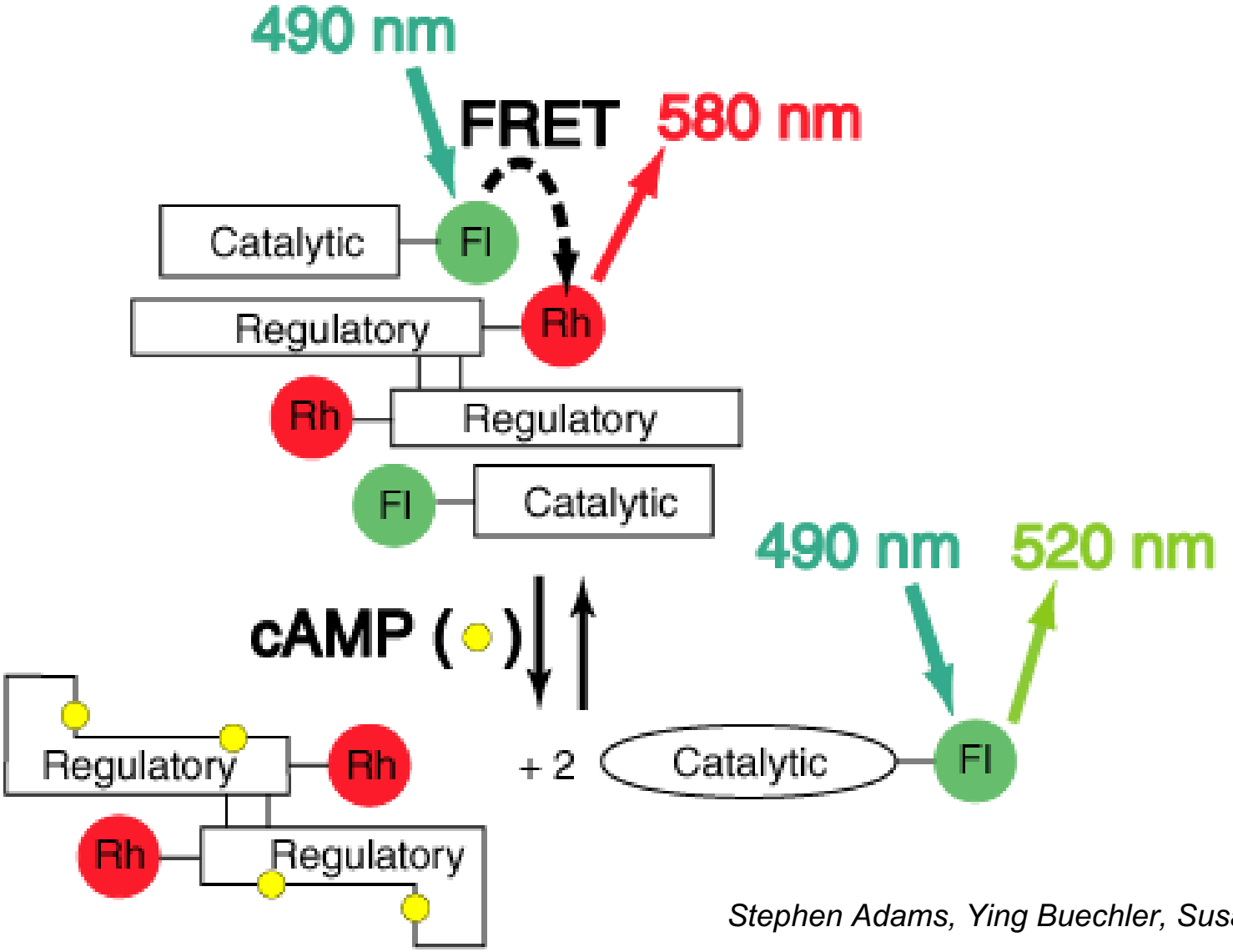


Stephen Adams, Ying Buechler, Susan Taylor

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

GENE 06296

Primary structure of the *Aequorea victoria* green-fluorescent protein

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

Douglas C. Prasher[a], Virginia K. Eckenrode[b], William W. Ward[c], Frank G. Prendergast[d] and Milton J. Cormier[b]

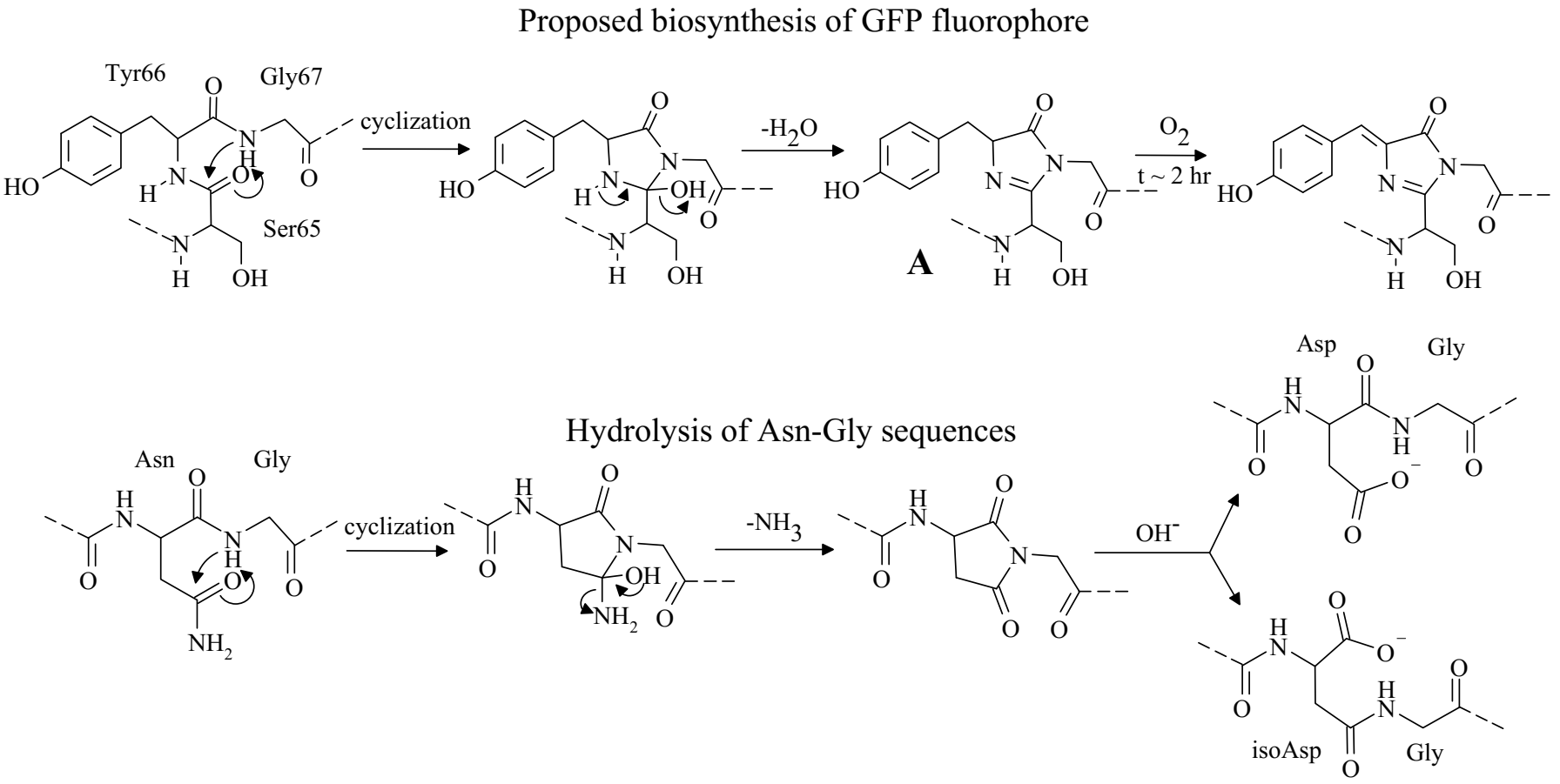
SUMMARY

Many cnidarians utilize green-fluorescent proteins (GFPs) as energy-transfer acceptors in bioluminescence. GFPs fluoresce in vivo upon receiving energy from either a luciferase-oxyluciferin 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 cnidarian, *Aequorea victoria*. The *gfp10* cDNA encodes a 238-aa-residue polypeptide with a calculated M_r of 26888. 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.

Correspondence to: Dr. D.C. Prasher, Redfield Bldg., Woods Hole Oceanographic Institution, Woods Hole, MA 02543 (U.S.A.)
Tel. (508)457-2000, ext. 2311; Fax (508)457-2195.

Supported in part by a Mellon Award from the Woods Hole Oceanographic Institution (27/50.44) and a grant from the American Cancer Society (NP640) to D.C.P.

