Fluorescence biology: Proteins and quantitative microscopy techniques

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Abstract:

Powerful tools of cell-imaging have emerged during the last decades. The combination of fluorescent proteins (FPs) with microscopy techniques have created a new fascinating area of research called fluoresce biology. Nowadays, FPs and quantitative microscopy techniques (QMTs) are widely used to answer cell-biological questions in bio-medicine and bio-molecular sciences. Since the remarkable discovery of two FPs, the green fluorescent protein (GFP) and the red fluorescent protein (DsRed), several mutations have been introduced in them allowing the development of a spectacular palette of FPs. In parallel, advances in optical components, filters, detectors, dichroic mirrors had allowed the development of microscopy techniques that can combine FPs to study, for instance, the interactions between proteins and the diffusion of relevant bio-molecules.

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The starting point of the fluorescence biology

Biomedical research has enormously benefited for the design of novel fluorescent probes and from innovations in fluorescence microscopy. The development of fluorescent-antibodies in 1940s provided the first breakthrough in visualizing the cellular proteins. However, these techniques require the cells to be fixed and permeabilized, which may cause alteration of the information [1]. As an alternative, direct labeling with organic fluorophores is possible; but this is laborious because it requires protein-purification, chemical labeling and microinjection into cells [2]. Consequently, there was a great need to develop a convenient approach which can allow the labeling and monitoring different molecules in living cells [1]. This was satisfied with the remarkable discovery in 1962 of the genetically encoded green fluorescent protein (GFP) [3], with its expression in prokaryotic and eukaryotic cells [4], and finally with its subsequent development [5]. Today, visible fluorescent proteins (FPs) are able in a variety of colors [6].

Advances in technology have been resulted in improved light sources for excitation, more versatile optical components (i.e. excitation/emission filters, dichroic mirrors) and faster and more sensitive detectors [2]. These allowed remarkable advances in digital imaging microscopy that enabled the utilization of FPs in quantitative microscopy. Currently, several cell-biological questions have been resolved using a combination of FPs with modern fluorescence microscopy techniques. Therefore, the aim of this review is to describe basic concepts of the current FPs as well as of the contemporary quantitative fluorescence microscopy techniques (QFMT) to illustrate their importance in cell-biology. Special attention will be given to GFP and other FPs

which have been applied to visualize the activities of bio-molecules in living cells. Furthermore, QFMT that have been widely used to study protein–protein interactions and the diffusion characterization of relevant bio-molecules will be covered. From them Fluorescence Resonance Energy Transfer (FRET) and Fluorescence Recovery After Photo-bleaching (FRAP) will be described in detail.

Fluorescent proteins

Discovery and development of the green and red fluorescent proteins

Shimomura *et al*, discovered the GFP as a companion monomeric protein to aqueorin, a quemiluminescent protein from the cnidarians *Aequorea victoria*. The same group published the excitation and emission spectrum of GFP, which picked at 395/475 and 508 nm respectively [3]. Remarkably, when the GFP was expressed alone in *Escherichia coli* or *Caenorhabditis elegans* retained its properties, showing that substrates or cofactors are not required for its fluorescence in prokaryotic or eukaryotic cells [4]. In 1992 the amino-acid sequence of GFP **(Figure 1, line 1)** was published [7]. Nowadays, the wild type gene has been re-synthesized with altered codons and improved translational initiation sequences to produce an enhanced GFP (EGFP) [8]. EGFP is the most widely used version of GFP in bio-molecular research, but because EGFP can be hindered by inefficient protein folding, resulting sometimes in protein aggregation and reduced fluorescence, efforts to improve folding properties, brightness, and photostability have been developed. Recently, Kremers GJ *et al*, produced by site direct mutagenesis the super GFP (SGFP) **(Figure 1, line 2)** which was based on EGFP.

SGFP has mutations V68L, S72A, M153T, V163A, S175G and A206K, which confer a faster and more efficient protein folding, accelerated chromophore oxidation *in vitro*, and a better photostability when is compared with EGFP [9]. The rest of mutations in SGFP are remnants from EGFP. Mutations F64L improve the folding at 37°C, S65T prevents the chromophore protonation, and the insertion of V at the position 2 improves the translational initiation in EGFP. Furthermore, some mutations in EGFP and consequently in SGFP are probably inadvertent and neutral *i.e.* H231L [10].

The second breakthrough for the development of FPs was the isolation of the tetrameric FP (DsRed) from *Discosoma striata*, a mushroom-shaped anemone. DsRed showed to have spectral characteristics completely different from GFP, with excitation and emission spectra of 558 and 583 nm respectively. As GFP, DsRed showed to have applications for *in vivo* analysis since DsRed could fluoresce when was expressed in mammalian cell cultures and *Xenopus* embryos [11]. However, the use DsRed has been restricted a cause of its poor maduration and because of its tetrameric form. The folding problem of DsRed have been overcome introducing the mutation N42Q resulting in the fast maturing variant DsRedT1 [12], but this protein was still prone to oligomerize by itself. This was solved by introduction of interface-disrupting mutations to create the monomeric red FP, mRFP1 [13].

