Fluorescent Proteins

Scott Olenych, PhD
solenych@zeiss.com
Aequorea Victoria, Green Fluorescent Protein:
The protein that started it all
Fluorescent Protein Timeline

Roger Tsien

Osamu Shimomura

Day and Davidson
38: 2887 (2009)

Martin Chalfie

Douglas Prasher
Fluorophores for Live-Cell Imaging, Superresolution & FRET

Live-Cell Imaging Requires High Specificity, Low Background Signal, and Photostability

Fluorophore photophysical & photochemical transitions critical for breaking diffraction barrier
Live-Cell Imaging for Temporal and Spatial Investigations

mKusabira Or – H2B in RK-13 Cells  mEYFP – ER in U2OS Cells

Laser Scanning Confocal Microscopy
30 second Time Lapse Interval

Spinning Disk Confocal Microscopy
2 Second Time Lapse Interval

MW Davidson, FSU NHMFL
Origin of Fluorescent Proteins: Nidogen/Entactin

- Cellular basement membrane protein
- Found in *C. elegans*, vertebrates and *D. melanogaster*
- G2 Fragment 11 stranded β barrel with a central α helix
- Not fluorescent

Nature Structural Biology, vol.8, 7, 2001, 634-640
Origin of Fluorescent Proteins:
The family tree

Architecture of *Aequorea victoria* GFP

*Aequorea victoria* GFP
238 Amino Acids

FP Barrel can reduce FRET Efficiency by ~ 40-60%

![Figure 1](image-url)
GFP Spectral Properties:
wtGFP and EGFP (S65T) and variants
GFP Spectral Properties:
wtGFP and EGFP (S65T) and variants
Improved GFPs Through Mutagenesis

~75% mutations occur in strands 7, 8, & 10

A206K avGFP common “monomerizing” mutation

λ mutations occur near central α-helix

Cyan and Yellow mutations at termini from CyPet & YPet

Folding mutations occur throughout the sequence

- Cyan and Yellow mutations at termini from CyPet & YPet
- A206K avGFP common “monomerizing” mutation
- ~75% mutations occur in strands 7, 8, & 10
- Folding mutations occur throughout the sequence
Formation of the EGFP Chromophore
## Properties of GFP variants

<table>
<thead>
<tr>
<th>Protein (Acronym)</th>
<th>Ex (nm)</th>
<th>Em (nm)</th>
<th>EC (x 10^3)</th>
<th>QY</th>
<th>Photostability</th>
<th>Quaternary Structure</th>
<th>Brightness (% of EGFP)</th>
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<td><strong>Blue Fluorescent Proteins</strong></td>
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<td>526</td>
<td>45.0</td>
<td>0.58</td>
<td>+</td>
<td>Monomer</td>
<td>78</td>
</tr>
</tbody>
</table>
High Performance Blue Fluorescent Proteins

**Sirius (GFP Derivative)**
- Emission Max: 424 nm
- Brightness: 3.6
- Photostability: ~75
- Phenylalanine Chromophore

**EBFP2 (GFP Derivative)**
- Emission Max: 448 nm
- Brightness: 18
- Photostability: 55
- EBFP + S30R + Y39N + T65S + S72A + N105T + I128V + V150I + D155V + I171V + N198S + A206V + V224R

**TagBFP2 (Aequorea macrodactyla Jellyfish Derivative)**
- Emission Max: 454 nm
- Brightness: 44
- Photostability: ~60
- LYG Chromophore

All BFPs can be imaged with DAPI Filter sets
Omega QMax Blue Best for BFPs
Cyan and Green Fluorescent Proteins

- **mTurquoise & mCerulean3 (mCerulean Derivatives)**
  - Emission Max = 475/503 nm
  - Brightness ~ 25
  - Photostability ~35

- **mTFP1 – Teal FP (Coral Derivative)**
  - Emission Max = 492 nm
  - Brightness = 54
  - Photostability = 110

- **Superfolder GFP (GFP Derivative)**
  - Emission Max = 510 nm
  - Brightness = 54
  - Photostability = 157

- **mEmerald (High-Performance EGFP Derivative)**
  - Emission Max = 509 nm
  - Brightness ~ 50
  - Photostability = 165
  - EGFP + 4 Folding Mutations (F64L / S65T / S72A / N149K / M153T / I167T)

Cyan proteins require special filter set – Green proteins use FITC
Architecture of the DsRed Variants
Oligomerization of the DsRed Variants: An inherent property

Oligomerization interferes with proper localization in fusions that form biopolymers.

