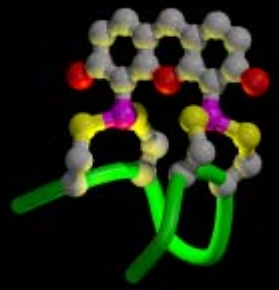
Genetically-encoded fluorescent tags for proteins

GFP

genetically targeted



biarsenical-
tetracysteine
(ReAsH & FlAsH)

Stephen R. Adams
Department of Pharmacology
University of California San Diego

The bioluminescent jellyfish *Aequorea victoria*, source of the blue-luminescent protein aequorin and its partner the Green Fluorescent Protein

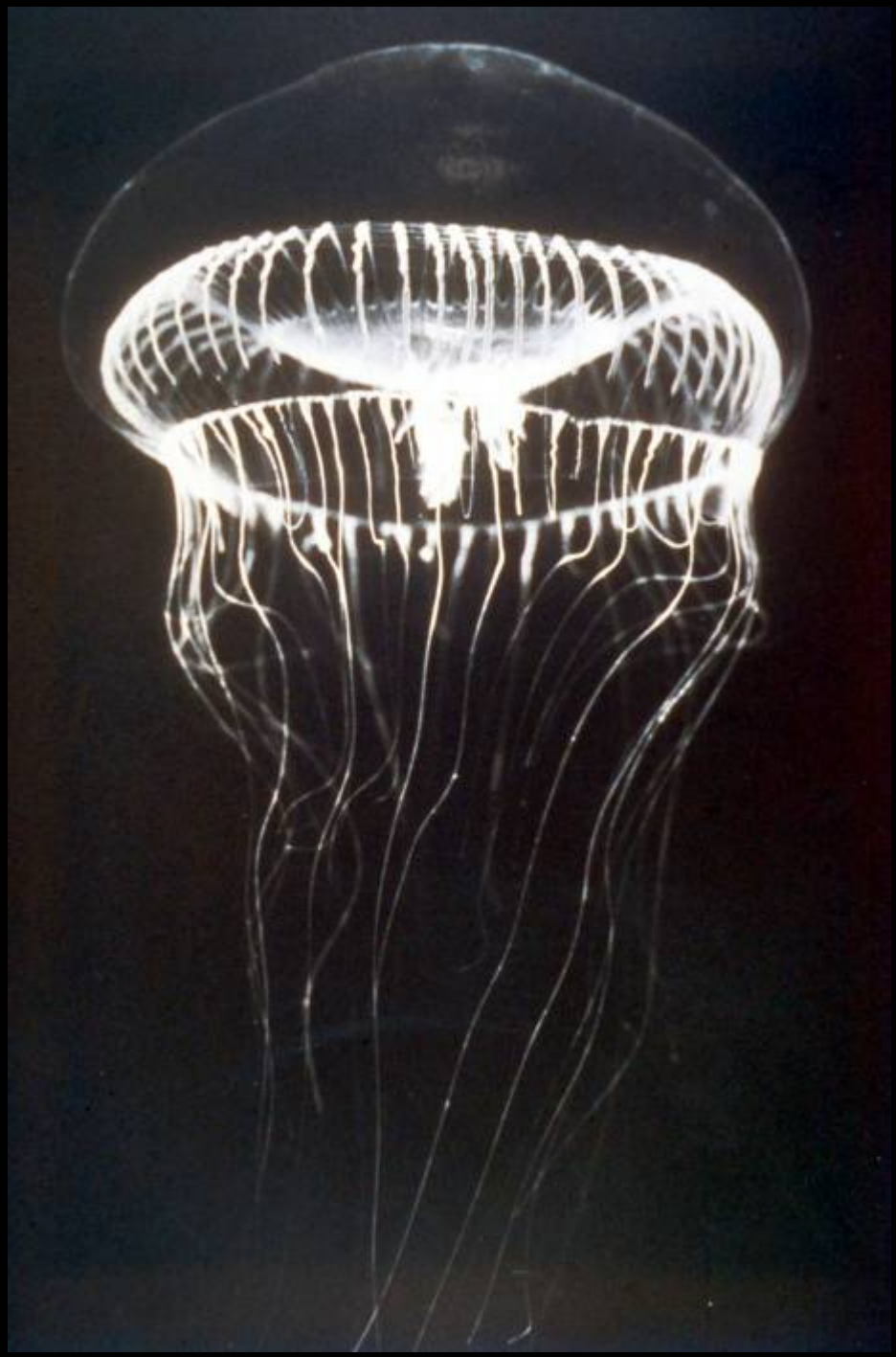
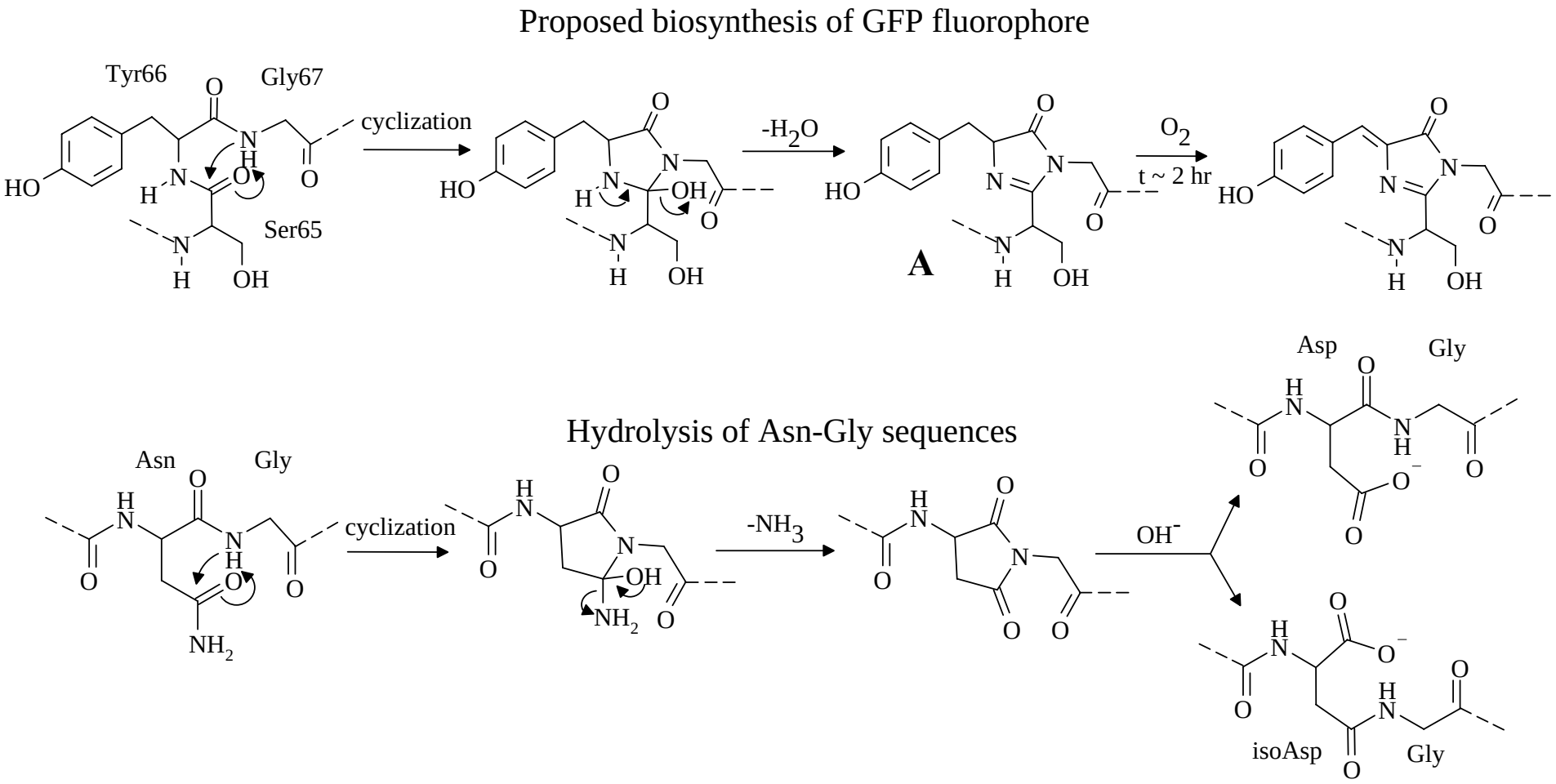


Photo courtesy of Claudia Mills & Douglas Prasher

GFP chromophore formation and its analogy to Asn-Gly hydrolysis



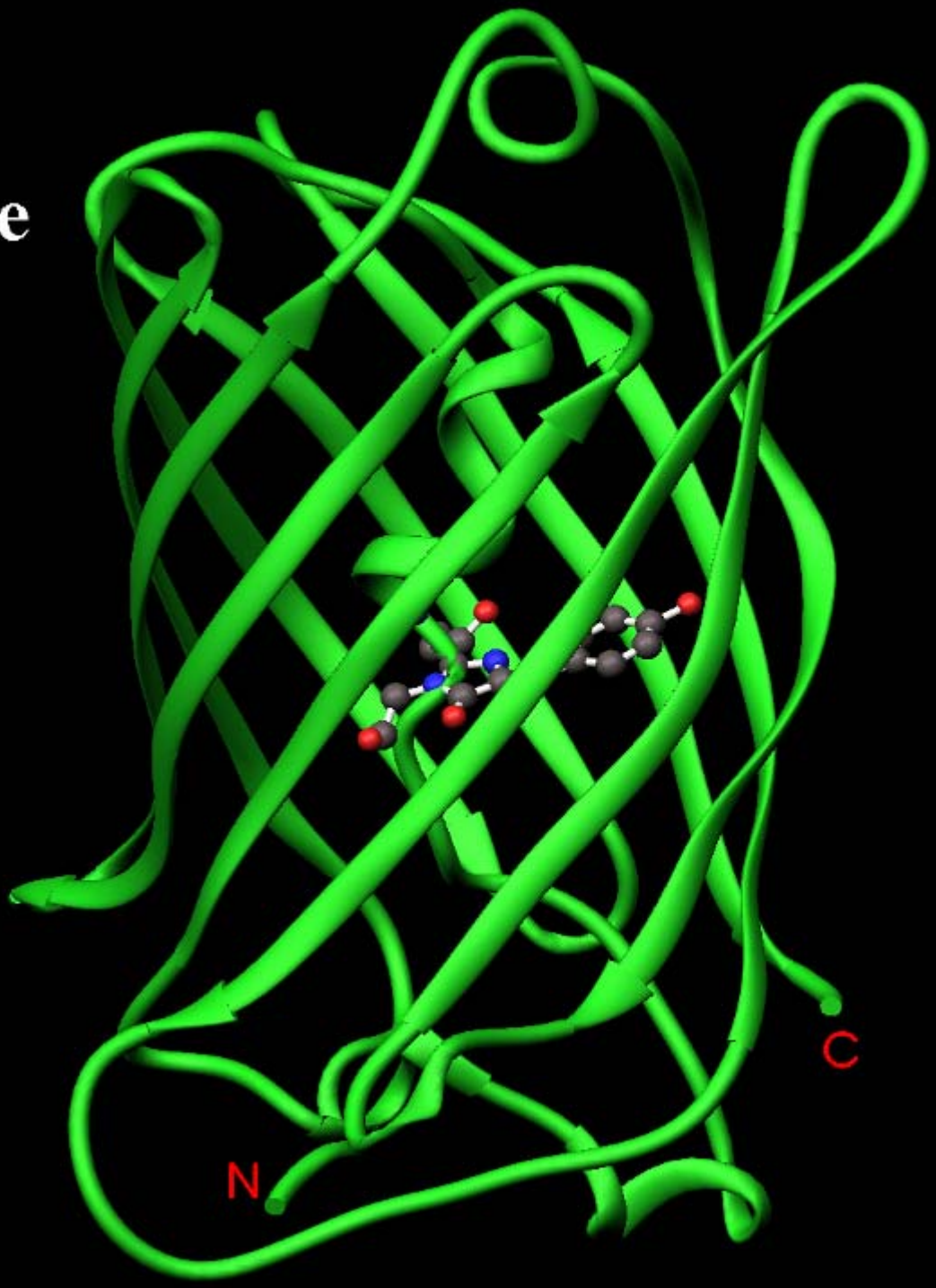
Note that one molecule of H_2O_2 should be generated for each molecule of GFP

Crystal structure of S65T GFP

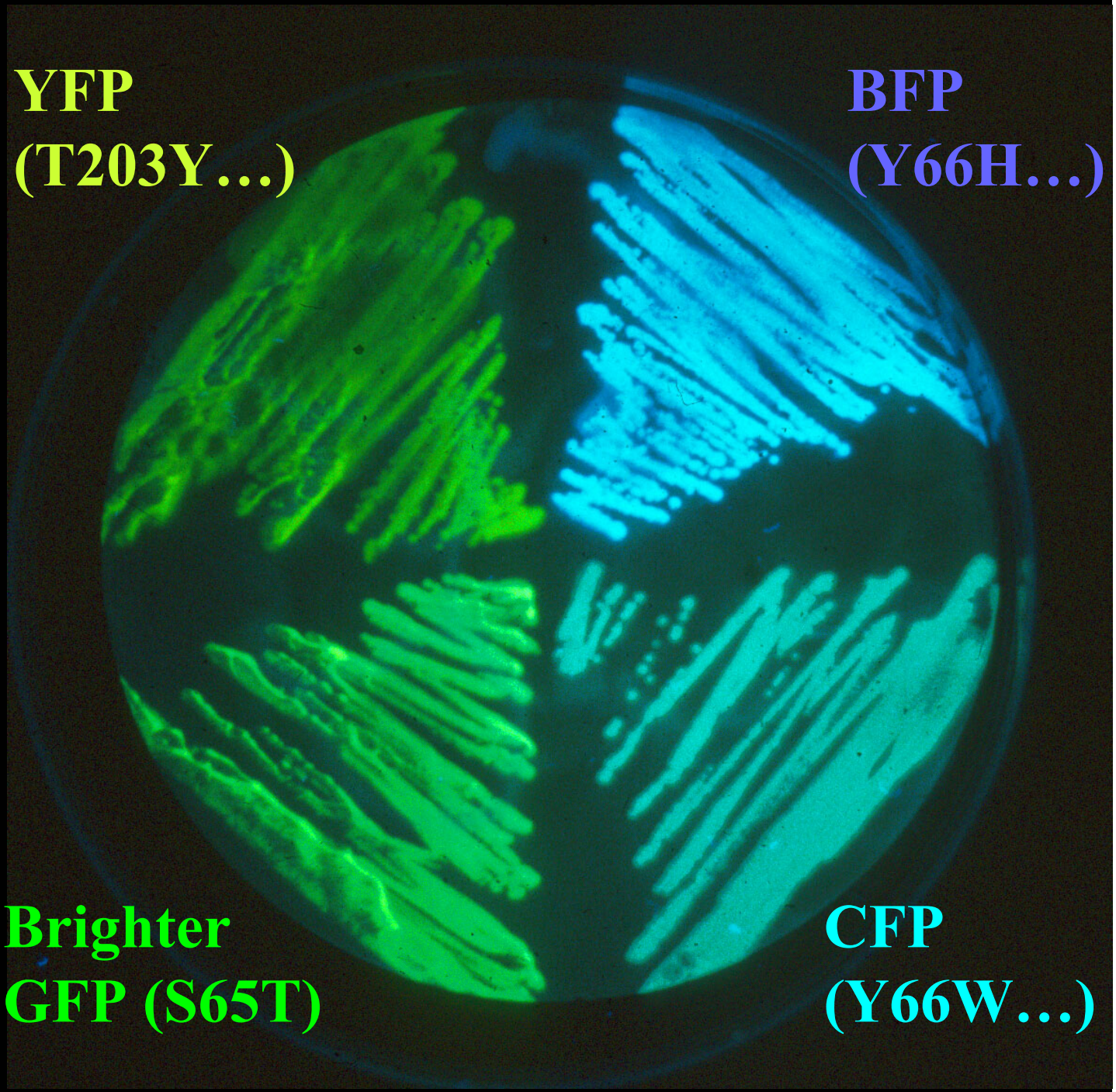
Mats Ormo
Karen Kallio
Jim Remington
(U. Oregon)

Andrew Cubitt
(Aurora Biosciences)

Roger Tsien
(HHMI, UCSD)

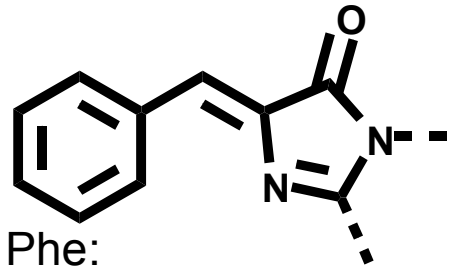


4 colors of GFP mutants expressed in *E. coli*

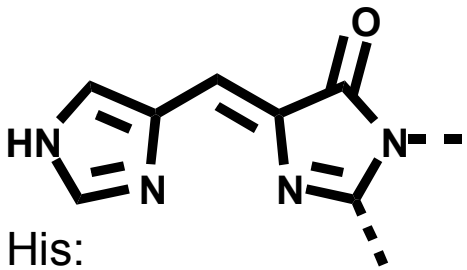


R. Heim, A. Cubitt

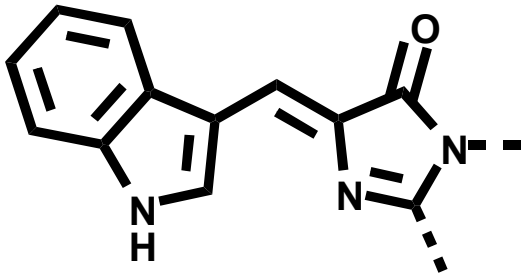
Wavelengths resulting from different aromatic amino acids at position 66 of GFP



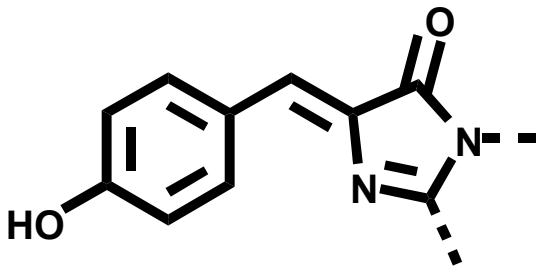
Phe:
exc 358, em 442



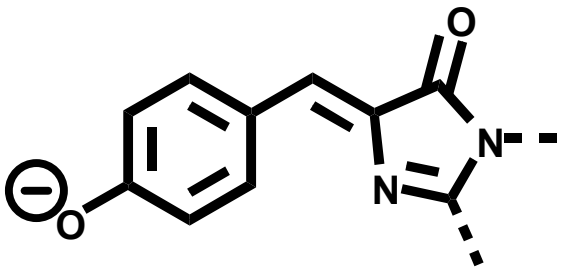
His:
exc 382, em 448



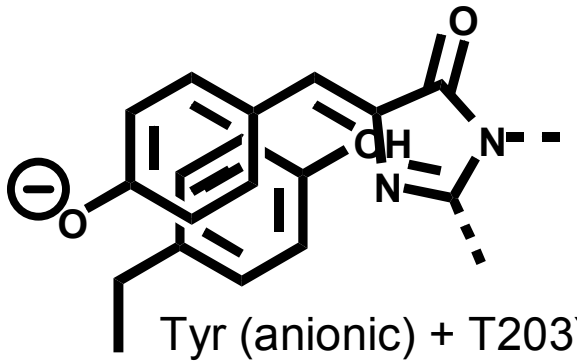
Trp:
exc 433, em 475



Tyr (neutral):
exc 395, em 508



Tyr (anionic):
exc 475, em 503



Tyr (anionic) + T203Y:
exc 513, em 527

R. Heim, A. Cubitt

Factors affecting the detectability of GFP

Total amount of GFP (picked out by antibodies, or by position on gel if GFP is abundant enough)

- Number of copies of gene, duration of expression
- Strength of transcriptional promotors and enhancers
- Efficiency of translation including Kozak sequence and codon usage
- Absence of mRNA splicing, protein degradation and export

Efficiency of posttranslational fluorophore formation

- Solubility vs. formation of inclusion bodies
- Availability of chaperones
- Hindrance to folding because of unfortunate fusions to host proteins
- Time, temperature, availability of O_2 , and intrinsic rate of cyclization/oxidation

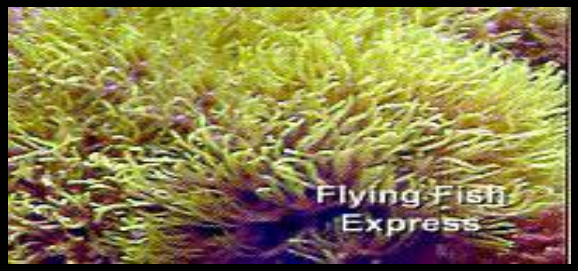
Molecular properties of mature GFP

- Wavelengths of excitation and emission
- Extinction coefficient and fluorescence quantum yield
- Susceptibility to photoisomerization/bleaching
- Dimerization

Competition with noise and background signals

- Autofluorescence of cells or culture media at preferred wavelengths
- Location of GFP, diffuse vs. confined to small subregions of cells or tissues
- Quality of excitation and emission filters and dichroic mirrors
- Sensitivity, noise, and dark current of photodetector

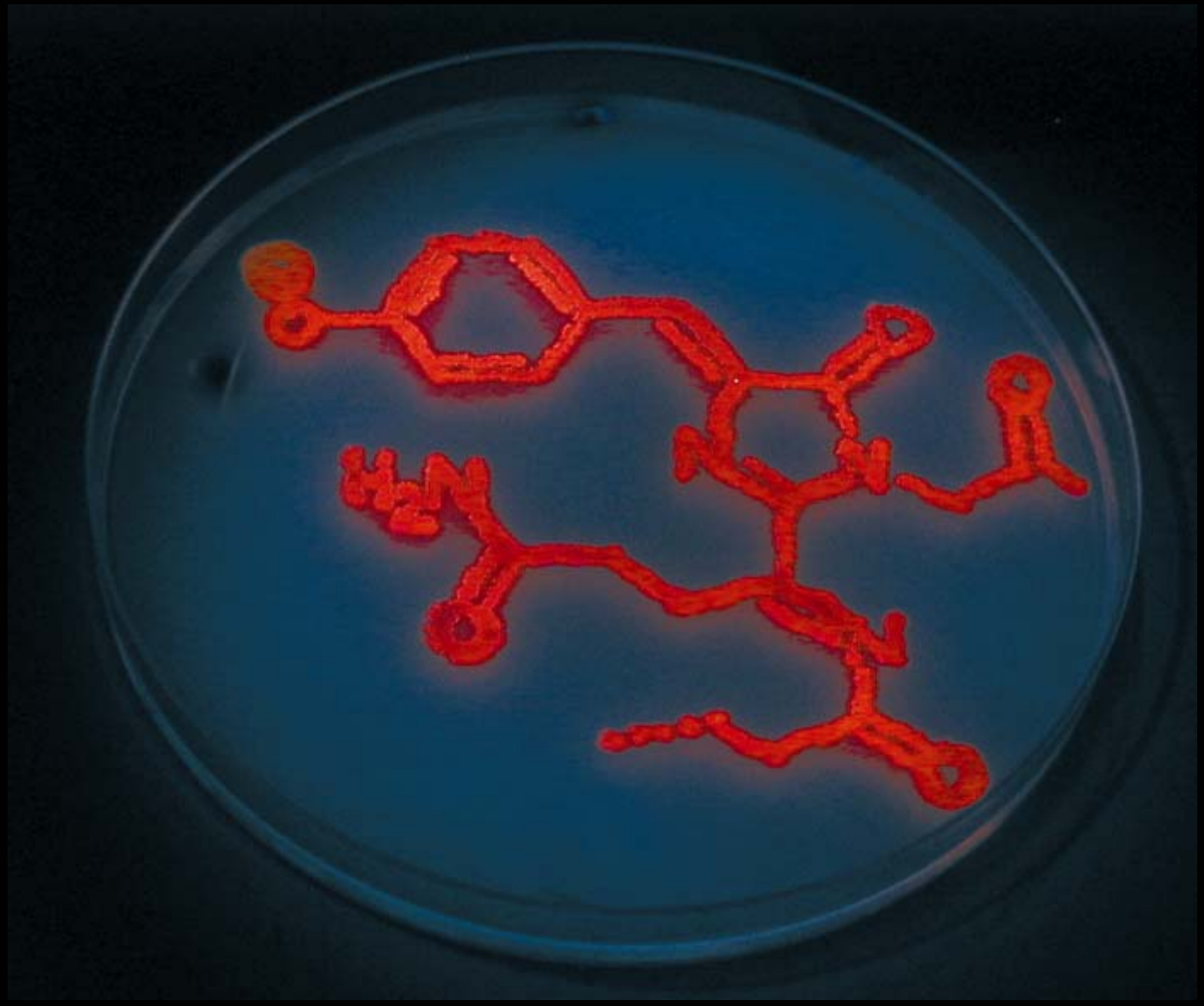
Many tropical corals contain fluorescent proteins



Why coral fluorescent proteins are potentially so valuable

- Multiplicity of coral species offers colors (yellows, reds, far-reds) not available from *Aequorea* GFP mutants
- RFP would be good FRET acceptor for GFP donor
  - GFP is spectroscopically superior to CFP, already incorporated into more fusions, better matched to standard lasers
- RFP should be good acceptor for resonance energy transfer (LRET, BRET) from Tb^{3+} or luciferases
  - LRET has less orientation dependence, lower spectroscopic cross-talk tolerating greater protein-protein distances, better rejection of autofluorescence than FRET has
- Longer wavelengths are more favorable for imaging in thick tissues and *in vivo*

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

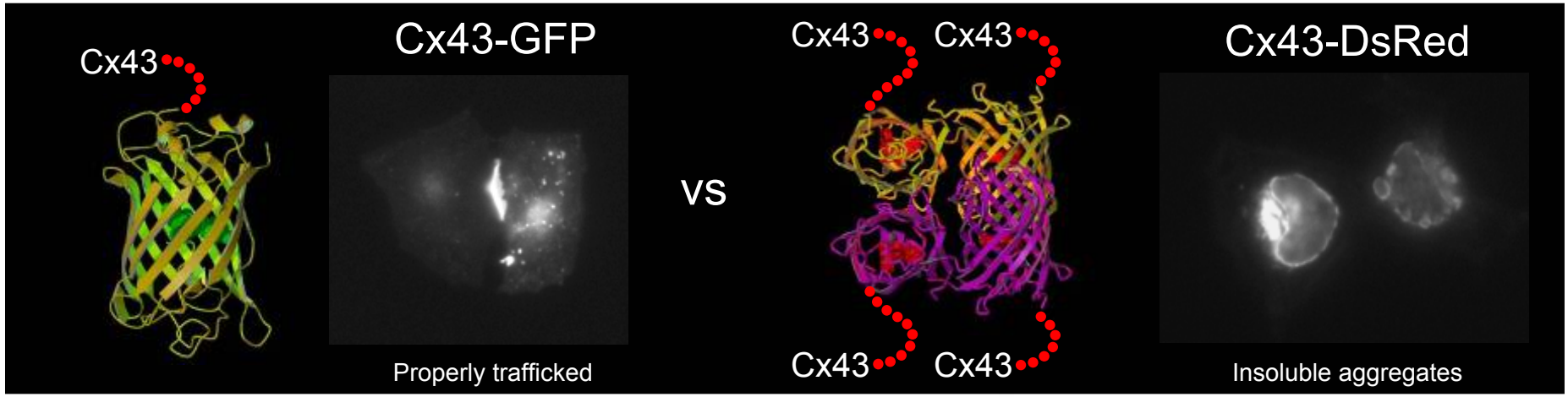


Structure detd. by Larry Gross, drawn by Varda Lev-Ram & Geoff Baird

The 3 problems with DsRed

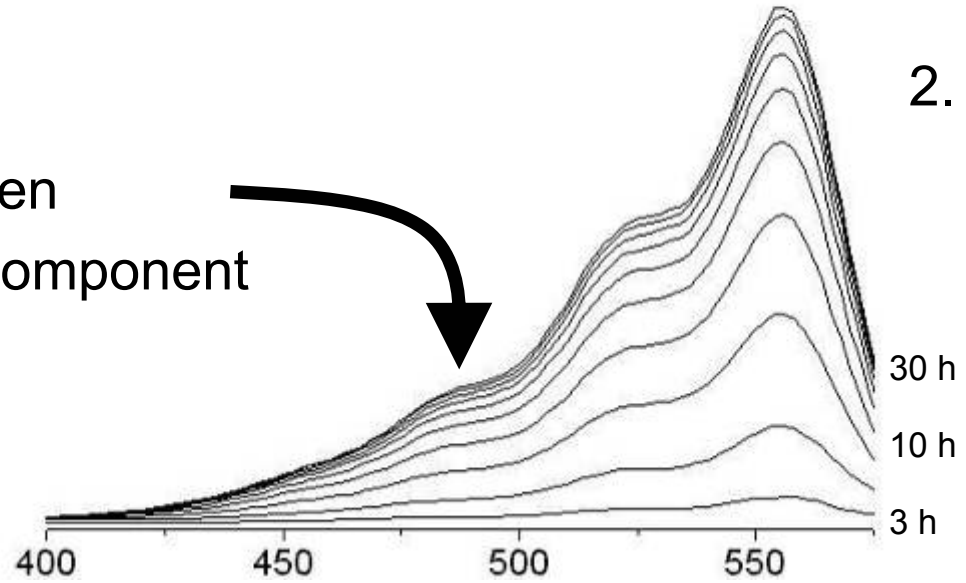
1. Tetrameric structure can prevent proper trafficking of fusions

   e.g. Fusion to the gap junction protein connexin43 (Lauf U. et al. *FEBS let.* (2001) **498**, 11-15).



