Glowing nature: Fluorescence emitting proteins and their scientific applications.

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Abstract

Belonging to a structurally homologous class of proteins, the green fluorescent protein (GFP) family of fluorescent proteins have the special ability to independently generate a visible wavelength chromophore from a specific sequence of amino acids present in their polypeptide chain. Their discovery was a breakthrough in the field of biology as these tools enable tracking of labeled cells and molecules, examining gene expression, visualizing dynamic processes, molecular interactions, molecule activity, and much more speeding up the research process. One of the most used fluorescent proteins is green fluorescent protein; an important tool which was first observed in 1962 in the jellyfish *Aequorea victoria*. From that point onward, GFP has enabled the observation of previously invisible processes such as the activity of cancer cells and the growth of nerve cells in the brain. Currently, a panoply of novel intrinsic or specific ligand activated fluorescent proteins including two novel families, halo- and snap-tag proteins, were used in scientific research with unprecedented outcomes in cell biology. This paper addresses properties of these different types of fluorescent proteins in a comparative fashion.

Introduction

Proteins are responsible for intricately regulating a variety of chemical reactions in the human body consequently a mechanical malfunction of these structures can result in disease. It is clearly a crucial necessity for bioscience to thoroughly understand the functions of proteins in different cell types in the body (Dramicanin *et al*., 2023). Allowing the tracking of protein locations, movements, and interactions, the Green Fluorescent Protein (GFP) historically played an indispensable role in the field of bioscience and still contributes greatly to the advancement of the medical field (Snapp *et al*., 2009; Alberts *et al*., 2002). Following the initial discovery of GFP, other intrinsically fluorescing proteins emitting fluorescence of different wavelengths as well as ligand-induced fluorescing proteins, such as the Snap-tag or Halo-tag proteins have been introduced to molecular and cellular research fields (Crivat *et al*., 2012). These tools guided researchers through the process of biological exploration helping them transform the invisible to visible at the molecular and single cell level. In this review paper, I will explore this miraculous magnificent tool, the fluorescent protein (Snapp *et al*., 2009; Crivat *et al*., 2012) with a comparative analysis of the intrinsically fluorescent GFP and the ligand activated fluorescing proteins Halo- and Snap-tag. Brief history of the discovery of fluorescent proteins

Researchers investigating the bioluminescent traits of the jellyfish, *A. victoria*, isolated aequorin along with GFP (Kremers *et al*., 2011). The jellyfish employs the small organs on its umbrella portion to produce the green bioluminescence. Animals often utilize this fluorescence for communication, deterring predators and attracting prey. Aequorin, a calcium-activated photoprotein, long before it was isolated from the animal, was subject to multiple research projects. Isolated alongside aequorin, GFP combines with aequorin to transform Ca^{2+}-induced luminescent signals into the jellyfish's distinctive green glow (Kremers *et al*., 2011). When Ca^{+2} binds to these indicators, aequorin emits blue light' the GFP then absorbs the blue light giving the protein its unique color as it glows green. After its cloning, GFP was initially

employed for tracking gene expression in bacteria and the sensory neurons of the nematode C. elegans, and it was further engineered to produce a more expanded colour palette (Campbell, 2008). Other colours included cyan, yellow, blue, orange, red, and other various colours (Kong *et al*., 2020; Kremers *et al.,* 2011). The entire group of these probes are referred to as fluorescent proteins. Shimomura led the discoveries after which followed the usage of GFP as an indispensable tool in biology. He was the first to identify GFP and isolate it from the *A. victoria*, and he also found that when exposed to ultraviolet light, this protein flashed green, and as a result he was awarded one-third of a Nobel prize in 2008 (www.nobelprize.org/prizes/chemistry/2008/shimomura/facts/).

Properties of Various Fluorescent Proteins

There are two distinct types of fluorescent proteins: those that are intrinsically fluorescing, such as the GFP and those that could be classified as self-labeling (ligand inducible) fluorescing proteins (Piston *et al*., 2024). The first group of proteins are structurally homologous fluorescent proteins, and they are used as genetic labels that could be produced inside the cell. This "built-in" structure can be achieved through transgenic approaches which involve recombinant DNA technology. This method eliminates the necessity for labeling with exogenous agents and such procedures that are required by other labeling agents. A clear approach is to fuse the fluorescent proteins with target proteins by using recombinant DNA methods (Dramicanin *et al*., 2023).

There are various types of fluorescent proteins that are currently available on the market that span almost the whole visible spectrum. They offer a wide range of options for cell studies and multicolor labeling. Among these diverse types of fluorescent proteins, the one that meets the requirement of the experiment should be precisely selected. Composed of about 200 different types of fluorescent proteins and chromoproteins, GFP-like proteins are a rapidly expanding family. In addition to green, these proteins can be found in a variety of other colors including blue, red, cyan, and yellow (Kremers *et al.,* 2011).

Properties of Halo-tag

Self-labeling proteins combine a small molecule fluorophore with a genetically encoded tag and recently they have gained a lot of interest since they can get around the drawbacks of fluorescent proteins. Halo tag is a type of self-labeling protein that is commonly utilized as it is a small, easily functionalizable ligand that is cell-permeant and exhibits quick, precise tagging (Los *et al*., 2008).

Halo-tag, among the many types of GFP, enables a single genetic construct with several functions to be used for a thorough analysis of protein function and interaction. This is achieved through a simple process where the Halo-tag ligand binds to the Halo-tag protein, typically linked to the protein of interest. This linkage process is rapid and irreversible as a covalent bond is formed between the protein and the ligand upon contact and it allows for rapid isolation and purification. The tagged protein can be employed with a range of substrates for multiple downstream applications after it has been cloned and produced, negating the need for additional cloning (Yazaki *et al*., 2020; Los *et al*., 2008).