GFP chromophore formation and its analogy to Asn-Gly hydrolysis

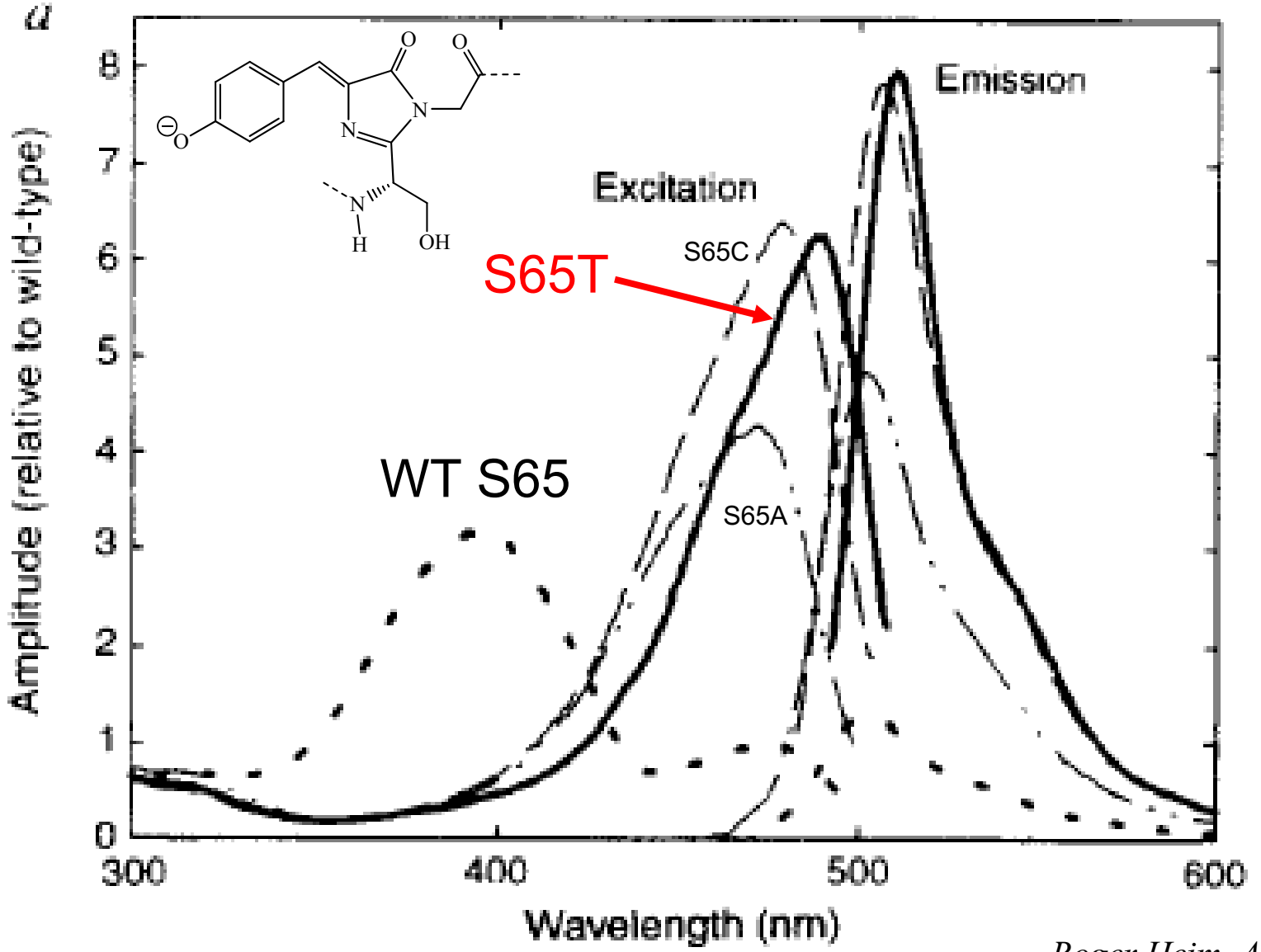


Note that one molecule of H_2O_2 is generated for each molecule of GFP
Newer work suggests that oxidation might precede dehydration (controversial)

