Minireview

Building and breeding molecules to spy on cells and tumors

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Abstract Imaging of biochemical processes in living cells and organisms is essential for understanding how genes and gene products work together in space and time and in health and disease. Such imaging depends crucially on indicator molecules designed to maximize sensitivity and specificity. These molecules can be entirely synthetic, entirely genetically encoded macromolecules, or hybrid combinations, each approach having its own pros and cons. Recent examples from the author's laboratory include peptides whose uptake into cells is triggered by proteases typical of tumors, monomeric red fluorescent proteins and biarsenical–tetracysteine systems for determining the age and electron-microscopic location of proteins.

Keywords: Molecular imaging; Fluorescence resonance energy transfer; Fluorescent protein; Polyarginine; Biarsenical; Tetracysteine

1. Introduction

The 20th century witnessed explosive progress in macromolecular biochemistry and genetics, which started with the rediscovery of Mendelian genetics and the recognition of biopolymers and culminated in the sequencing of complete genomes. However, genome sequences alone lack spatial and temporal information and are therefore as dynamic and informative as census lists or telephone directories. The challenge for the 21st century is to figure out how these casts of molecular characters work together to make living cells and organisms and how such understanding can be harnessed to improve health and wellbeing. I believe this quest will depend heavily on molecular imaging, which shows when and where genetically or biochemically defined molecules, signals or processes appear, interact and disappear, in time and space. Therefore, molecular imaging synergistically draws upon physics, chemistry, anatomy, physiology, biochemistry and genetics.

Our first significant contribution to molecular imaging was a series of organic chemical buffers and indicators (reviewed in [1,2]) for intracellular calcium (Ca^{2+}), which is a crucially important messenger inside cells. We also synthesized novel molecules to measure other intracellular signals such as sodium [3] and proton concentrations [4], gene expression [5] and membrane potential [6–8].

In the mid-1990s, we began to put most of our effort into developing genetically encoded macromolecular indicators, which are usually administered not as ready-made proteins but as genes, which tell the cell or organism to make the indicators to our specifications. The key building blocks for such genetically encoded indicators are mutants of the green fluorescent protein (GFP) [9] from the jellyfish *Aequorea victoria*. GFP was discovered as a protein by Shimomura [10], the gene was cloned by Prasher [11], and Chalfie's and Tsuji's labs [12,13] first reported heterologous expression. GFP and its relatives have become tremendously useful in many areas of molecular and cell biology because they provided the first means by which a simple gene could give rise to bright visible fluorescence. Whenever scientists want to make a cellular protein fluorescent, their first thought nowadays is to fuse the gene for their favorite protein to the gene for a fluorescent protein (FP), then put this composite gene back into the cell or organism of interest. If all goes well, the hybrid generates a chimeric protein in which the host component goes about its normal business, while the attached FP fluoresces and reports the presence and position of the pair [9] (see Fig. 1).

Dr. Roger Heim in my lab started working in 1992 on the GFP gene provided by Prasher [11]. We very much wanted to create mutants with brighter fluorescence, because the original GFP was dim, fickle and spectrally impure. We also wanted different colors to enable fluorescence resonance energy transfer (FRET), which inherently requires a pair of colors. Heim developed the first mutants in which the spectrum of GFP was simplified and enhanced [14]; one of these mutations (S65T), right next to the chromophore, is at the heart of the optimized GFPs now routinely used around the world. Heim also created blue and cyan-emitting mutants, BFPs and cyan fluorescent protein (CFPs), respectively [15,16]. Later we helped Prof. Jim Remington's group to solve the X-ray crystallographic structure of GFP, which immediately suggested a

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Abbreviations: GFP, green fluorescent protein; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; mRFP1, monomeric red fluorescent protein, 1st version; K_d, dissociation constant; ROS, reactive oxygen species; CaM, calmodulin; ER, endoplasmic reticulum; cAMP, cyclic adenosine 3′,5′-monophosphate; PKA, protein kinase A or cAMP-dependent protein kinase; AKAR2, A-kinase activity reporter, 2nd version; FlAsH, fluorescein-based arsenical hairpin binder; ReAsH, resorufin-based arsenical hairpin binder; PF, cerebellar parallel fiber; PC, Purkinje cell; LTP, long-term potentiation