Figure 1. GFP protein sequences.
Wild type GFP, line 1, and SGFP, line 2, (NCBI accession numbers AAA27722 and ABM97851 respectively) were aligned using Clone Manager Suite 7. Matches and non-matches are indicated in sky blue and green respectively [7, 9].

Visible fluorescent proteins

Several mutants of GFP have been described that have altered spectroscopic properties. This has resulted in a variety of color variants of GFP ranging from blue to greenish-yellow colors [2]. One of the first color variants of GFP was the blue FP (BFP), a Y66H mutant of GFP with excitation and emission spectra of 380 and 450 nm respectively.

BFP has a dim brightness and is susceptible to be photo-bleached. Consequently, making several point mutations in BFP, the enhanced blue FP (EBFP) was created [15]. Other proteins derived from GFP are yellow FP (YFP) and cyan FP (CFP), which are a good pair for FRET applications. Mutations Y66W and V163A in CFP conferred a blue-shifted excitation and emission spectra (435/474 nm) [14] as well as accommodation of the bulkier chromophore [15]. Interestingly, in contrast to the other VFPs that were developed using random mutagenesis, YFP was rationally designed based on the crystal structure of GFP [16]; **Figure 1**. Currently, the most used variant of YFP in biomedical sciences is Venus, having an excitation and emission spectra of 514 and 526 nm respectively, is the fast maturing FP and well expressed at 37°C. [17] Direct evolution of RFP1 has resulted in six monomeirc proteins called mFruits, which exhibit an emission maxima ranging from 540 to 610 nm and include mHoneydew, mBanana, mOrange, mTangerine, mStrawberry, and mCherry. Because of its brightness and photo- stability the most promising FPs from the mFruits group are mOrange, mCherry and mStrawberry, *i.e*. mCherry shows faster maturation and higher photostability when is compared with its parental protein, RFP1 [18, 19].

Other types of visible fluorescent proteins

Other types of fluorescent proteins have been developed for whole body mapping and super resolution imaging. EGFP expression in animal tissues is stable over indefinite time periods, allowing, *i.e.* quantitative imaging of tumor growth, metastasis formation as well as their inhibition by agents of all types [20]. However, since in biological tissues light penetration is efficient in the region of 650 and 1100 nm of great importance is the development of far red FPs. The tetrameric fluorescent protein eq611 isolated from the anemone *Entacmaea quadricolor* is the most red shifted native FP known until now with its emission maximum at 611 nm [21]. Similarly, the most far red shifted monomeric FP is mPlum with an excitation and emission maxima at 590 and 649 nm, respectively [22]. Although the spectroscopic properties of the red shifted FPs, research efforts should be done to improve their weakly fluorescent [2].
Super resolution imaging is now possible since the development of photoactivable FPs (PFPs). PFPs can be turn on at will upon intense illumination with violet light of around 400 nm. There are currently three PFPs, PAGFP, PS-CFP and PAmRFP1. PFPs contain mutations that prevent the chromophore deprotonation, *i.e*. PAGFP was created by introducing the mutation T203H in GFP, thereby enabling the excitation at 488 nm after the irradiation at 400 nm [23].

The 3D structure of the fluorescent proteins

The crystal structure of GFP revealed that the fold of this FP consists of an eleven-stranded β-barrel with a co-axial helix; **figure 2**. The barrel forms a nearly perfect cylinder of 42 Å long and 24 Å in diameter. Interestingly, the fold structure of GFP is highly conserved in all FPs, but not its sequence, suggesting a convergent evolution between them [24]. Moreover, the chromophore in GFP is contained in the helix and is composed by three aminoacids S65, Y66 and G67 in a ring like structure [16].

Quantitative fluorescence microscopy

Fluorescence resonance energy transfer

FRET is a non-radiative transfer of energy from a donor fluorophore to an acceptor fluorophore that are typically < 80 Å away. FRET occurs only, if the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor, *i.e.* in the FRET pair, CFP-YFP; **figure 3** [25].

Under these conditions energy (E) is transferred from the donor to acceptor with an efficiency defined by the **Equation 1**, in which r is the distance between the two fluorophores (donor and acceptor), Ro is the Förster distance at which 50% of the energy transfer takes place and is dependent on the extended of the spectral overlap between the donor and acceptor, the orientation of the fluorescent probes, and the quantum yield of the donor [25].

Equation 1:

$$E = \frac{1}{1 + (r/Ro)^6}$$

Equation 2:

$$D = \frac{\beta A}{4 * t_{hr}}$$

FRET with FPs is well suited to measure and study protein-protein interactions **(figure 3)**, protein folding and concentration of ions within the cells [6]. The occurrence of FRET can be visualized by microscopy measuring the quenching of donor fluorescence and sensitized emission of the acceptor by imaging of donor and acceptor fluorescence intensity [26].