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

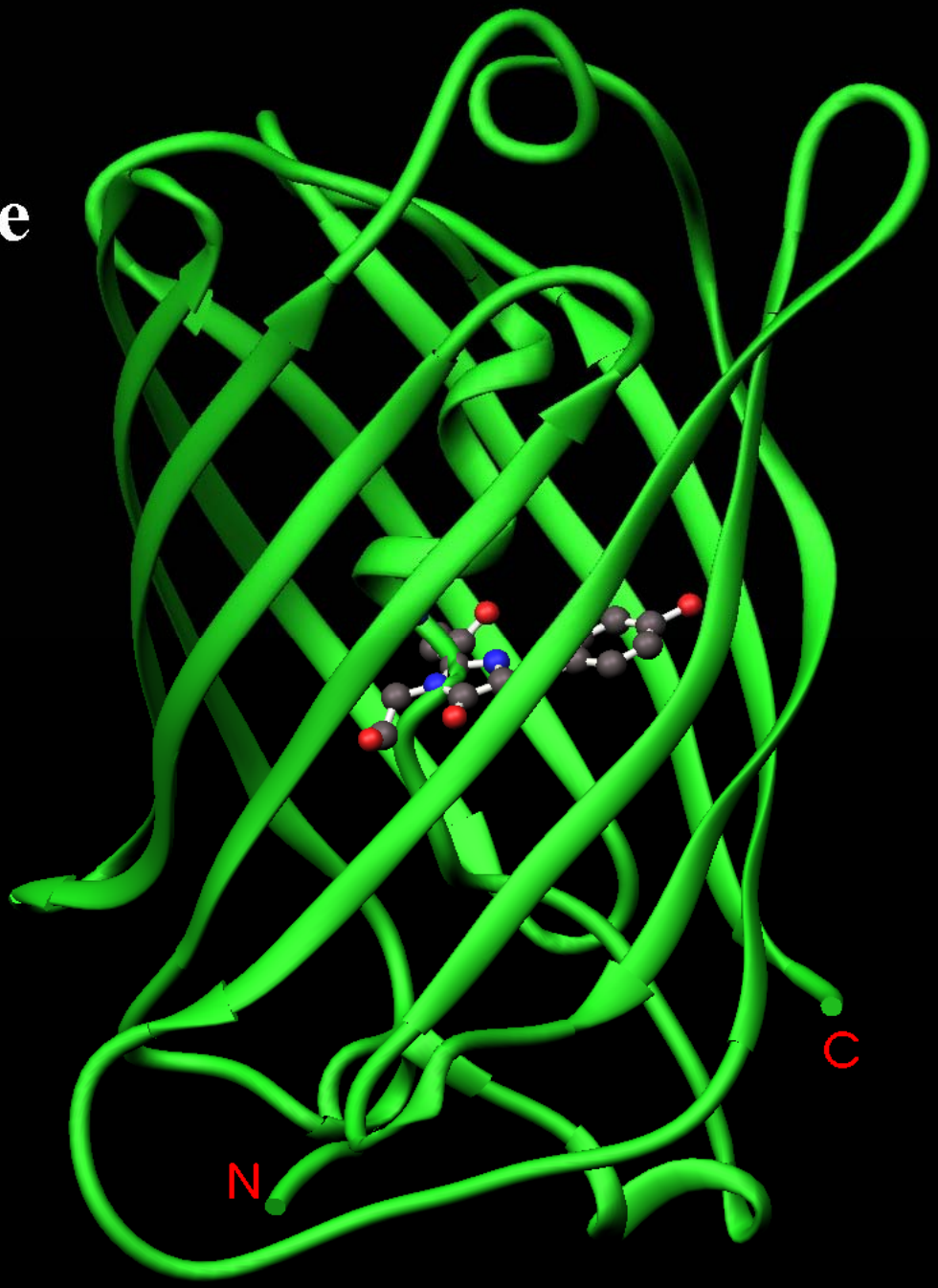


Roger Heim, Andrew Cubitt

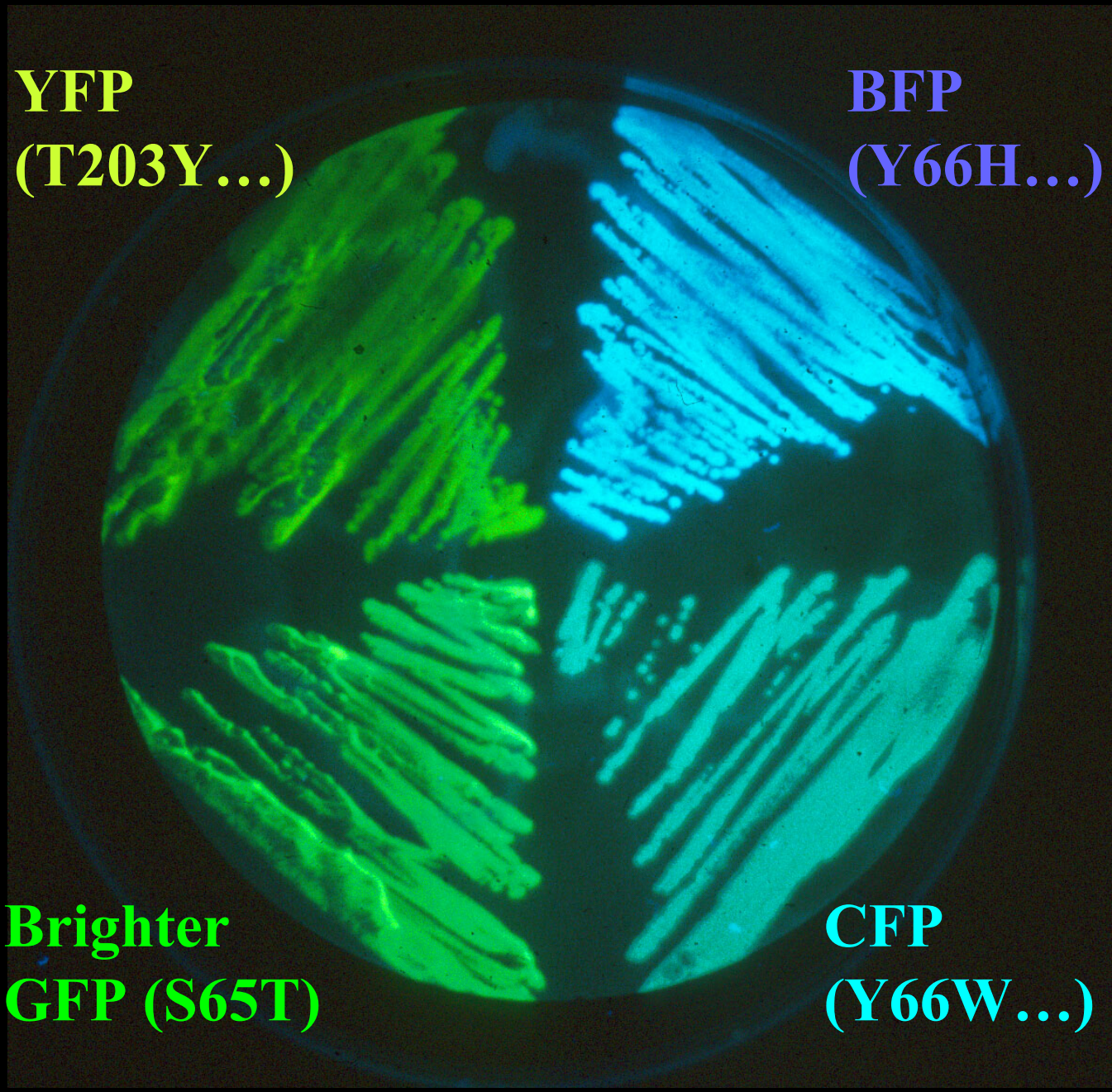
Crystal structure of S65T GFP

Mats Ormo
Karen Kallio
Jim Remington
(U. Oregon)

Andrew Cubitt
(Aurora Biosciences)

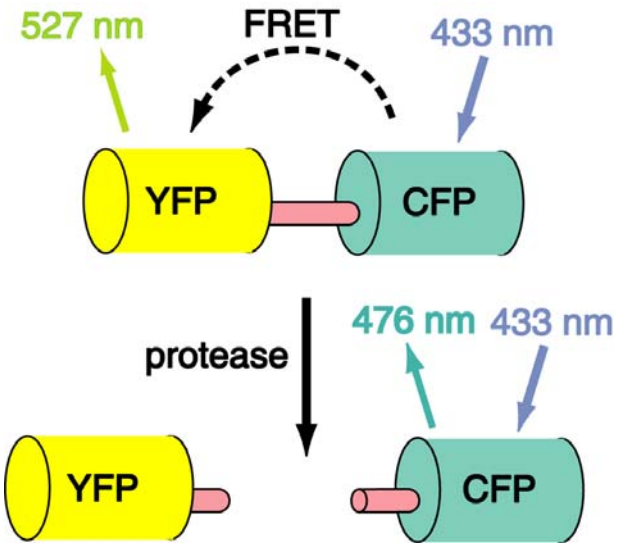


4 colors of GFP mutants expressed in *E. coli*

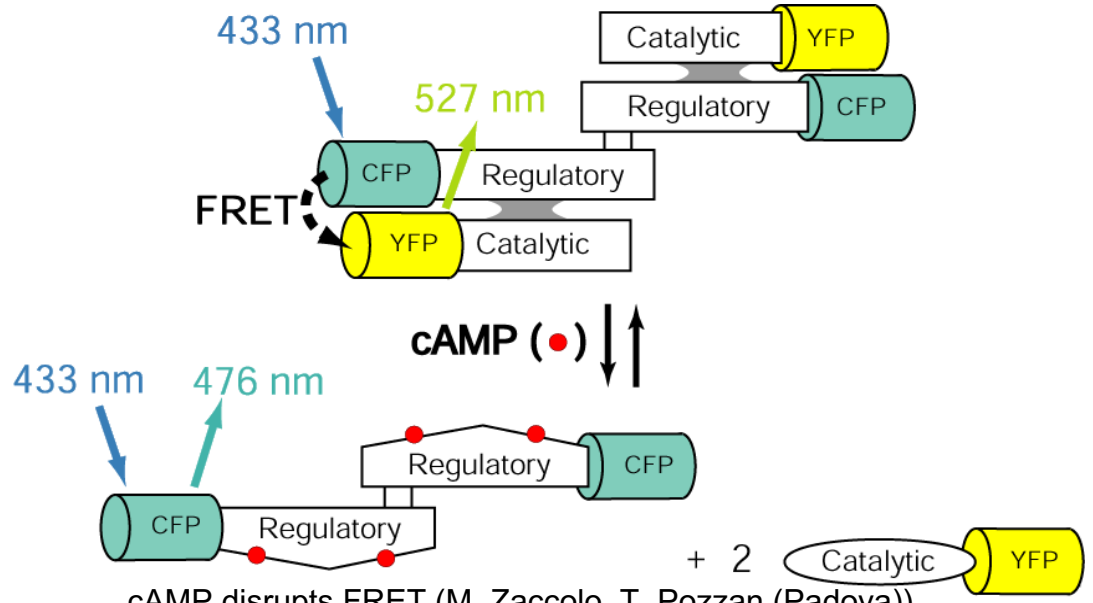


R. Heim, A. Cubitt

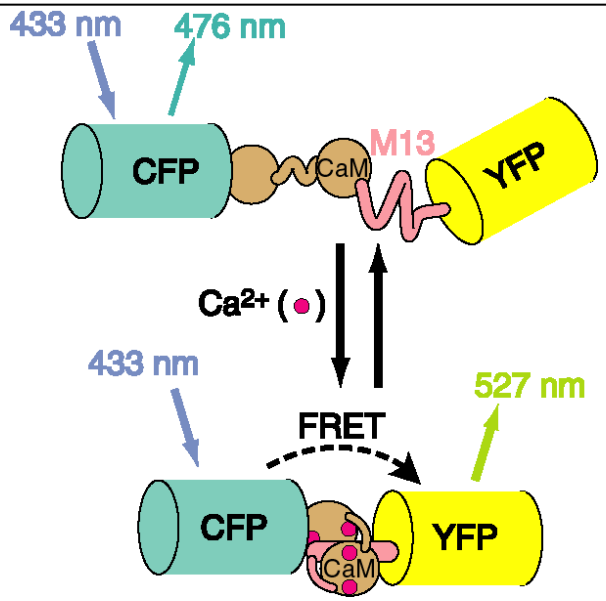
Examples of genetically encoded FRET sensors