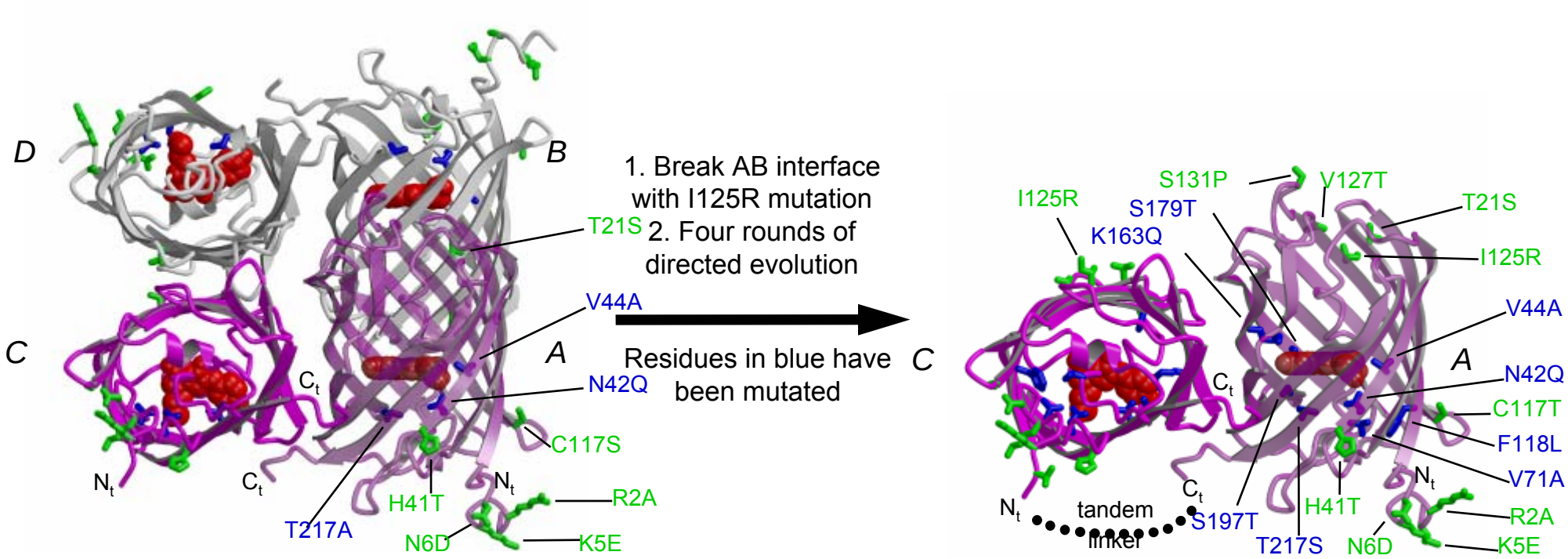
2. Slow maturation

3. Residual green fluorescent component



Baird, G. S. et al. *Proc. Natl. Acad. Sci. USA* (2000) **97**, 11984-11989

Evolution of a bright red fluorescent dimer of DsRed



T1* (fast tetramer)

3 mutations inside barrel

6 mutations outside barrel

0 dimer interface mutations

9 mutations total

dimer2

8 mutations inside barrel

7 mutations outside barrel

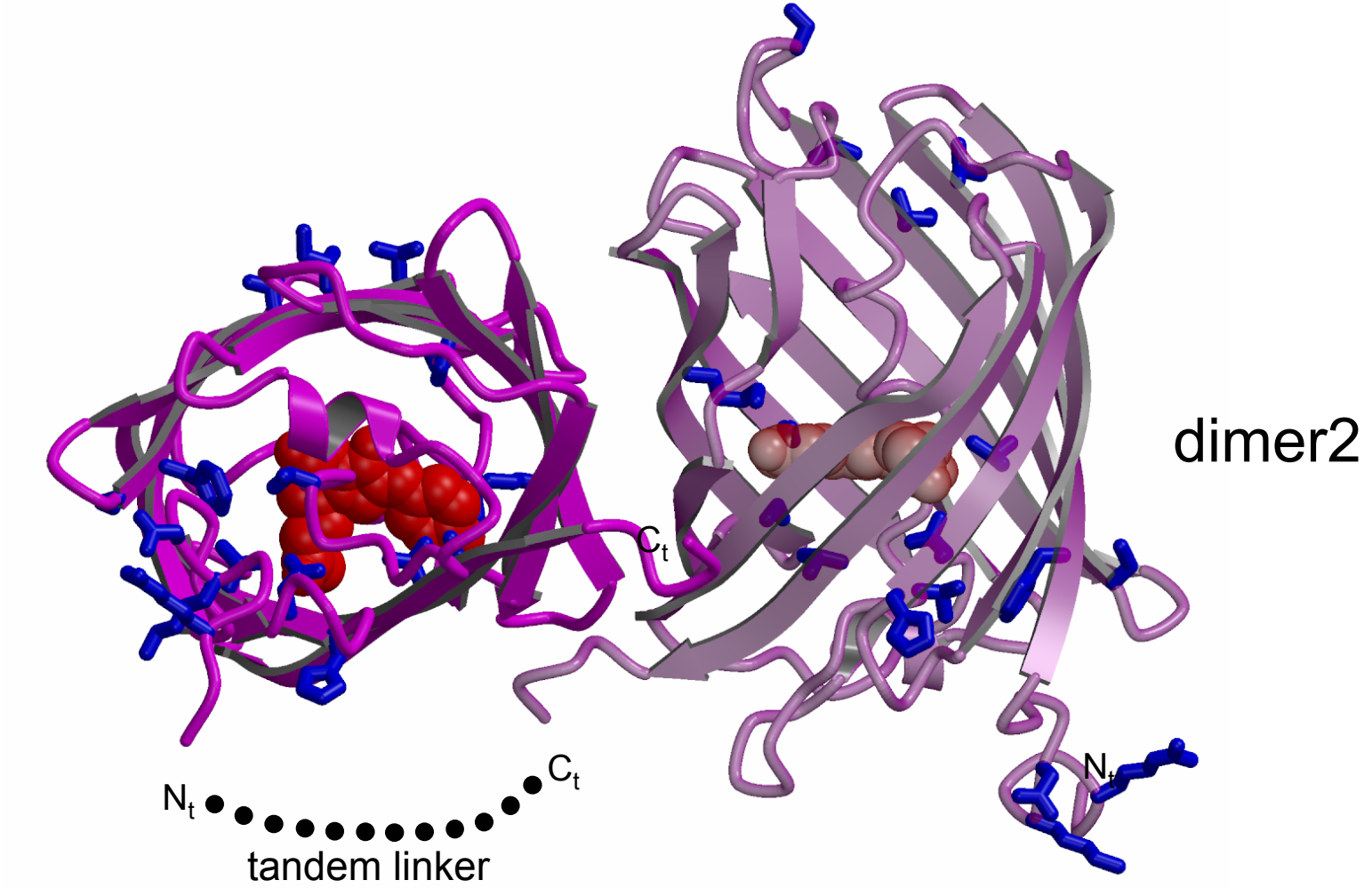
2 AB dimer interface mutations

17 mutations total

*Bevis, B.J. and Glick, B.S. *Nature Biotech*. (2002) **20**, 83-87

Robert Campbell

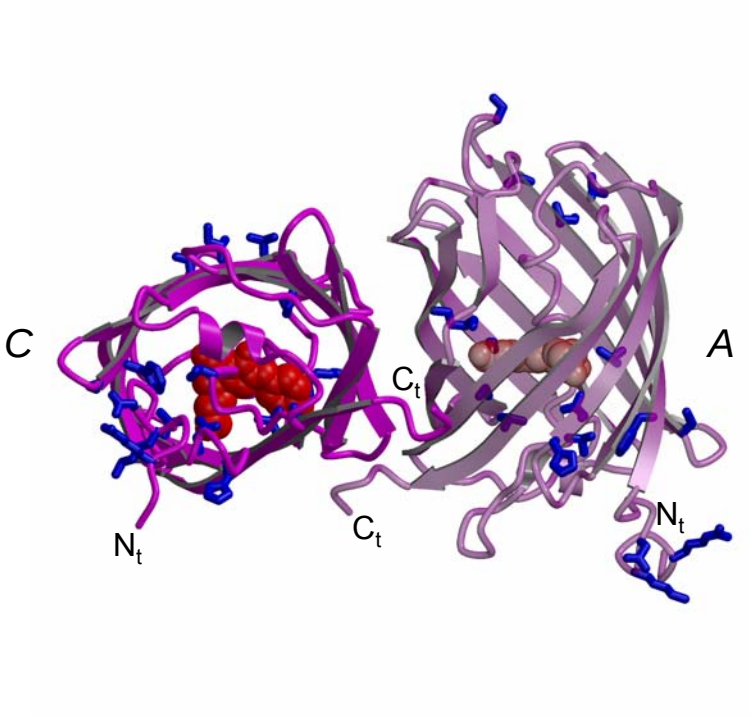
A fused ‘tandem dimer’ provides an effective monomer



A 12 amino acid linker (–GHGTGSTGSGSS-) appears to be the minimal length that does not adversely effect the fluorescence

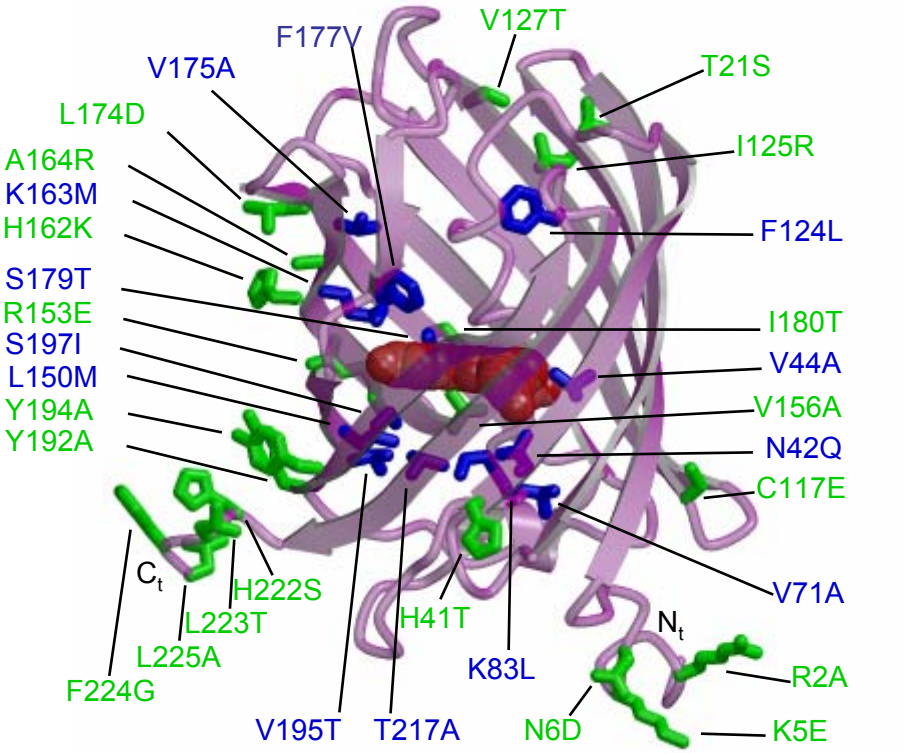
Robert Campbell

Evolution of a monomeric DsRed



1.Break remaining AC interface
2.Seven rounds of directed evolution

Residues in blue have been mutated



<u>Dimer1 (1st generation of dimer)</u>

6 mutations inside barrel

5 mutations outside barrel

1 AB dimer interface mutation

12 mutations total

<u>mRFP1</u>

14 mutations inside barrel

9 mutations outside barrel

2 AB dimer interface mutations

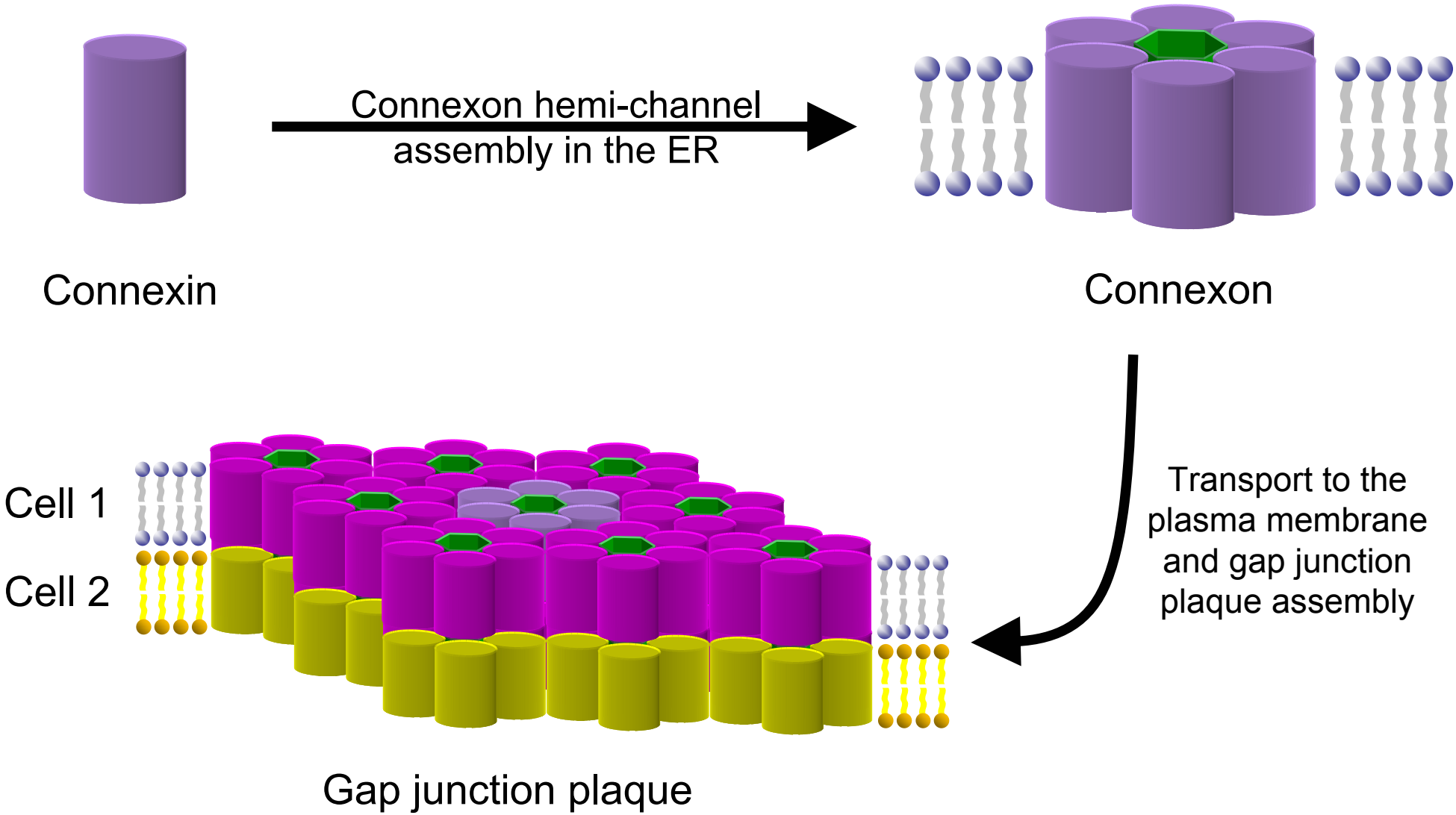
8 AC dimer interface mutations

33 mutations total, 15% of protein

>1500 requests processed!

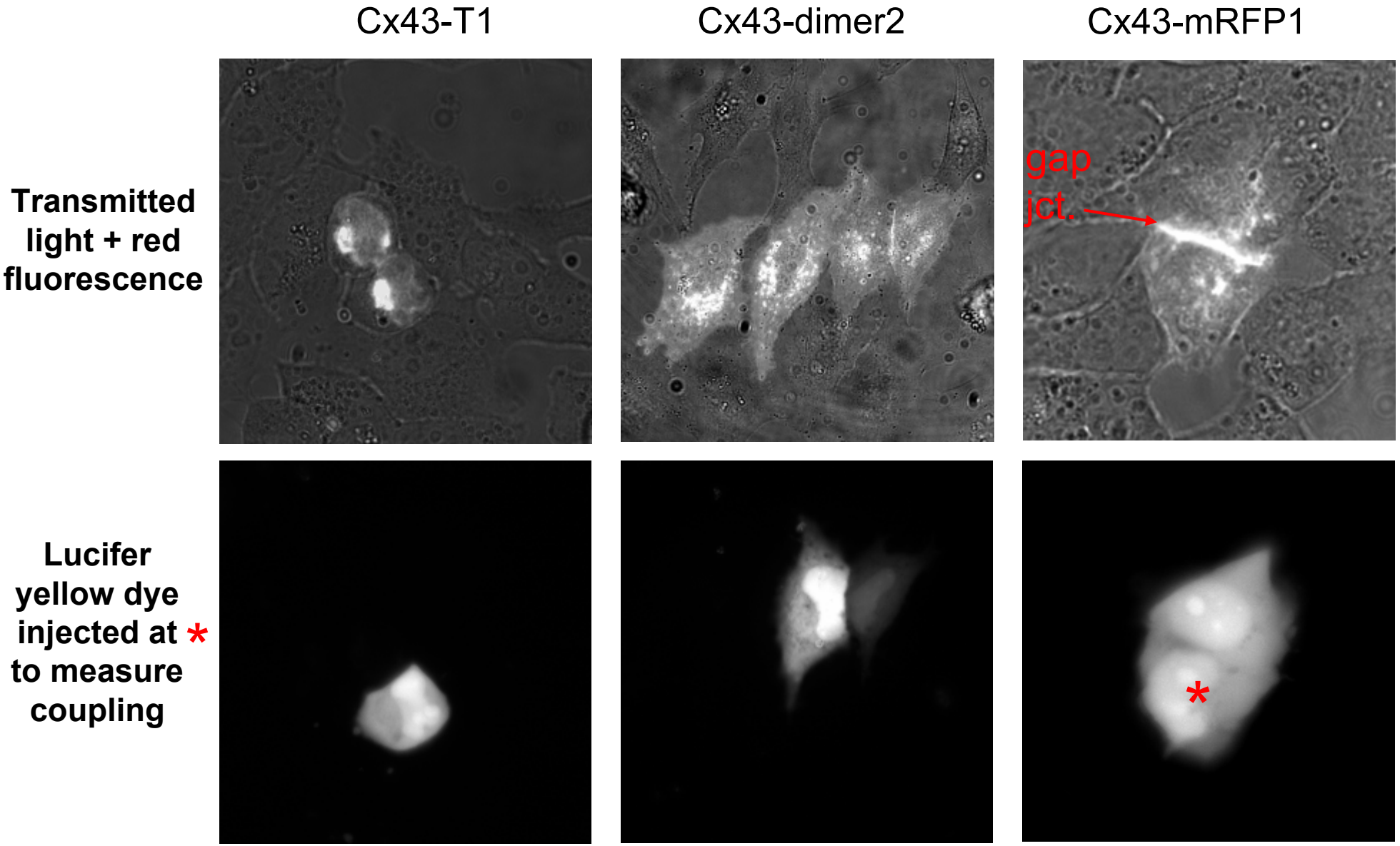
Robert Campbell

Gap junction assembly



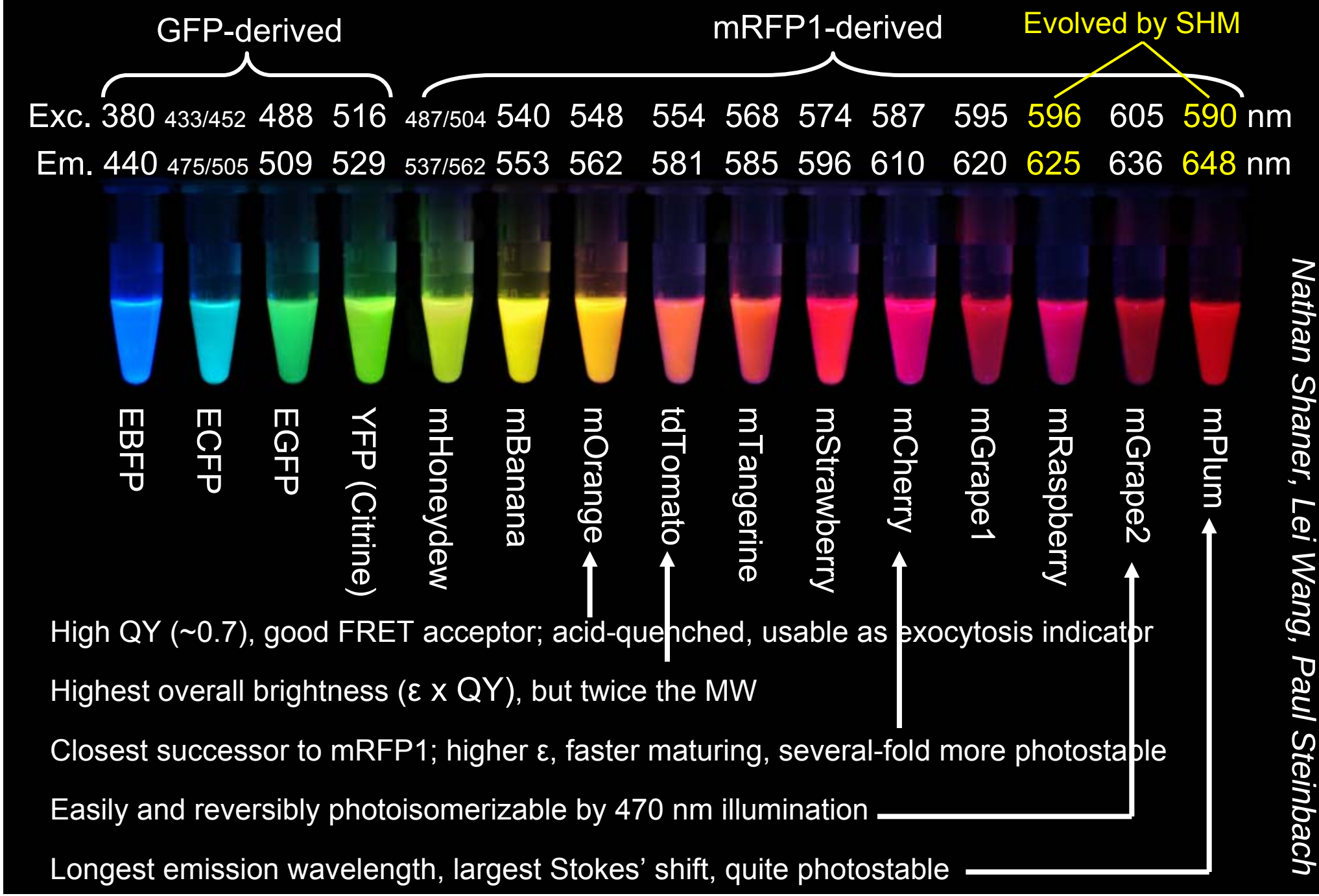
Falk, M.M. *Eur. J. Cell Biol*. (2000) **79**, 564-574

DsRed tetramer fusions are dead, dimer fusions are crippled, monomer fusions are fully functional



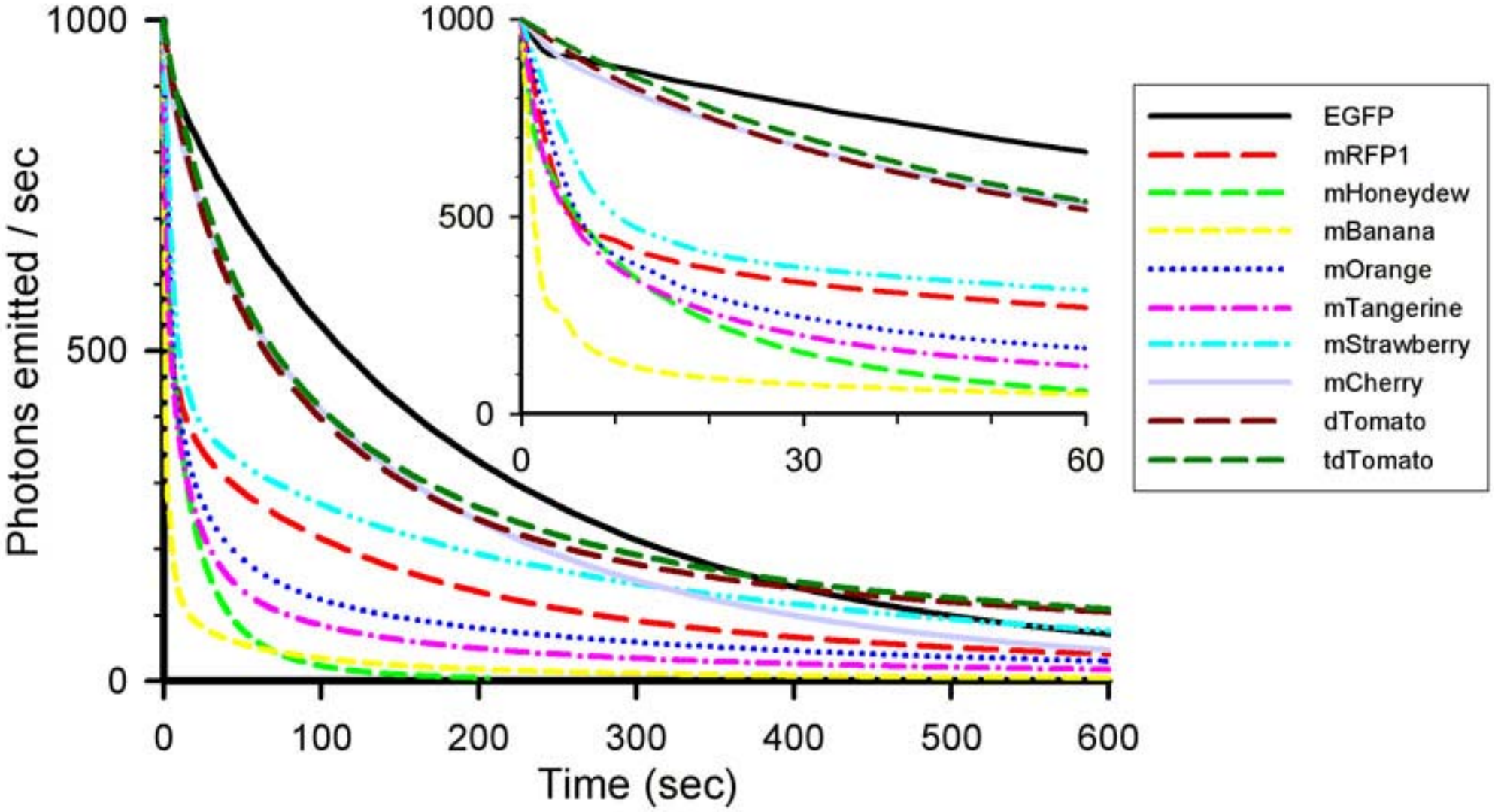
Oded Tour, Robert Campbell

The 2004 palette of nonoligomerizing fluorescent proteins



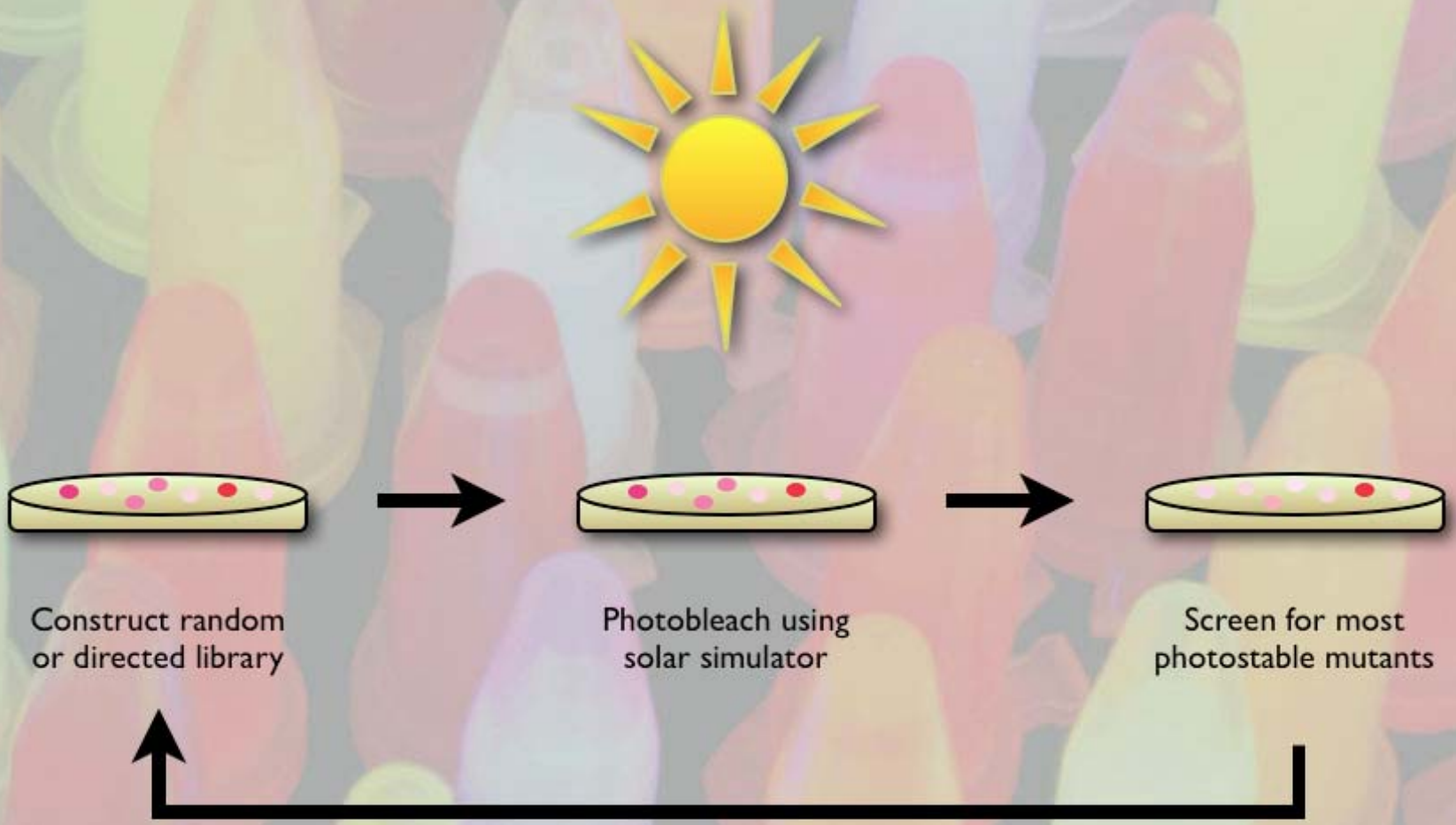
Photobleaching kinetics

Normalized so that each molecule in the population starts out emitting 1000 photons/s

