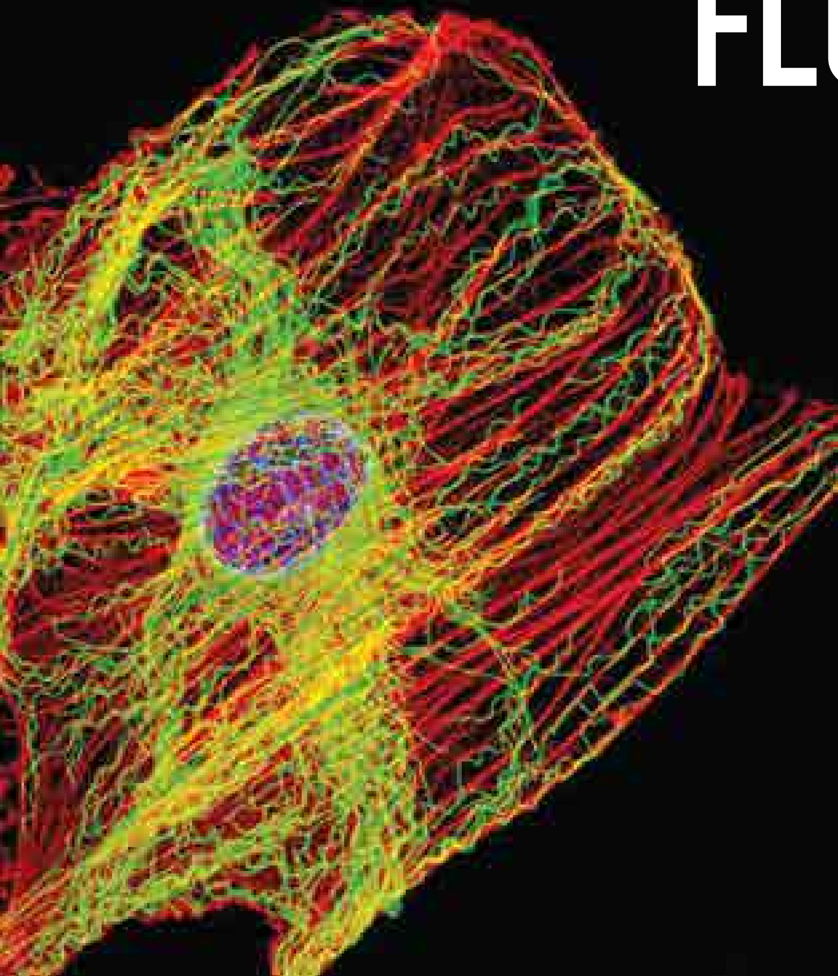


FLUORESCENT TAGS: INDISPENSABLE TOOLS IN MEDICINE

NEERAJA DASHAPUTRE

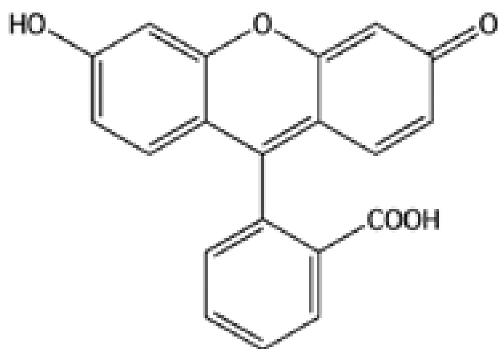
The use of fluorescent tags in imaging and disease detection has been part of many important scientific discoveries. What is fluorescence? How was it discovered? How are fluorescent molecules used to detect disease?

In the year 2008, the Nobel Prize in Chemistry was jointly awarded to Osamu Shimomura, Martin Chalfie, and Roger Tsien for discovering and using a fluorescent jellyfish protein to study the inner workings of a cell. In their announcement, the Nobel Foundation emphasized the role these contributions had played in making it possible to *"watch processes that were previously invisible, such as the development of nerve cells in the brain or how cancer cells spread"*[1]. In doing so, it acknowledged the rapid strides that the discovery of fluorescent molecules and their applications as tags have precipitated in other fields, particularly biology and medicine.

What are Fluorescent Tags?