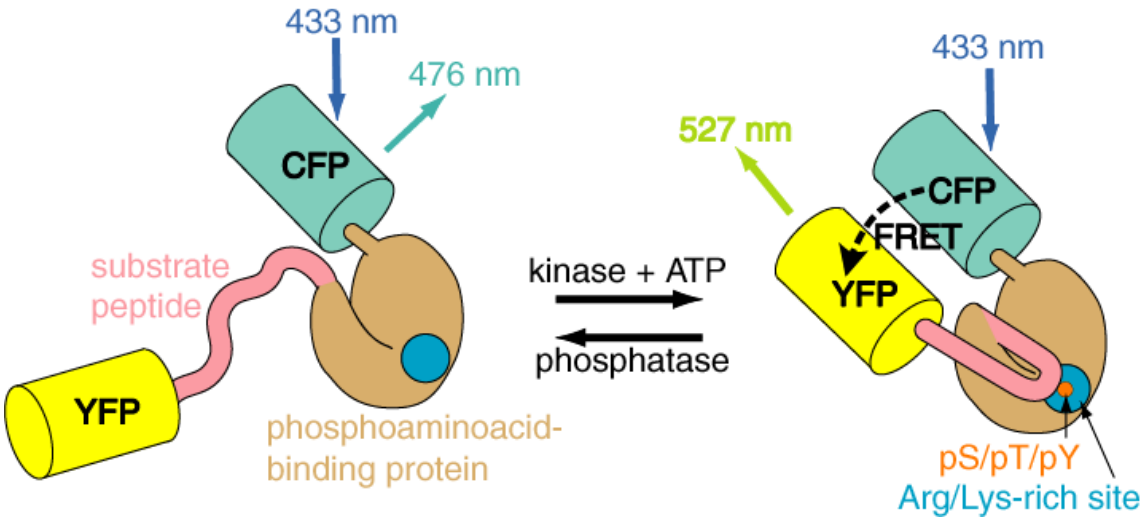
Protease disrupts FRET (R. Heim)



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

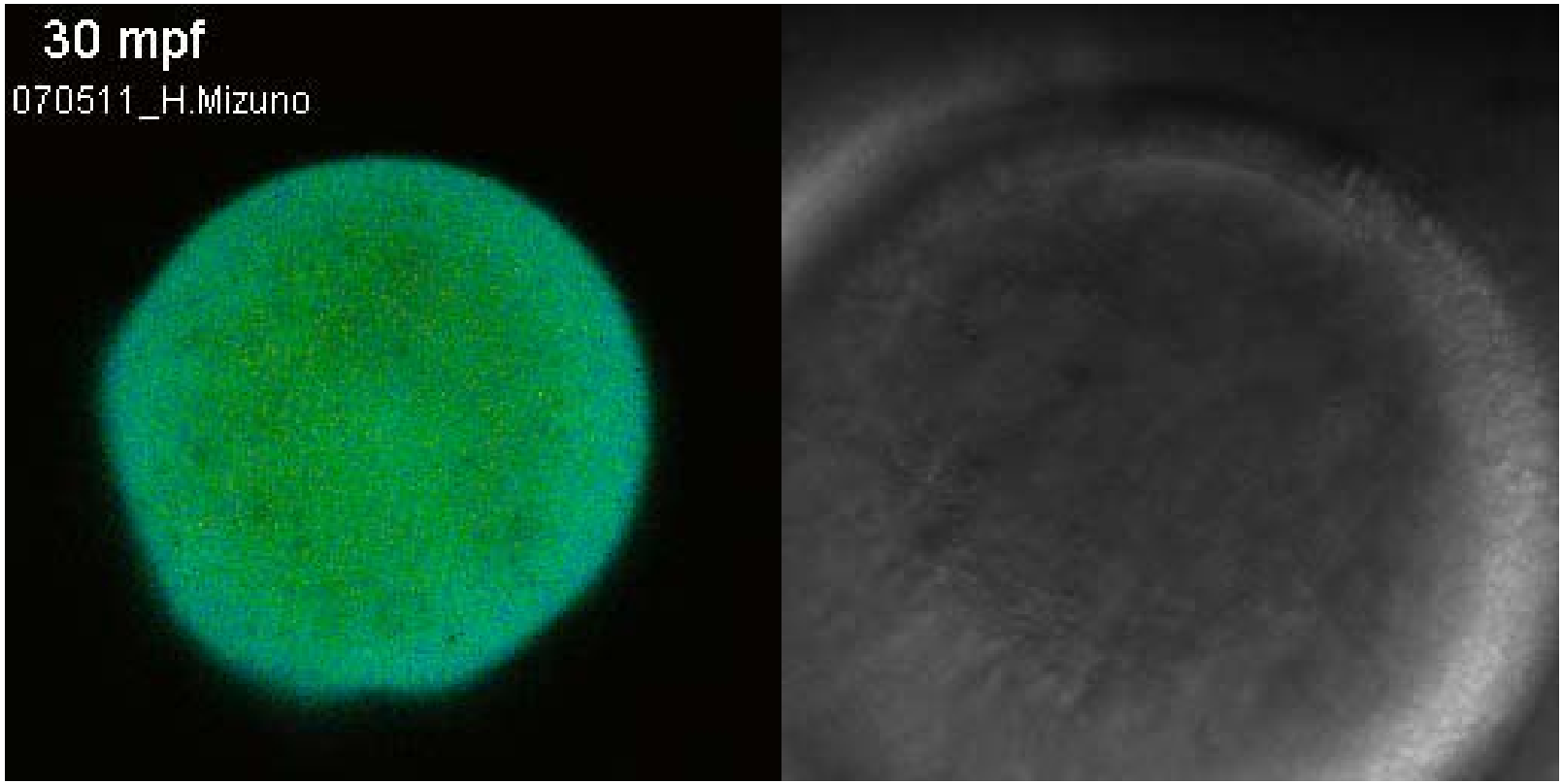


Ca^{2+} increases FRET (A. Miyawaki)



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

Cytosolic Ca^{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

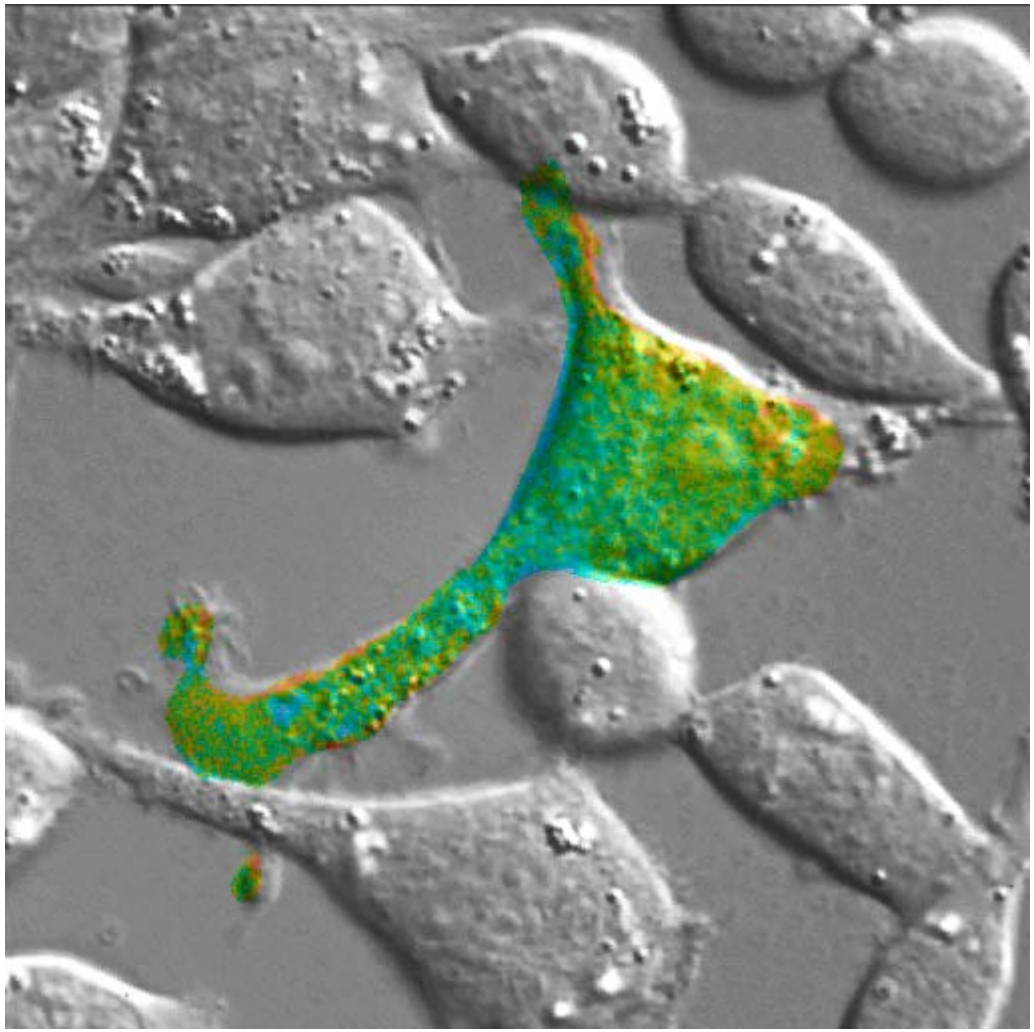
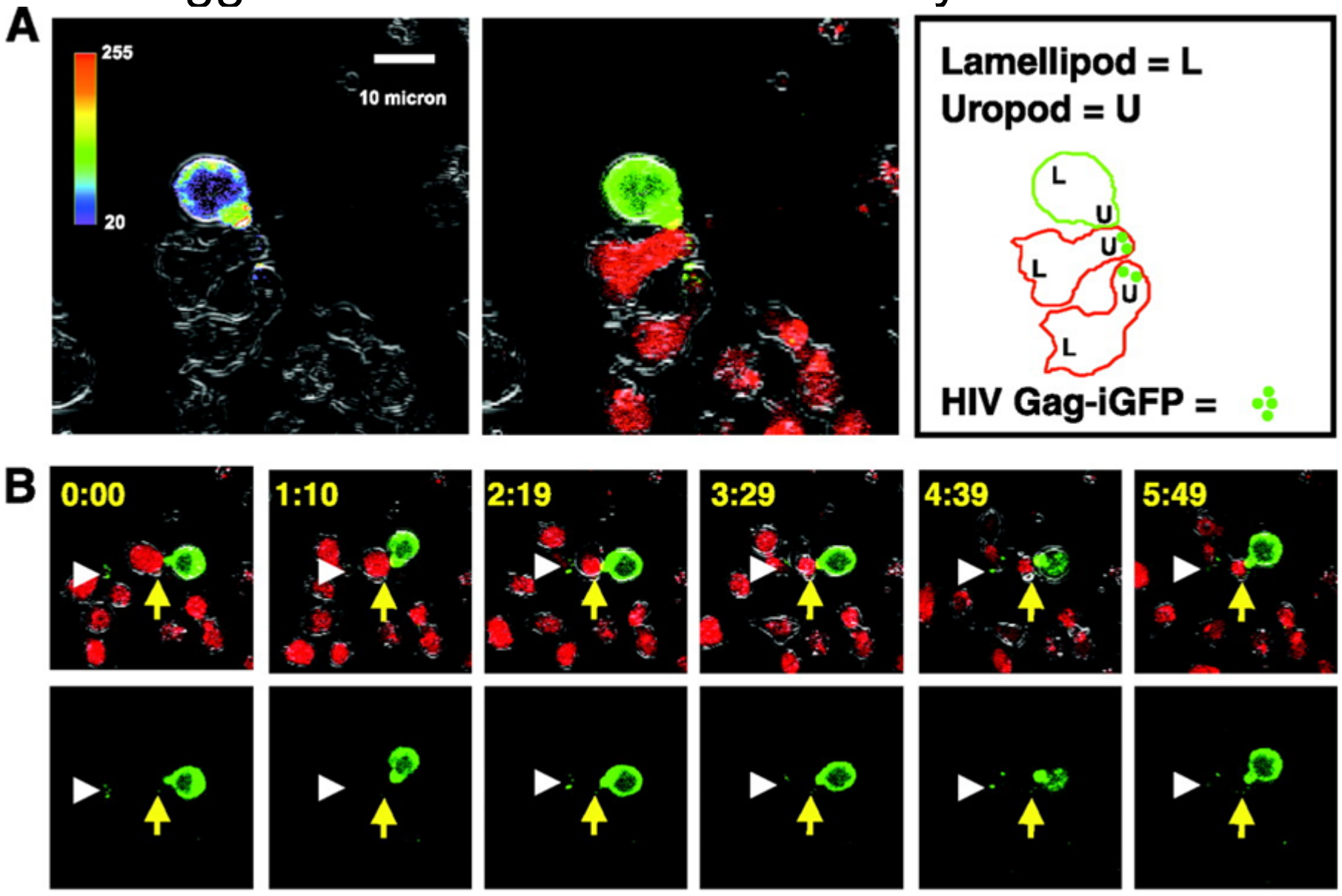