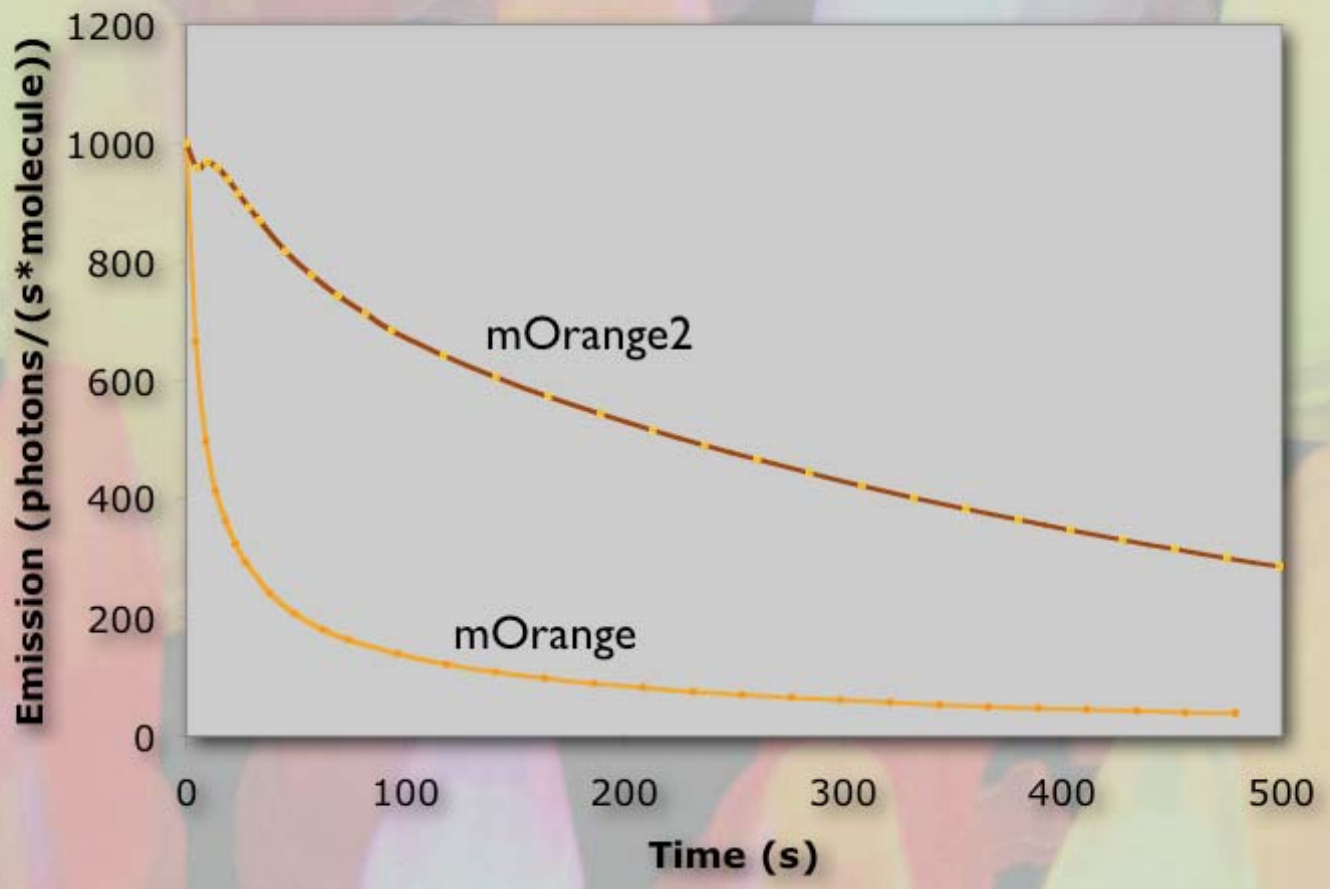


Nathan Shaner, Paul Steinbach

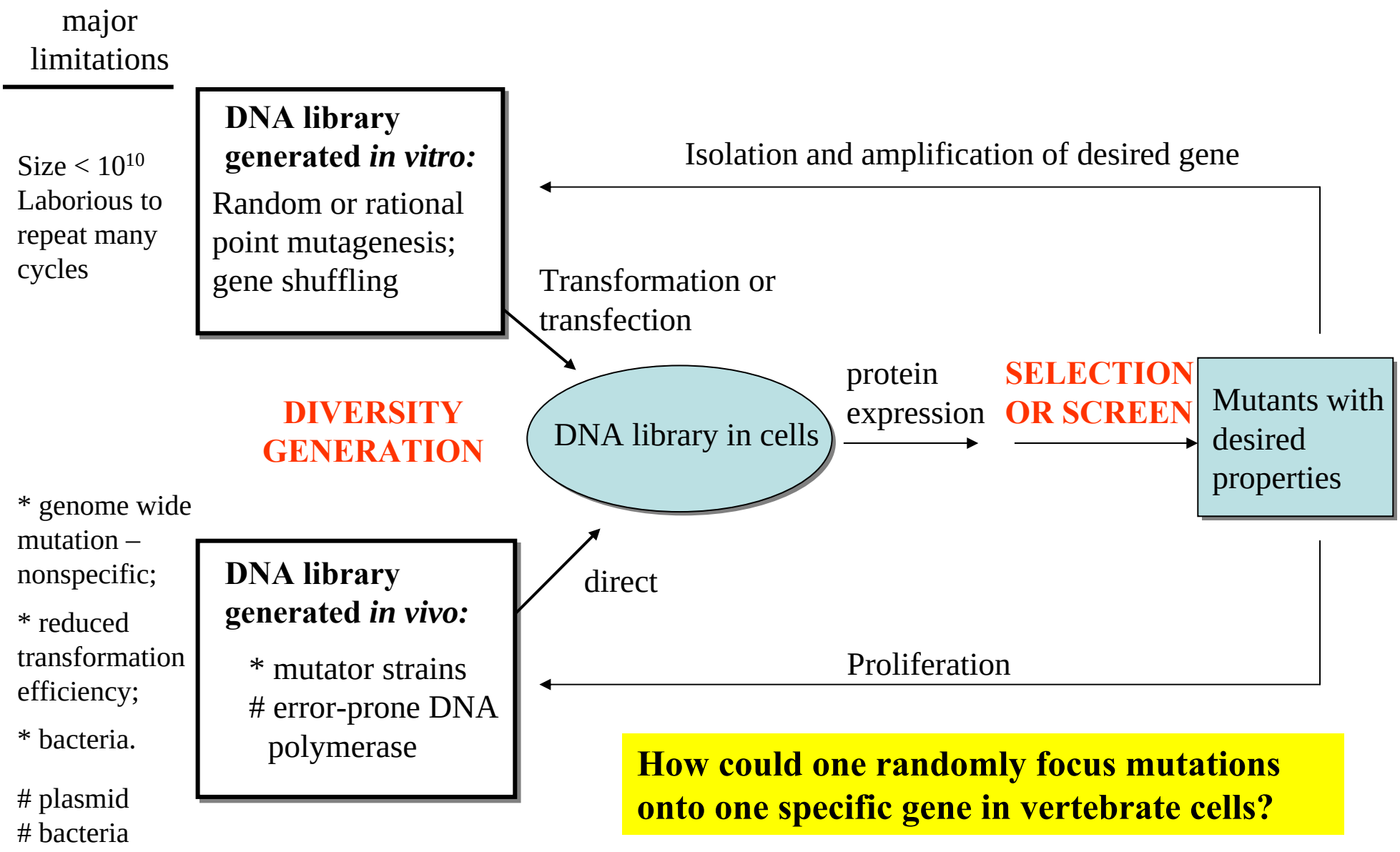
Optimization of photostability



mOrange2


Emission (photons/(s*molecule))
1200
1000
800
600
400
200
0
mOrange2
mOrange
0
100
200
300
400
500
Time (s)

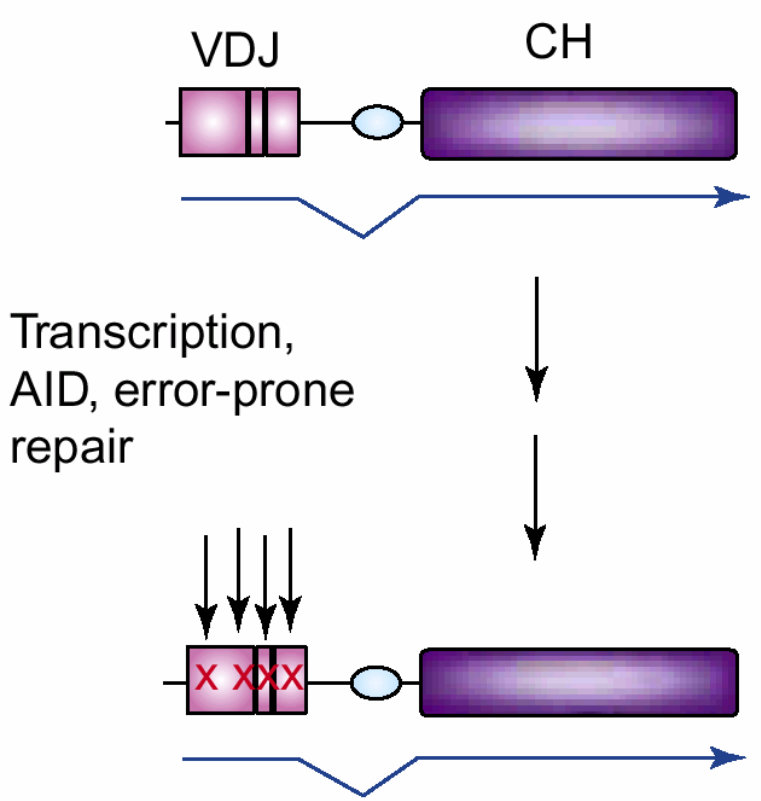
Directed evolution *in vitro* vs. *in vivo*



Lei Wang

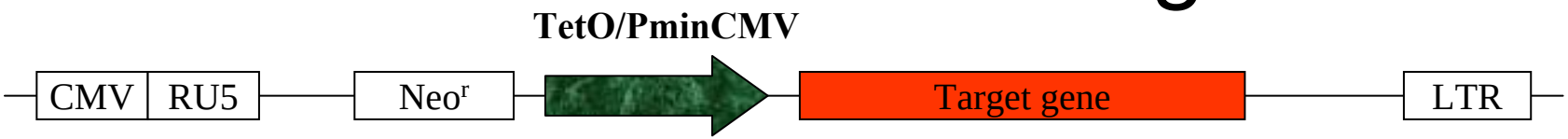
Somatic Hypermutation (SHM)

After B cells encounter and become activated by antigen, the rearranged V regions of immunoglobulin undergo a high rate of point mutation that creates additional diversity within the expanding clone of B cells.



- Centroblast stage of B-cell differentiation
- High frequency: 10^{-3} – 10^{-4} bp/generation (10^{6} times higher than background genomic mutation).
- Predominance of point mutations, occasional deletions or duplications
- Transition (G ⇔ A; T ⇔ C) >> transversion
- Mutation biased to GC base pairs, hot spot RGYW/WRCY. (R = A or G; Y = C or T; and W = A or T)
- Mechanism under intensive study in immunology laboratories

Protein Evolution Using SHM



Transfect into Ramos cells, because they already have AID and accessory error-prone DNA repair enzymes

Add doxycycline to induce high-level gene expression and SHM

Cells with mutant library

Selection/screen, ideally by FACS

Cells with selected phenotype

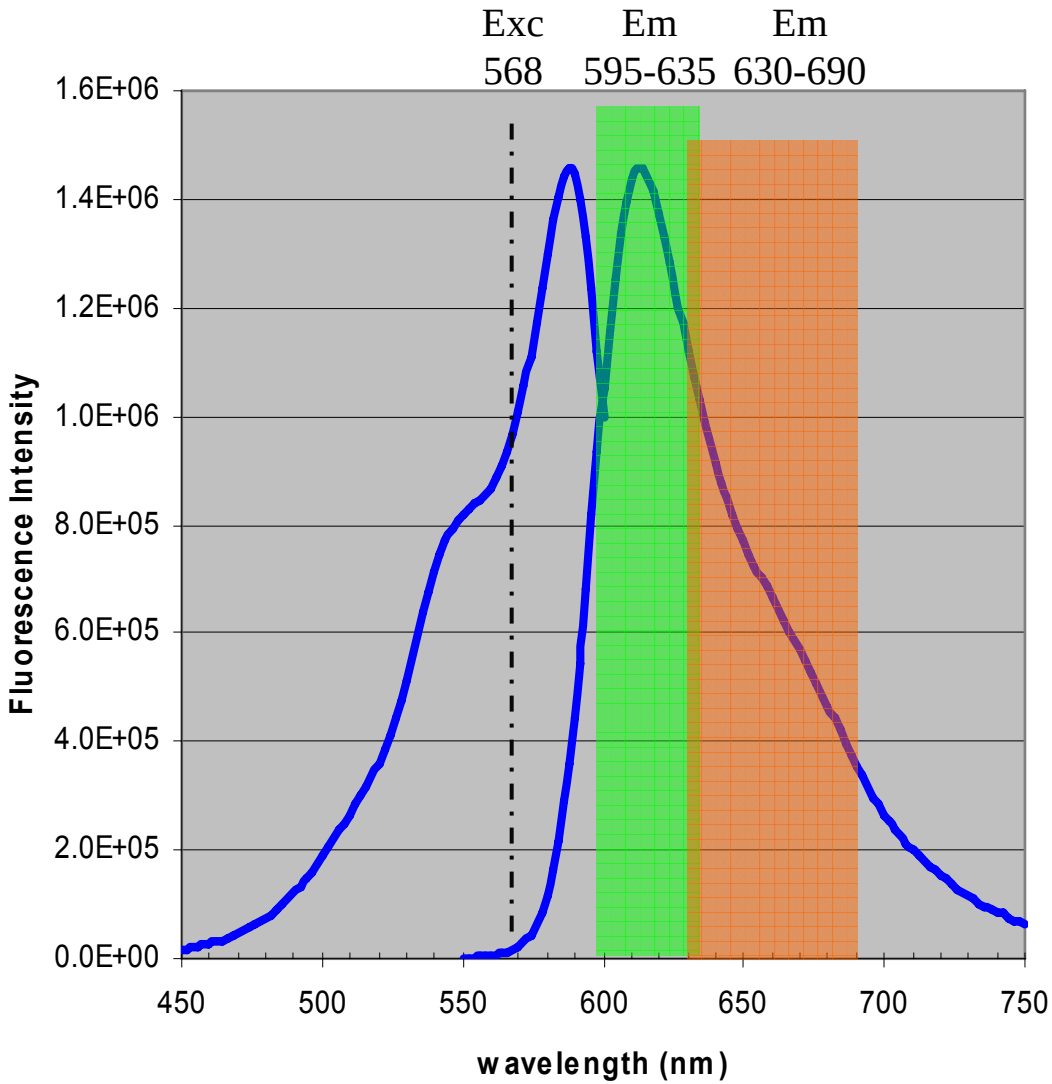
Allow proliferation in the absence of doxycycline to restrict SHM

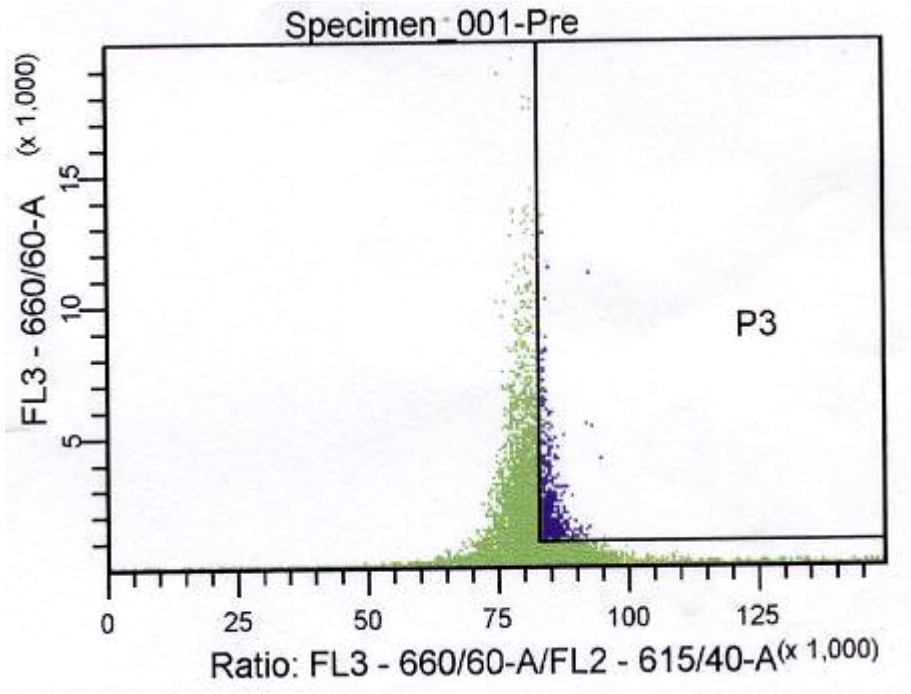
Isolate mRNA, RT-PCR, sequence the mutant

Mutants with desired properties

Lei Wang

Ratio sorting to further red-shift mRFP1

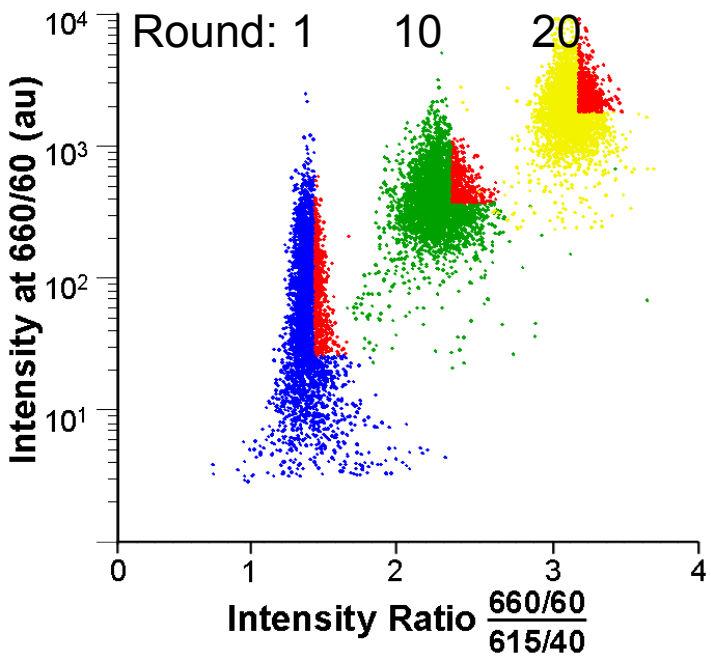


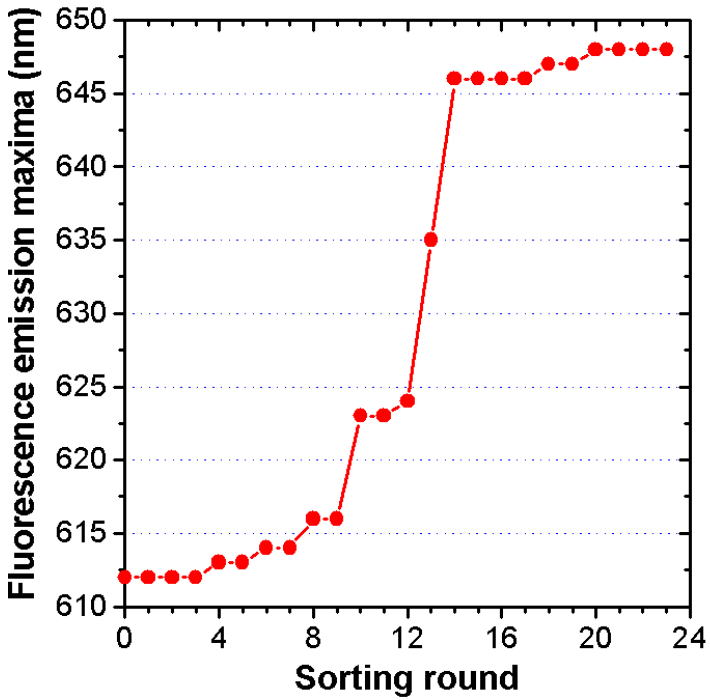


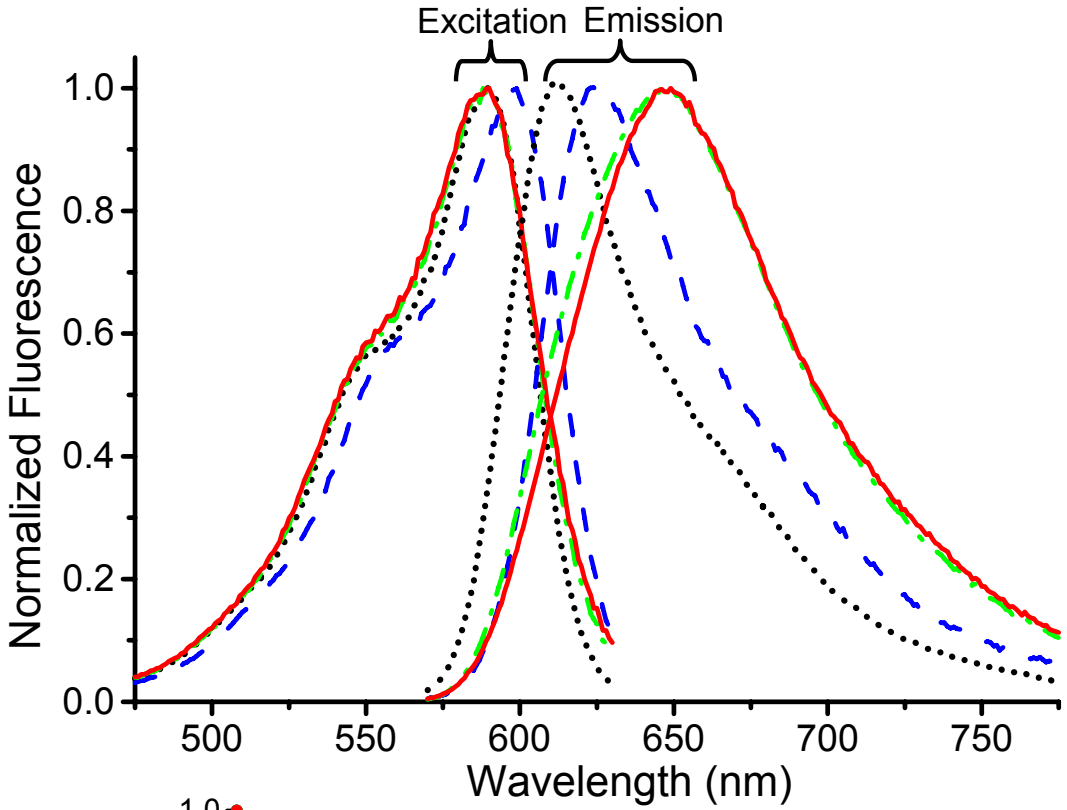
$$\text{Ratio} = \frac{I_{630\text{-}690\ \text{nm}}}{I_{595\text{-}635\ \text{nm}}}$$

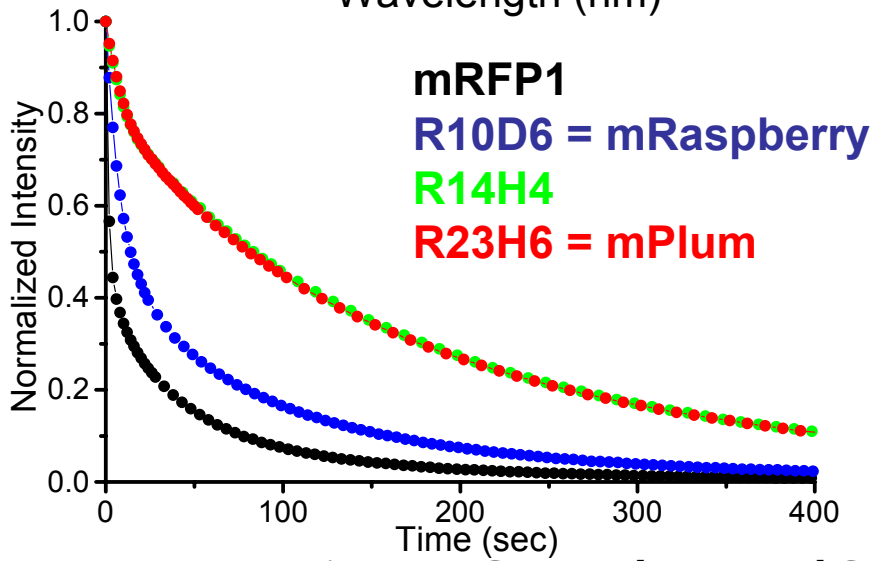
Lei Wang, Coyt Jackson

Progressive red-shifting in Ramos cells









Lei Wang, Coyt Jackson, Paul Steinbach

Summary of somatic hypermutation on mRFP1

- Exogenously introduced genes undergo mutation in hypermutating Ramos cells;
- Mutations are well distributed in the target gene, allowing for large sequence space to be sampled;
- Although G/C mutations are most common, A/T mutations are also observed and beneficial ones accumulate after multiple rounds of selection;
- Several mRFP mutants with emission peaks red-shifted to different wavelengths as high as 648 nm have been evolved. Somatic hypermutation mostly found mutations different from those chosen in semi-rational mutation.
- Deliberate introduction of other amino acids at positions located by SHM has failed to produce further gains, i.e. SHM already found the best substitutions there

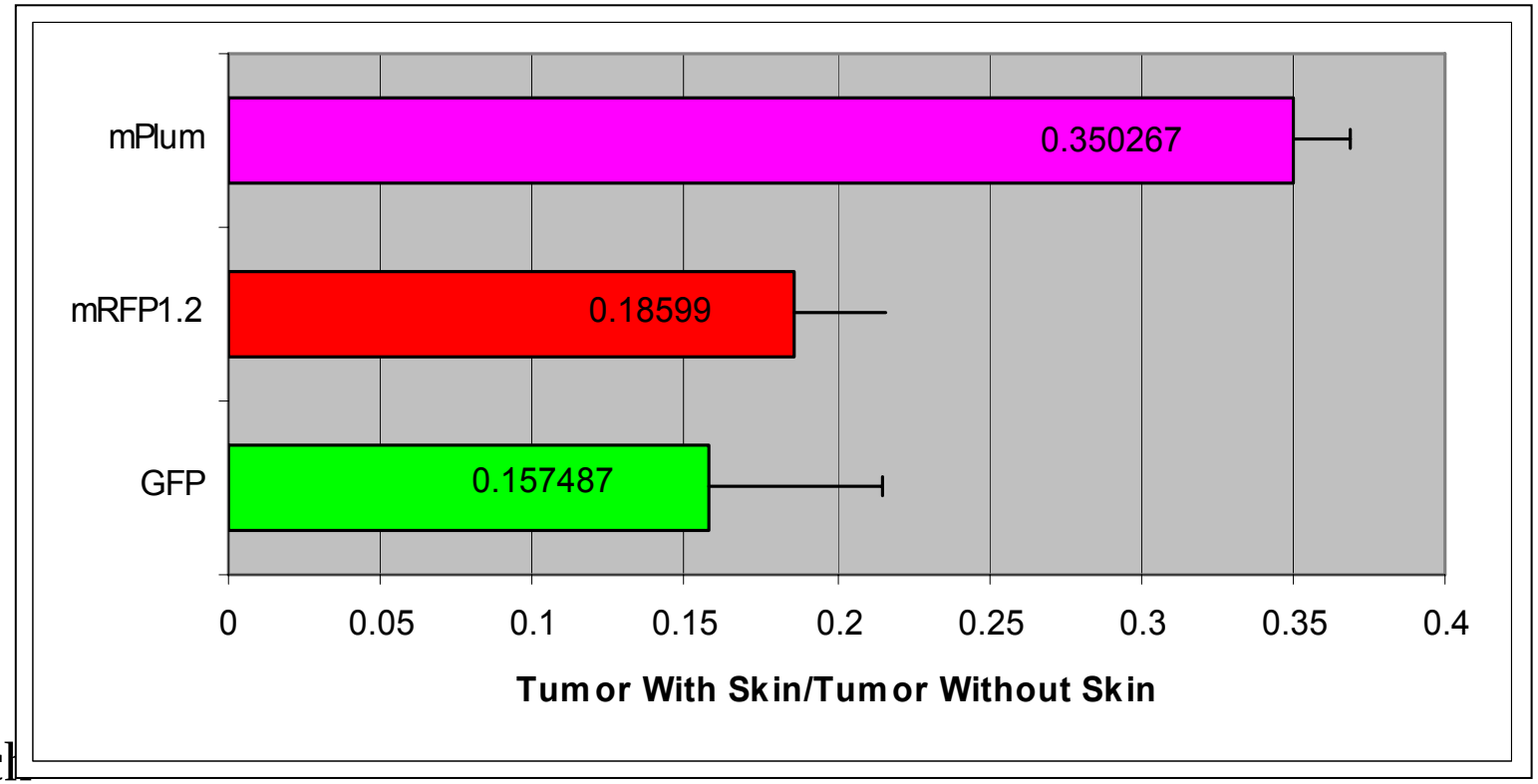
Next steps

- Introduce sex, i.e. recombination via DNA shuffling
- Excite at longer wavelengths to allow excitation wavelength to red-shift too
- Select for higher intensity/quantum yield instead of just wavelength
- Apply this strategy to other fluorescent proteins

Lei Wang

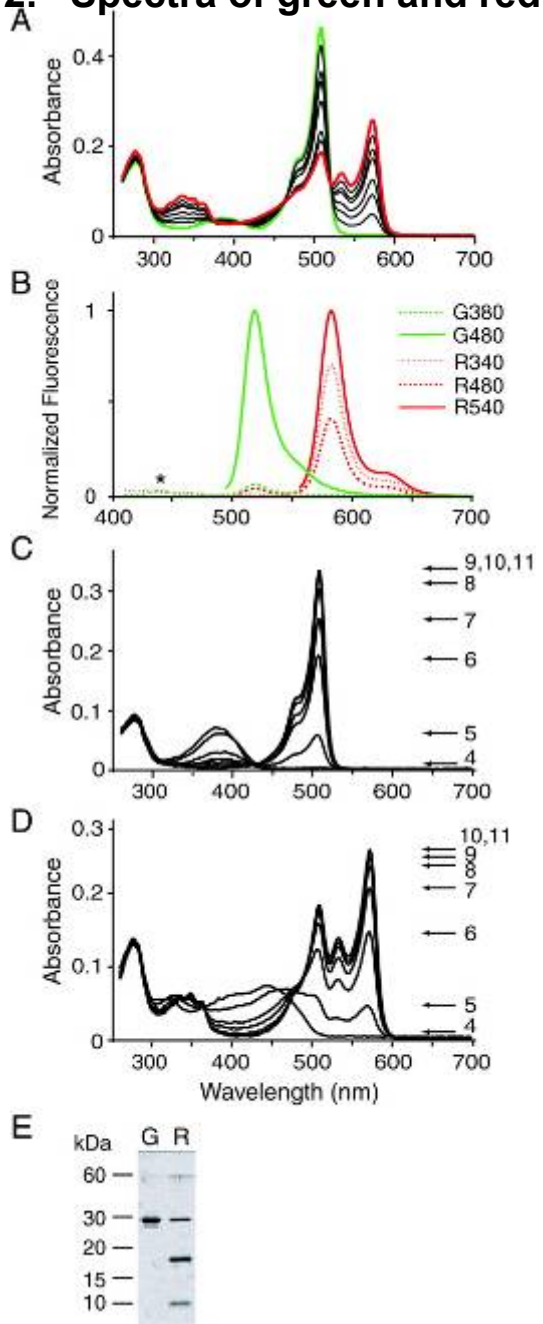
mPlum is less affected by tissue attenuation

- mPlum attenuation by skin is significantly less than that of GFP and mRFP1.2 ($p<0.05$, 2-tailed t-test, 6 animals)
- GFP and mRFP2.1 are not significantly different than each other!



Emmi Olson, Lei Wang

Fig. 2. Spectra of green and red states of Kaede



Proc Natl Acad Sci U S A. 2002 Oct 1;99(20):12651-6. Epub 2002 Sep 23.

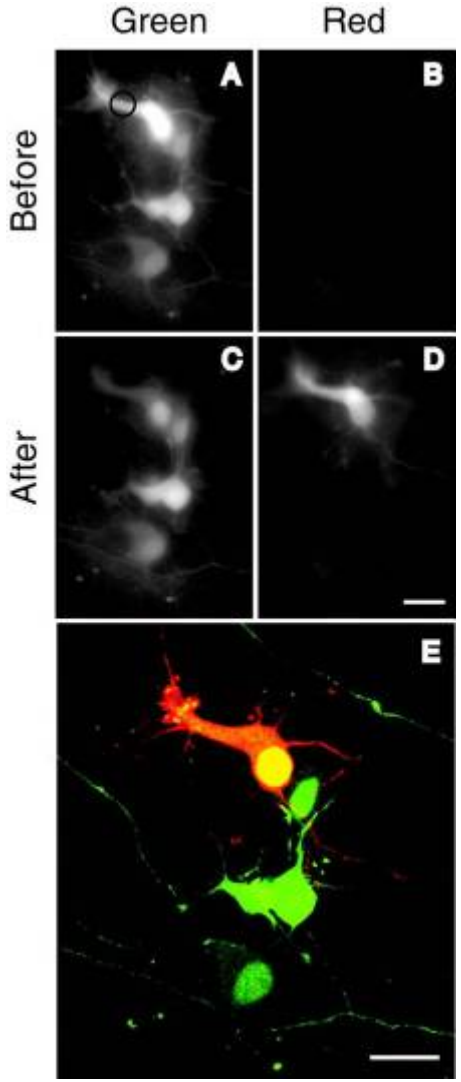
An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein.