Fluorescence recovery after photoblaching

All FPs can be destroyed after the exposure of a high intensity of light. This phenomenon has been used to measure the diffusion behavior several kinds of bio-molecules in a method called FRAP. In this technique a fluorescence region of interest (FROI) is bleached with an intense pulse of light and then the movement of non-bleached surrounded molecules is measured over the time [27]; **figure 4.**

Under these conditions the diffusion coefficient (D) is defined by the **Equation 2**, in which β is a constant equals to 1 for a confocal microscope, A is the surface of the bleached area, and $\mathbf{t_{hr}}$ is the half time of recovery in the FROI [27].

FRAP1 is well suited to study several kinds of biological phenomena, *e.i*, DNA repair, transcription and biological-machineries assembly [28].

Concluding remarks

Quantitative microscopy techniques (QMTs) and fluorescent proteins (FPs) constitute an essential part of fluorescent biology, which is a powerful toll to answer cell-biological questions. For instance, protein-protein interactions, folding and ion concentration within cells can be well studied using fluorescence resonance energy transfer (FRET) and FPs. Moreover, Fluorescence recovery after photobleaching with green fluorescent protein (GFP) can be used to study for example, transcription or biological-machineries assembly. Now is clear the importance of QMTs and FPs in bio-medical and bio-molecular sciences.

Conflict of interest

None declared by author.

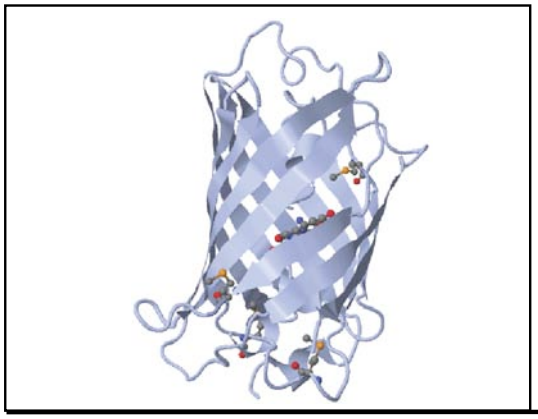

Figure 2. Ribbon diagram of the GFP crystal structure
The chromophore is buried in the protein's interior and shown in sticks representation (PDB entry 1EMA) [16].

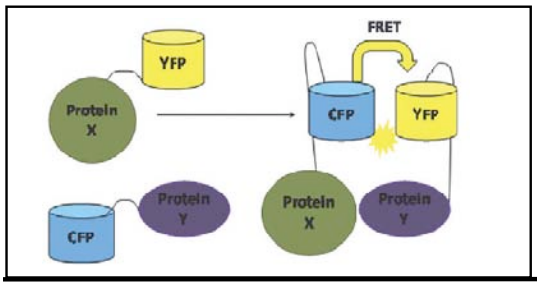

Figure 3. Design of FRET-based fluorescent proteins.
Hypothetical cellular proteins (X and Y) can be labeled with donor (CFP) and acceptor (YFP) FPs to study their interactions within the cell. FRET between CFP and YFP will only occur if protein X and Y interact. Proteins X, Y, CFP and YFP are indicated in green, purple, sky-blue, and yellow correspondingly [6].

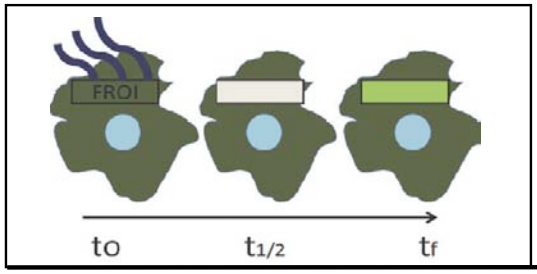

Figure 4. Design of FRAP-based EGFP fusion proteins.
An hypothetical cytoplasmatic protein can be labeled with EGFP to study its diffusion. The FROI can be belached using an intense pulse of light at t_0. Biomolecules of interest tagged with EGFP will disappear at $t_{1/2}$. Non-bleached molecules from the rest of the cytoplasmatic region will cover the FROI at t_f. Initial, mid and final times and light are represented as t_0, $t_{1/2}$, t_f and blue-wavelengths correspondingly [27]

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Biología fluorescente: Proteínas y técnicas cuantitativas de microscopia

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Resumen

Importantes herramientas de biología celular se han desarrollado durante estas últimas décadas. La combinación de proteínas fluorescentes (PF) con microscopia ha creado una nueva y fascinante área llamada biología fluorescente. Actualmente, las PF así como las técnicas cuantitativas de microscopia (TCM) son ampliamente usadas para responder preguntas de biología celular en bio-medicina y ciencias bio-moleculares. Desde el descubrimiento de dos PF: proteína fluorescente verde (green fluorescent protein, GFP) y proteína fluorescente roja (red fluorescent protein, DsRed), varias mutaciones han sido introducidas en ellas permitiendo el desarrollo de una variedad espectacular de PF. Paralelamente, los últimos avances en componentes ópticos, filtros y detectores han permitido el desarrollo de nuevas técnicas de microscopia que combinan PF para el estudio de la interacción de proteínas y la difusión de moléculas biológicas.

Palabras clave:
Proteínas fluorescentes (FPs), Componentes ópticos, Filtros, Detectores, Técnicas cuantitativas de microscopia (TCM).