Fluorescence refers to a physical phenomenon where certain materials emit light when (and as long as) they are exposed to electromagnetic (visible, ultraviolet etc.) radiations. This process involves three stages. Fluorescent materials have certain molecules (usually aromatic) called fluorophores that absorb photons (or energy) from the radiation. Consequently, electrons in these fluorophores jump to a higher and more unstable energy state. Unable to remain there for longer than a few nanoseconds, the electrons fall back to lower and more stable energy states by releasing some of the excess energy into the environment as light. As a result, such objects look like they are glowing from within.

Fig. 1. Fluorescein.



(a) Structure.

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(b) A red dye in powder form.

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(c) The bright green glow of fluorescein dissolved in water, as seen under UV light.

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The earliest description of fluorescence dates back to 1565, when the Spanish physician and botanist Nicolas Monardes observed the bright blue glow that the bark of *Lignum nephriticum* (Mexican Kidneywood) showed in water. This glow came from the fluorophore matlaline, naturally produced by the oxidation of flavonoids in the tree bark. The fact that synthetic materials could also exhibit fluorescence was first demonstrated by Adolf Von Bayer, a German chemist, in 1871. Bayer produced a red dye, called Fluorescein, that became bright green when diluted with water (refer **Fig. 1**). An investigation into Fluorescein's structure showed a network of alternating single and double bonds, known as conjugation. Chemists have, today, used this knowledge to synthesize a library of conjugated fluorescent dye molecules that display a wide spectrum of colors.

One of the most famous uses of Fluorescein was discovered during the Second World War. German pilots were instructed to open vials of Fluorescein from their emergency kits to indicate their locations when they were shot down into the ocean. On being diluted, Fluorescein would produce a bright patch of fluorescent green in the surrounding waters that could be detected by air sea rescue operations over long distances. This ability of some fluorescent materials to act as 'tags' has found extensive applications. For example, apart from being used in sea rescue operations even today, Fluorescein is also used in detecting oil and water leakages. But, more importantly, both natural and synthetic fluorescent materials have found extensive applications in science as fluorescent tags, probes or labels. These tags can be chemically or enzymatically attached to biomolecules like amino acids, proteins, antibodies or nucleic acids. When exposed to certain wavelengths of light, the tags fluoresce revealing the presence, location and patterns of the linked biomolecules in cells, tissues or organs (refer **Fig. 2**).

A key breakthrough in fluorescence imaging involved the discovery of naturally occurring fluorescent proteins in 1962. Osamu Shimomura, a Japanese organic chemist and marine biologist, was studying the blue-green glow of a jellyfish species *Aequorea victoria*. While isolating a bioluminescent protein (capable of

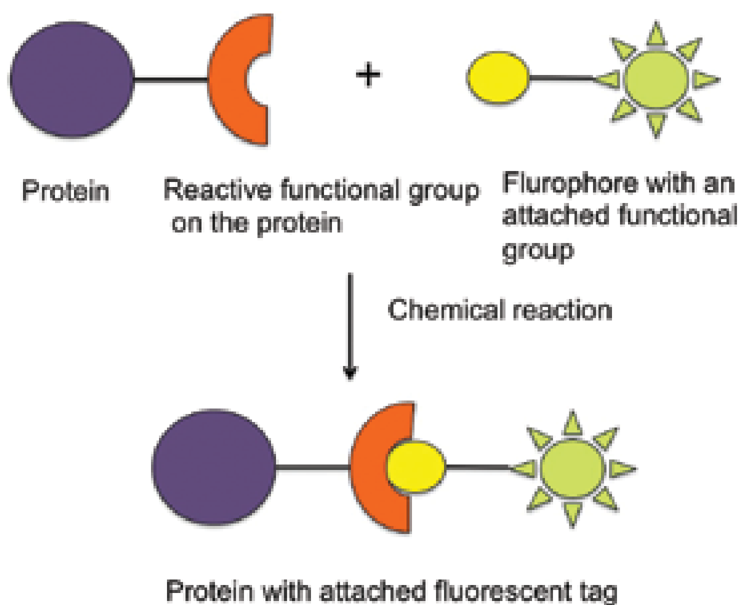

Fig. 2. A schematic showing the synthesis of a fluorescently tagged protein.