Ando R, Hama H, Yamamoto-Hino M, Mizuno H, Miyawaki A.

Laboratory for Cell Function and Dynamics, Advanced Technology Development Center, Brain Science Institute, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan.

We have cloned a gene encoding a fluorescent protein from a stony coral, Trachyphyllia geoffroyi, which emits green, yellow, and red light. The protein, named Kaede, includes a tripeptide, His-Tyr-Gly, that acts as a green chromophore that can be converted to red. The red fluorescence is comparable in intensity to the green and is stable under usual aerobic conditions. We found that the green-red conversion is highly sensitive to irradiation with UV or violet light (350-400 nm), which excites the protonated form of the chromophore. The excitation lights used to elicit red and green fluorescence do not induce photoconversion. Under a conventional epifluorescence microscope, Kaede protein expressed in HeLa cells turned red in a graded fashion in response to UV illumination; maximal illumination resulted in a 2,000-fold increase in the ratio of red-to-green signal. These color-changing properties provide a simple and powerful technique for regional optical marking. A focused UV pulse creates an instantaneous plane source of red Kaede within the cytosol. The red spot spreads rapidly throughout the cytosol, indicating its free diffusibility in the compartment. The extensive diffusion allows us to delineate a single neuron in a dense culture, where processes originating from many different somata are present. Illumination of a focused UV pulse onto the soma of a Kaede-expressing neuron resulted in filling of all processes with red fluorescence, allowing visualization of contact sites between the red and green neurons of interest.

Fig. 7. Visualization of an individual neuron in a hippocampal primary culture

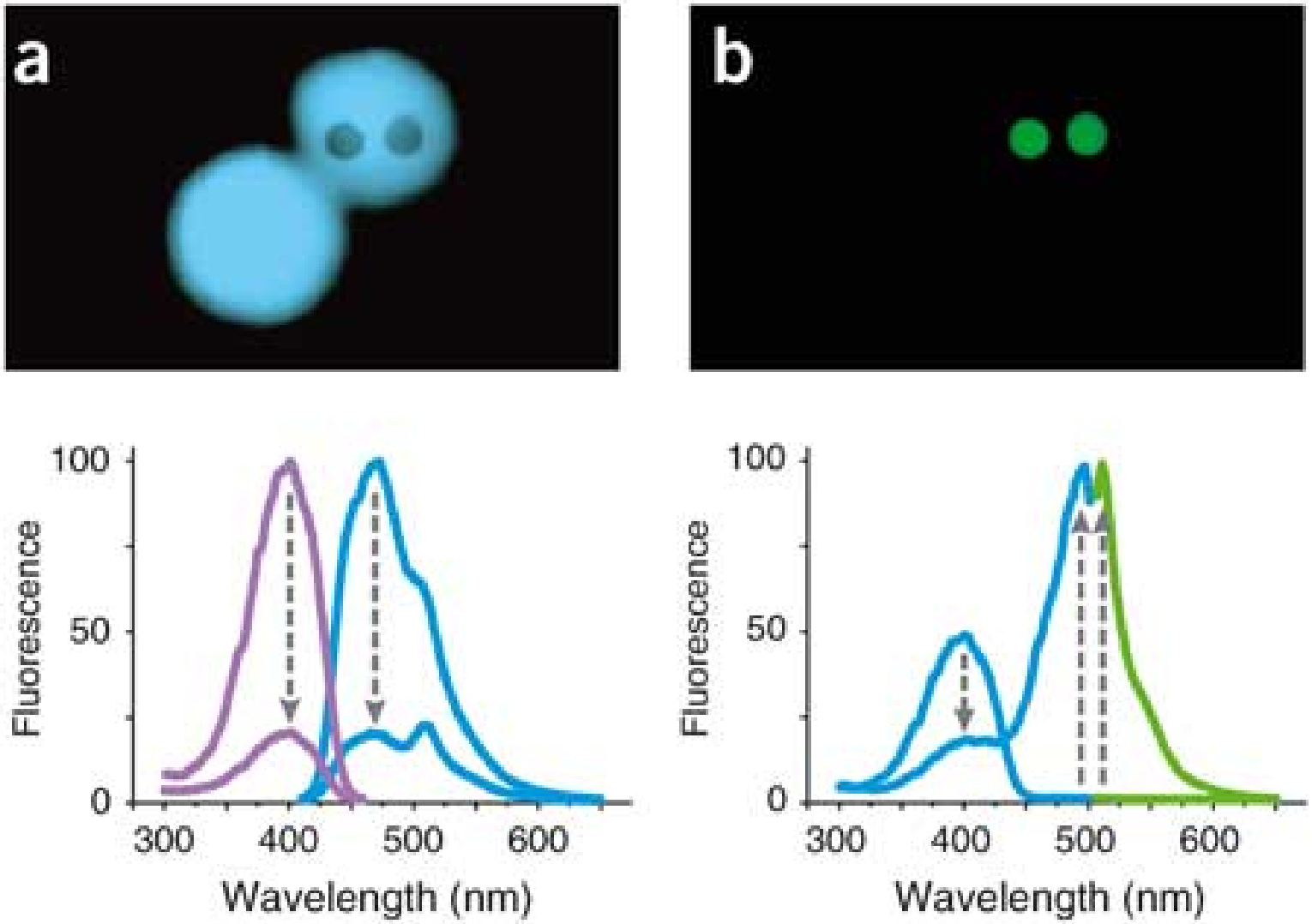


Ando, Ryoko et al. (2002) Proc. Natl. Acad. Sci. USA 99, 12651-12656

PNAS

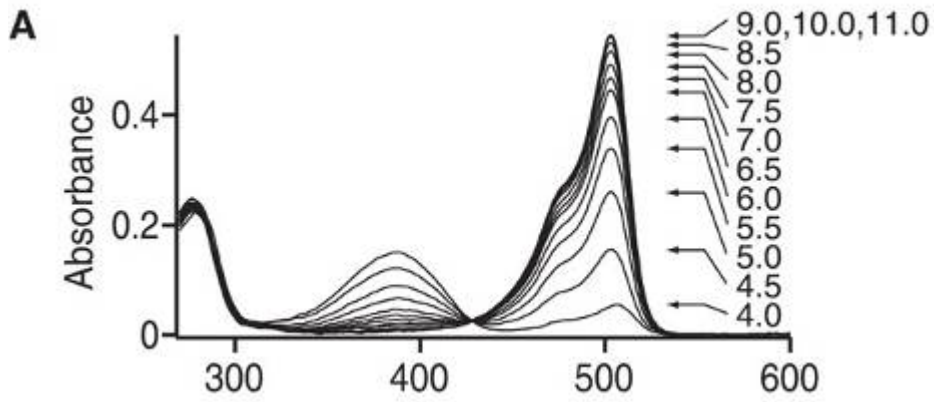
PS-CFP: a monomeric fluorescent protein that changes color upon illumination

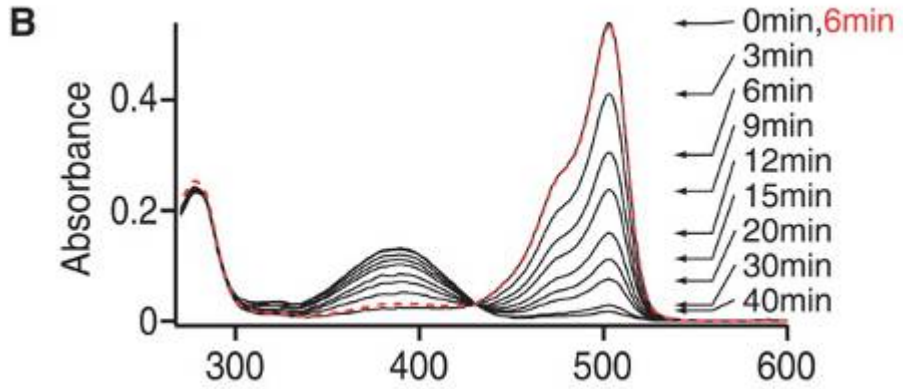
Chudakov *et al. Nature Biotech.* **22**: 1435-1439 (2004)

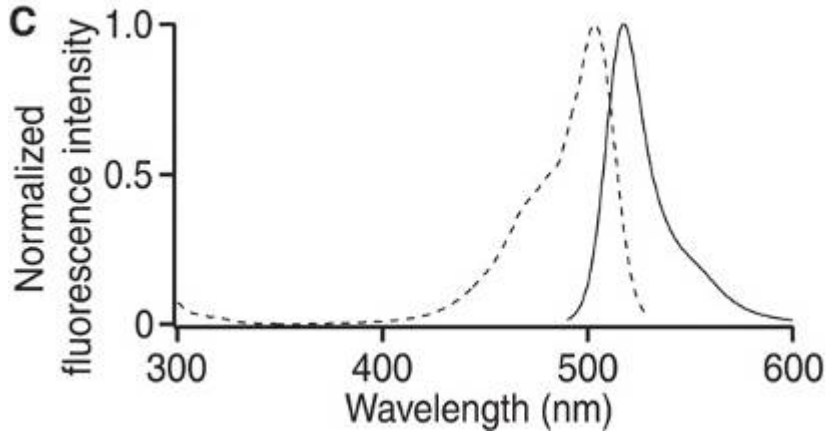


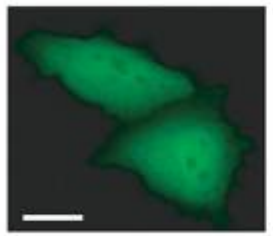
Dronpa: a reversible protein highlighter

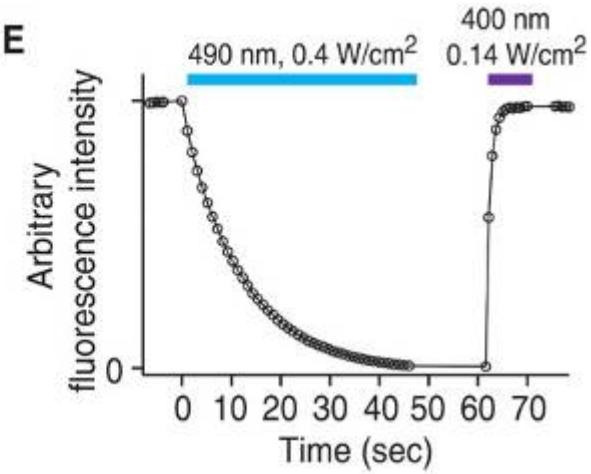
Ando *et al*, *Science* **306**: 1370-1373 (2004)

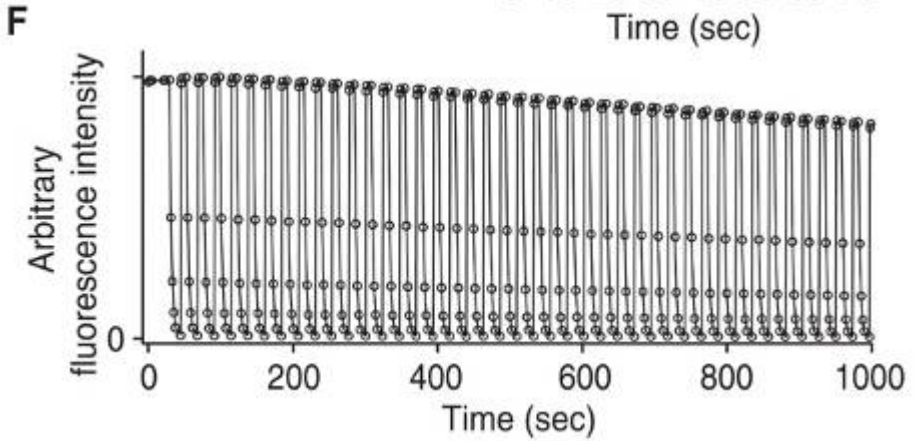








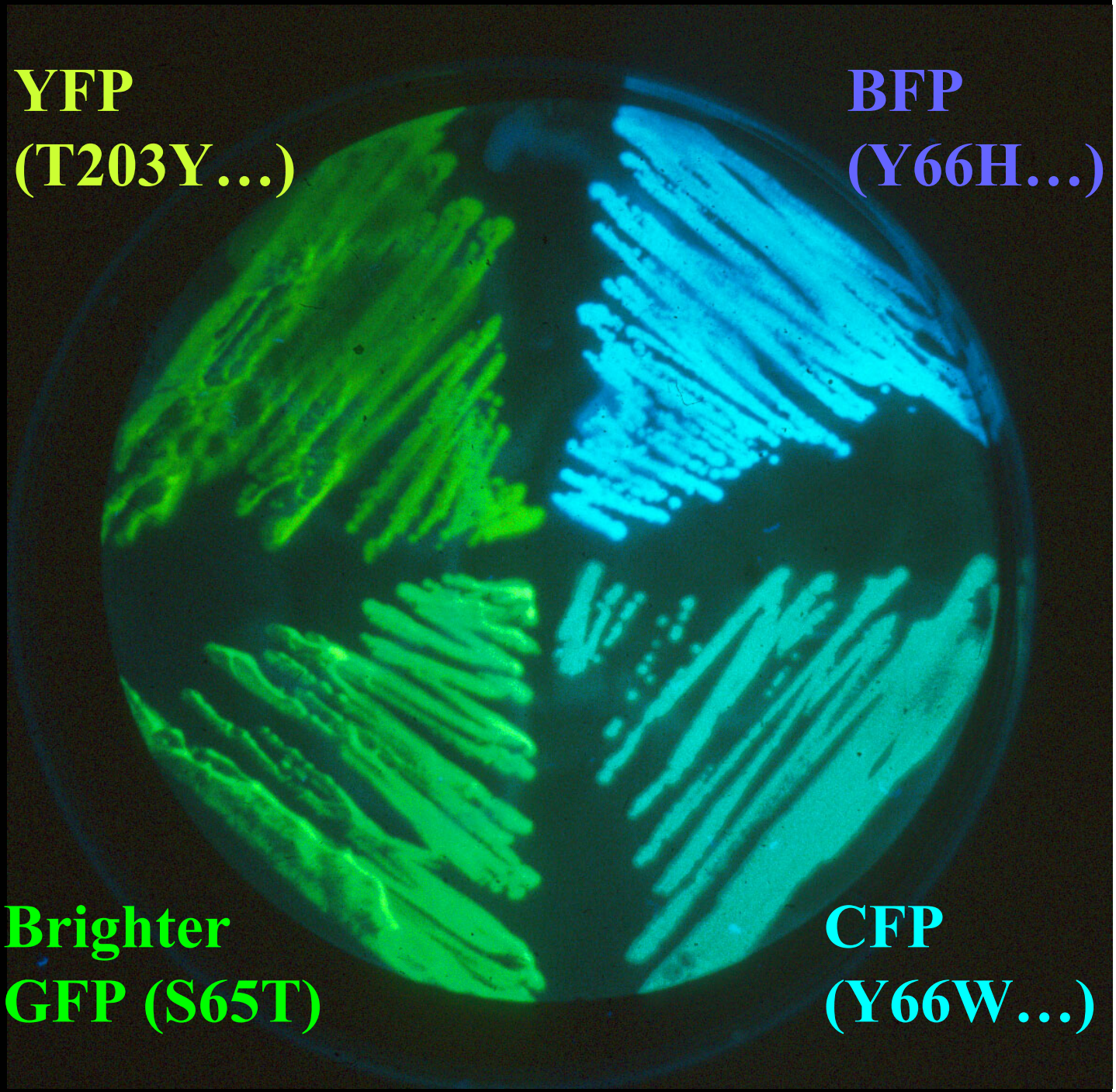




Genetically targeted labels and indicators can now monitor:

- Membrane potential
- Intracellular Ca^{2+}
- Other second messengers, e.g. cyclic nucleotides
- Redox status (thiol/disulfide)
- Exocytosis & endocytosis
- Neurotransmitters
- Protein conformational changes
- Kinase/phosphatase activities
- Protein-protein interaction
- Gene expression / protein turnover / trafficking
- Protease activation

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



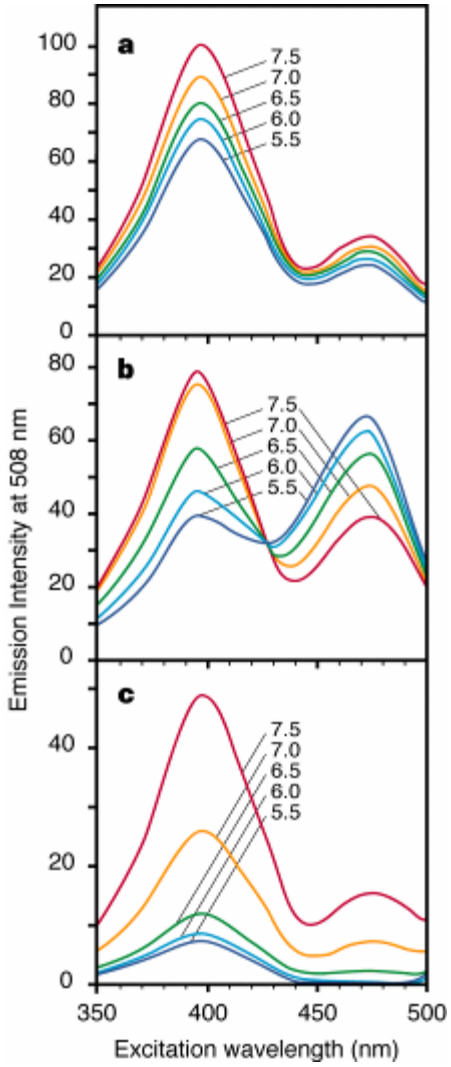
R. Heim, A. Cubitt

pH-sensitive GFPs in synaptic vesicles to monitor neurotransmission

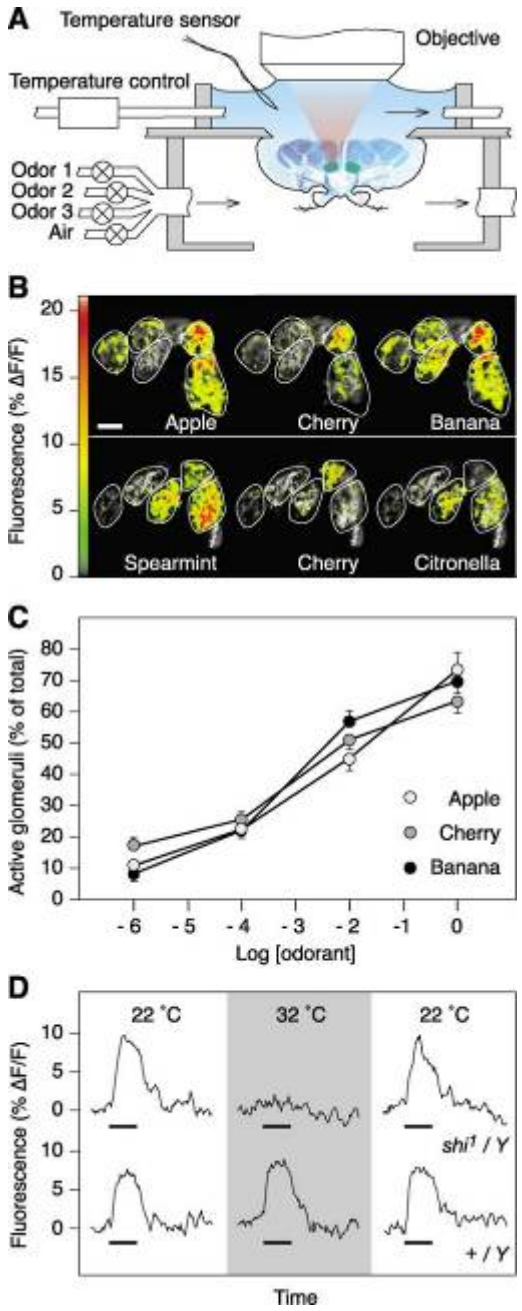
WT
GFP

Ratiometric
pHluorin

Ecliptic
pHluorin

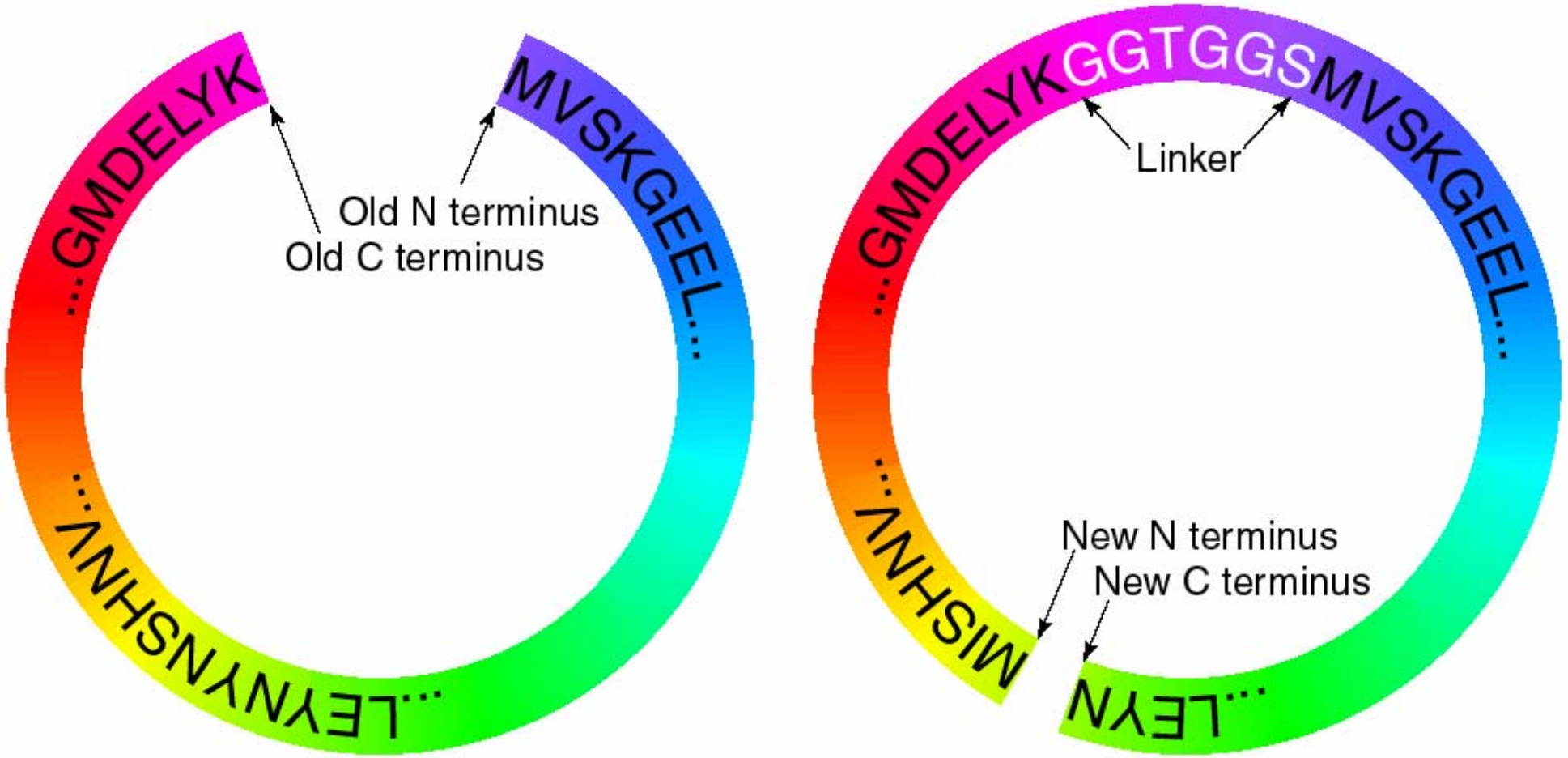


Drosophila
antennal lobe

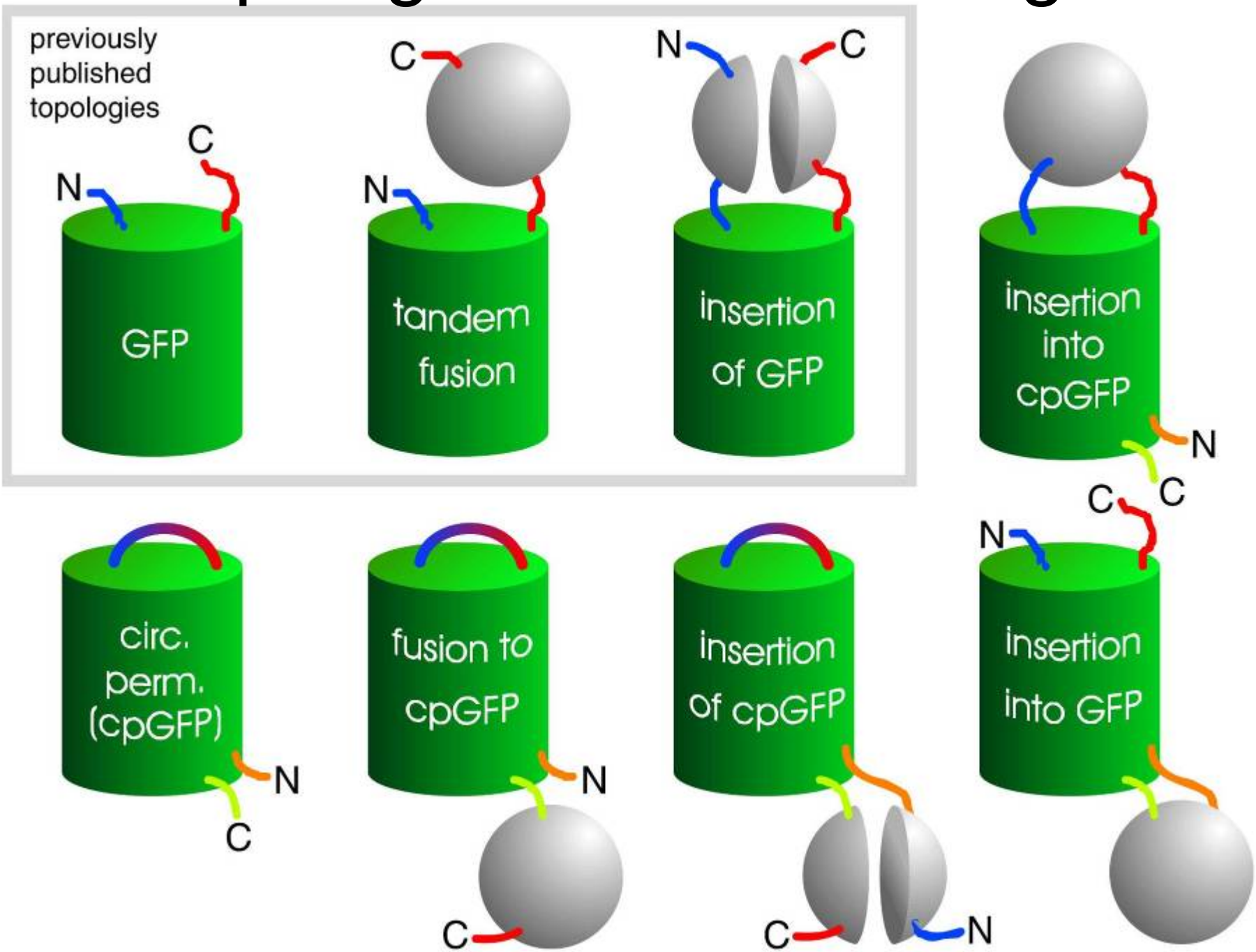


Miesenbock *et* al (1998) *Nature* **394**: 192-5
Ng *et al* (2002) *Neuron* **36**: 463-74

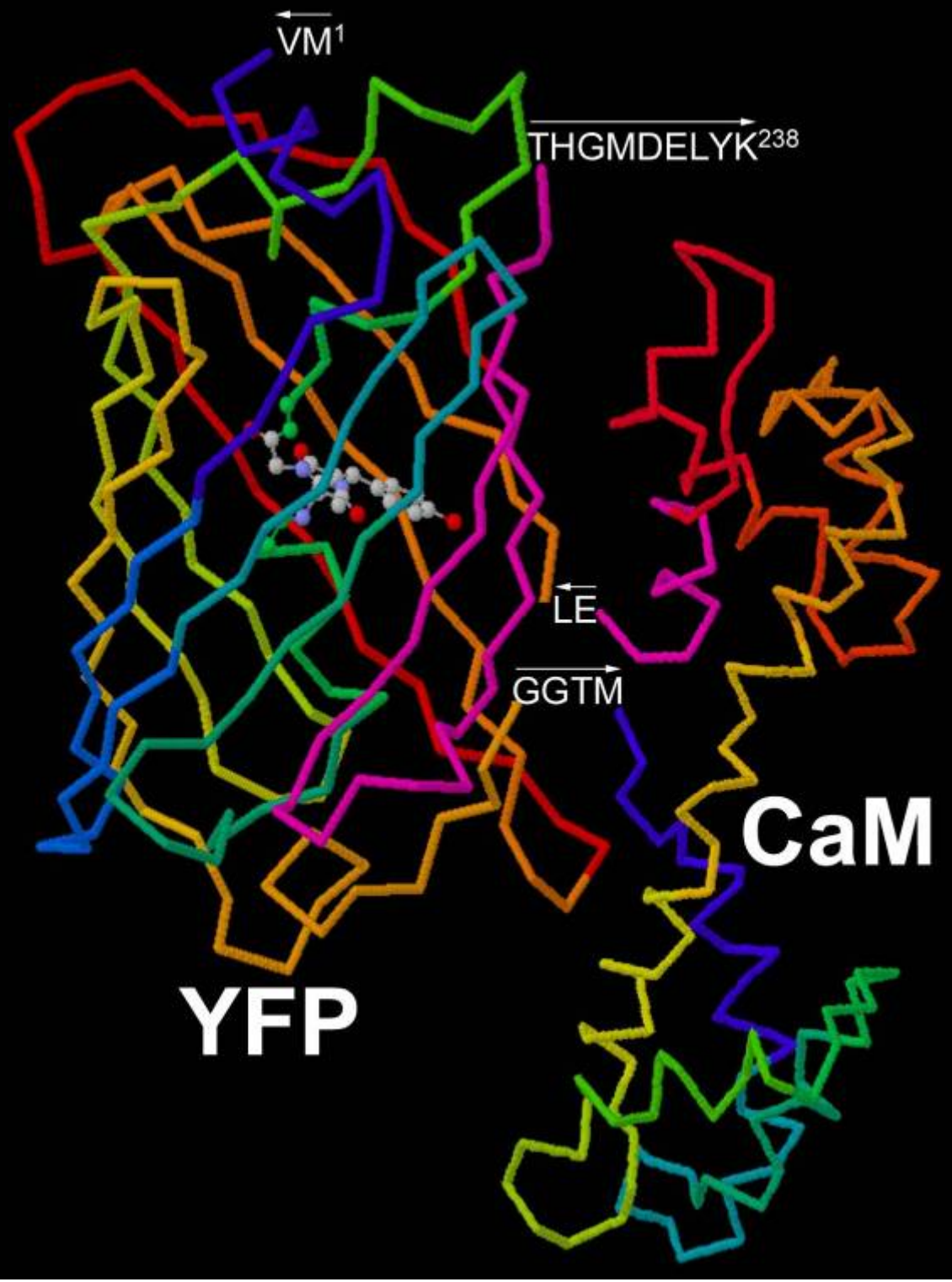
Circular permutation of GFP requires linking old terminii and creating new ones