Image taken every 5 sec;
Collected over 20 min

Alice Ting

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



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.

*J. Virology (*2007) **81:** 12582–12595

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

Kim Woojin, Kim Yunkyoung, Min Jaeki, Kim Dong Jin, Chang Young-Tae* and Michael H. Hecht (2006) *ACS Chem. Biol.* **1**: 461–469

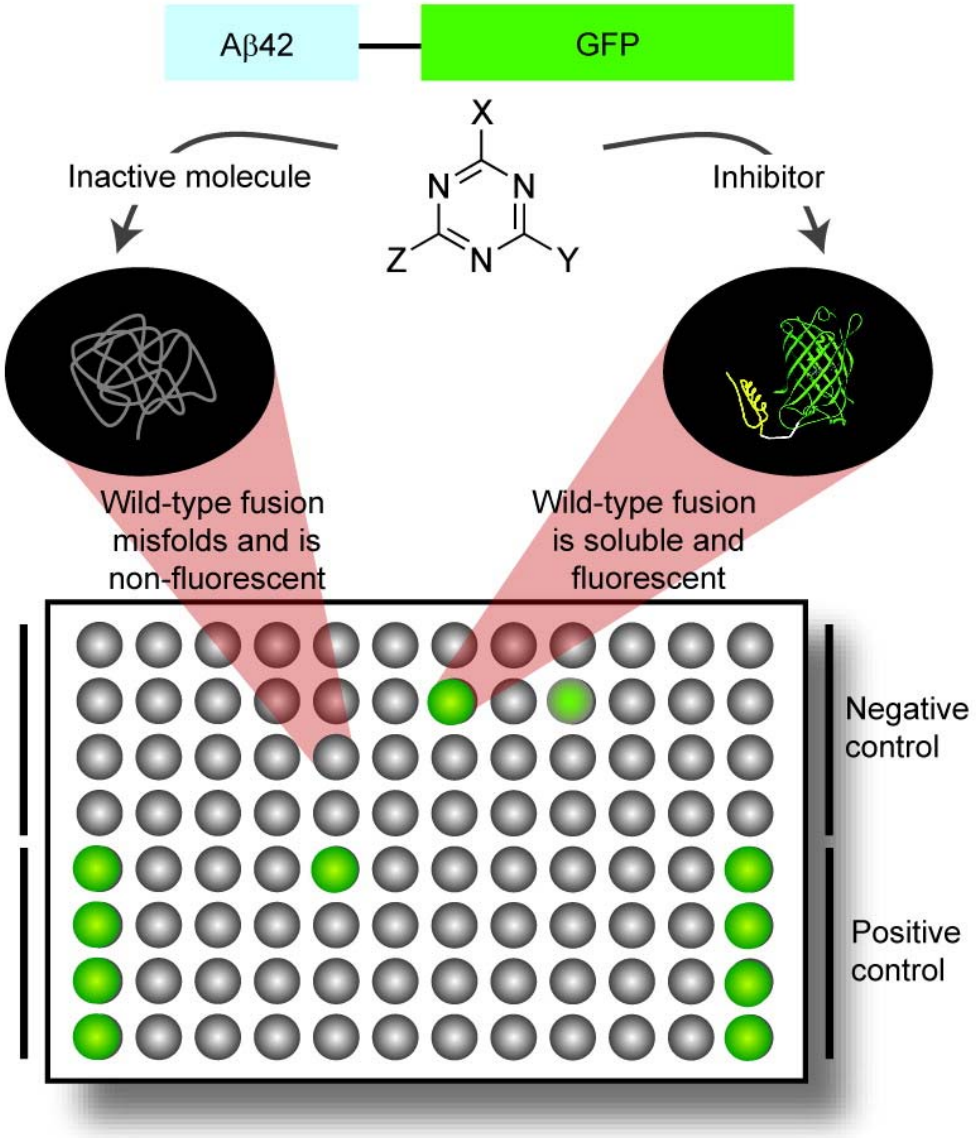


Figure 1. Fluorescence-based screen using the Aβ42–GFP fusion. In the absence of inhibition, the Aβ42 portion of the fusion aggregates rapidly and causes the entire Aβ42–GFP fusion to misfold and aggregate (left). Therefore, no fluorescence is observed. However, inhibition of Aβ42 aggregation enables 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.