Oligomerization produces aggregation artifacts in live cells.

Figure 2
Overcoming Oligomerization of the DsRed Variants

In some cases, tetramers and dimers don’t affect localization.

Tandem Dimer may work similar to a monomer but at twice the size.
Many red proteins contain “monomerizing” mutations in Strands 6, 8 & 9.

mCherry mutations occur near chromophore.
Anthozoa Fluorescent Protein Variants:
DsRed Chromophore Formation
Orange and Red Fluorescent Proteins

mKO2 (Kusabira Orange; Coral Derivative)
Emission Max = 565 nm  Brightness = 40  Photostability = 100

tdTomato (dsRed Derivative)
Emission Max = 581 nm  Brightness = 95  Photostability = 75

mApple (mOrange Derivative; 18 Mutations)
Emission Max = 592 nm  Brightness = 37  Photostability ~ 100

mCherry (mRFP1 Derivative)
Emission Max = 610 nm  Brightness = 16  Photostability ~ 100

Orange FPs can be imaged with a TRITC or Cy3 filter set
Red FPs require Texas Red or Longpass filter set
Red and Far Red Fluorescent Proteins

**mKate and Derivatives (Coral Derivatives)**
Emission Max = 635 nm   Brightness = 9 (15)   Photostability ~ 166

\[ \text{mKate} = \text{mTagRFP + R70K - N146S - F177L - H200R} \]

**mRuby (eqFP611 Derivative)**
Emission Max = 605 nm   Brightness = 39   Photostability ~ 100

\[ \text{mRuby} = \text{eqFP611 + 28 mutations} \]

**mNeptune (mKate Derivative)**
Emission Max = 650 nm   Brightness = 13   Photostability ~ 150

\[ \text{mNeptune} = \text{mKate + 5 mutations} \]

Red FPs are best imaged with a Texas Red or Longpass filter set
The “mFruit” Proteins are Ideal for Live-Cell Imaging

mCherry – Actin fusion expressed in Rabbit Kidney (RK-13) Cells

543 nm; LSCM; 30-second TL; 24 hour observation; 40x Oil

MW Davidson, FSU NHMFL
Optical Highlighters

Protein tracking
Parameters determined:
- Movement rate and direction
- Diffusion coefficient
- Mobile and immobile fractions
- Time parameters of compartmental residency and exchange between compartments
- Rate of turnover

Organelle tracking
Parameters determined:
- Movement rate and direction
- Rate of content interchange
- Fission and fusion events

Cell tracking
Parameters determined:
- Movement rate and direction
- Cell localization
- Rate of cell division
- Shape and volume of cells

Nat Rev Mol Cell Biol 2005 vol. 6 (11) pp. 885-890
Optical Highlighters

**Green to Red**

- Photoconversion
  - Kaede – Tetramer
  - Eos – Tandem Dimer
  - mEos2 – Monomer
  - Dendra – Monomer
  - KikGR – Tetramer
  - mKikGR – Monomer
  - mClavGR – Monomer

**Photoactivation**

- PA-CFP – Monomer
- PS-CFP – Monomer
- PA-mCherry1 – Monomer
- PA-TagRFP – Monomer

**Photoswitching**

- Dronpa – Monomer
- KFP1 – Tetramer
- mTFP0.7 – Monomer
Photoactivatible Fluorescent Proteins: PA-GFP

PA-GFP is activated with 405 nm light

Prior to activation, will fluoresce at 8% brightness of EGFP (effectively “off”)

Will fluoresce at 40% brightness after activation
Photoconvertible Fluorescent Proteins: tdEos Photoconversion

RK-13 cells expressing tdEos Mitochondria

MW Davidson, FSU NHMFL
Photoconvertible Fluorescent Proteins
Photoconversion may be Common

8 of 12 proteins tested displayed some photoconversion

Conversion rate increases supralinearly with laser power

Nature Methods, 2009,(6), 5,355-360
J Schwartz/Duke
Photoswitching Fluorescent Proteins: Dronpa