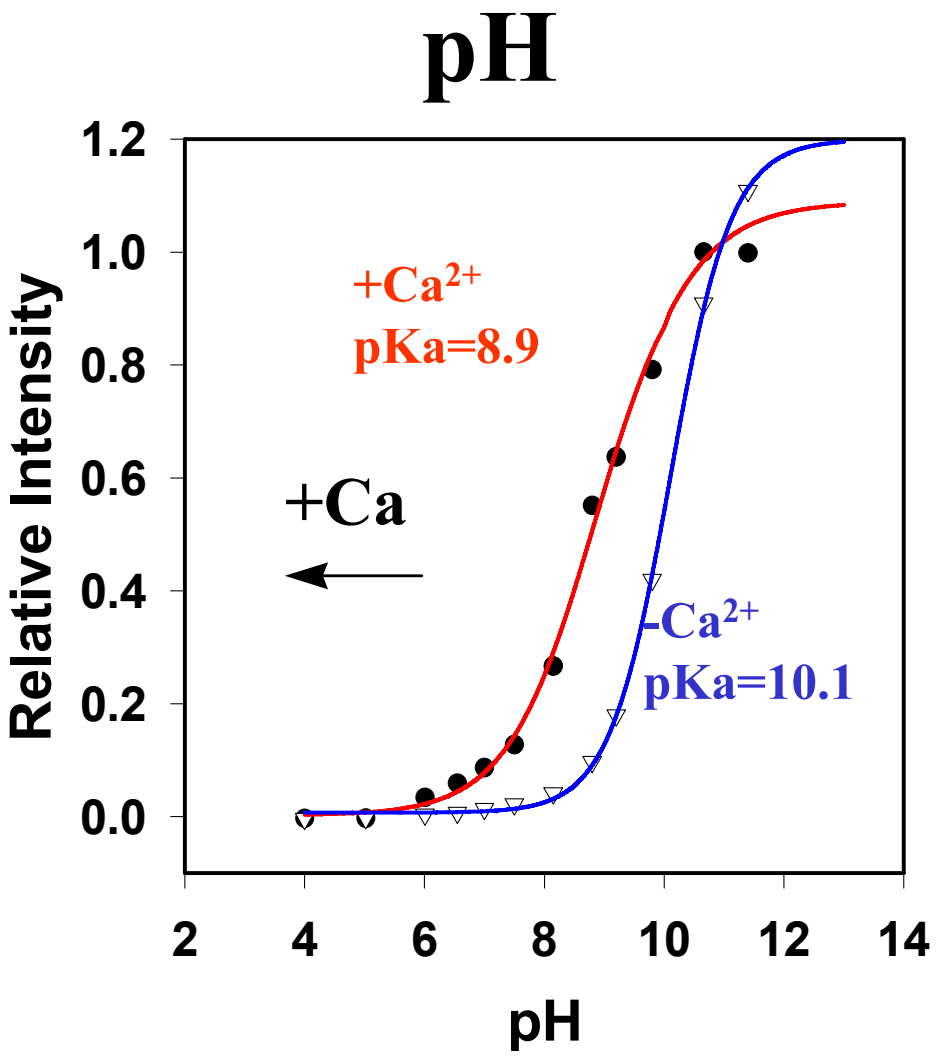
8 topologies for GFP linkages

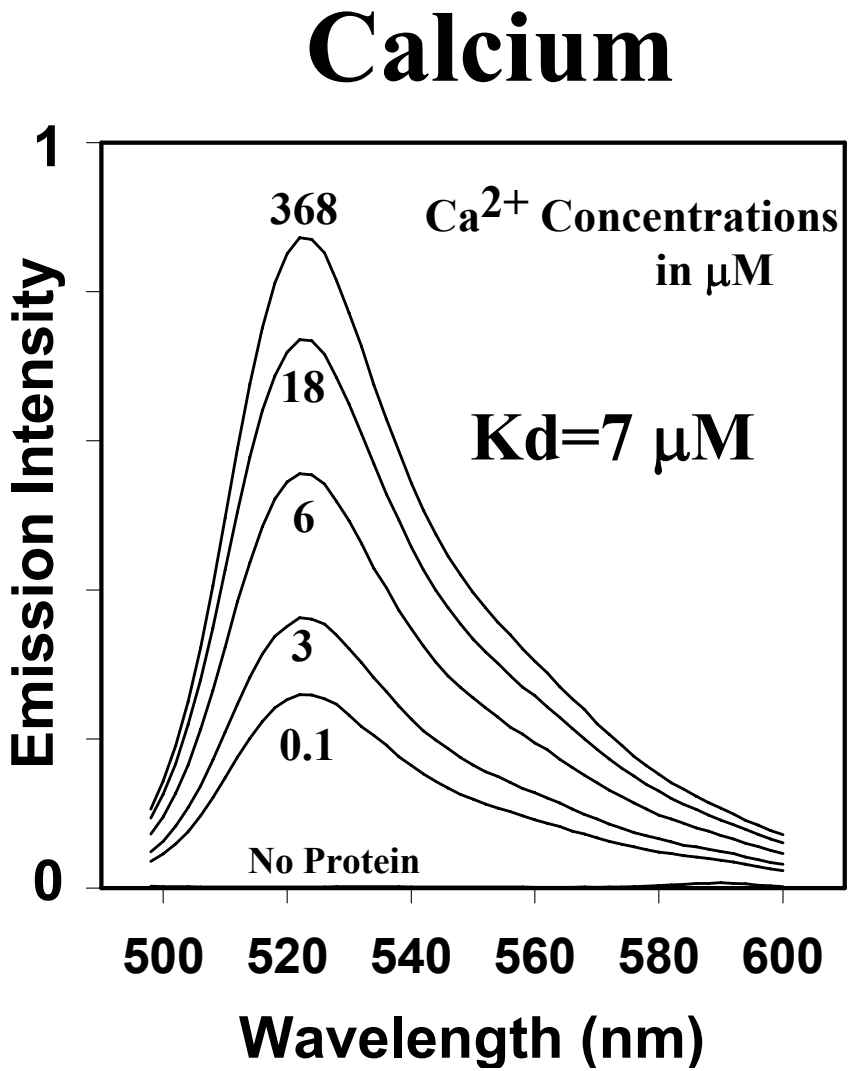


Splicing CaM into YFP creates…

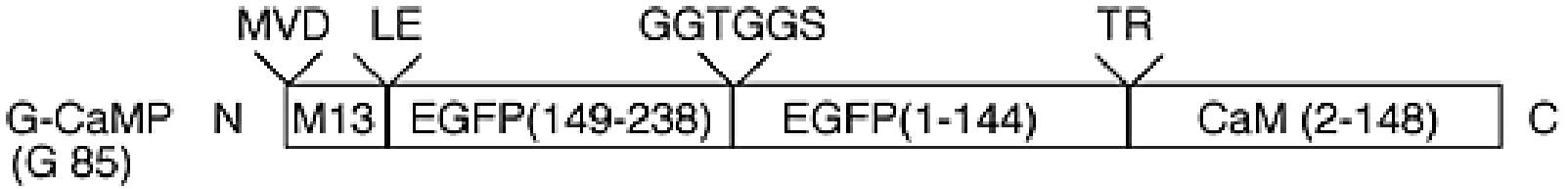


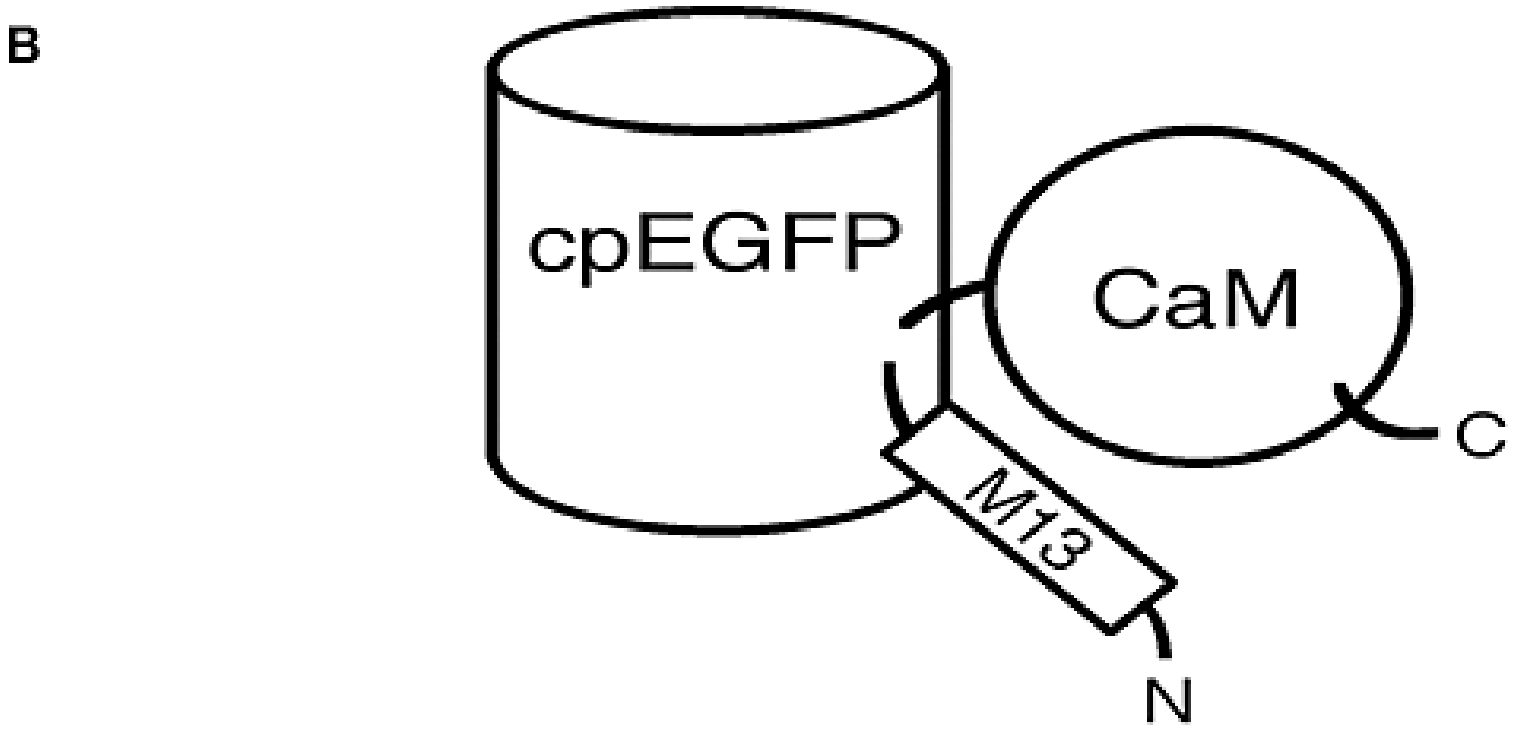
CaMgaroo pH and Ca^{2+} titrations





Generic structure of Ca^{2+}-indicators based on circularly permuted GFP (GCaMP and pericams)

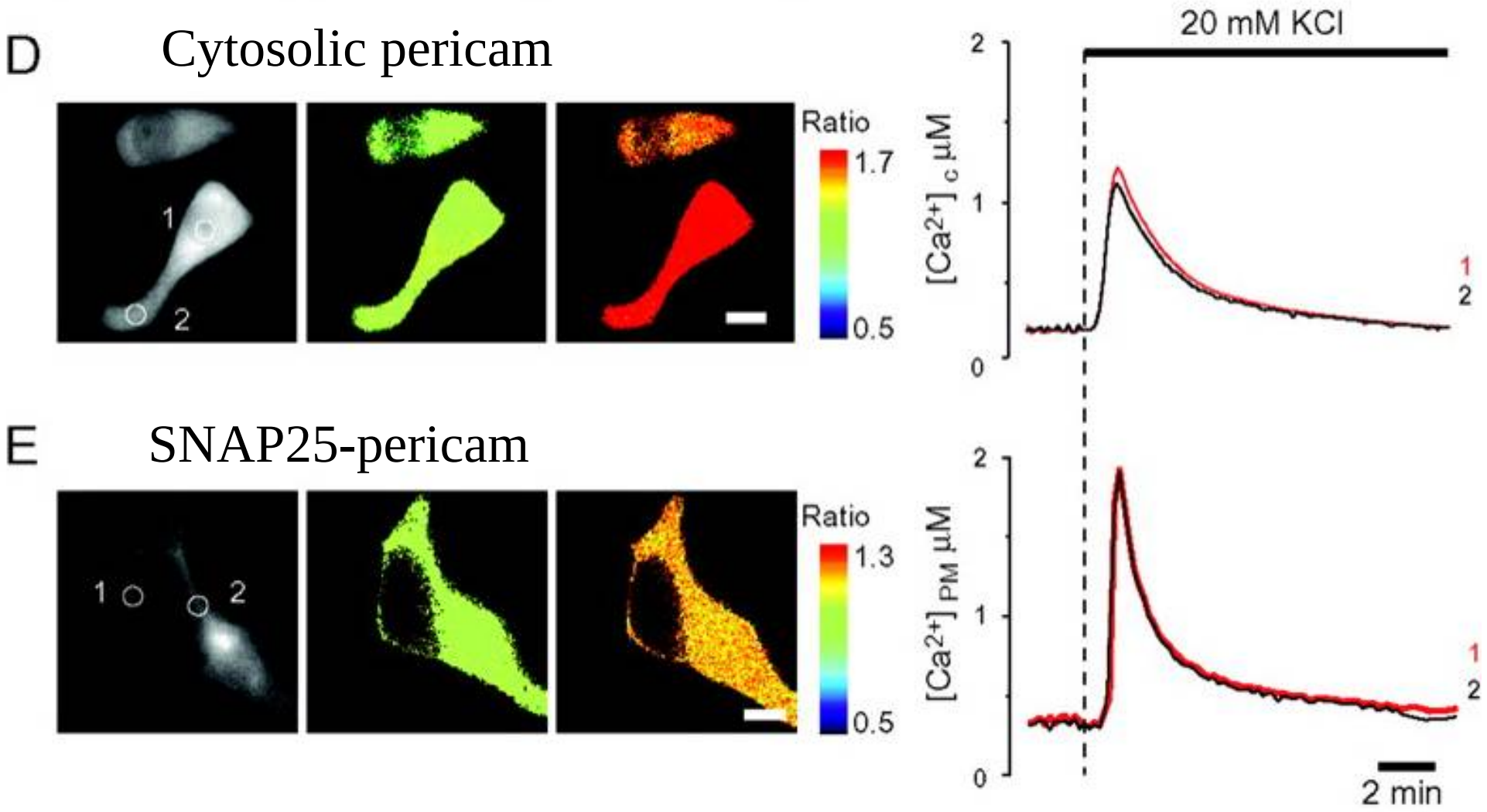




Nakai *et al* (2001) *Nature Biotechnology* **19**:137-41

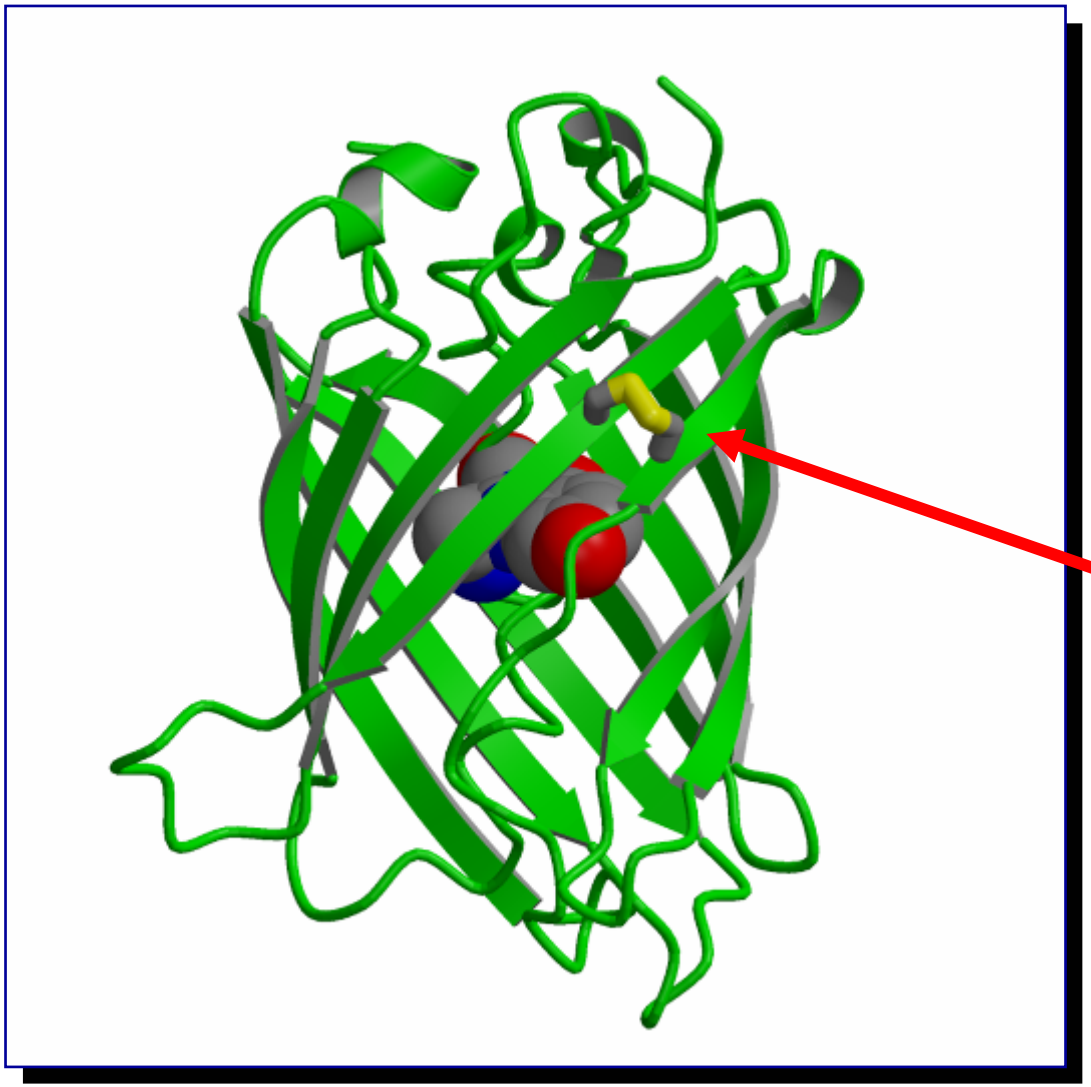
Nagai *et al* (2001) *Proc. Natl. Acad. Sci. USA* **98**: 3197-3202

Membrane-targeted pericam reports higher Ca^{2+} than cytosolic pericam in KCl-stimulated pancreatic beta cells



Pinton *et al* (2002) *J. Biol. Chem.* **277**:37702-10

Redox-sensitive indicators based on GFP (roGFPs)



Mutations in roGFP1:

Remove surface cysteine:

Cys48→Ser

Redox-sensing mutations:

Ser147→Cys

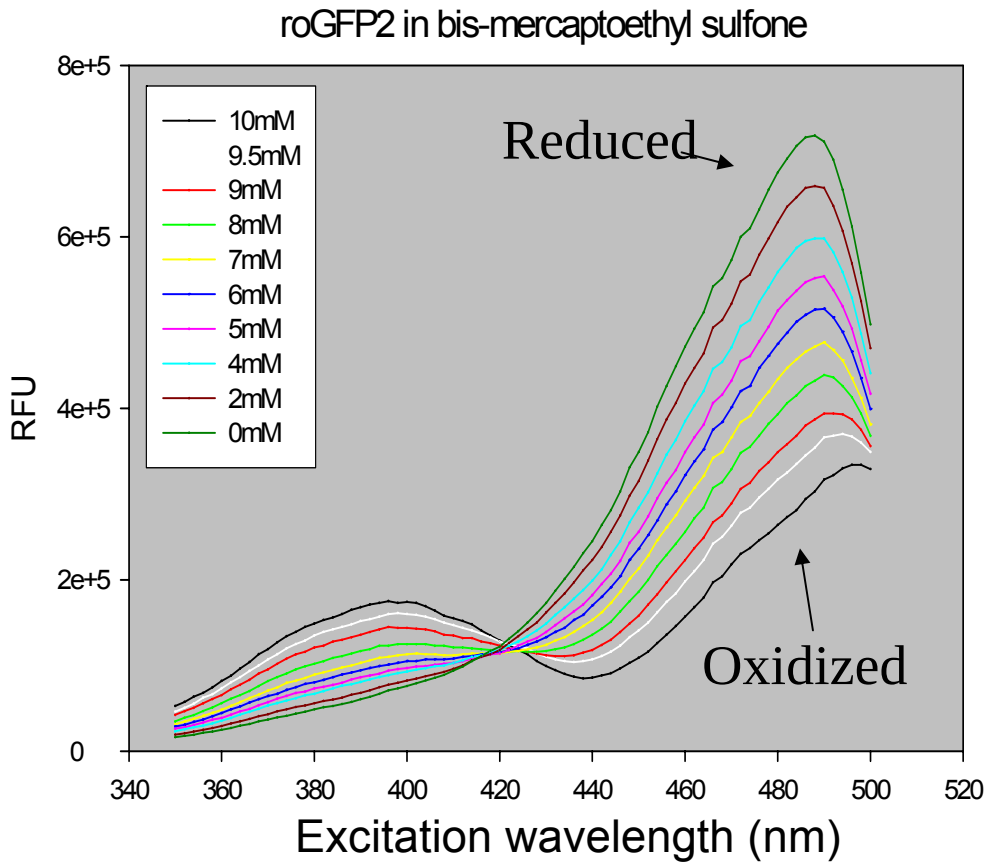
Gln204→Cys

roGFP2 has the above plus

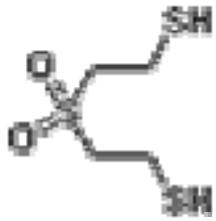
Ser65→Thr

George Hanson, S. James Remington (U. Oregon)

Determination of midpoint potential by titration in BMES buffer



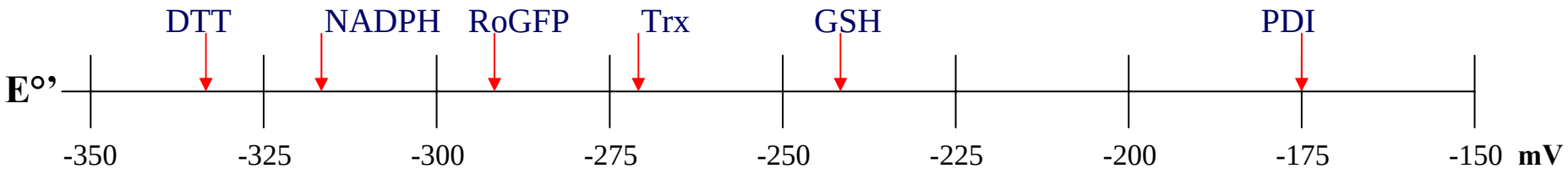
BMES



Nernst equation:

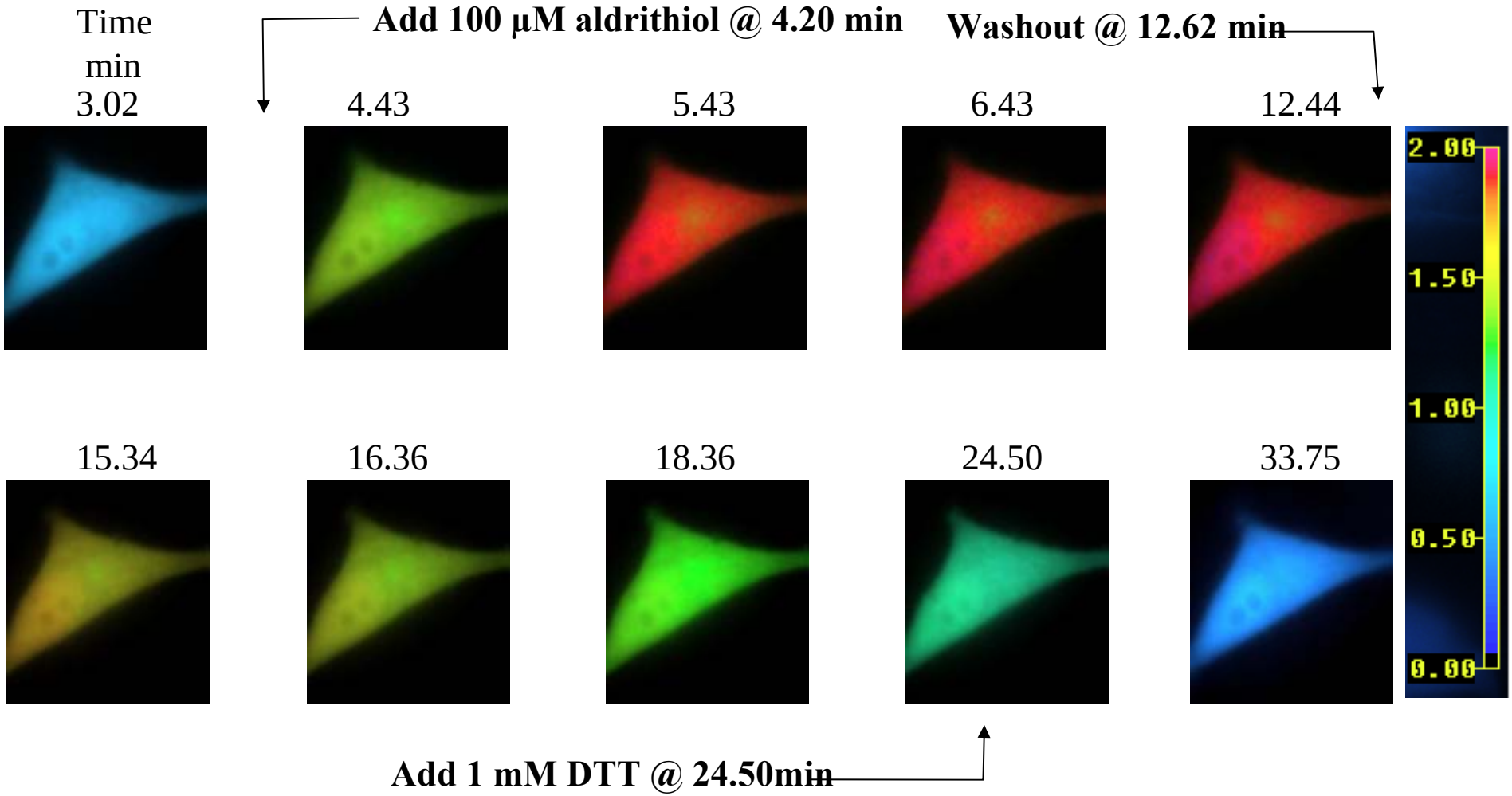
$$E' = E^{o'} - \frac{RT \ln K}{nF}$$

Redox Buffer	roGFP1 E' (mV)	roGFP2 E'(mV)
Lipoic acid	-293 ± 4	-284 ± 7
BMES	-294 ± 3	-288 ± 1



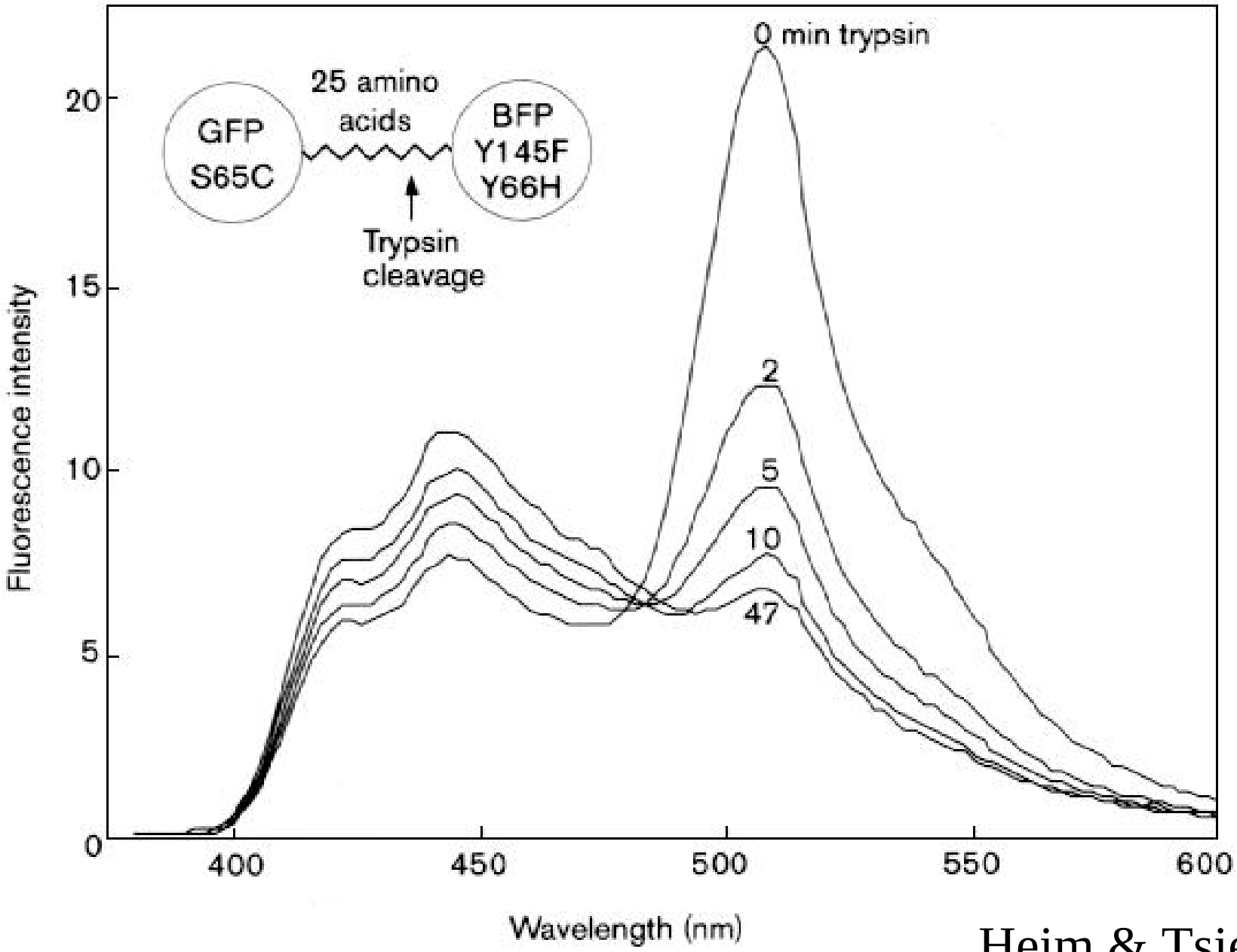
Colette Dooley

Aldrithiol (=2,2'-pyridyl disulfide) causes reversible oxidation of HeLa cells expressing roGFP1