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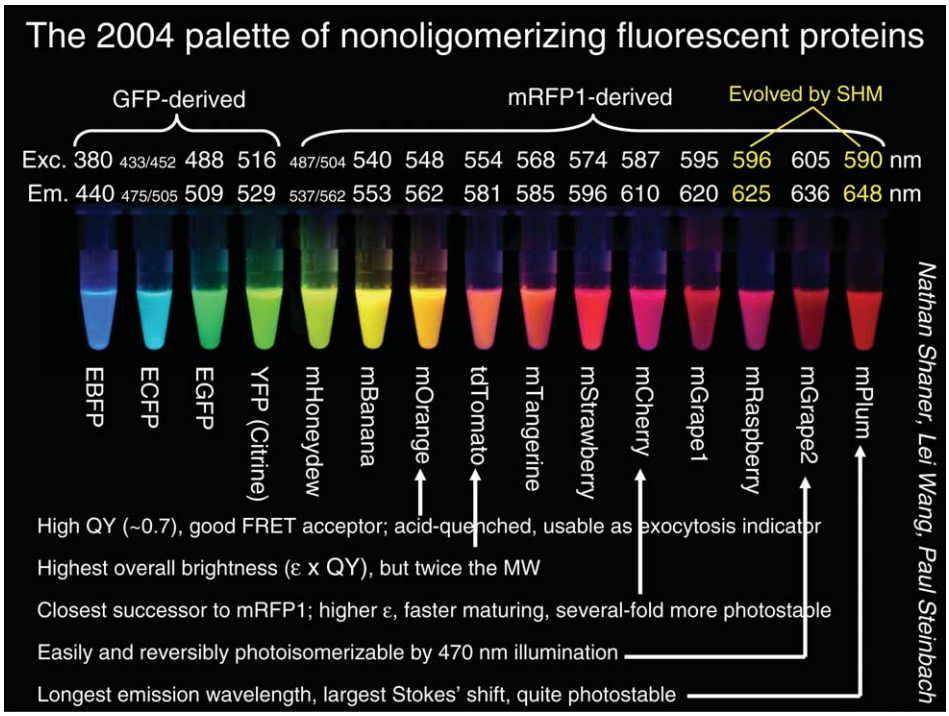

Fig. 1. Engineered fluorescent proteins cover the full visible spectrum of emissions. Protein samples were purified from *E. coli* expression systems, excited at wavelengths up to 560 nm and photographed by their fluorescence. The excitation and emission maxima are listed above the sample tubes and the names below the tubes.

way to push the emission to somewhat longer wavelengths [17]. Andrew Cubitt made the appropriate mutants, whose yellowish appearance led to the name of yellow fluorescent protein (YFPs). Currently, the best pair for FRET consists of the cyan and the yellow mutants, CFP and YFP, respectively.

2. Recent progress (1999–2004) in understanding and improving fluorescent proteins

For most applications of YFPs, it is important to minimize their sensitivity to pH, [Cl^-] and photo bleaching. We found a mutant of YFP (Q69M) with greater resistance to these factors and solved the crystal structure, which showed that the Met filled a cavity in previous YFPs [18]. We destroyed the weak dimerization of CFP/YFP to probe loose association of membrane-anchored versions in or out of lipid rafts [19].

The cloning of the first red fluorescent protein, DsRed [20], revealed nothing about its post-translational biochemistry. We showed that DsRed is an obligate tetramer, which first becomes green-fluorescent before finally maturing slowly and incompletely to red [21]. Such tetramerization explained why most attempts to fuse DsRed to other proteins produce mistargeting or toxicity. Mass spectrometry told us the covalent structure of the chromophore, formed from the GFP chromophore by an unprecedented dehydrogenation of a Cα–N bond [22]. X-ray crystallographers [23,24] confirmed the structure by observing the subtle change of that Cα from tetrahedral to trigonal. The intimacy of the intersubunit contacts led to predictions that the tetramer would be impossible to break [25], but we succeeded [26]. The resulting monomeric red fluorescent protein, "mRFP1" has become very popular for many in vivo applications [27–30].

Although others have discovered coral fluorescent proteins of varied colors, all have been wild-type tetramers. Also improvements in mRFP1 brightness and photostability would be desirable. Therefore, Nathan Shaner has evolved mRFP1 into brighter monomers ranging in emission between 534 and 638 nm. For example, "mCherry" matures more quickly and completely than mRFP1, giving higher extinction coefficient and brightness, yet bleaches about 10-fold more slowly [77].

Lei Wang developed an alternative method for evolving novel protein properties directly in mammalian cells by harnessing somatic hypermutation, the process by which B lymphocytes constitutively mutate genes for immunoglobulin or other highly expressed proteins up to 10^6 faster than normal [31]. mRFP1 was stably transfected into the B-cell line Ramos with expression controllable by doxycycline. Cells with progressively red-shifted fluorescence emissions were selected by fluorescence-activated cell sorting. After over 23 generations of selection, the emission peak gradually shifted to 648 nm, 36 nm longer than parental mRFP1 and beyond any found by semi-rational in vitro mutagenesis [78].

3. Fluorescent-protein-based indicators

Redox. James Remington's lab invented redox indicators consisting of GFP mutants with cysteine pairs, whose reversible oxidation to a disulfide profoundly shifts the excitation spectrum to shorter wavelengths, enabling ratiometric measurement [32]. Colette Dooley [33] characterized and applied these indicators in live cells, targeted them to cytosol, plasma membrane and nucleus and increased their oxidation sensitivity by placing positive charges next to the cysteines to lower the pK_a of the latter. The redox indicators become substantially