Problems with cAMP sensor

• Original cAMP sensor required:
  – expression and purification of R and C subunits of PKA in *E. coli*;
  – labeling with rhodamine and fluorescein *in vitro* without destroying protein function;
  – reconstitution of holoenzyme
  – microinjection into living cells

• Wanted general means to fluorescently label genetically designated proteins in living cells
  – Fuse naturally fluorescent proteins (ideally 2 colors), or
  – Devise a motif unique enough to trap small membrane-permeant dye molecules
    • Discussions with Alex Glazer in Berkeley regarding phycobiliproteins ca. 1989? Phycocyanobilin lyase required
The bioluminescent jellyfish *Aequorea victoria*, source of the blue-luminescent protein aequorin and its partner the Green Fluorescent Protein.

Photo courtesy of Claudia Mills, Friday Harbor Laboratory
Bioluminescence
in the sea pansy
*Renilla koellikeri*

Geoff Baird, San Diego
Prasher et al (1992) clone GFP

Gene, 111 (1992) 229–233
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Primary structure of the Aequorea victoria green-fluorescent protein

(Bioluminescence; Cnidaria; aequorin; energy transfer; chromophore; cloning)


SUMMARY

Many cnidarians utilize green-fluorescent proteins (GFPs) as energy-transfer acceptors in bioluminescence. GFPs fluoresce in vivo upon receiving energy from either a luciferase-oxy Luciferin excited state complex or a Ca$^{2+}$-activated photoprotein. These highly fluorescent proteins are unique due to the chemical nature of their chromophore, which is comprised of modified amino acid (aa) residues within the polypeptide. This report describes the cloning and sequencing of both cDNA and genomic clones of GFP from the cnidian, Aequorea victoria. The gfp10 cDNA encodes a 238-aa-residue polypeptide with a calculated $M_\text{r}$ of 26,888. Comparison of A. victoria GFP genomic clones shows three different restriction enzyme patterns which suggests that at least three different genes are present in the A. victoria population at Friday Harbor, Washington. The gfp gene encoded by the λGFP2 genomic clone is comprised of at least three exons spread over 2.6 kb. The nucleotide sequences of the cDNA and the gene will aid in the elucidation of structure-function relationships in this unique class of proteins.

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GFP chromophore formation and its analogy to Asn-Gly hydrolysis

Proposed biosynthesis of GFP fluorophore

Hydrolysis of Asn-Gly sequences

Note that one molecule of H$_2$O$_2$ is generated for each molecule of GFP
Newer work suggests that oxidation might precede dehydration (controversial)
What was wrong with wild-type GFP?

- Main excitation peak in the UV (395 nm), minor excitation at ~475 nm
  - Broad exc. spectrum prevents usage as FRET acceptor
  - Ratio between two exc. peaks depends on protein concentration and past illumination
- Poor folding efficiency above room temp.
- Slow formation of fluorescence (>2 hr)
- Nonoptimal codon usage for mammals
- Cryptic splice site in plants (Haseloff et al)
Mutations of Ser65 improve excitation spectra

Roger Heim, Andrew Cubitt
Crystal structure of S65T GFP

Mats Ormo
Karen Kallio
Jim Remington
(U. Oregon)

Andrew Cubitt
(Aurora Biosciences)
4 colors of GFP mutants expressed in *E. coli*

*R. Heim, A. Cubitt*
Examples of genetically encoded FRET sensors

Protease disrupts FRET (R. Heim)

Ca\(^{2+}\) increases FRET (A. Miyawaki)

Ca\(^{2+}\) increases FRET (A. Miyawaki)

Phosphorylation increases or decreases FRET (J. Zhang, A. Ting)

cAMP disrupts FRET (M. Zaccolo, T. Pozzan (Padova))

Phosphorylation increases or decreases FRET (J. Zhang, A. Ting)
Cytosolic Ca\textsuperscript{2+} waves trigger contraction at cleavage furrows during embryonic development

Transgenic zebrafish embryo expressing yellow cameleon 3.60
Single confocal z-plane, imaged every 5 sec (‘mpf’ = minutes post fertilization)
Hide Mizuno & Atsushi Miyawaki, RIKEN
Phosphorylation-dependent emission ratio of EGFR reporter, overlaid on DIC image

EGF added; FRET increases

EGF washed out; FRET decreases

Image taken every 5 sec; Collected over 20 min

Alice Ting
GFP-tagged HIV can be transmitted by cell-cell contact

A

B

Predominant Mode of Human Immunodeficiency Virus Transfer between T Cells Is Mediated by Sustained Env-Dependent Neutralization-Resistant Virological Synapses. Ping Chen, Wolfgang Huebner, Matthew A. Spinelli, and Benjamin K. Chen.