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producing light by absorbing energy from a chemical reaction) from the jellyfish, Shimomura stumbled upon the discovery of another protein that seemed to be bound to it. He named the bioluminescent protein Aequorin after the genus of jellyfish it was isolated from; and the other one **Green Fluorescent Protein (GFP)** for its bright green fluorescence under UV light. Further analysis showed that the jellyfish's greenish-blue fluorescence was produced by Aequorin and GFP together — Aequorin produces blue light by bioluminescence and GFP absorbs this blue light to produce green fluorescence. Being a natural fluorophore, produced by a sequence of amino acids, GFP is much easier to incorporate in cells than synthetic fluorophores. However, the tediousness of extracting pure forms of this protein from jellyfish limited its applications till Douglas Prasher, an American molecular biologist, was successful in cloning the GFP gene in 1992. This opened up immense possibilities — GFP could now be attached as a tag to proteins or cell organelles, or coded into the genome of the cell and expressed on demand. Martin Chalfie, an American neurobiologist, was the first to report this potential in 1994 through his results from a series of experiments on identifying specific neural circuits in the roundworm *Caenorhabditis elegans*. For example, in one of his first experiments, Chalfie used Prasher's clone to color and track six individual cells in the transparent body of the worm. In 1994, Roger Tsien, a Chinese-American biochemist, identified the chemical basis of GFP's fluorescence. Based on this understanding, he and his collaborators created a range of genetic and structural variants of GFP to intensify its natural fluorescence and expand its colour palette (to, for example, pink, yellow, red and blue fluorescence). Today, GFP and its derivatives are routinely used for imaging and have played a key role in many scientific discoveries (refer **Fig. 3**).

Applications of fluorescent tags

(a) In research: How does a cell function? How do different organelles in a cell communicate with each other? How does a cell respond to an attack by a pathogen? These are just some of the questions that have always puzzled scientists. Although cells can be visualized under a microscope, understanding submicroscopic molecular interactions and cellular processes in real-time is not possible by microscopy alone. This is where fluorescent tags play a significant role.

Fig. 3. Green Fluorescent Protein.

(a) The jellyfish *Aequorea victoria* exhibiting blue light using bioluminescence.

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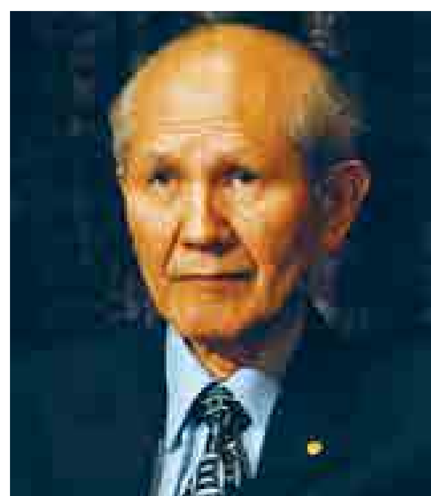

(b) Osamu Shimomura.

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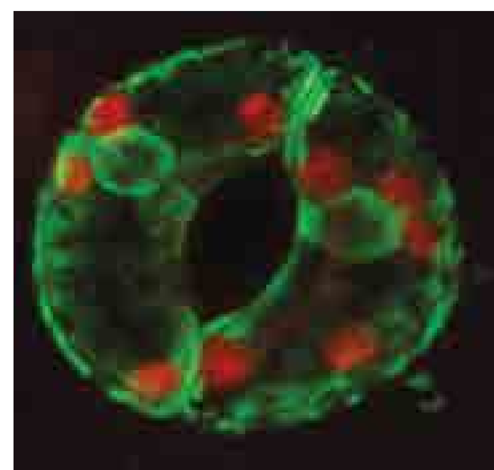

(c) A fluorescence microscopy image showing tubulins, mitochondria and the nucleus of a cell bound to fluorescent proteins.