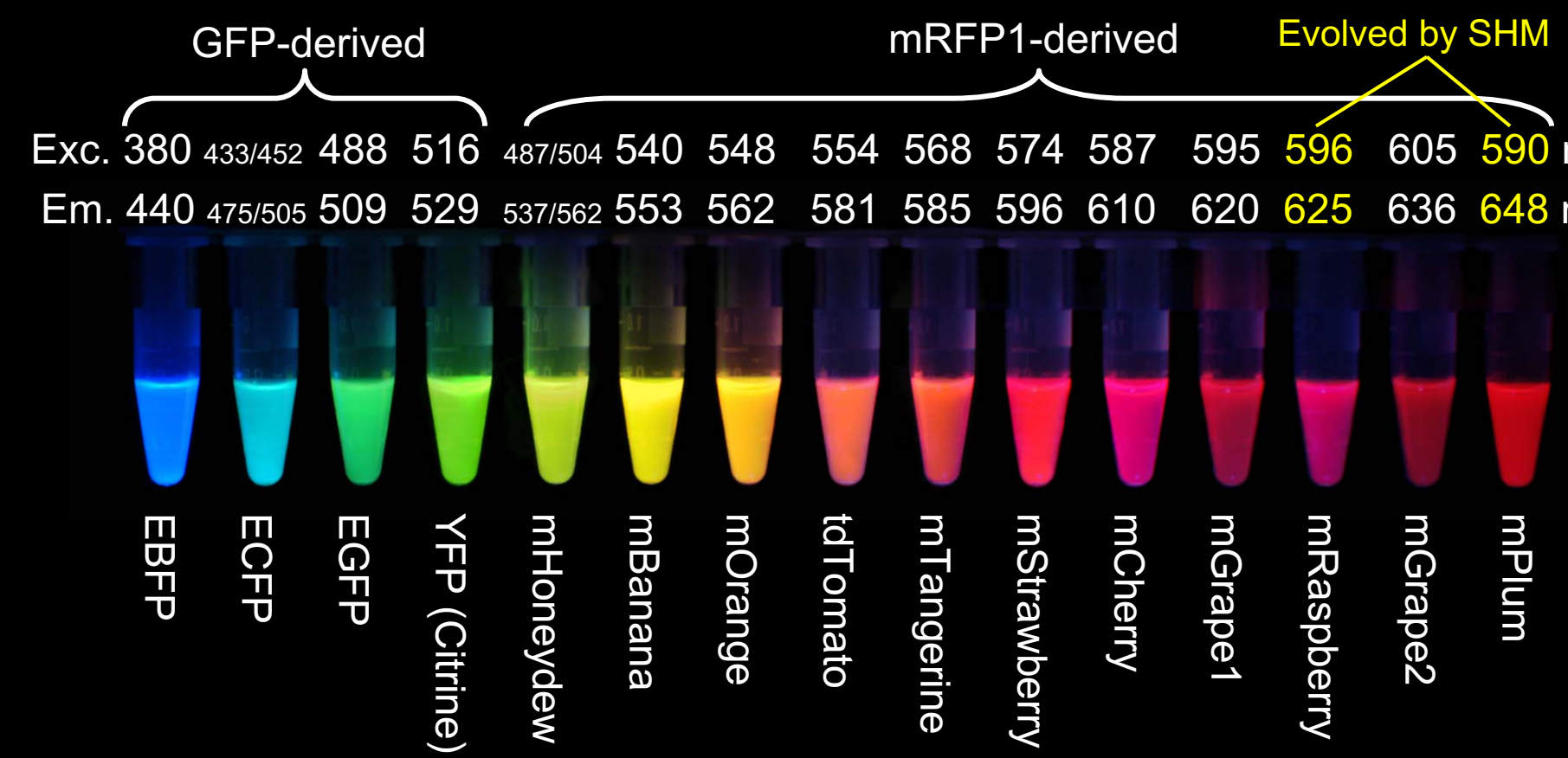
Many tropical corals contain fluorescent proteins

First discovered by Lukyanov lab: Matz et al (1999) Nature Biotech. 17: 969-973

The DsRed structure drawn using *E. coli* expressing DsRed as “ink”

Structure detd. by Larry Gross, drawn by Varda Lev-Ram & Geoff Baird

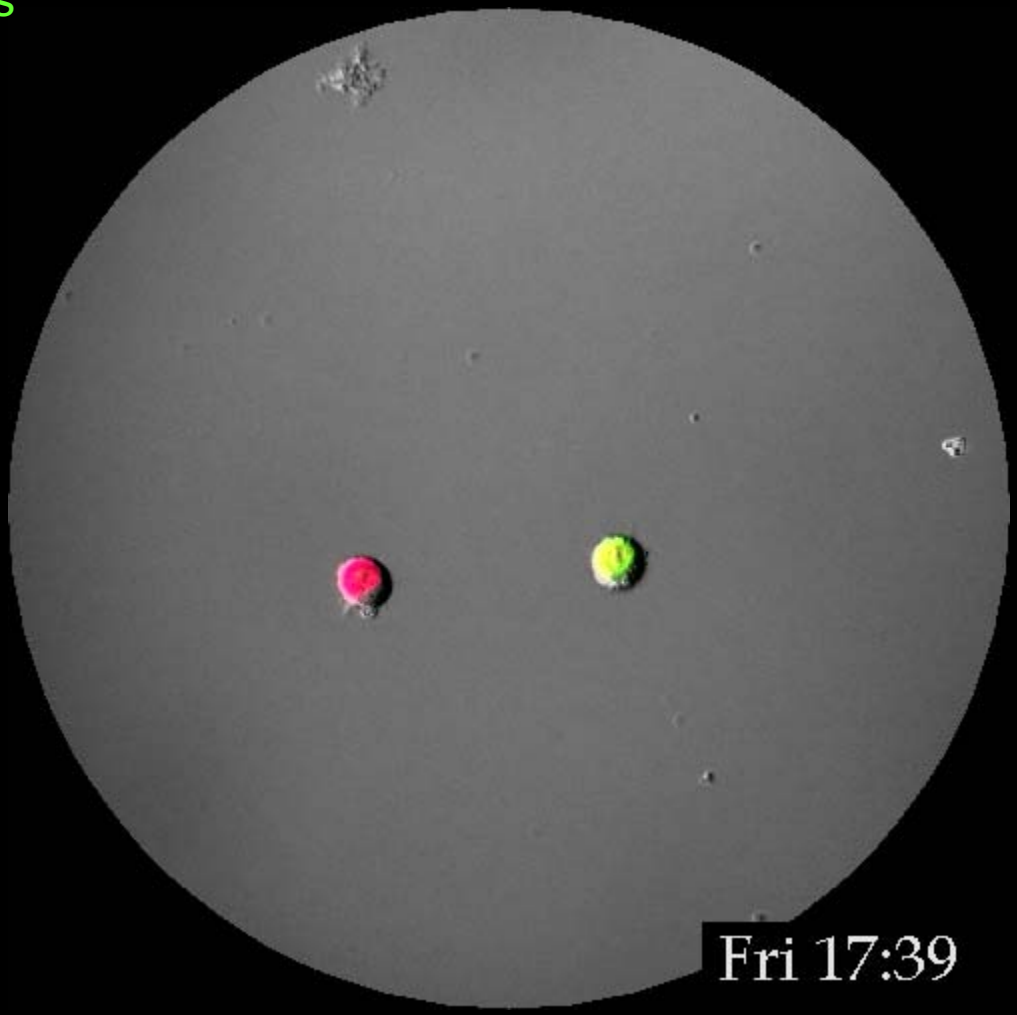
The 2004 palette of nonoligomerizing fluorescent proteins



Nathan Shaner et al (2004) *Nature Biotech.* **22**: 1567-1572
Lei Wang et al (2004) *Proc. Natl. Acad. Sci. USA* **101***:* 16745-16749

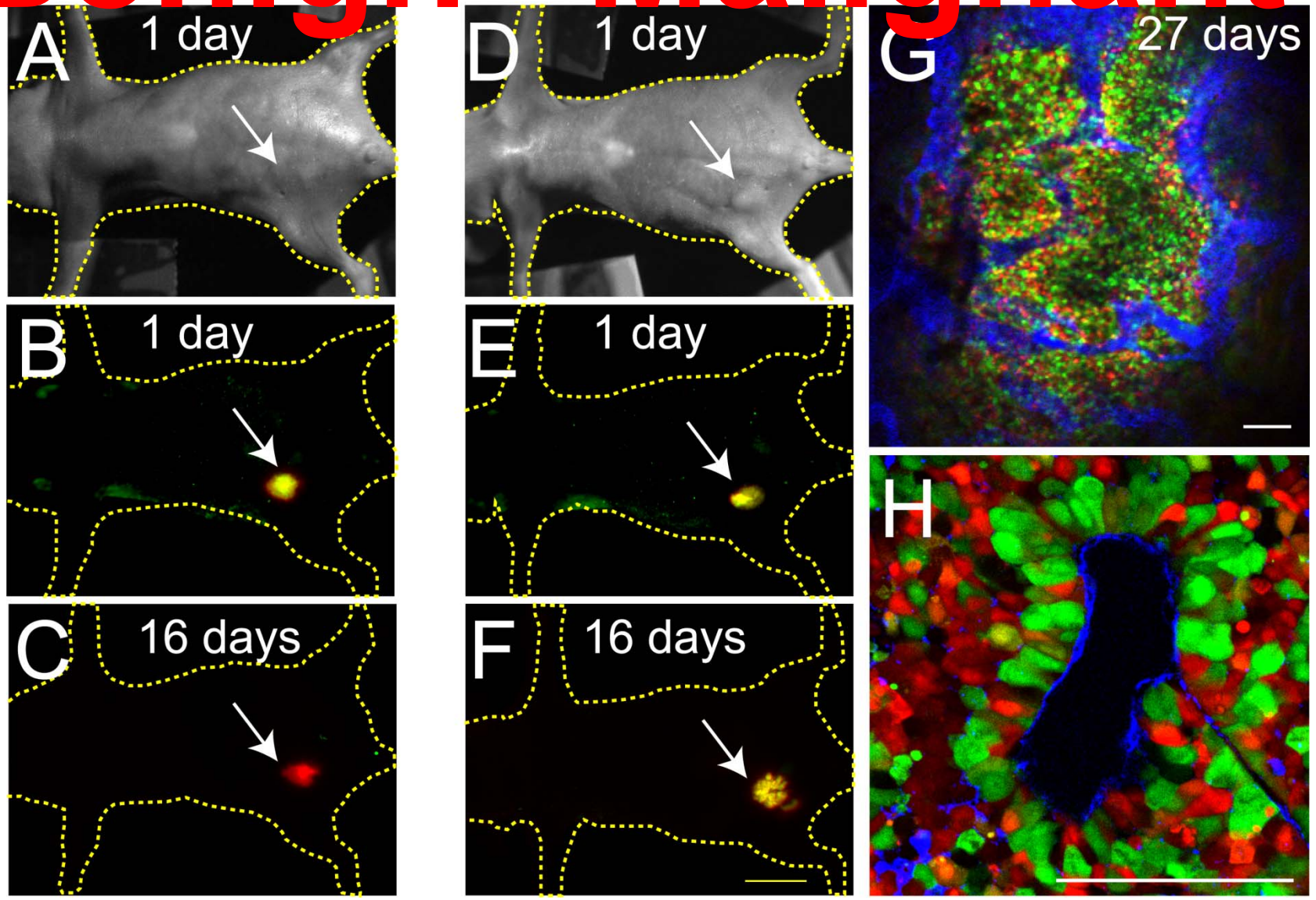
Cell cycle indicator using YFP and mCherry