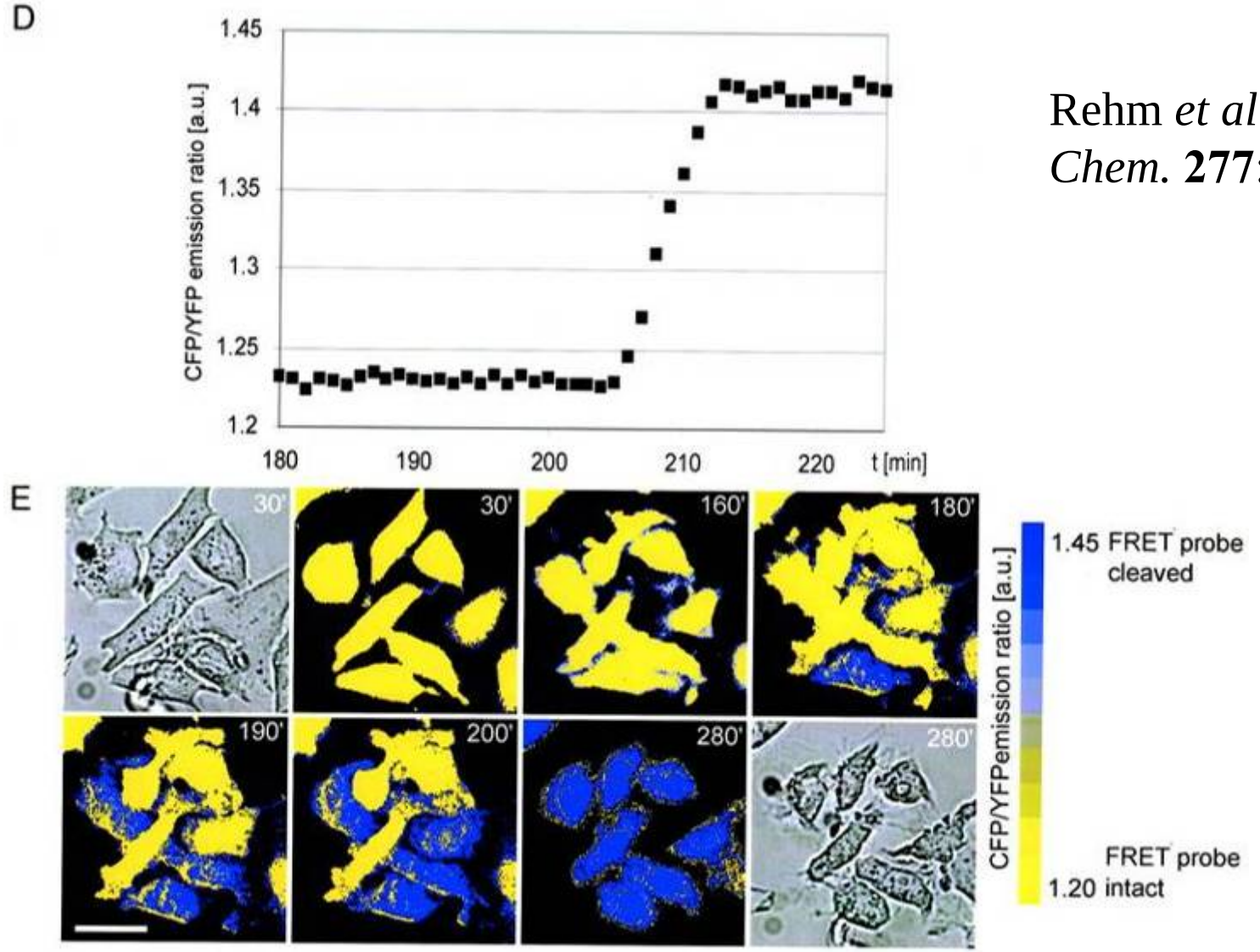
Colette Dooley

1st GFP FRET assay for protease activity



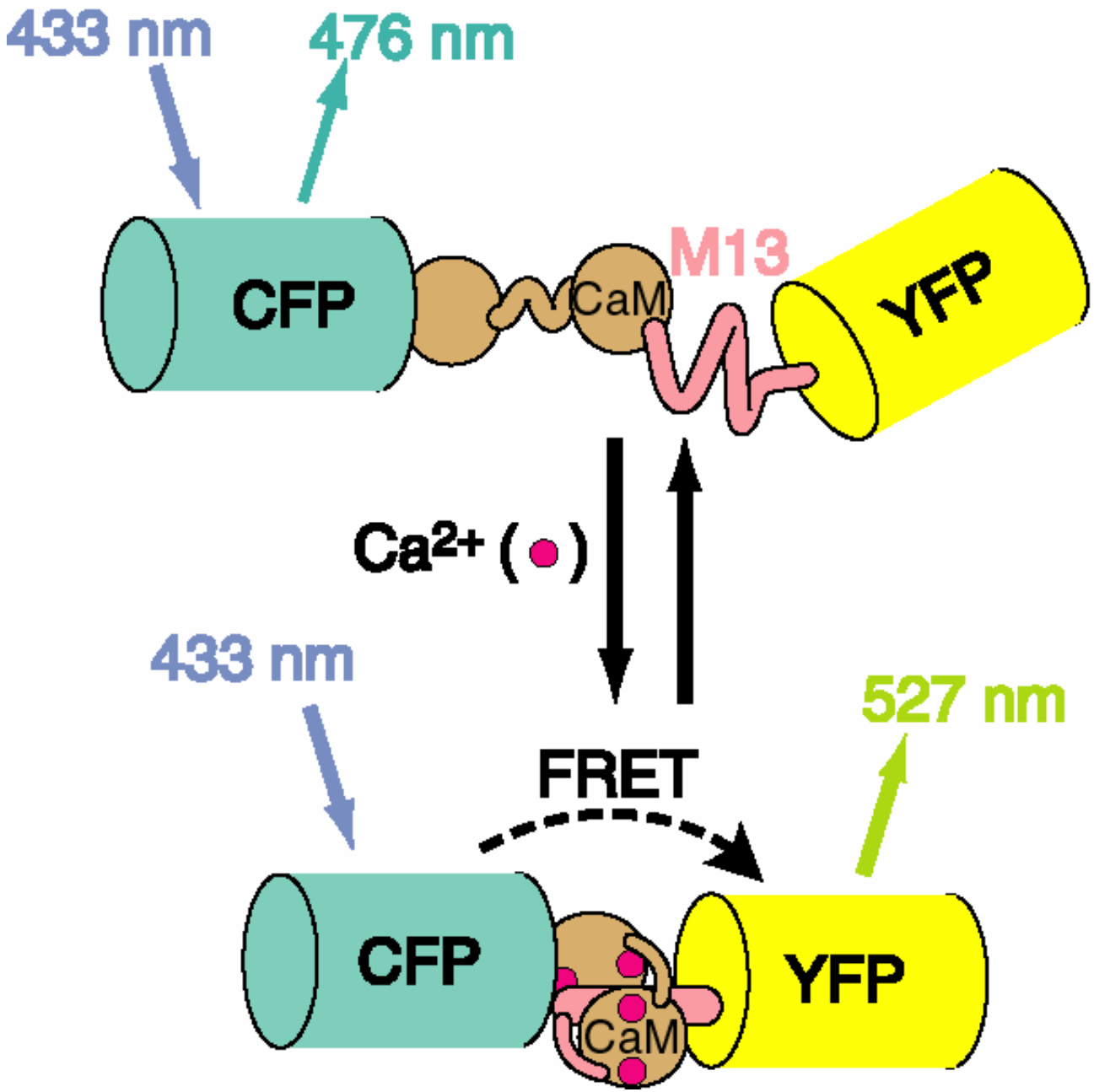
Heim & Tsien (1996)

Cleavage of CFP-DEVD-YFP during apoptosis is a sudden event in single cells

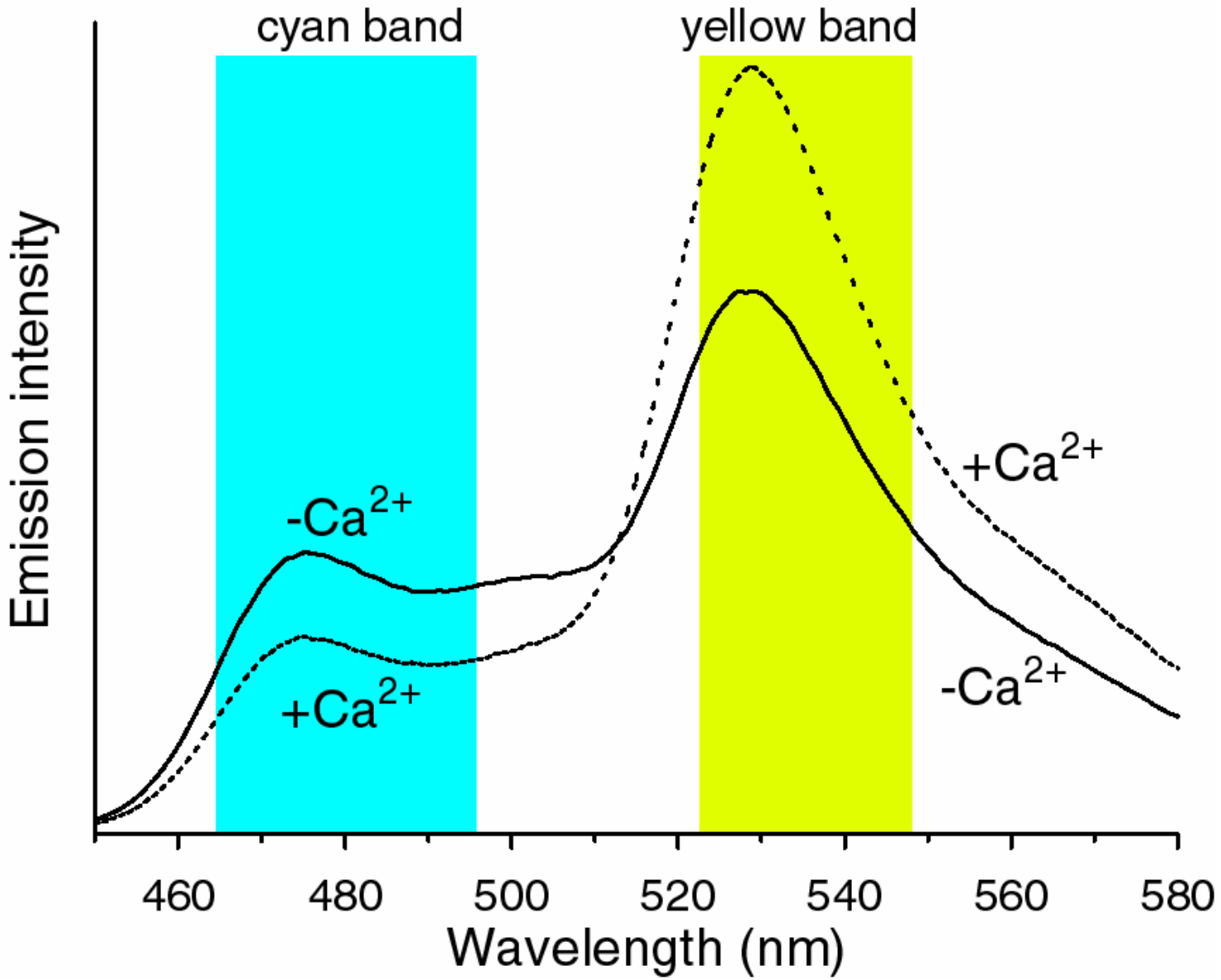


Rehm *et al* (2002) *J. Biol. Chem.* **277**: 24506-24514

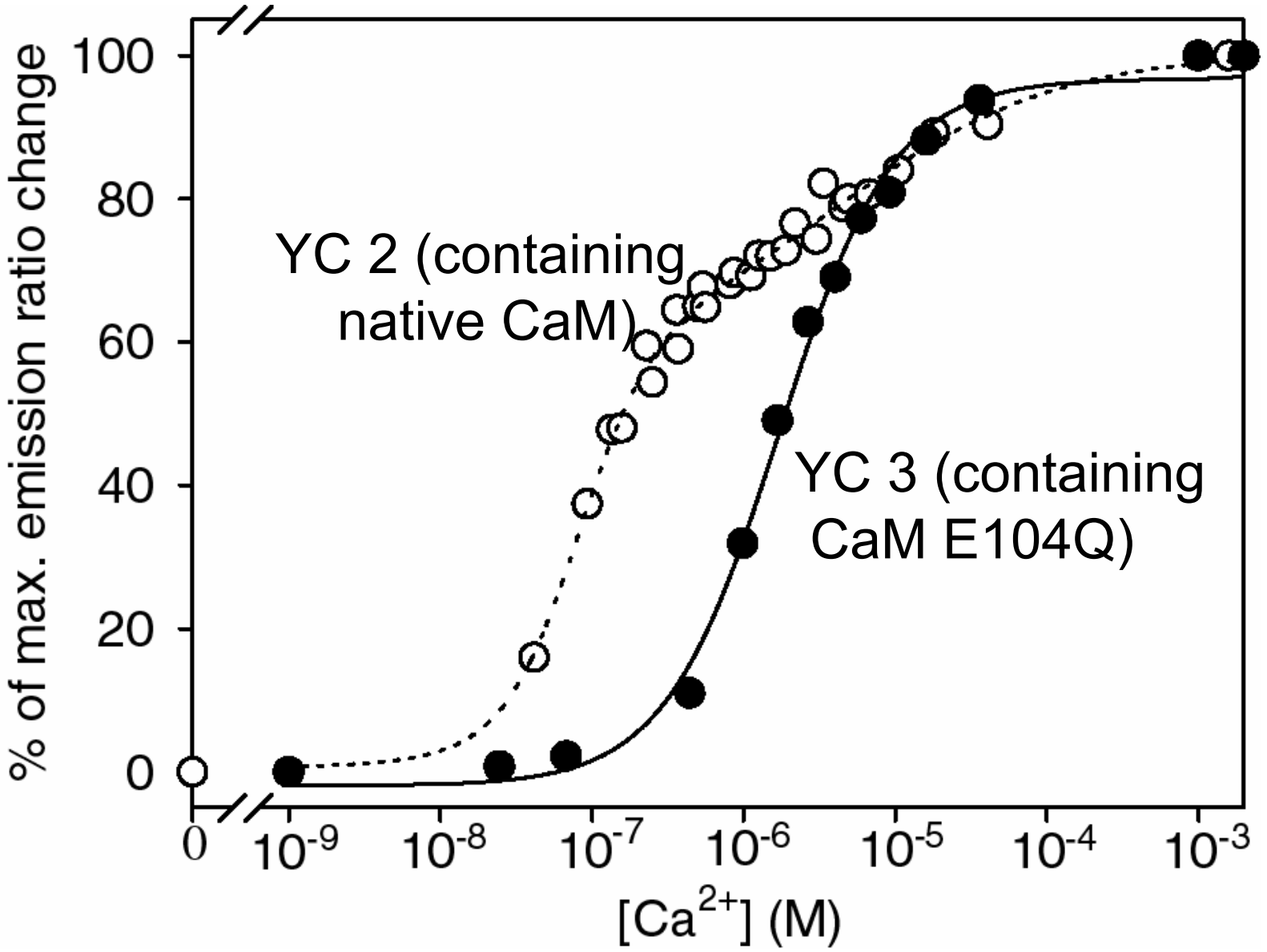
Cameleons: Ca^{2+} indicators based on CaM + GFP mutants



Emission spectra of yellow cameleons ± Ca^{2+}

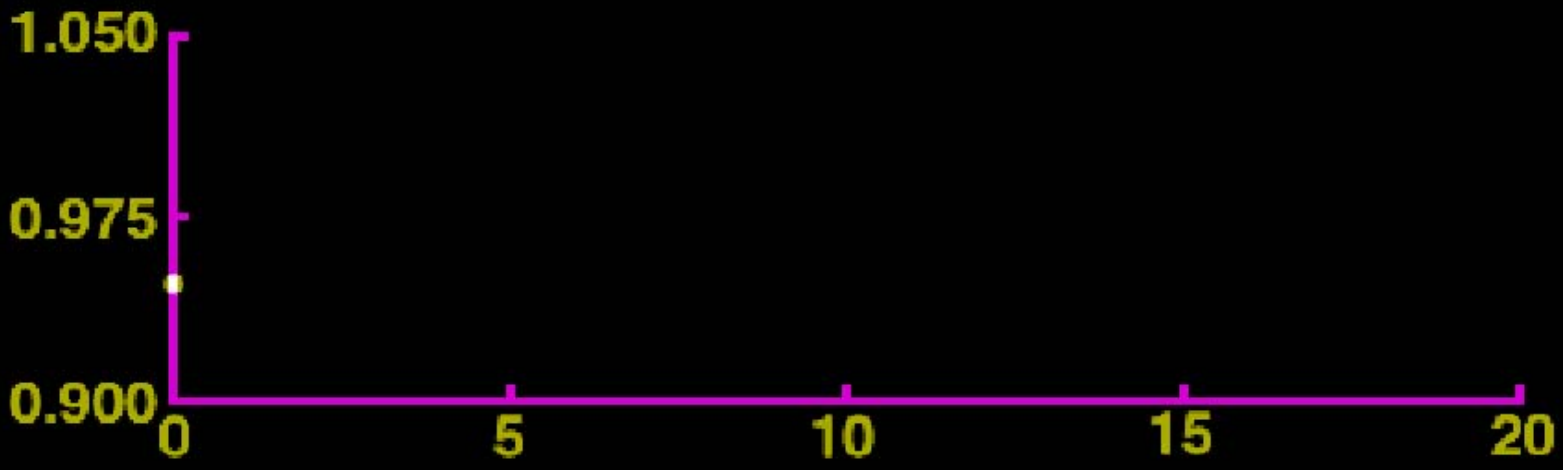


Ca^{2+} titration curves for YC 2 and 3



Yellow cameleon 3 in cardiac myocytes of transgenic mice

Varda Lev-Ram, Randa Hilal-Dandan,
Lawrence Brunton



Advantages of GFP-based fluorescent indicators

1) Applicable to nearly all organisms; no need for ester permeation and hydrolysis

2) Can be targeted to specific tissues, cells, organelles, or proteins

3) Unlikely to diffuse well enough to blur spatial gradients

4) Modular construction is readily modified/improved by mutagenesis

5) Good optical properties: visible excitation, emission ratioing, high photostability

6) cDNAs or improved sequences are very cheap to replicate and distribute

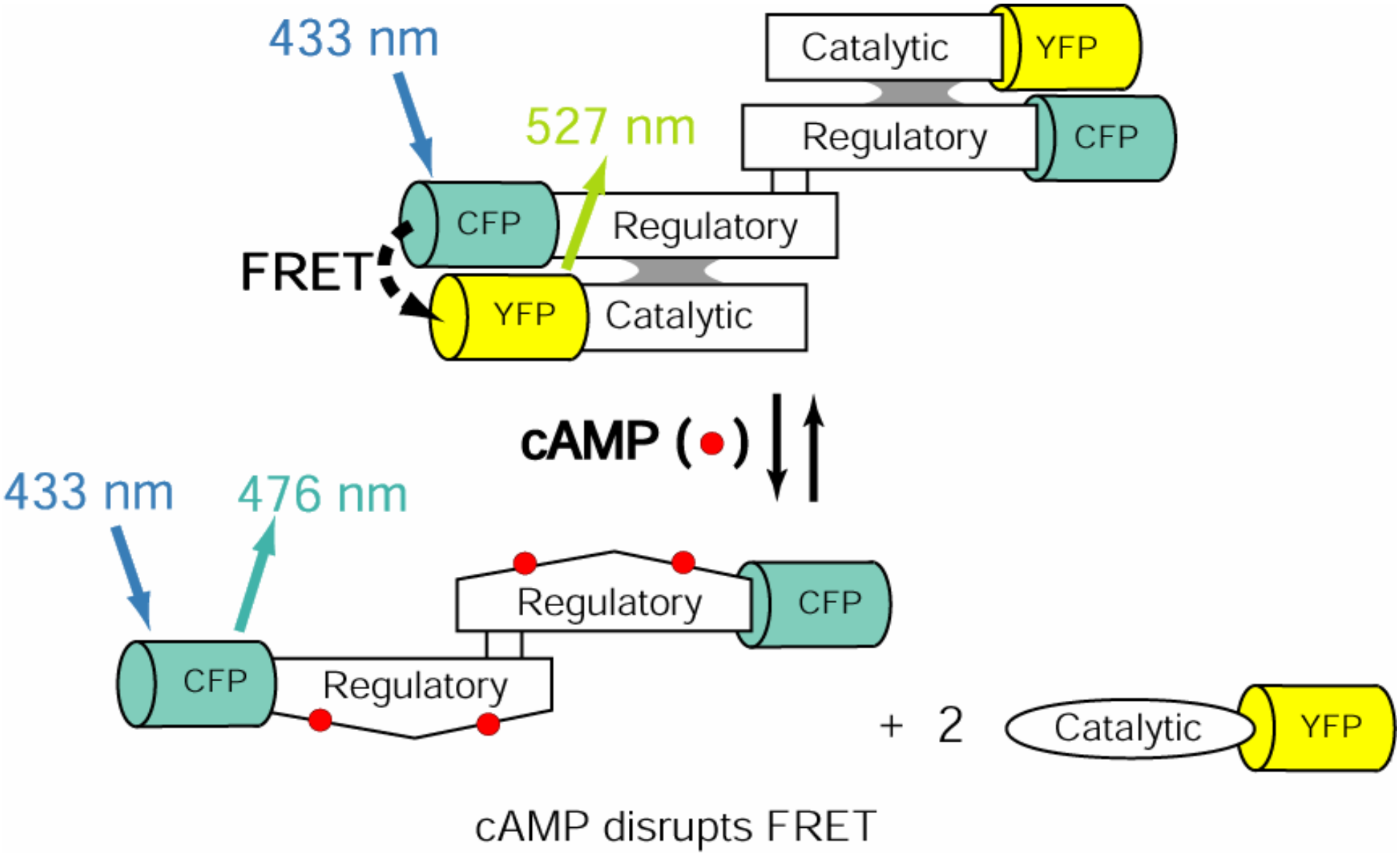
7) Should be generalizable to measure many bioactive species other than Ca^{2+}, as long as a conformationally sensitive receptor is available

Disadvantages of GFP-based sensors (for Ca^{2+})

1) Gene transfection required
2) The maximum change in emission ratio is currently less than for small-molecule dyes
3) The binding kinetics are somewhat slower
4) The CaM or M13 might have some additional biological activity

A genetically encodable cAMP indicator

using GFP mutants fused to subunits of cAMP-dependent protein kinase



cAMP disrupts FRET

Microcompartmentation of cAMP in cardiac myocytes stimulated with norepinephrine

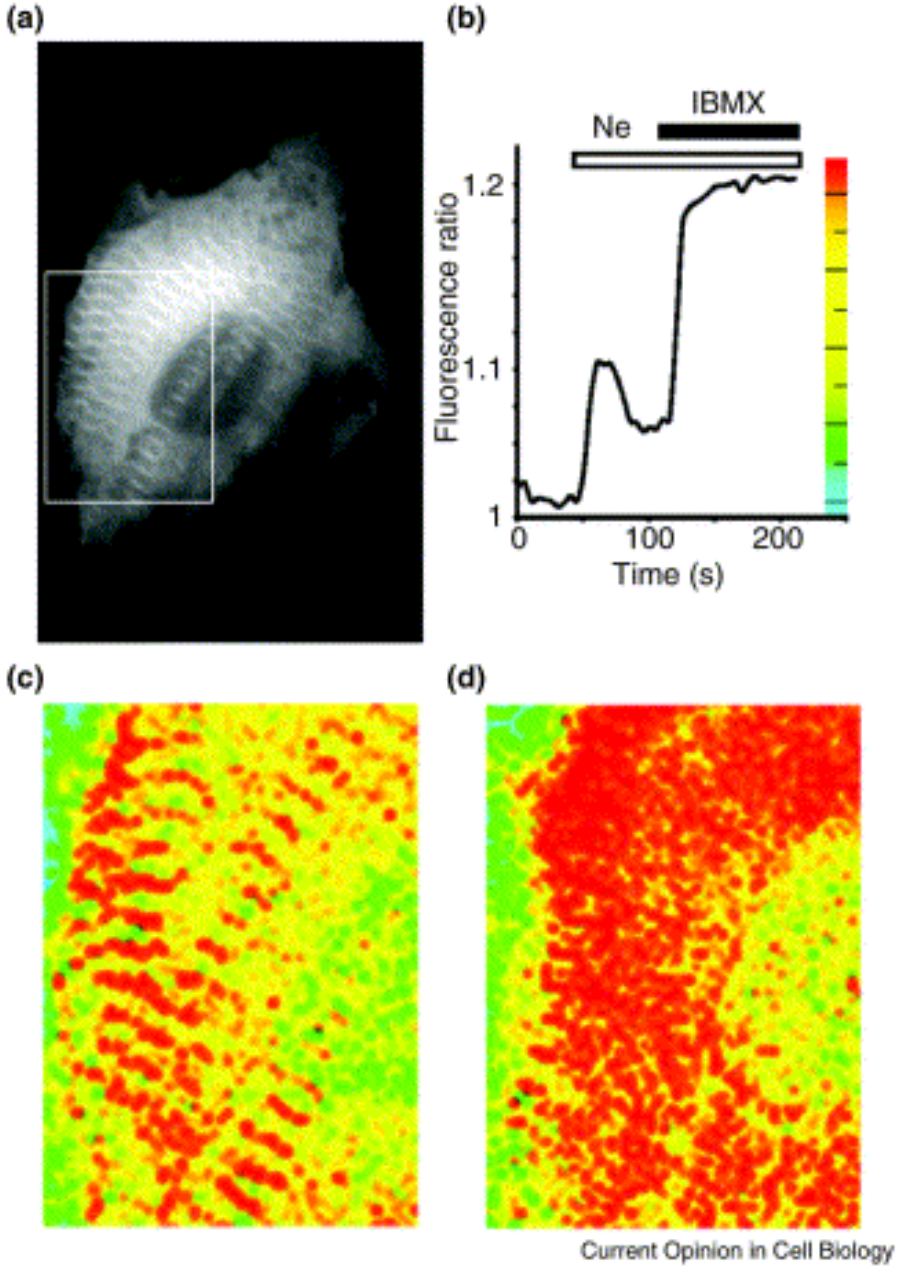
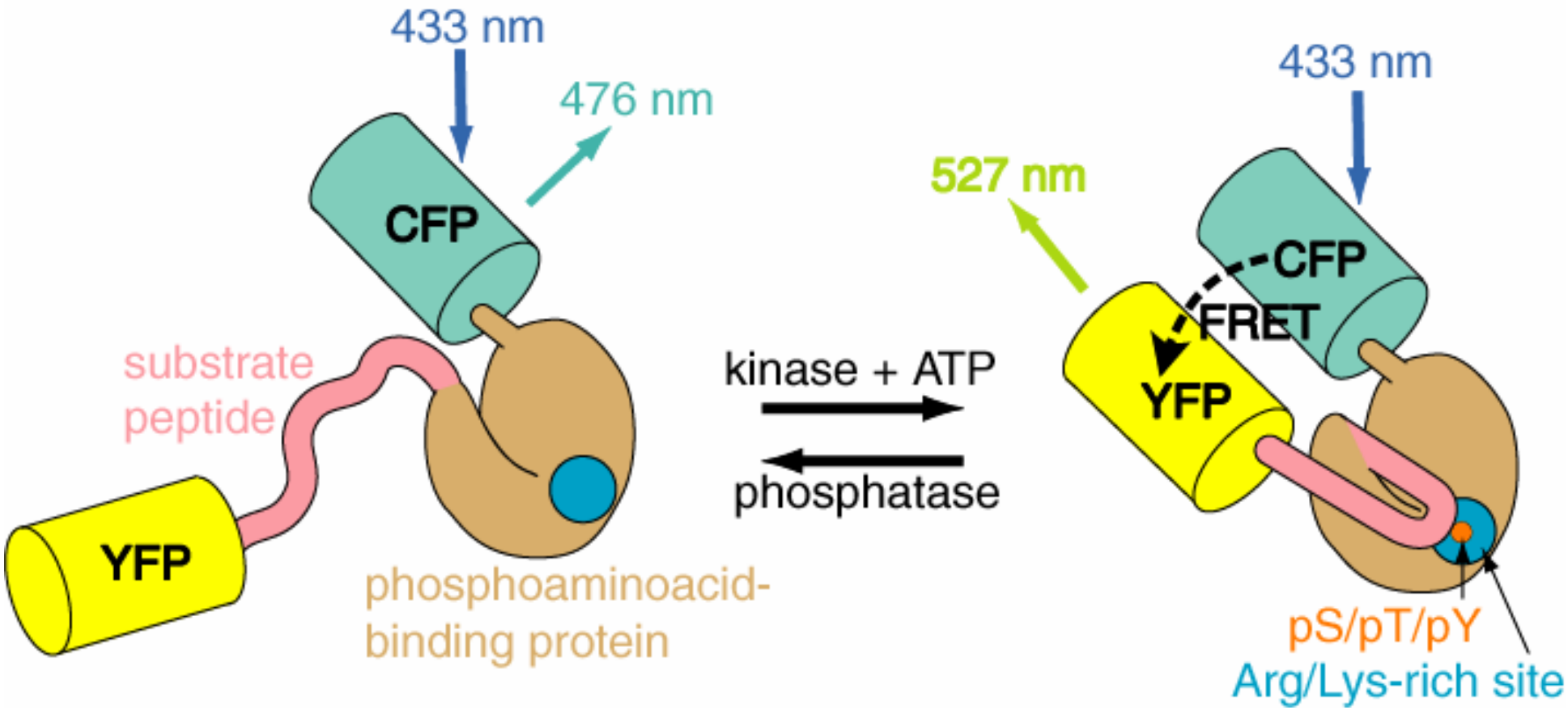


Fig. 3. Microdomains of cAMP monitored in live cardiac myocytes using a cAMP sensor based on FRET and PKA. (a) A cardiac myocyte transfected with C–YFP (C, the catalytic subunit of PKA) and R–CFP (R, the regulatory subunit of PKA) shows a localisation of the probe in thin parallel striations in correspondence of the sarcomeric Z line. (b) The effect of the -adrenergic agonist norepinephrine (Ne, white bar) and of the broad range PDE inhibitor IBMX (black bar) on the concentration of cAMP is monitored by measuring FRET changes. The cell is excited at 440 nm (the CFP excitation wavelength). Fluorescence emission of both CFP (at 480 nm) and YFP (at 545 nm) is collected and the 480 nm: 545 nm fluorescence ratio value is plotted against time. The values shown in (b) are the mean ratio values calculated over the entire area of the cell. (c) and (d) The detail delimited by the box in panel (a) is shown in pseudocolour. Different colours correspond to different fluorescence ratio values, according to the colour bar indicated in panel (b). (c) The cell was imaged at the peak of the response to norepinephrine (time = 60 s) and red, corresponding to the sarcomeric Z lines, indicates localised regions of high cAMP levels. Addition of IBMX abolishes this subcellular heterogeneity, and the level of cAMP rises uniformly in the cytosol, as indicated in (d), which illustrates the same cell imaged at the plateau of its response to IBMX (time = 200 s). Curr Opin Cell Biol 2002 Apr;14(2):160-6

Zaccolo & Pozzan (2002)
Science **295**: 1711-5
Zaccolo *et al* (2002) *Curr. Opin. Cell Biol.* **14**: 160-6

A generic design for indicators of kinase/phosphatase activity



Reporters for:

PKA†, Akt/PKB — *Jin Zhang* — †*PNAS* 98: 14997 (2001)

