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Structure-guided wavelength tuning in far-red fluorescent proteins

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In recent years, protein engineers have succeeded in tuning the excitation spectra of natural fluorescent proteins from green wavelengths into orange and red wavelengths, resulting in the creation of a series of fluorescent proteins with emission in the far-red portions of the optical spectrum. These results have arisen from the synergistic combination of structural knowledge of fluorescent proteins, chemical intuition, and high-throughput screening methods. Here we review structural features found in autocatalytic far-red fluorescent proteins, and discuss how they add to our understanding of the biophysical mechanisms of wavelength tuning in biological chromophores.

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Introduction

Red is the color of blood, and I will seek it. – Conrad Aiken

Fluorescent proteins (FPs), originally identified as biological curiosities in sea creatures such as jellyfish [1], coral [2] and anemone [3], have become central tools of cell biology research. FPs, as genetically encoded fluorescent labels, are used as real-time protein tags, gene expression reporters, and cell lineage tracers [4]. FPs have also served as surprisingly adaptable templates for engineering chemical sensors for pH and ions [5], membrane voltage potential [6,7], and metabolites [8]. A broad palette of engineered FPs has enabled Forster resonance energy transfer experiments to characterize protein–protein interactions and various biochemical phenomena associated with protein conformational changes [9,10]. FPs are fundamental tools powering exciting new fields such as optogenetics and super-resolution microscopy [11,12].

Two motivations drive efforts to develop FPs with excitability beyond 600 nm. First, excitation beyond 600 nm would facilitate non-invasive imaging of cells in vascularized tissues. Hemoglobin is the primary absorber of light in mammalian tissue, with broad absorbance up to wavelengths to 600 nm [13], thus use of excitation light beyond 600 nm should enable better detection of fluorescently labeled cells through tissue. The second reason is to provide an additional channel for multi-wavelength imaging. The availability of a FP that can be efficiently excited at 633 nm would allow the use of this common and less phototoxic laser wavelength for imaging multiple events in living samples.

A note on nomenclature may be useful. Some orange FPs have historically been called red FPs (RFPs). In this review, we will use 'red' to describe FPs with average (not peak) emission above 620 nm, a commonly accepted orange/red boundary [14]. As there is no standard definition of 'far red', we will use a 650 nm average emission as a cutoff to be consistent with existing far-RFP naming. This differs from the plant literature, where 650–700 nm light is designated red and 700–750 nm light is designated far red [15].

Recent engineering efforts have successfully produced a number of FPs with excitation maxima above 600 nm and average emission in the far-red. Here we review structural and biophysical features of red-shifting of both excitation and emission in RFPs and far-RFPs of the GFP superfamily.

Evolutionary relationships of RFPs and far-RFPs

Far-red FPs from coral

Autocatalytic far-red FPs are derived by mutagenesis of tetrameric orange or red FPs. DsRed from *Discosoma* sp. coral, was the first orange-red FP discovered. DsRed is the natural parent of some of the most commonly used RFPs (Table 1). DsRed, which emits in the orange (peak excitation/emission 558/583 nm) [2], was engineered into the dimeric orange dTomato [16•], with similar spectra and brightness, and into the monomeric mRFP1 [17•],