Green = in mitosis
Red = interphase



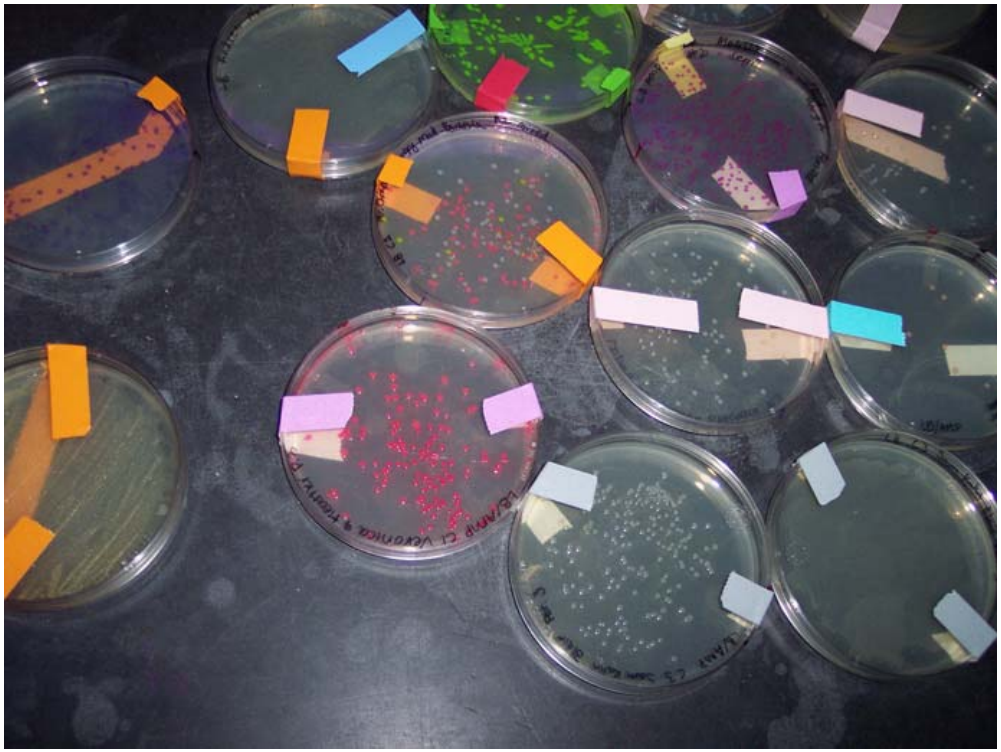
A. Sawano & A. Miyawaki, RIKEN

Benign Malignant



Asako Sawano & Atsushi Miyawaki, RIKEN

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*

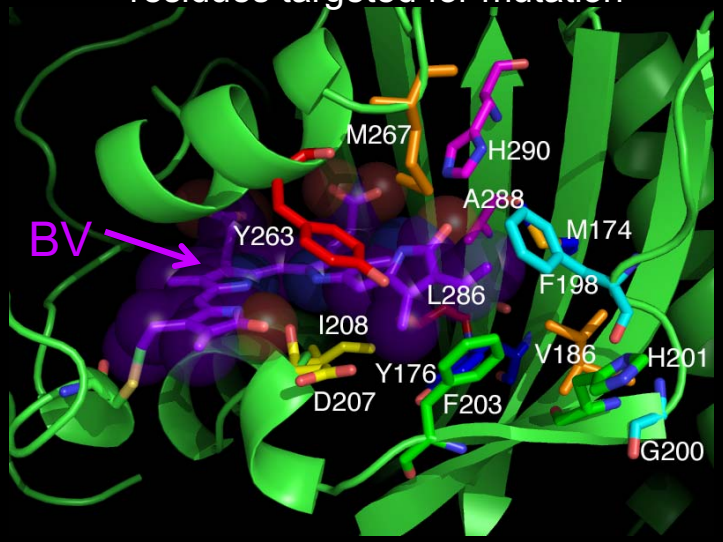
- Whole-body scanning requires other imaging techniques, e.g. magnetic resonance
- Gene transfer required, not yet feasible in humans and many other species

  → *Develop synthetic probes localizing a variety of contrast agents at sites of high proteolytic activity*

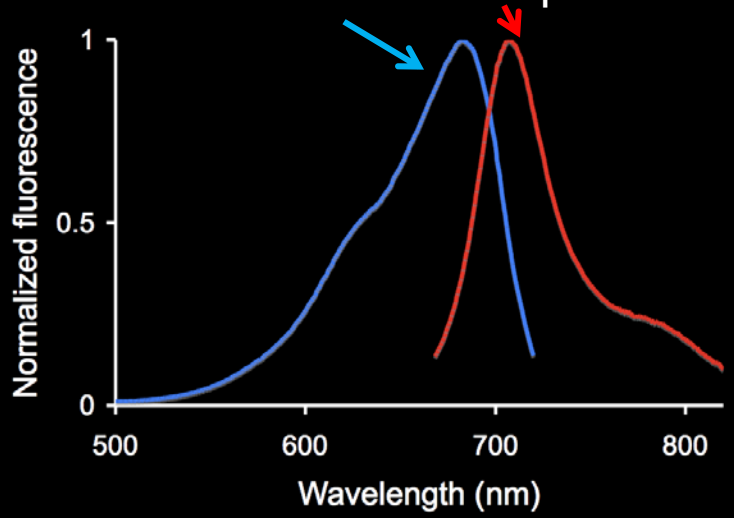
(More detail @ 4:15 PM lecture 12 Dec. 2008, G-salen, Arrhenius Laboratory, Stockholm Univ.)

Infrared fluorescent protein based on biliverdin-binding bacterial phytochrome improves *in vivo* imaging