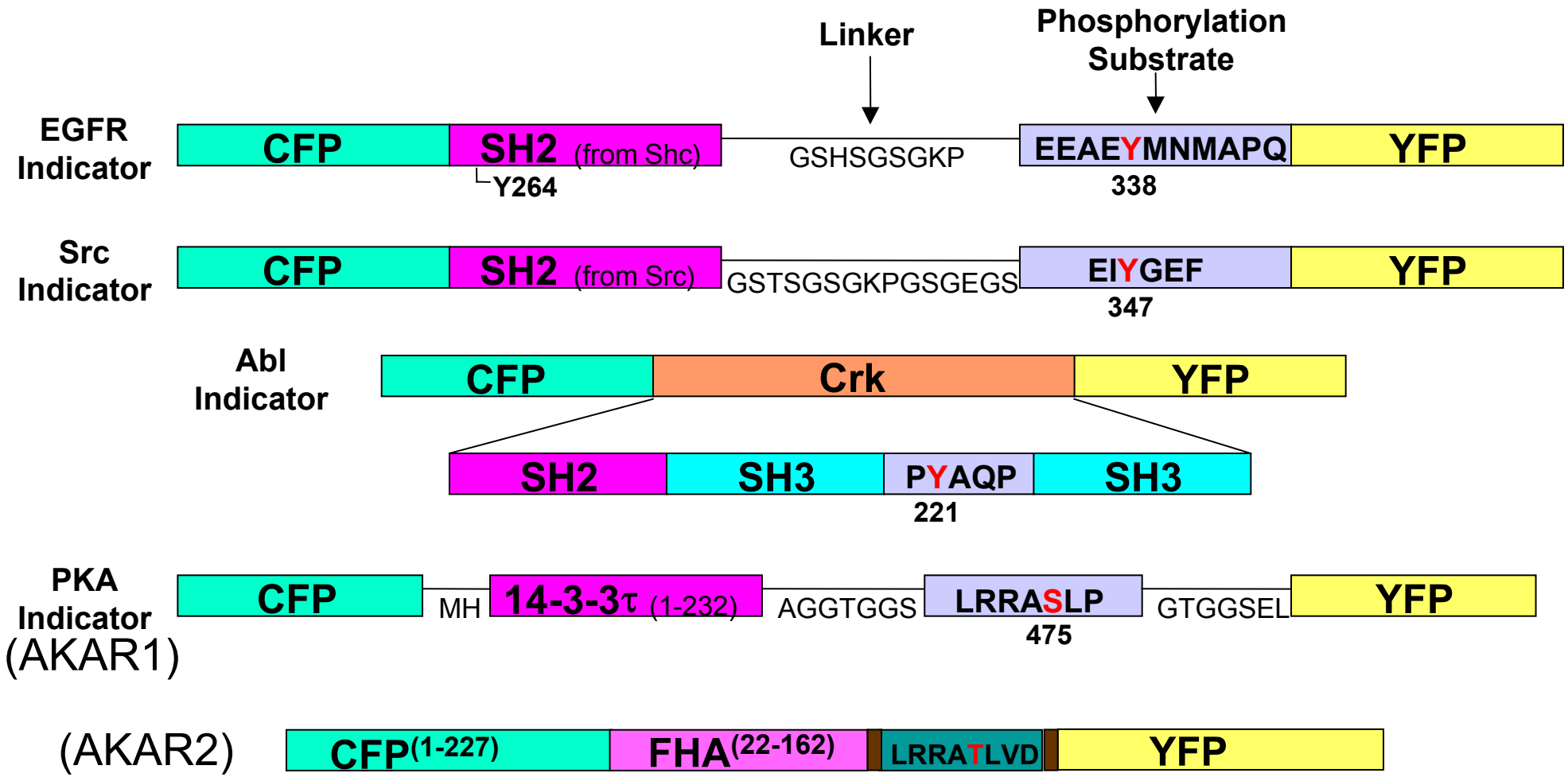
PKC — *Jon Violin, Alexandra Newton (UCSD)*

EGF receptor*, Src*, Abl*: *Alice Ting* — **PNAS* 98: 15003 (2001)

Abl: see also Kurokawa et al, *JBC* 276: 31305 (2001)

InsR: see also Sato et al, *Nature Biotech.* 20: 287 (2002)

Modular construction of phosphorylation indicators



Alice Ting, Jin Zhang

Phosphorylation-dependent emission ratio of EGFR reporter, overlaid on DIC image

EGF added;
FRET increases

EGF washed out;
FRET decreases

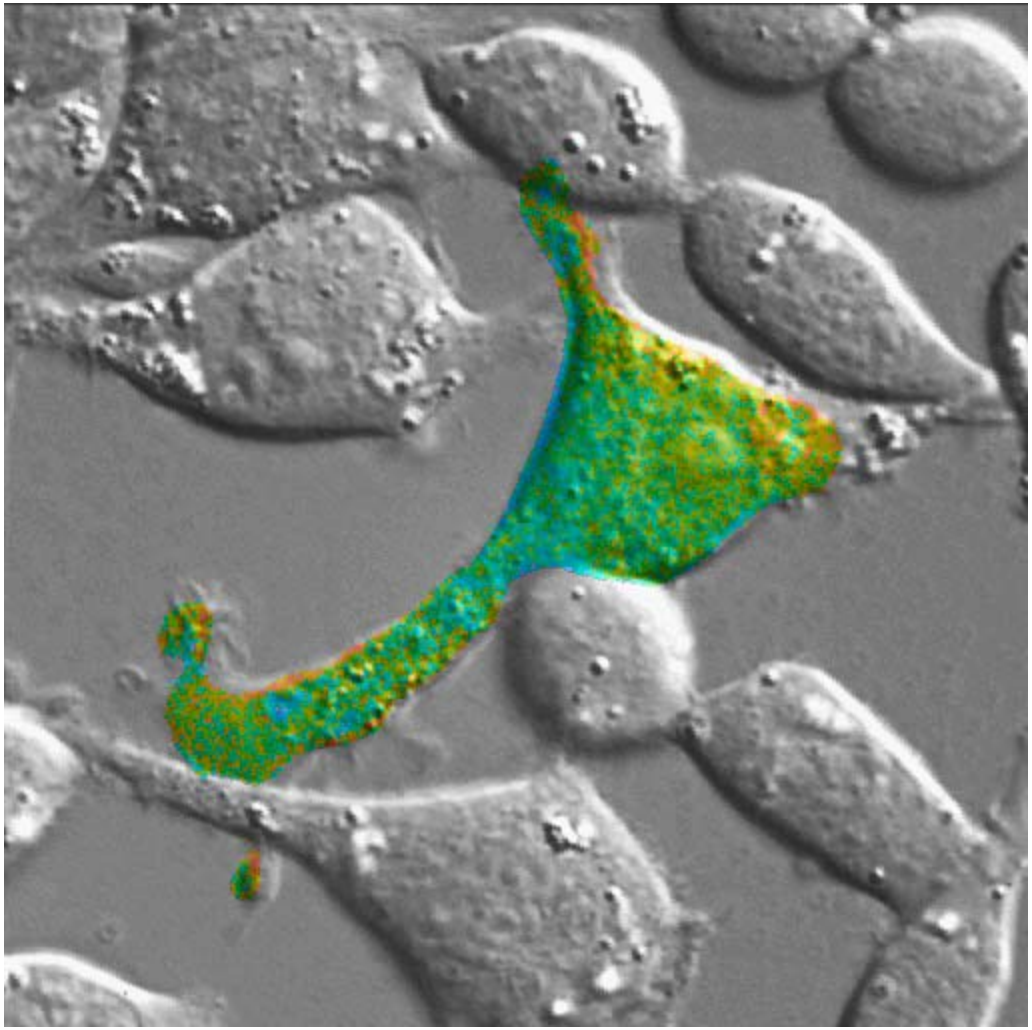
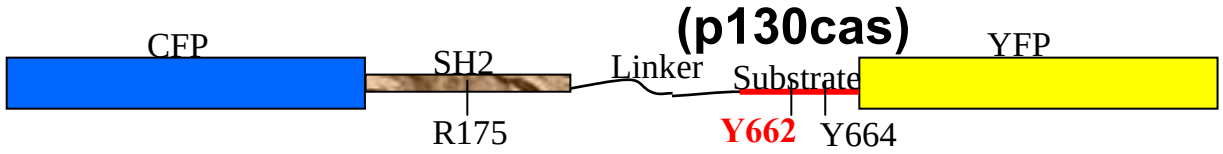
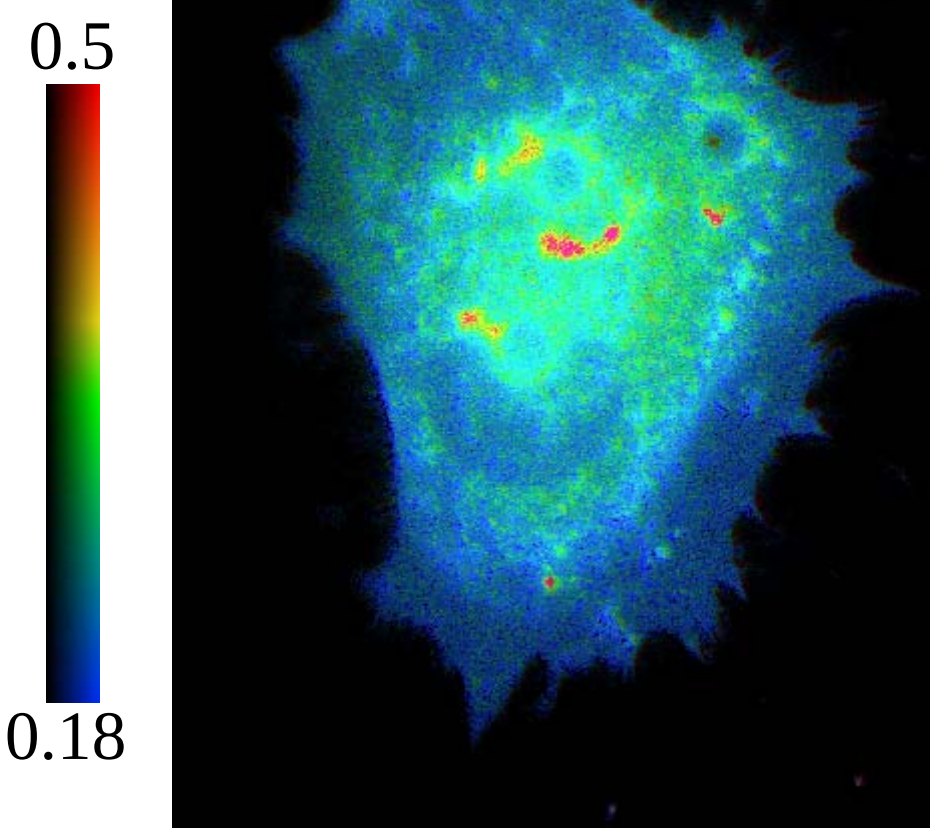


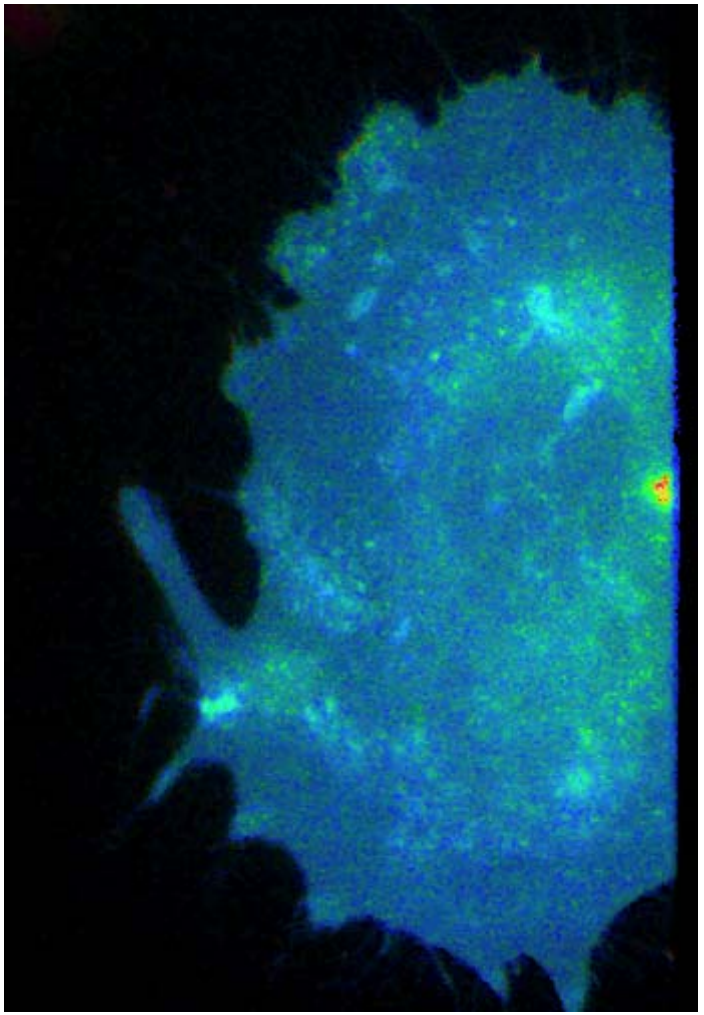
Image taken every 5 sec;
Collected over 20 min

Alice Ting

Pulling Fn-coated beads with laser tweezers activates a wave of Src activation, seen with lyn-tagged Src reporters

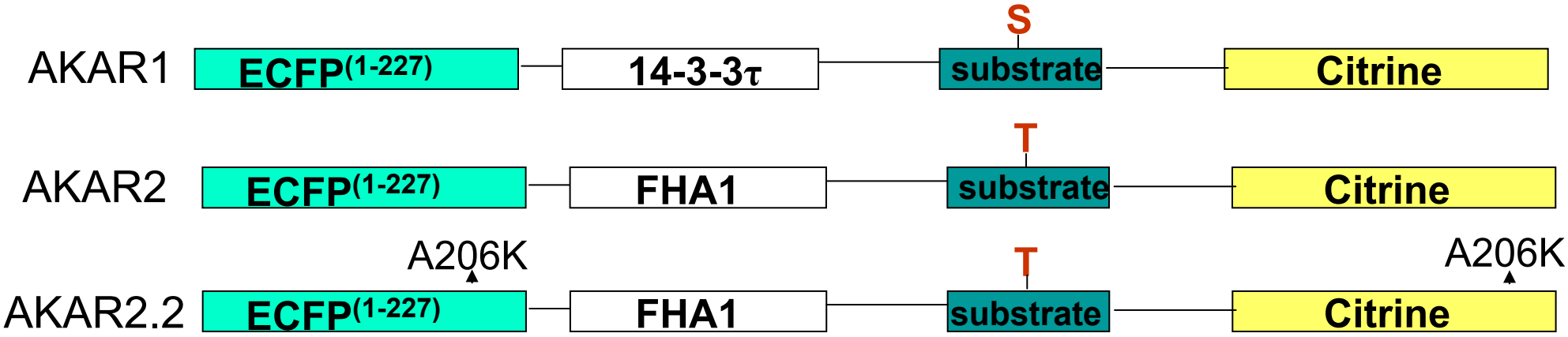


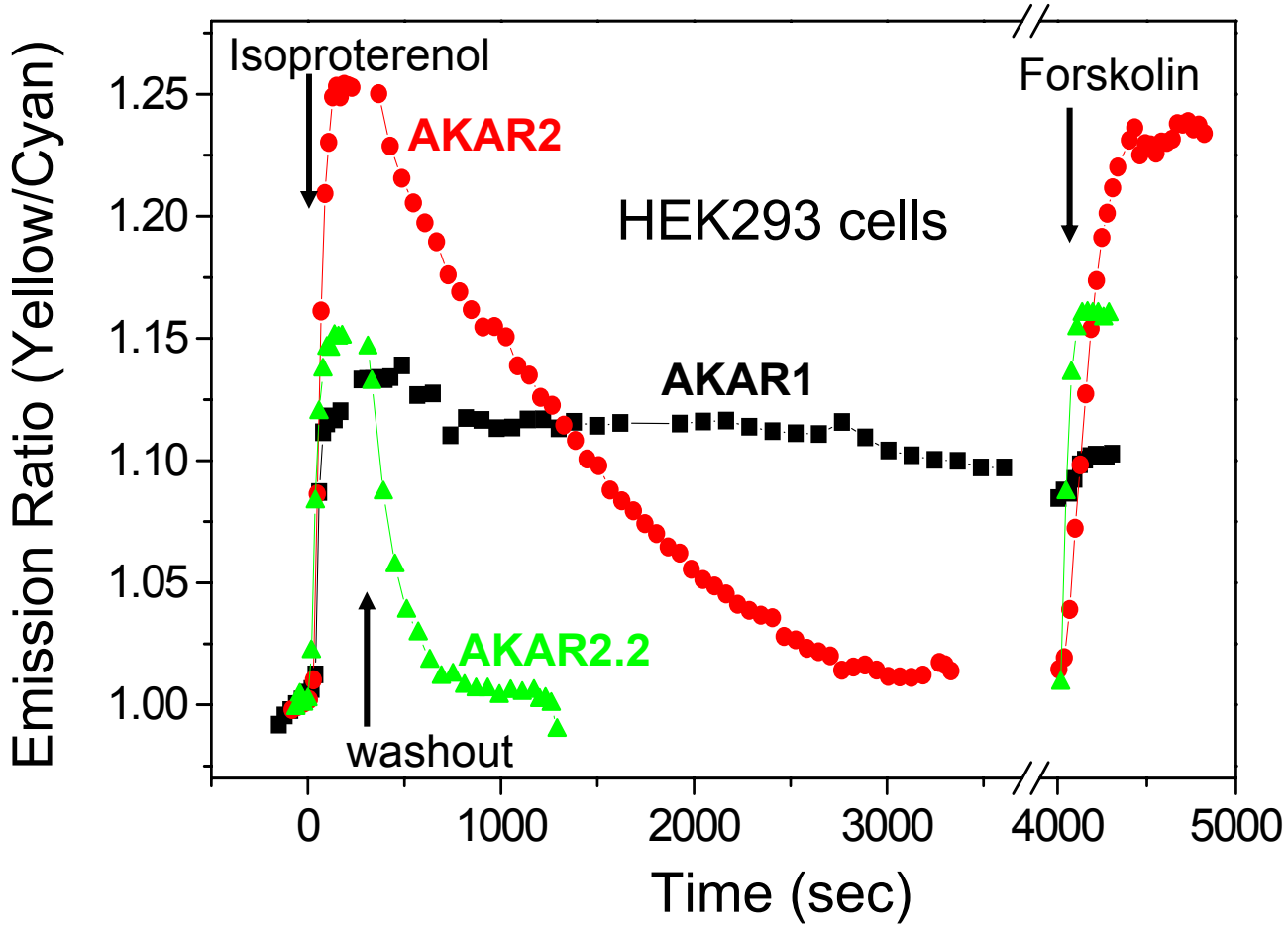


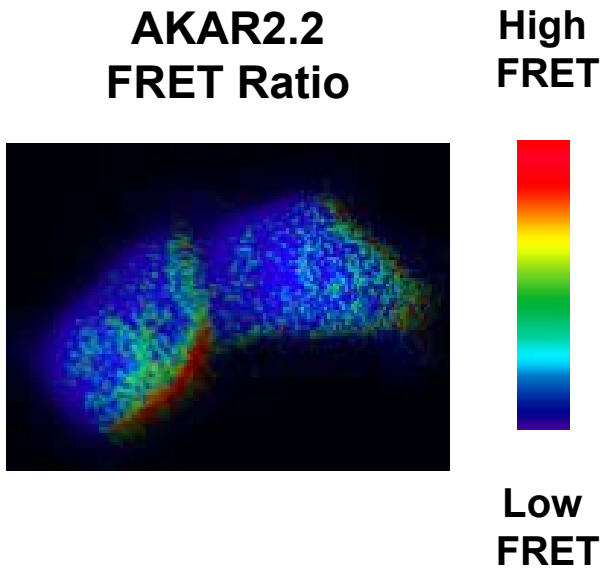


Peter Wang, Shu Chien (UCSD)

Increasing reversibility from AKAR1 to AKAR2 and 2.2



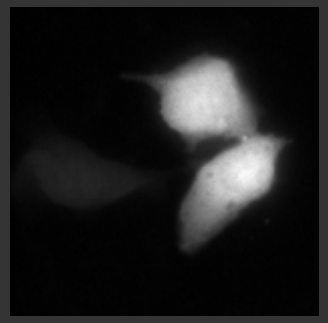


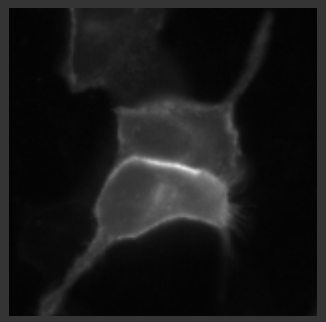


Jin Zhang

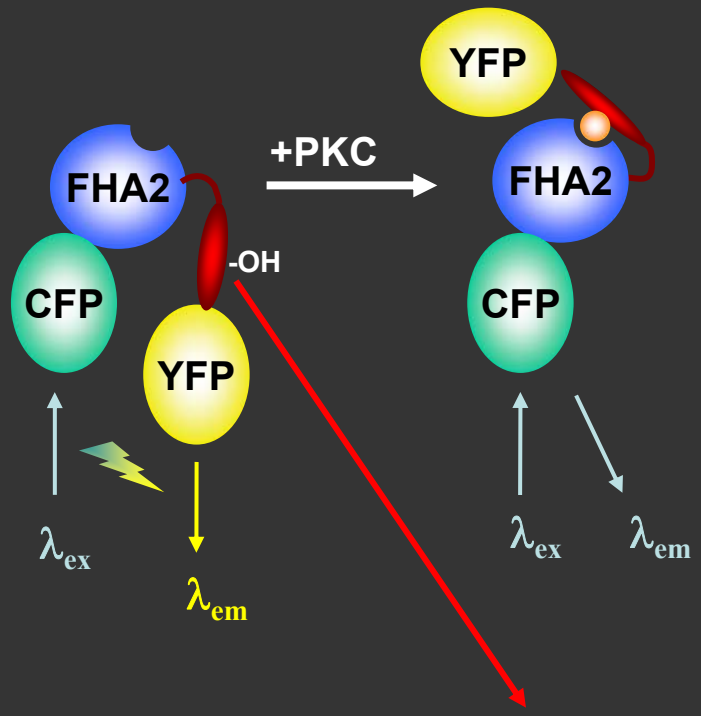
CKAR: C Kinase Activity Reporter

cytosolic CKAR

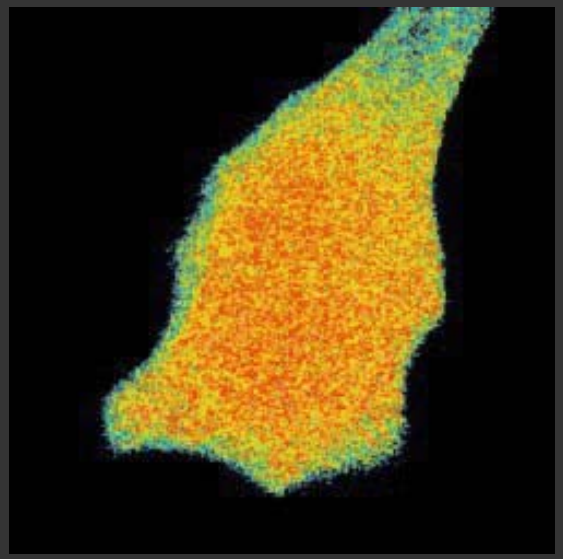




PM-anchored CKAR



CKAR FRET Ratio



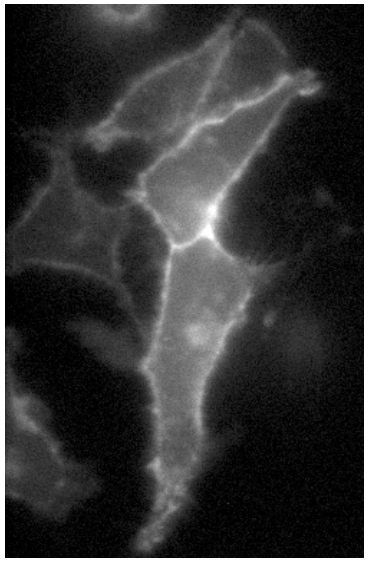
60min, 450x Frame Rate

GGSGGRFRRFQTLKIKAKAGGSGG

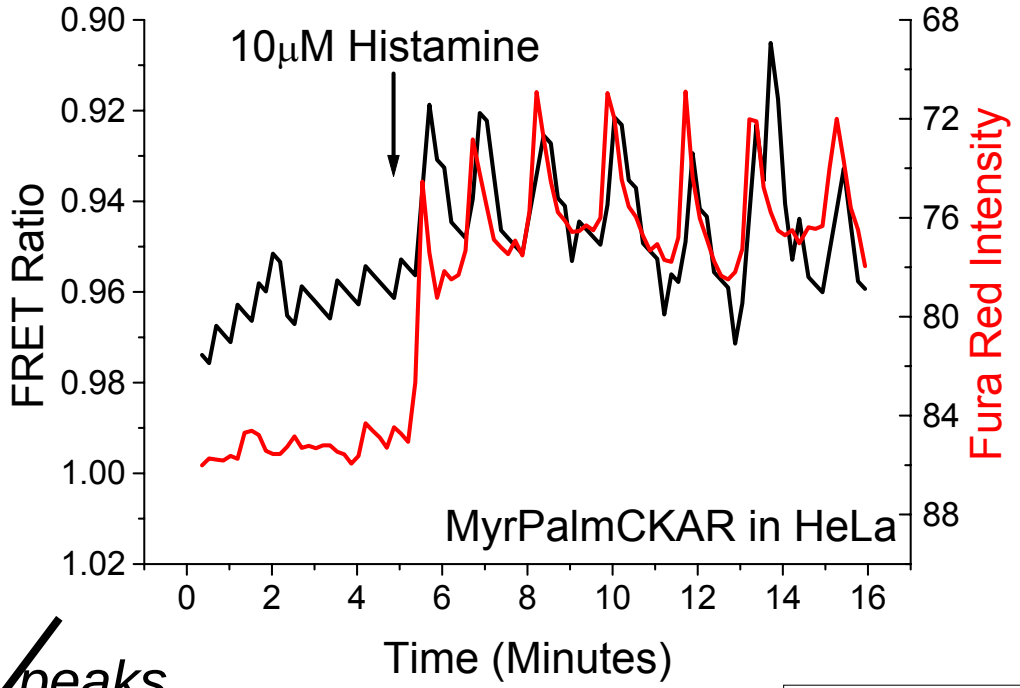
substrate sequence designed with help from http:/scansite.mit.edu

Jon Violin, Alexandra Newton (UCSD)

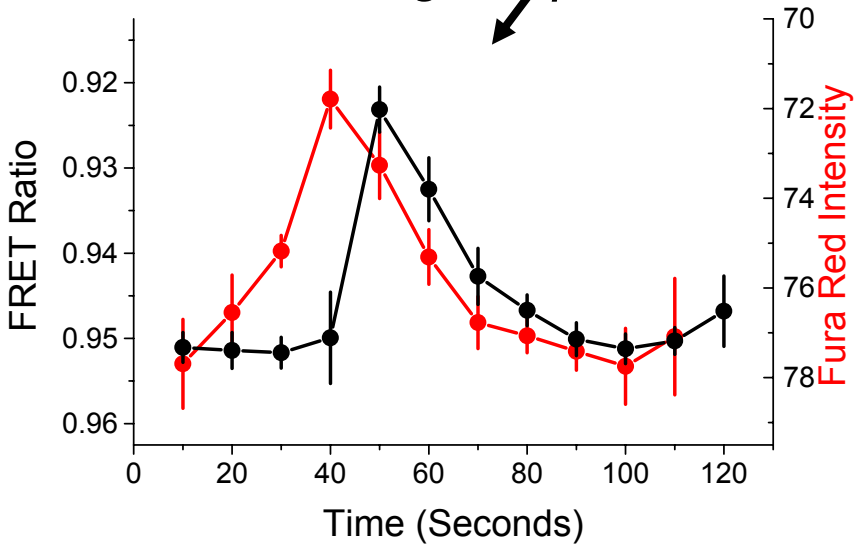
CKAR targeted to plasma membrane by acylation detects agonist-stimulated oscillations slightly lagging $[Ca^{2+}]_c$

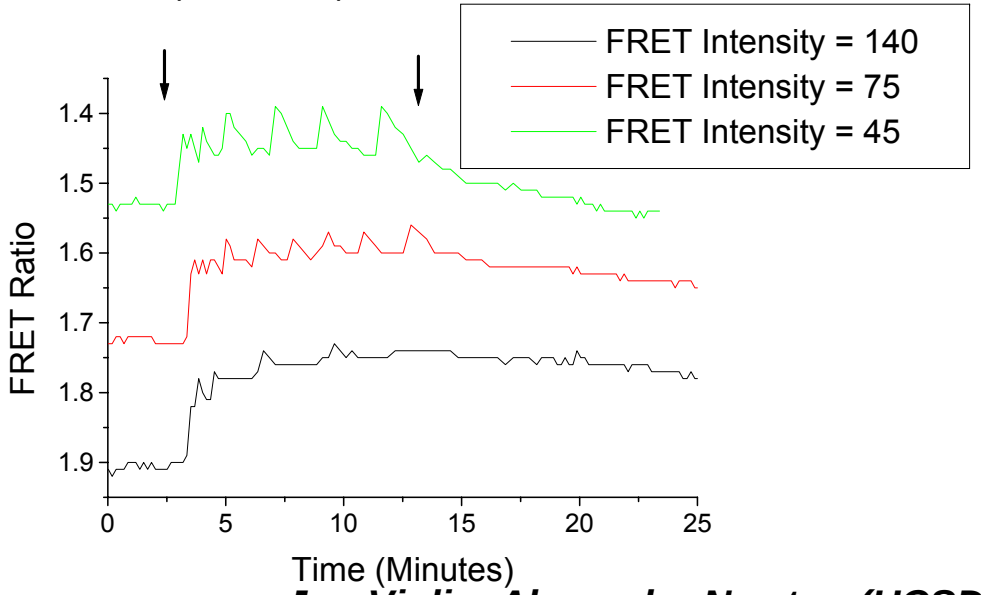


MyrPalm-CKAR



averaged peaks





Jon Violin, Alexandra Newton (UCSD)

What are genetically targetable sensors good for?