- A7r5 cells expressing Dronpa-Actin
- Capable of hundreds of cycles

MW Davidson, FSU NHMFL
Photoswitching Fluorescent Proteins
Photoswitching Fluorescent Proteins:
rsEGFP

$\varepsilon$ (M$^{-1}$cm$^{-1}$)
47,000
QY
0.36
~50% GFP

Optical Highlighter Proteins:
Palm - Photoactivated Localization Microscopy

Widefield TIRF Images  PALM Image

A B C

Tandem dimer-Eos-Vinculin in Fox Lung Fibroblast Cells
Optical Highlighter Proteins:
PALM - Photoactivated Localization Microscopy

1. Photoactivate and image PA species single molecules with a high degree of precision
2. Photobleach and repeat step 1 until all molecules are expended
3. Localize single molecule centers and construct super-resolution image
Fluorescent Protein Timers:
Fast-FT, Medium-FT and Slow-FT

Nat Chem Biol, 2009 vol. 5 (2) pp. 118-126
Fluorescent Protein Timers:
Fast-FT, Medium-FT and Slow-FT

Table 1: Properties of the blue and red forms of the purified FTs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Excitation peak (nm)</th>
<th>Emission peak (nm)</th>
<th>Extinction coefficient (M⁻¹ cm⁻¹)</th>
<th>Quantum yield</th>
<th>pKₐ</th>
<th>16 °C</th>
<th>25 °C</th>
<th>37 °C</th>
<th>45 °C</th>
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<tbody>
<tr>
<td>Fast-FT</td>
<td>Blue form</td>
<td>403</td>
<td>466</td>
<td>0.30</td>
<td>2.8</td>
<td>1.6</td>
<td>0.58</td>
<td>0.25</td>
<td>0.18</td>
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<tr>
<td></td>
<td>Red form</td>
<td>583</td>
<td>606</td>
<td>75,300</td>
<td>0.09</td>
<td>42</td>
<td>18</td>
<td>7.1</td>
<td>4.2</td>
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<tr>
<td>Medium-FT</td>
<td>Blue form</td>
<td>401</td>
<td>464</td>
<td>44,800</td>
<td>0.41</td>
<td>2.7</td>
<td>2.2</td>
<td>1.6</td>
<td>1.2</td>
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<tr>
<td></td>
<td>Red form</td>
<td>579</td>
<td>600</td>
<td>73,100</td>
<td>0.08</td>
<td>4.7</td>
<td>23</td>
<td>8.8</td>
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<td>Slow-FT</td>
<td>Blue form</td>
<td>402</td>
<td>465</td>
<td>33,400</td>
<td>0.35</td>
<td>2.6</td>
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<td>20</td>
<td>9.8</td>
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<td>Red form</td>
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<td>604</td>
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<td>0.05</td>
<td>4.6</td>
<td>108</td>
<td>69</td>
<td>28</td>
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</table>

Characteristic times correspond to fluorescence maxima for the blue forms, and to maturation half-times for the red forms (Fig. 2c).

Nat Chem Biol, 2009 vol. 5 (2) pp. 118-126
FUCCI:
Fluorescent Ubiquitination Cell Cycle Indicator

• mAzami Green Geminin expressed during S/G2/M Phases

• mKusabira Orange 2 Cdt1 expressed during G1 Phase

• Ubiquitination targets proteins for destruction during other cell Cycle phases via the proteosome

Chemistry and Biology 15, 2008, 97-98
FUCCI:
Fluorescent Ubiquitination Cell Cycle Indicator

- HeLa cells expressing the mAG-Geminin and mKO2 Cdf1 plasmids
- All phases of cell cycle are visible

Cell, 132, 3, 2008, 487-498
FUCCI:
Fluorescent Ubiquitination Cell Cycle Indicator

- Transgenic mice embryos expressing FUCCI have balanced red and green expression
- Green/Red ratio decreases over time

Cell, 132, 3, 2008, 487-498
Imaging with Fluorescent Proteins:
Neptune – Optimized for Intravital Imaging
# Imaging with Fluorescent Proteins: Neptune – Optimized for Intravital Imaging