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The presence and interactions of a biomolecule of interest can be studied by fluorescence tagging it through the formation of a covalent bond with a fluorophore (fluorescent core of the dye). For example, a chemically modified fluorophore can be linked to the functional group (like amines, hydroxyl and thiols) of an amino acid or protein of interest (refer **Fig. 4**). Similarly, Ethidium Bromide (EtBr) can intercalate (stacks along the hydrogen bonds) with DNA molecules to produce a bright orange glow under UV light. Thus, specific cellular processes can be visualized in real-time by selectively tagging the specific biomolecules involved in them. For example, when attached to the genome of a virus, GFP can be used to track the pathogen's movement inside a cell. These robust and easy-to-monitor methods are widely applicable to a large range of fluorophores.

(b) In diagnosis: Diagnostic methods in the field of pathology help doctors and medical care specialists confirm if a patient suffers from a particular infection. Historically, the detection of disease has been a time-intensive process, taking anywhere between a couple of days to a few weeks. This process involves the isolation of infected samples from the patient, culturing cells from the infected sample outside the body, and confirming the presence and nature of the pathogen. Speed and precision in diagnostic assays can, however, play a significant role in determining the efficacy of treatment.

More rapid diagnostic assays used today are designed to identify specific antibody-antigen interactions. On exposure to a pathogen, specific cells (called B lymphocytes) in our body