The Halo-tag system requires only a single genetic construct, the assembly of numerous DNA sequences that are transferred and incorporated into the host genome. This is because varying Halo-tag ligands can be employed to explore various characteristics of the protein of interest. Moreover, this Halo-tag technology can be applied to the imaging of living and fixed cells due to its ability to withstand and remain stable in harsh conditions, making it ideal for a variety of in vitro and in vivo protein analysis applications.

Labelling with the Snap-tag

Like the Halo-tag, the Snap-tag is another protein-tag which is covalently attached to a protein of interest by using the recombinant DNA technology (Cole *et al*., 2013). It is modified from the human O6-alkylguanine-DNA-alkyltransferase (AGT), the DNA repair enzyme. Since most SNAP-tag substrates are chemically inert towards other proteins, using the SNAP-tag technique allows for the avoidance of nonspecific labeling. Using various methodologies to express target proteins as an N- or C-terminal fusion; however, to label the tag with cell impermeable SNAP-Surface substrates, it must be exposed to the plasma membrane's

extracellular surface (Cole *et al*., 2013).

Fluorescent Proteins v.s. Halo- and Snap-tag Comparison

Although fluorescent proteins have significantly improved the field of fluorescence microscopy, they still do have certain downsides. They are inherently pH sensitive, have limited brightness and photostability, particularly in the red-shifted region, and need oxygen for chromophore maturation. Much work has gone into creating synthetic fluorophores that are suitable for labeling proteins in biological systems to get around these problems. This is where protein tags like Halo-tag come into the picture. The protein tag technology, enabling selective binding to a synthetic ligand that can be easily appended to small-molecule fluorophores, combines the genetic specificity of the protein tag with the highly attractive photophysical properties of synthetic dyes (Los *et al*., 2008; Crivat *et al*., 2012; Cole *et al*., 2013). An example

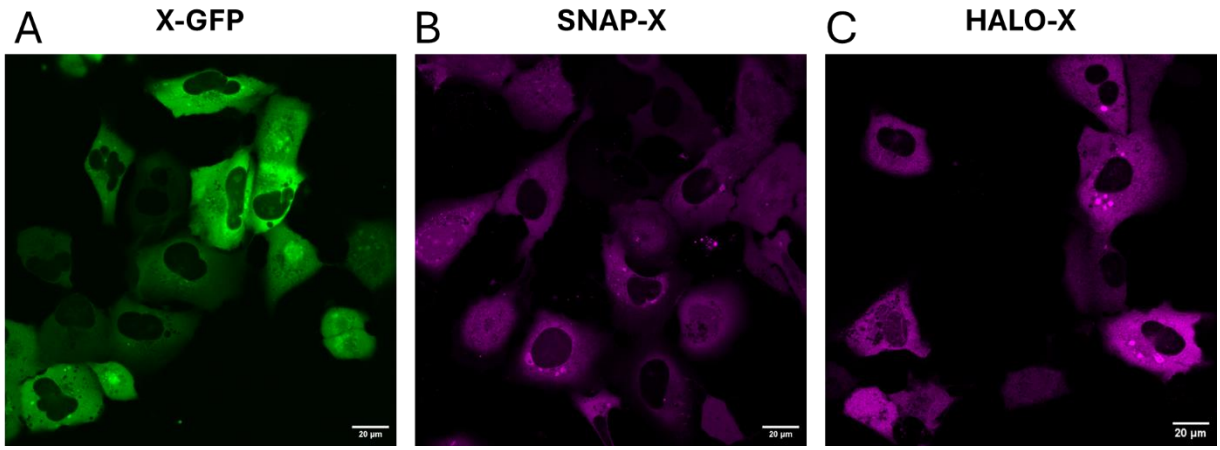

Figure 1: Comparative analysis of the subcellular localization of the same protein when tagged with different fluorescent proteins. Osteosarcoma cells (U2OS) were transfected with vector DNA containing a recombinant protein gene linked to; **A,** GFP at its C-terminus; **B,** SNAP-tag at its N-terminus; **C,** Halo-tag at its N-terminus and cells were monitored using a Zeiss-880 LSM confocal microscope. Bar indicates 20 um. A similar distribution of the X-protein was detected im all three types of fluorescent protein-labelling (Photo courtesy of Dr. Agit Çetinkaya, Gebze Technical University).

of labelling the same intrinsically disorder containing RNA binding protein (named as X-protein for simplicity) is shown in Fig.1 (above). In this experiment, protein-X was labelled with three different tags in different U2OS cell colonies, and they were analyzed by confocal microscopy. As expected, all three fluorescent protein tags indicated a similar subcellular localization of the disorder region containing RNA-binding protein of interest (Fig.1).

Discussion

Fluorescent proteins have revolutionized the field of cell biology, profoundly transforming the way researchers study cellular processes and structures. Prior to their invention, it was very difficult to investigate the dynamic processes that occur within living cells, frequently necessitating invasive methods that might disturb the cell's natural state. The discovery of fluorescent proteins, such as the GFP and its various other types, diminished these problems by providing non-invasive ways to perform these investigations on cellular structures. Alongside GFP, Halo-tag and Snap-tag are also labeling agents that are commonly utilized as they are small and exhibit quick, precise tagging.

In conclusion, all these advancements regarding fluorescent proteins have gotten the field of cell biology to where it is now, propelling it to unprecedented levels of discovery and innovation. Fluorescent proteins are poised to have an even greater impact on cell biology as they continue to develop, leading to deeper comprehension and significant advancements in the scientific understanding of life at the molecular level.

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