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**Table 1. Characteristics of Far-Red Fluorescent Proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Excitation Peak&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Emission Peak&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Φ&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Φ&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Brightness Excited at Peak&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Brightness Excited at 633 nm&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Photostability&lt;sup&gt;f&lt;/sup&gt;</th>
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<tr>
<td>dTomato&lt;sup&gt;g&lt;/sup&gt;</td>
<td>554</td>
<td>581</td>
<td>69,000</td>
<td>0.69</td>
<td>48</td>
<td>0</td>
<td>64</td>
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<td>mCherry&lt;sup&gt;g&lt;/sup&gt;</td>
<td>587</td>
<td>610</td>
<td>72,000</td>
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<td>16</td>
<td>0.084</td>
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<td>625</td>
<td>86,000</td>
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<td>13</td>
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<td>15</td>
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<td>mGrape3</td>
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<td>646</td>
<td>40,000&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>63,000</td>
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<td>0.56</td>
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<td>650</td>
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<td>650</td>
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<td>0.20</td>
<td>13</td>
<td>1.8</td>
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<sup>a</sup> Wavelength (nm)

<sup>b</sup> Emission efficiency

<sup>c</sup> Quantum yield

<sup>d</sup> Relative fluorescence

<sup>e</sup> Relative fluorescence intensity at 633 nm

<sup>f</sup> Photostability

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Chemistry and Biology, 16, 2009, 1169-1179
Imaging with Fluorescent Proteins:
Neptune – Optimized for Intravital Imaging

Chemistry and Biology, 16, 2009, 1169-1179
Imaging with Fluorescent Proteins: Infrared Fluorescent Proteins – IFP 1.4

- Bacteriophytochrome of *Deinococcus radiodurans*
  Biliveridin as chromophore

- More than 1500 bacteriophytochrome like sequences identified

Science, May 8 (324), 2009, 804-807
Imaging with Fluorescent Proteins:
Infrared Fluorescent Proteins – IFP 1.4

Science, May 8 (324), 2009, 804-807
Imaging with Fluorescent Proteins: mCerulean3

<table>
<thead>
<tr>
<th>Variant</th>
<th>ε (M⁻¹cm⁻¹)</th>
<th>Quantum Yield</th>
<th>Brightness</th>
<th>relative to EGFP</th>
<th>Bleaching (t₁/₂) (s)</th>
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<td><strong>34.8</strong></td>
<td><strong>1.09</strong></td>
<td><strong>1,100</strong></td>
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<td>EGFP</td>
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<td>0.52</td>
<td>49.92</td>
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</table>

- mCerulean3
- brighter than EGFP
- 20X more photostable than previous CFPs
- no reversible photoswitching

Markwardt, M et al, 2011, PlosONE

Courtesy of Mark Rizzo, mrizz001@umaryland.edu
Imaging with Fluorescent Proteins: mCerulean3 – Performance in Live Cells

- COS7 cells were imaged continuously for 200 frames
- mCerulean3 shows little bleaching in live cells
Imaging with Fluorescent Proteins:
MiniSOG for Correlative Microscopy

~16% brightness of GFP

Shu, X et al, Plos Biology, 2011
Imaging with Fluorescent Proteins:
Scale Clearing Tissues

- Scale solution inexpensive mix of Urea, Glycerol and Triton X-100
- Preserves fluorescence of FPs
- Imaging depth limited by working distance!