Table 1

Characteristics of selected far-red fluorescent proteins

Fluorescent protein	Quarternary structure	Ex. peak[a]	Em. peak[a]	Extinction coefficient[b]	Quantum yield[c]	Peak brightness[d]	PDB number
DsRed	Tetramer	558	583	75	0.79	59	1GGX
E2-Crimson	Tetramer	611	646	59	0.12	7.0	
dTomato	Dimer	554	581	69	0.69	48	
mRFP1	Monomer	584	607	50	0.25	13	
mCherry	Monomer	587	610	72	0.22	16	2H5Q
mPlum	Monomer	590	649	41	0.10	4.1	2QLG
AQ143	Tetramer	595	655	90	0.04	3.6	4OHS
mRaspberry	Monomer	598	625	86	0.15	13	
mGrape3	Monomer	608	646	40[e]	0.03	1.2	
eqFP578	Dimer	552	578	102	0.54	55	3PIB
Katushka2	Dimer	588	633	67	0.44	29	3PJ7
eqFP650	Dimer	592	650	65	0.24	16	4EDO
eqFP670	Dimer	605	670	70	0.06	4.2	4EDS
TagRFP	Monomer	555	584	98	0.41	40	3M22
mKate	Monomer	585	635	42	0.30	13	3BXB
mKate2	Monomer	586	630	50	0.36	18	
TagRFP675	Monomer	598	675	56	0.08	4.5	4KGF
mNeptune2.5	Monomer	599	643	95	0.28	27	
mNeptune2.5	Monomer	599	643	95	0.28	27	
mNeptune	Monomer	600	650	67	0.20	13	3IP2
mCardinal	Monomer	604	659	87	0.19	17	4OJ0, 4OQW
TagRFP657	Monomer	611	657	34	0.10	3.4	

[a] Excitation and emission maxima in nm.
[b] Maximum extinction coefficient per chromophore in $mM^{-1}\,cm^{-1}$ measured by the alkali denaturation method.
[c] Quantum yield of fluorescence.
[d] Calculated as peak extinction coefficient per chain in $mM^{-1}\,cm^{-1}$ multiplied by quantum yield.
[e] After photoactivation by 470 nm light. See text for references. FPs are grouped into DsRed and eqFP578 clades. Within each clade, they are ordered first by quarternary structure then by excitation peak.

with true red emission but with brightness reduced to 22% of dsRed (Table 1).

From mRFP1, further red-shifted RFPs were engineered. mCherry, perhaps the most used RFP, shows slightly redder spectra and slightly improved brightness [16•]. mRaspberry and mPlum feature more dramatically red-shifted spectra with mPlum being the first FP with average emission in the far-red [18•]. Raspberry impressively is as bright as its parent, but, the red-shifted emission of mPlum came at the cost of reduced brightness. mGrape3, in which a π–π interaction was introduced at the chromophore for the purpose of red-shifting excitation (discussed below), exhibited a red-shifted excitation peak at 608 nm, but only transiently after illumination by 470 nm light [19••]. mGrape3 is also rather dim, retaining only 2% of the brightness of dsRed (Table 1).

The same π–π interaction as in mGrape3 was also introduced in a DsRed variant, creating the tetrameric E2-Crimson [20•], which was brighter than mGrape3, retaining 12% of the brightness of dsRed (Table 1). The dimness of mGrape relative to E2-Crimson mirrors the dimness of mRFP1 relative to DsRed, and in both cases are primarily due to decreased quantum yield. This suggests a role for tetramerization in stiffening the chromophore pocket and thereby improving quantum yield.

Far-red FPs from anemone corals

An orange FP from the bubble-tip anemone Entacmaea quadricolor, eqFP578 [21•], has served as a highly successful scaffold for engineering red and far-red FPs (Table 1). The dimeric eqFP578 was red-shifted to create the dimeric RFP Katushka [22•], which was further evolved into the dimeric far-RFPs eqFP650 and eqFP670 [23] (Table 1). Meanwhile eqFP578 was also monomerized to create TagRFP, which was evolved into monomeric RFPs mKate [22•] and mKate2 [24]. mKate then served as the parent for the far-RFPs mNeptune [19••], mNeptune2.5 [25••], TagRFP657 [26], and TagRFP675 [27]. Finally, mNeptune was further red-shifted to create the bright mCardinal [25••] and a series of mNeptune variants with significantly redder emissions but lower brightness similar to TagRFP675 [28] (Table 1). Thus eqFP578 has been exceptionally productive as a scaffold for the engineering of far-red FPs.

Interestingly, eqFP578-derived RFPs and far-RFPs generally show brighter fluorescence than their DsRed-derived counterparts at the same excitation wavelengths. For example, mKate is brighter than mCherry, and mNeptune is brighter than mRaspberry (Table 1). The trend to higher brightness of eqFP derivatives has extended to even redder excitation wavelengths, so that the eqFP578-derived mCardinal and TagRFP657 are both