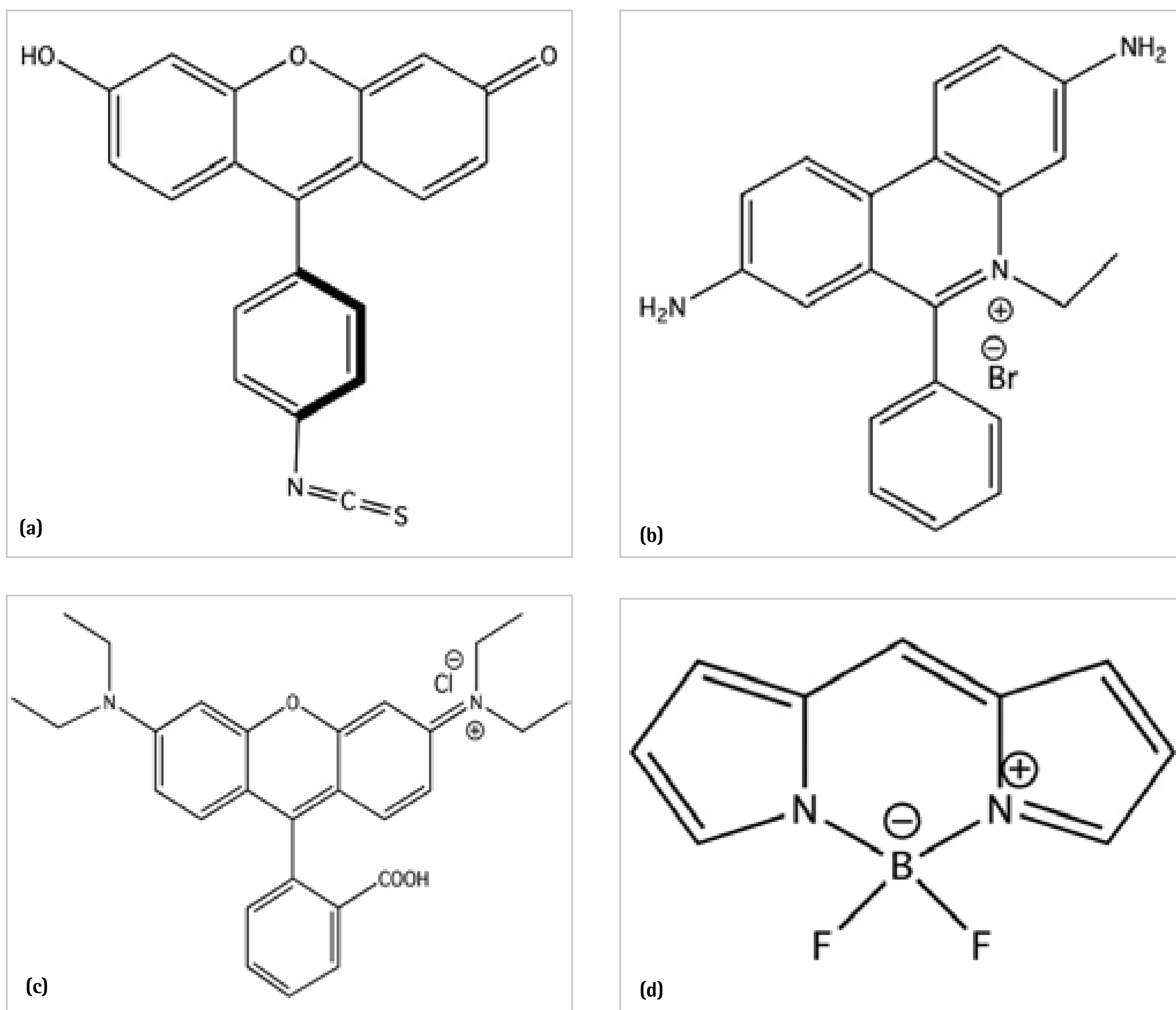

Fig. 4. The various fluorophores commonly used as protein tags. (a) Fluorescein isothiocyanate. **(b)** Ethidium bromide. **(c)** Rhodamine dyes. **(d)** BODIPY dyes.

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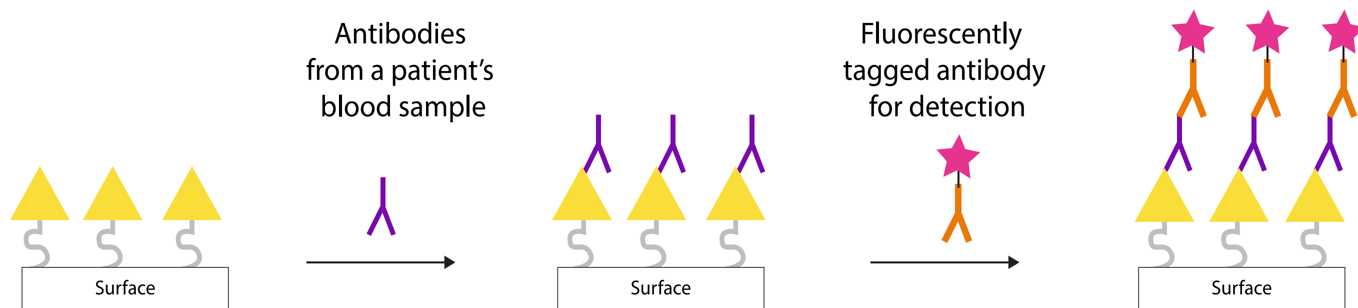

Fig. 5. Schematic of an antibody-antigen assay to detect presence of antibodies
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produce special Y-shaped proteins (called antibodies) that can bind to specific molecules (called antigens) like proteins, polysaccharides or lipids on the surface of the pathogen. The synthesis and binding of antibodies to antigens is rapid and specific.

To develop a diagnostic test, antibodies or antigens specific to a pathogen are either isolated and/or synthesized. Antibodies specific to a pathogen are immobilized/adsorbed on a solid support to which a patient's blood or serum sample is added. If the patient is infected with the pathogen, the antigens present in her blood sample bind to the antibody attached to the surface. The solid support is washed to remove any free, unbound antigen. Then, a second antibody tagged with a fluorescence marker and capable of recognizing the bound antigen is added. Special instruments are used to detect and measure the presence and intensity of fluorescence, allowing us to determine the presence and quantity of pathogen in a patient's sample. This 'sandwich' immunosorbent assay is far more rapid than conventional tests. In some cases, a viral infection is detected by adsorbing antigens unique to the pathogen onto a solid surface. These bind the antibodies specific to the blood sample of an infected person. After the unbound antibody is washed off the support, a second fluorescently tagged antibody that recognizes the bound antibody is used to detect the presence of the pathogen (refer **Fig. 5**).

To conclude

Through its many applications in diagnostics and medicine, fluorescence tagging has made immense contributions to health care. Fluorescent tags have shed light on many biological processes and enabled the study of functions of various proteins. Immunosorbent assays that use fluorescent tags offer many advantages over solution-based detection tests as they require less sample volume, are more accurate and less time-intensive. However, the vast potential of fluorescence tagging is yet to be explored fully and is likely to prove to be an indispensable tool in the future.

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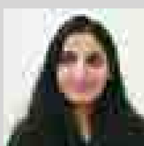

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