Deinococcus radiodurans phytochrome residues targeted for mutation



IFP1.4 Exc. & em. spectra



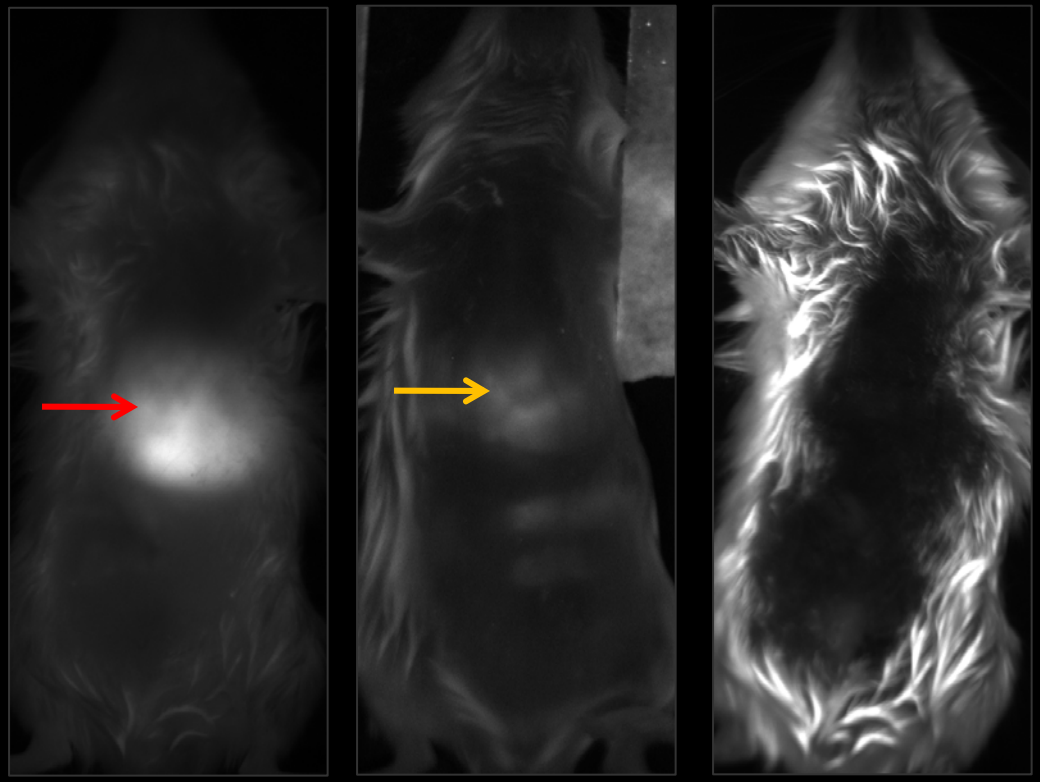
Adenovirally transfected livers in intact mice

IFP1.1 + BV

mKate

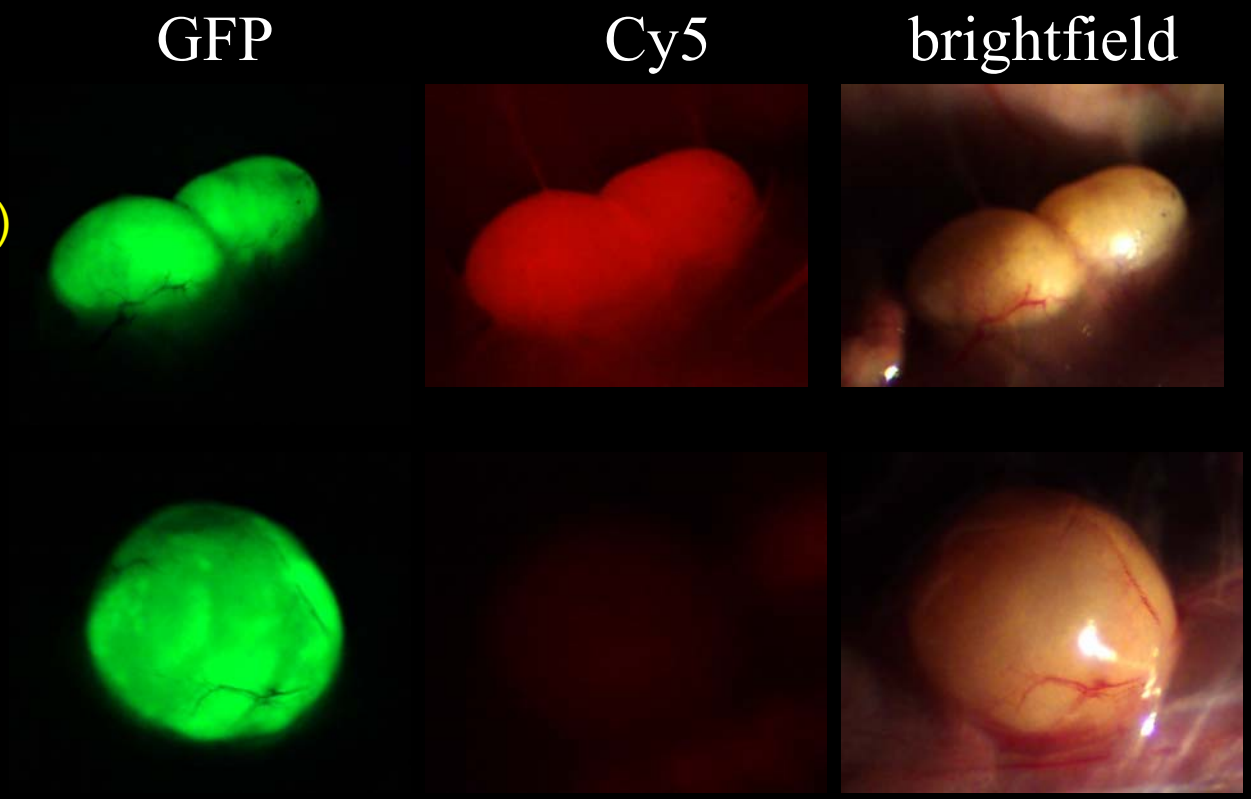
GFP

brightened 5 fold rel. to IFP



Xiaokun Shu, Antoine Royant, Michael Lin, Todd Aguilera

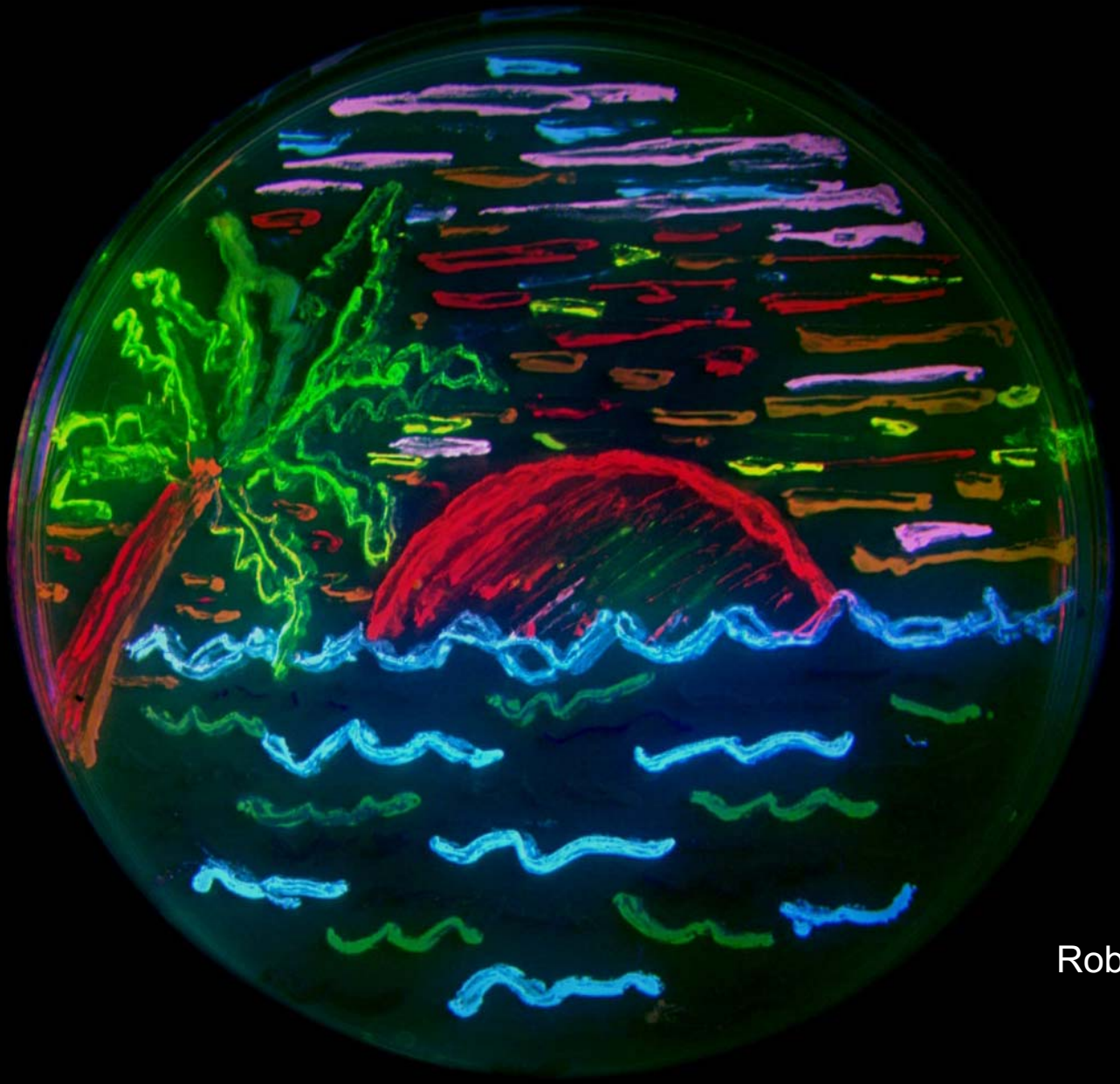
ACPP colocalizes with GFP-transfected Hep2 xenografts: high magnification, after removal of skin



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?



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

Sunset with green flash as viewed from a California lab