## Popular Fluorescent Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference</th>
<th>Excitation, nm</th>
<th>Emission, nm</th>
<th>Brightness, % of EGFP</th>
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<tr>
<td>Sirius</td>
<td>Tomosugi et al., Nat. Methods, 2009</td>
<td>355</td>
<td>424</td>
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<td>EBFP2</td>
<td>Ai et al., Biochemistry, 2007, 46, 5904</td>
<td>383</td>
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<td>ECFP</td>
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<td>AzamiGreen</td>
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<td>T-Sapphire</td>
<td>Zapata-Hommer et al., BMC Biotechnol., 2003, 3</td>
<td>399</td>
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<td>mKO2</td>
<td>Tsutsui et al., Nat. Methods, 2008, 5, 683.</td>
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<table>
<thead>
<tr>
<th>Protein</th>
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<th>Brightness, % of EGFP</th>
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<tr>
<td>mOrange/mOrange2</td>
<td>Shaner et al., Nat. Biotechnol., 2004, 22, 1524</td>
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<td>DsRed2 (tetramer)</td>
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<td>TagRFP/TagRFP-T</td>
<td>Merzlyak et al., Nat. Methods, 2004, 22, 1524</td>
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<td>DsRed-monomer</td>
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<td>mCherry</td>
<td>Shaner et al., Nat. Biotechnol., 2004, 22, 1524</td>
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<td>Platkevich et al., PNAS, 2010, in press.</td>
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<td>Katushka2 (dimer)</td>
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<td>mKate2 (tagFP635-2)</td>
<td>Shcherbo et al., Nat. Methods, 2007, 4, 741</td>
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<td>E2-Crimson (tetramer)</td>
<td>Strack et al., Biochemistry, 2009, 48, 8279</td>
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<td>Wang et al., PNAS, 2004, 101, 16745</td>
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<td>mNeptune (dimer?)</td>
<td>Lin et al., Chem. Biol., 2009, 16, 1169</td>
<td>600</td>
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</table>
Imaging with Fluorescent Proteins:
Fluorescent Protein Summary

FPs considered obsolete:
- DsRed variants, mRFP1
- EYFP (weak dimer)
- ECFP (weak dimer)

Replacement
- mCherry, mApple
- mCitrine, YPet, mVenus
- mCerulean3, mTurquoise

New Colors
- Sirius; Azurite; EBFP2; mTagBFP2 (blue)
- mTFP1 (teal)
- sfGFP; mWasabi (green)
- mKusabira Orange (mKO; mKO2 yellow-orange)
- TagRFP (orange-red); TagRFP-T
- tdTomato (orange-red); mOrange2
- mApple; mRuby (red)
- mPlum; tdKatushka; mKate; mKate2; mNeptune (deep red)
Web Resources:
Fluorescent Proteins and Microscopy

Fluorescence Microscopy and Live-Cell Imaging

Fluorescent Proteins

The discovery and development, over the past decade, of fluorescent proteins from a wide variety of marine organisms has initiated a revolution in the study of cell behavior by providing convenient markers for gene expression and protein targeting in living cells and organisms. The most widely used of these fluorescent proteins, the green fluorescent protein (GFP), first isolated from the jellyfish _Aequorea victoria_, can be attached to virtually any protein of interest and still fold into a fluorescent molecule. The resulting GFP fusion product can be used to localize previously uncharacterized proteins or to visualize and track known proteins to further understand cellular events. The use of fluorescent proteins as a minimally invasive tool for studying protein dynamics and function has been stimulated by the engineering of genetic variants with improved brightness, photostability and expression properties (see Figure 1). Cells that express gene products tagged with fluorescent proteins can be imaged with low light intensities over many hours to provide useful information about changes in the steady-state distribution of a protein over time.
Fluorescent Protein Technology

It took over thirty years, and the advent of recombinant DNA as well as vastly improved molecular biological approaches to see the pioneering work of Osamu Shimomura developed into a useful tool for live-cell imaging by Doug Prasher and Martin Chalfie. Just in the past decade, however, we have witnessed a truly remarkable explosion in the fluorescent protein palette, largely driven by the innovative studies from Roger Tsien’s laboratory. Most of the fluorescent proteins that are commonly used today have been modified through mutagenesis to optimize their expression in biological systems. Continued efforts using directed evolution approaches will no doubt improve the spectral characteristics, photostability, maturation time, brightness, acid resistance, and utility of the fluorescent protein tags for cellular imaging.

Review Articles

Introduction to Fluorescent Proteins - The current thrust of fluorescent protein development strategies is centered on fine-tuning the current palette of blue to yellow variants from jellyfish, while simultaneously developing monomeric fluorescent proteins emitting in the orange to far-red regions of the visible light spectrum.

Fluorescent Proteins Derived from Aequorea victoria - We now have jellyfish proteins that span an 80-nanometer portion visible spectrum from deep blue to yellow-green, providing a wide range of genetically encoded markers for studies in cell biology.
We make it visible.