A High-Throughput Screen for Compounds That Inhibit Aggregation of the Alzheimer’s Peptide


Figure 1. Fluorescence-based screen using the Aβ42–GFP fusion. In the absence of inhibition, the Aβ42 portion of the fusion aggregates rapidly and causes the entire Aβ42–GFP fusion to misfold and aggregate (left). Therefore, no fluorescence is observed. However, inhibition of Aβ42 aggregation enables GFP to form its native green fluorescent structure (right). (The green part of the ribbon diagram shows the structure of GFP; the yellow part is merely a schematic representation of a nonaggregated form of Aβ42.) The triazine scaffold is shown at the center of the figure. Combinatorial diversity was introduced at sites marked X, Y, and Z. A 96-well plate is shown at the bottom of the figure. Compounds were added to each well, followed by E. coli cells expressing the Aβ42–GFP fusion.
Many tropical corals contain fluorescent proteins

The DsRed structure drawn using *E. coli* expressing DsRed as “ink”
The 2004 palette of nonoligomerizing fluorescent proteins

- **GFP-derived**
  - Exc. 380 433/452 488 516
  - Em. 440 475/505 509 529

- **mRFP1-derived**
  - Exc. 487/504 540 548 554 568 574 587 595
  - Em. 509 537/562 553 562 581 585 596 610 620 625 636 648

- **EBFP**
- **ECFP**
- **EGFP**
- **YFP (Citrine)**
- **mHoneydew**
- **mBanana**
- **mOrange**
- **tdTomato**
- **mTangerine**
- **mStrawberry**
- **mCherry**
- **mGrape1**
- **mRaspberry**
- **mGrape2**
- **mPlum**

*Evolved by SHM*

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Cell cycle indicator using YFP and mCherry

Green = in mitosis
Red = interphase
Fluorescent proteins are also good educational tools in the high school classroom

Jeremy Babendure
Major limitations of fluorescent proteins

- Sometimes FPs are too big (>200 aa)
  → Develop small peptides (≤ 12 aa) that selectively bind small synthetic molecules
- Excitation wavelengths <600 nm do not penetrate far through mammalian tissue
  → Develop FPs with 600-700 nm excitation
- Whole-body scanning requires other imaging techniques, e.g. magnetic resonance
- Gene transfer required, not yet feasible in humans and many other species
  → Develop synthetic probes localizing a variety of contrast agents at sites of high proteolytic activity

(More detail @ 4:15 PM lecture 12 Dec. 2008, G-salen, Arrhenius Laboratory, Stockholm Univ.)
Infrared fluorescent protein based on biliverdin-binding bacterial phytochrome improves *in vivo* imaging

*Deinococcus radiodurans* phytochrome residues targeted for mutation

Adenovirally transfected livers in intact mice

IFP1.1 + BV

mKate brightened 5 fold rel. to IFP

GFP

Xiaokun Shu, Antoine Royant, Michael Lin, Todd Aguilera
ACPP colocalizes with GFP-transfected Hep2 xenografts: high magnification, after removal of skin

(Suc)\(e_8\)-XPLGLAG-\(r_9\)-c(Cy5)

d-amino acid control: (Suc)\(e_8\)-Xplglag-\(r_9\)-c(Cy5)

Quyen Nguyen & Anticancer, Inc.; Tao Jiang
Lessons and conclusions

• Deliberate design and synthesis of molecules (both small and macro) is fun chemistry and can have a significant impact on cell biology and neurobiology
• Biology, chemistry, and instrumentation must be closely integrated
• Small teams of 1-2 postdocs/students in an academic lab of 3-15 can make basic progress in 0.5-5 yrs (huge teams not required)
• Find the right collaborators (senior and junior)!
• Most major biochemical signals can now or will soon be visualized in live cells
• Cells (especially neurons) are highly individualistic; spatial organization (microscopic and submicroscopic) and temporal patterning are all-important
• The joy of fishing?
Sunset with green flash as viewed from a California lab

**Early work on GFP:**
Douglas Prasher & Virginia Eckenrode (WHOI), Roger Heim, Andrew Cubitt, S. James Remington (U. Or.)

**cAMP imaging:**
Stephen Adams, Susan Taylor (UCSD), Tullio Pozzan (Padova), Jin Zhang

**Other CFP/YFP FRET sensors:**
Atsushi Miyawaki, Varda Lev-Ram, Alice Ting

**RFPs and IFPs:**
Geoffrey Baird, Larry Gross, Robert Campbell, Nathan Shaner, Lei Wang, Xiaokun Shu