- GFP-based transfectable indicators exist in principle for pH, Cl^-, Ca^{2+}, Zn^{2+}, cAMP, cGMP, redox potential, membrane potential…
- Protein-protein interactions, transcriptional activation, proteases, kinase/phosphatase activities can also be sensed
- Genetic encoding greatly simplifies delivery into any organism, tissue and subcellular localization, mutagenic improvement, and reagent distribution
- Hybrid genetic/organic (tetracysteines/biarsenical) systems allow determination of protein age, electron-microscopic location, rapid inactivation

Acknowledgments

GFPs sensitive to thiol-disulfide redox potential

Colette Dooley, Timothy Dore George Hanson, S. James Remington (U. Oregon)

Cameleons: Transfectable Ca^{2+} indicators based on Calmodulin and GFPs

Atsushi Miyawaki, Juan Llopis, Roger Heim

Imaging of $[Ca^{2+}]_i$ with cameleons in cardiac myocytes from transgenic animals

Varda Lev-Ram, Atsushi Miyawaki Randa Hilal-Dandan, Lawrence Brunton (UCSD)

Red Fluorescent Protein (DsRed) properties

Geoffrey Baird, David Zacharias, Larry Gross Kim Baldridge (SDSC)

Reporters of kinase/phosphatase activity

Jin Zhang, Alice Ting, Peter Wang Alexandra Newton, Shu Chien (UCSD)

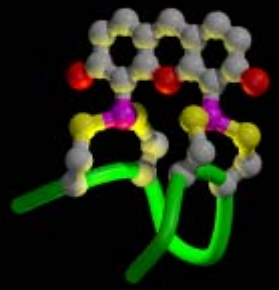
Monomerization and wavelength shifting of a tetrameric coral red fluorescent protein

Robert Campbell, Nathan Shaner, Lei Wang, Coyt Jackson, Paul Steinbach

Genetically-encoded fluorescent tags for proteins

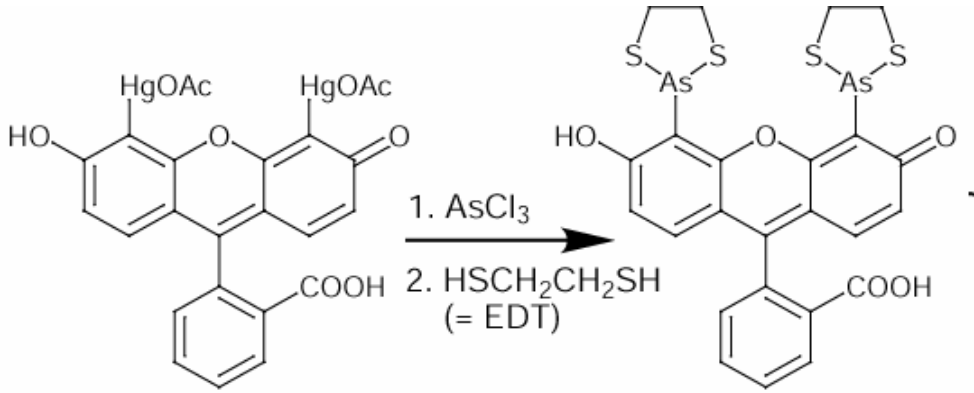
GFP

genetically targeted



biarsenical-
tetracysteine
(ReAsH & FlAsH)

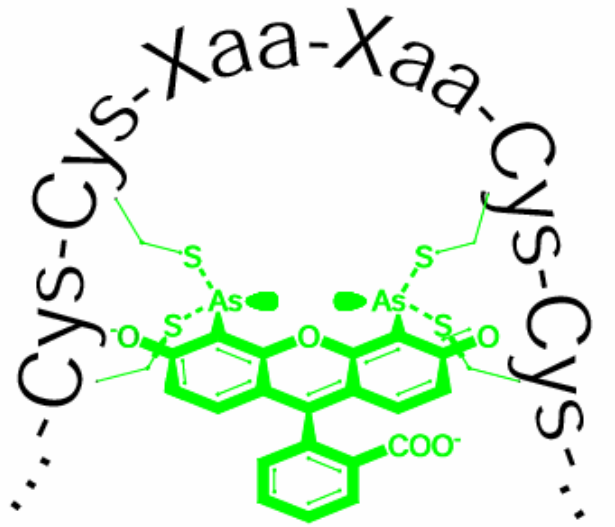
FlAsH = fluorescein-based arsenical hairpin binder



4,5-bis(acetoxy-mercuri)fluorescein

$FLASH\text{-}EDT_2$
(nonfluorescent)

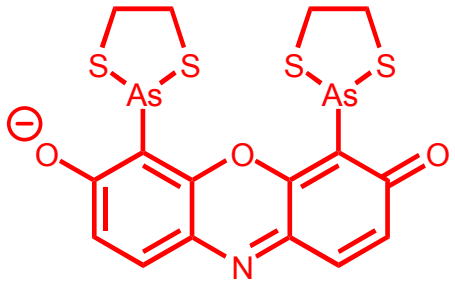
...Cys-Cys-Xaa-Xaa-Cys-Cys...
(genetically encoded tetracysteine motif)



fluorescent complex

K_d < 20 pM

ReAsH = resorufin-based arsenical hairpin binder (red)

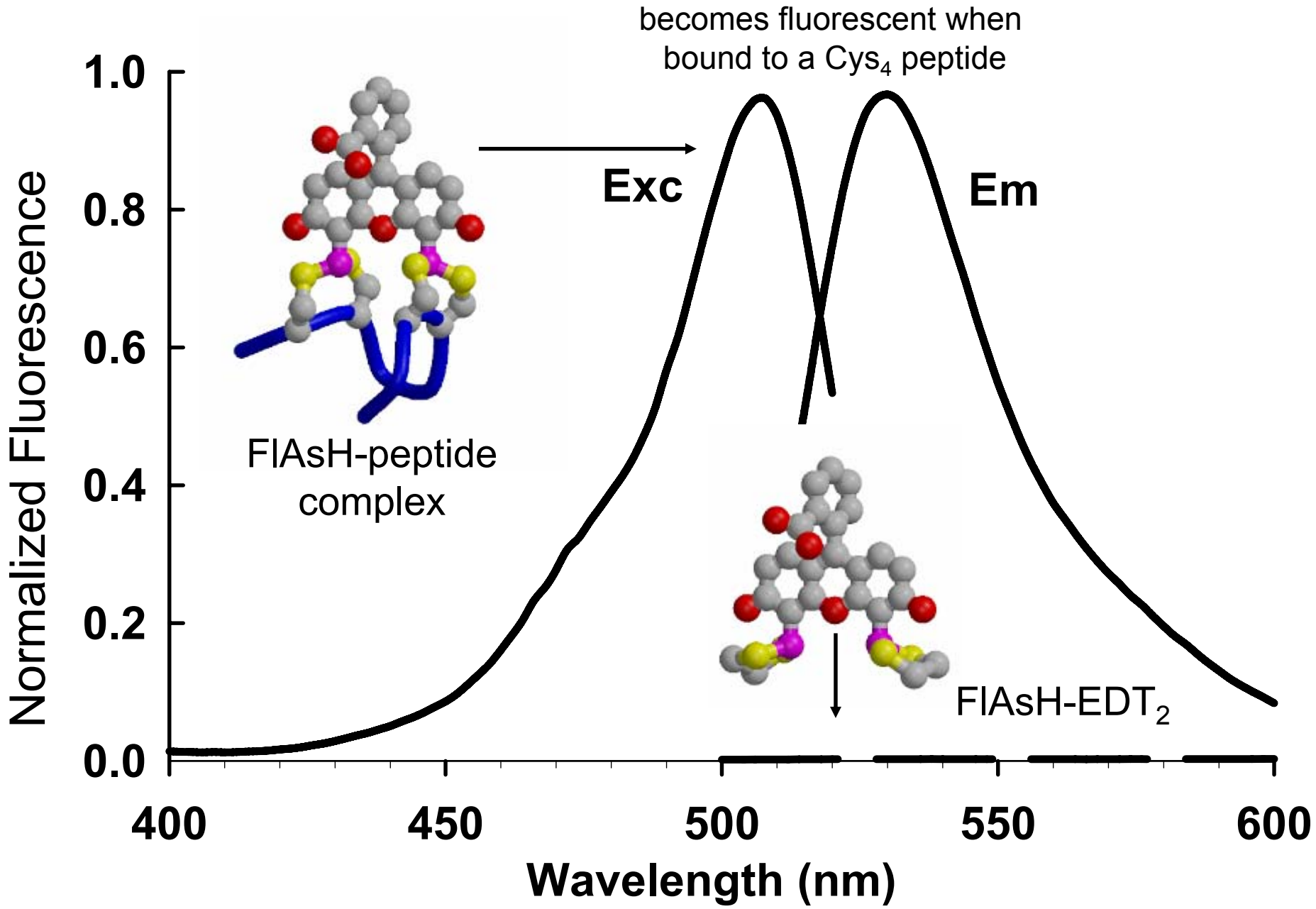


$ReAsH\text{-}EDT_2$

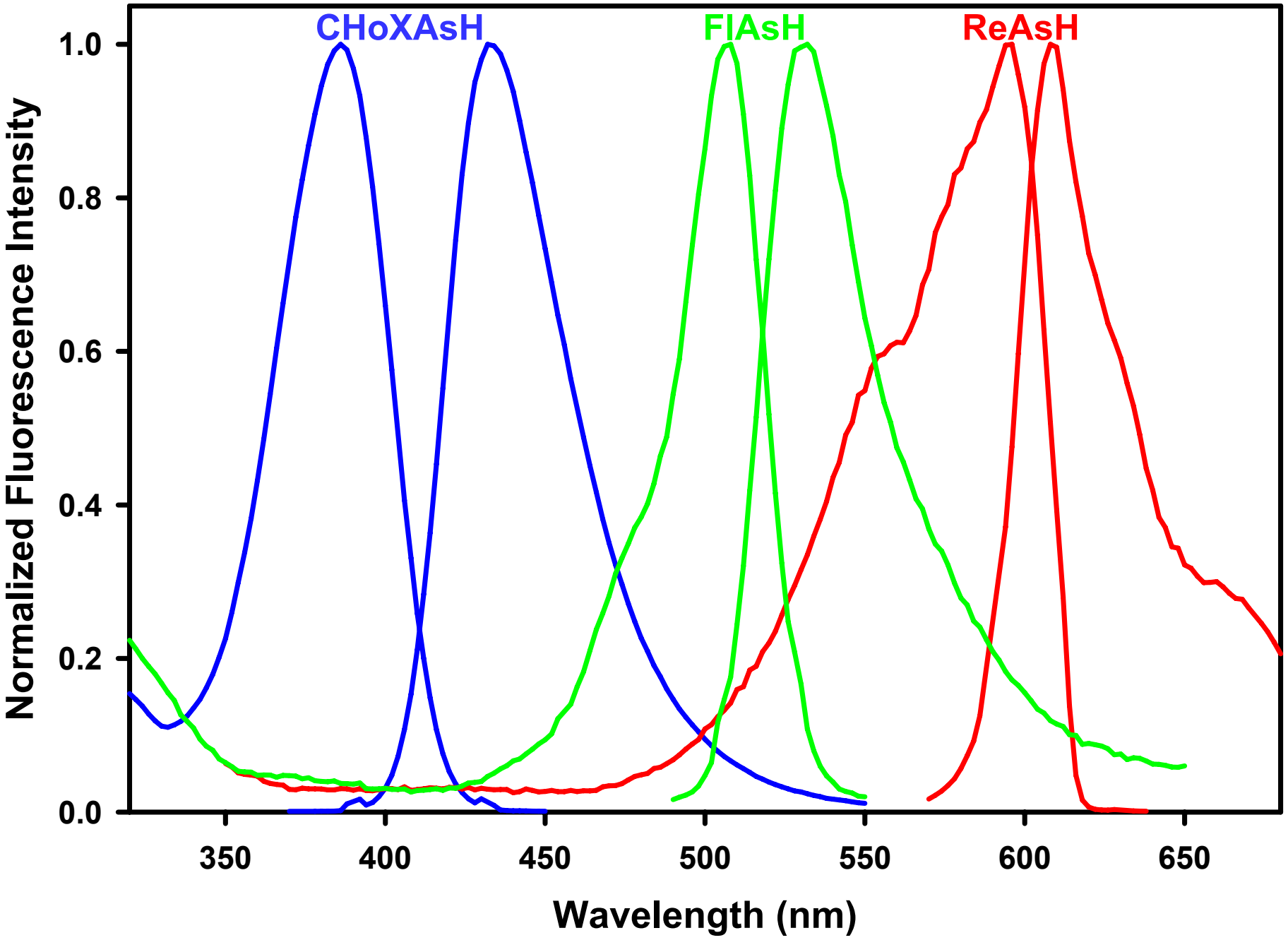
FlAsH and ReAsH now available from Invitrogen as "TC-FlAsH" and "TC-ReAsH"

Albert Griffin

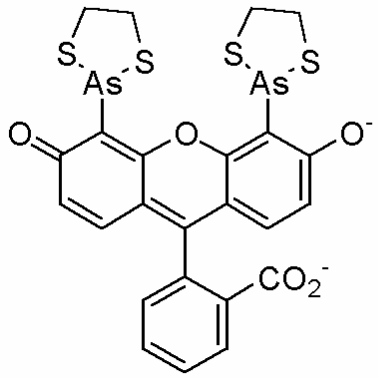
FlAsH = fluorescein-based arsenical hairpin binder



Blue, green and red biarsenical-peptide complexes

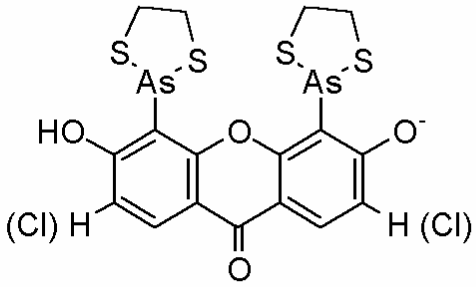


Ligands for tetracysteine tags



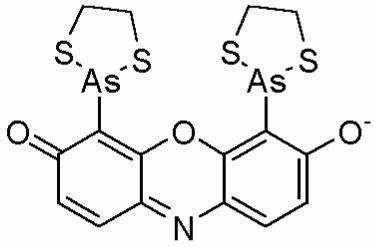
FlAsH-EDT$_2$

green fluorescence, FRET from CFP



(C)HoXAsH-EDT$_2$

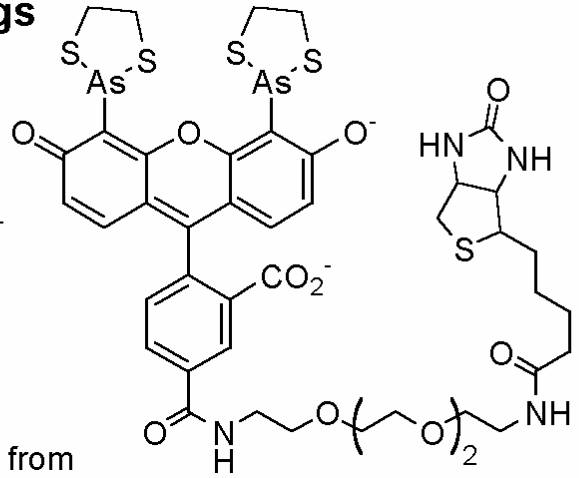
blue fluorescence, FRET to GFP,YFP



ReAsH-EDT$_2$

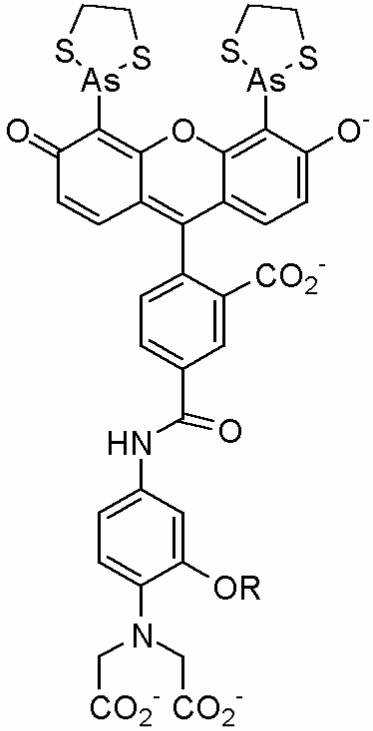
red fluorescence, FRET from GFP, YFP

photoconversion of diaminobenzidine for electron microscopy



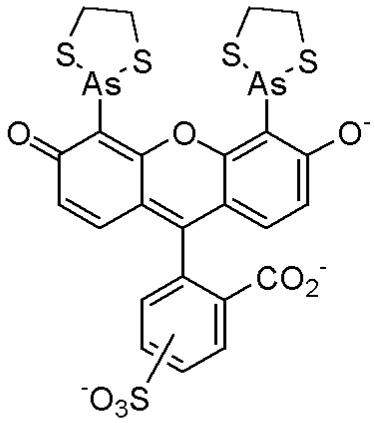
FlAsH-biotin

green fluorescence, ligand for streptavidin-quantum dots



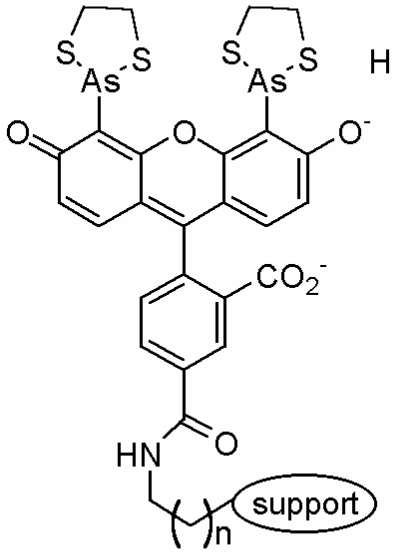
Calcium Green FlAsH-EDT$_2$

low affinity fluorescent Ca^{2+} indicator



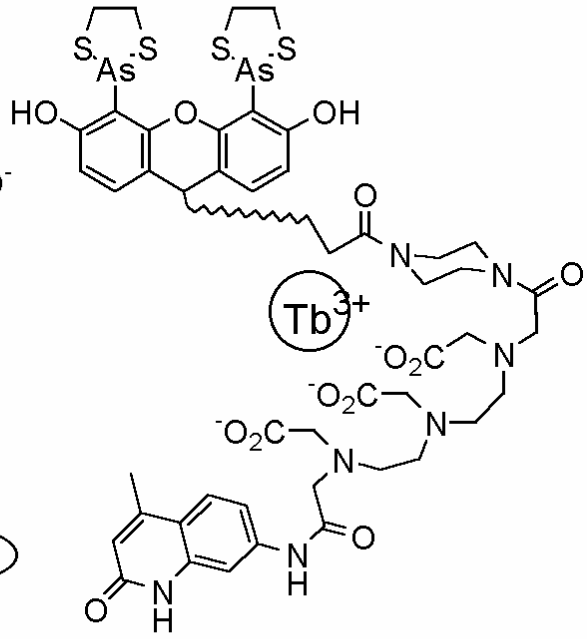
sulfoFlAsH-EDT$_2$

membrane impermeant ligand for extracellular proteins



Immobilized FlAsH-EDT$_2$

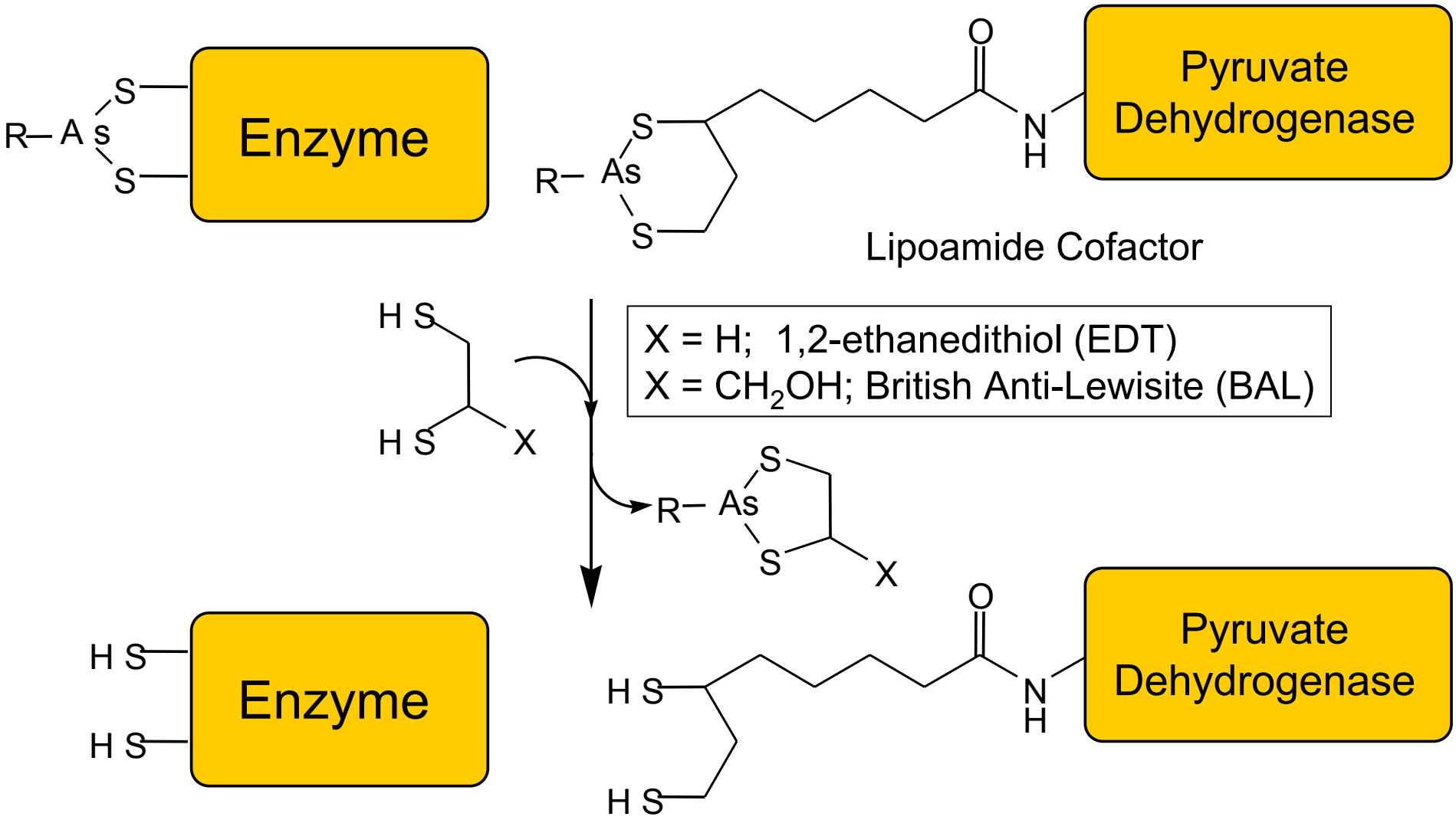
affinity chromatography



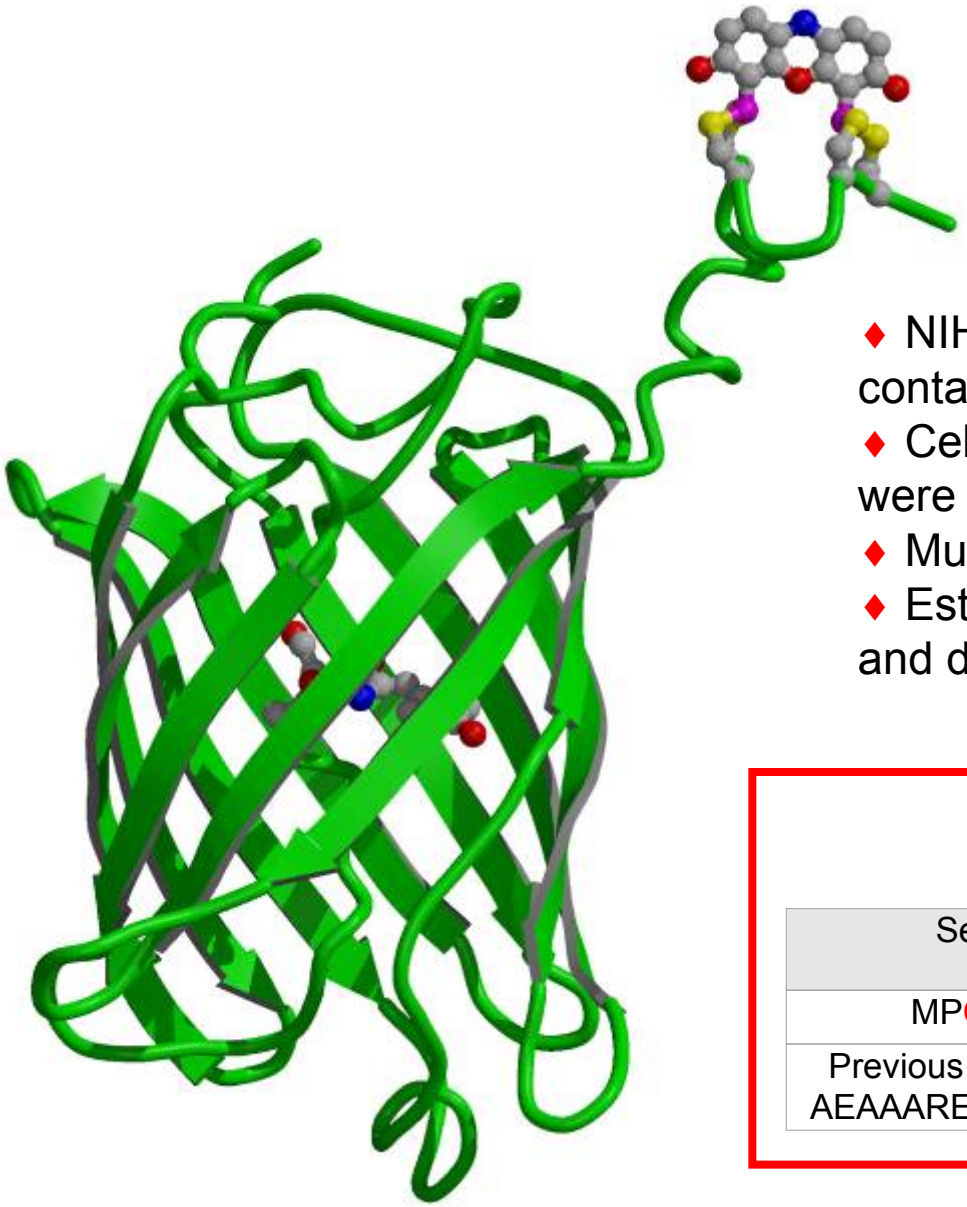
SpLAsH-Tb

luminescent, LRET to GFP

Antidotes such as EDT and BAL Prevent Arsenic Toxicity and Binding to Endogenous Thiols



A Library Strategy for Tetracysteine Evolution in Mammalian Cells



EGFP-XXCCXXCCXX + ReAsH

X = NNK nucleotides
Nucleotide diversity = $1x10^9$
a.a. Diversity = $6.4x10^7$

- NIH 3T3 cells infected with a retroviral library containing EGFP-XXCCXXCCXX.
- Cells stained with ReAsH and cells with high FRET were selected by FACS.
- Multiple cycles of sorting and expansion.
- Establish monoclonal cell lines of high FRET cells and determine a.a. sequence of tetracysteine motif

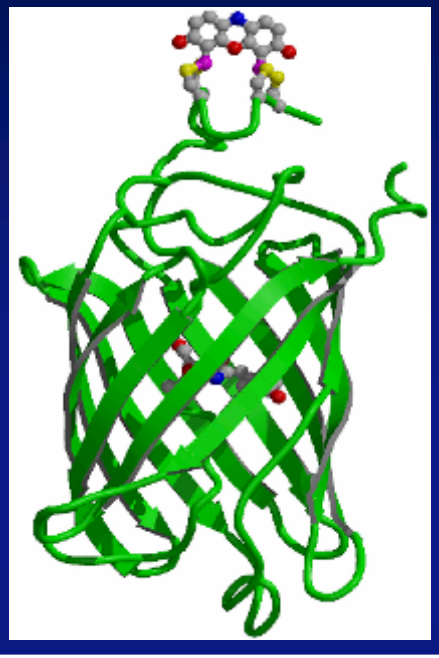
ReAsH Retroviral Library Results

Sequence	IC_{50}	Fold Improvement
MPCCPGCCGC	1100	9.2
Previous best sequence AEAAAREACCPGCCARA	550	4.6

Brent Martin

Expanding the consensus tetracysteine motif using ultra-high throughput FACS

MPCCPGCC: > 20 fold improvement over WEAAAREACCRECCARA



Second Generation Library

XXXCCPGCCXXX

N-terminal GFP

BamHI *NotI* **Emerald GFP …**

ATG GCC ***GGA TCC*** TAA □***GC GGC CGC*** AGC AAG GGC GAG GAG CTG

M A G S ***

↓ **Insert Library Cassette**

N-terminal PG GFP ReAsH Retroviral Library

BamHI *NotI* **Emerald GFP…**

ATG GCC ***GGA TCC*** NNK NNK NNK TGC TGC **CCC GGG** TGC TGC NNK NNK NNK G***GC GGC CGC*** AGC AAG GGC GAG GAG CTG

M A G S **X X X C C P G C C X X X** G G R S K G E E L

Theoretical nucleotide diversity = 1.07×10^9, actual nucleotide diversity = 8.88×10^8

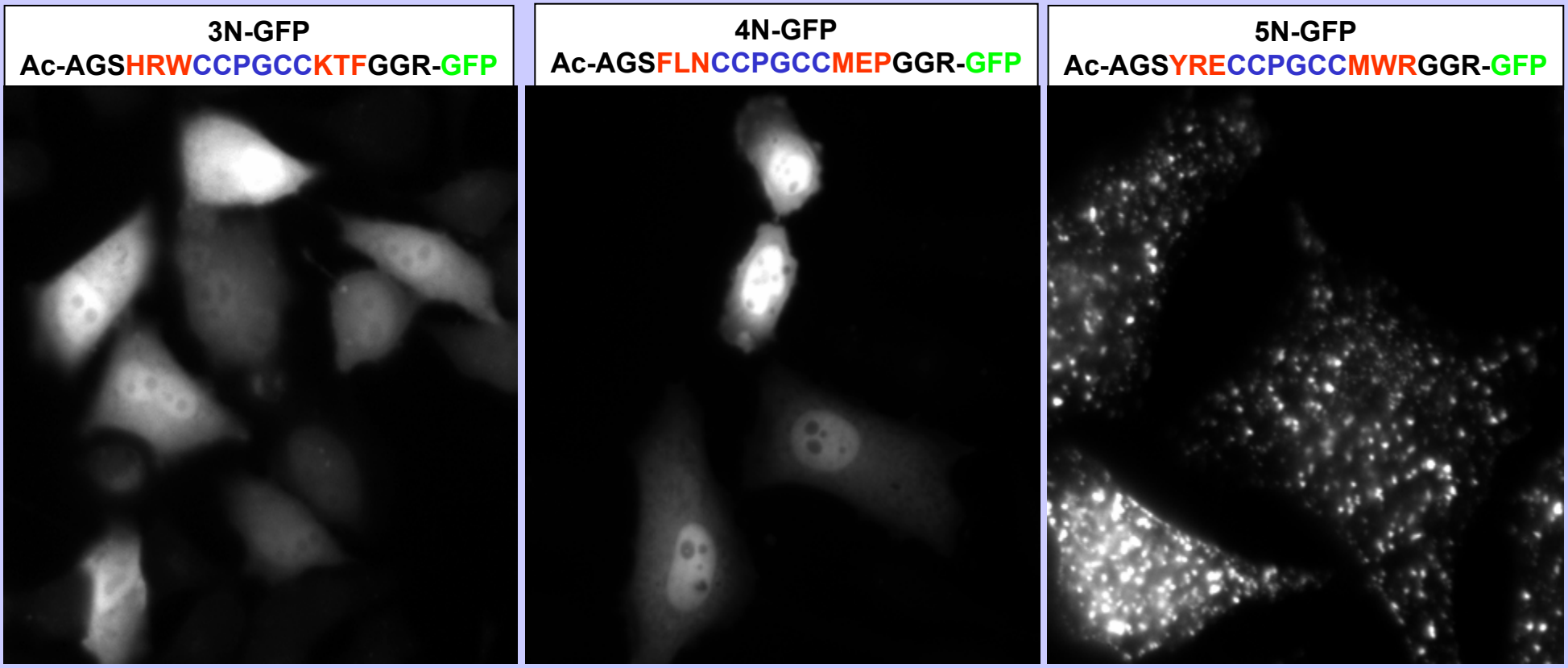
Peptide diversity = 6.4×10^7, cloned library size = ~8.3×10^8

After multiple rounds of fluorescence-activated cell sorting (FACS) at progressively higher [EDT] and [BAL], clones isolated with ~2X higher quantum yield and >10X higher resistance to dithiols

Current favorite: FLNCCPGCCMEP: QYs for FlAsH=0.78 (N), 0.70 (C); for ReAsH, 0.47 (N), 0.42 (C)

Brent Martin, W. Coyt Jackson

Sequence Convergence to 3 Highly Dithiol Resistant Clones



x480/30, m653/95

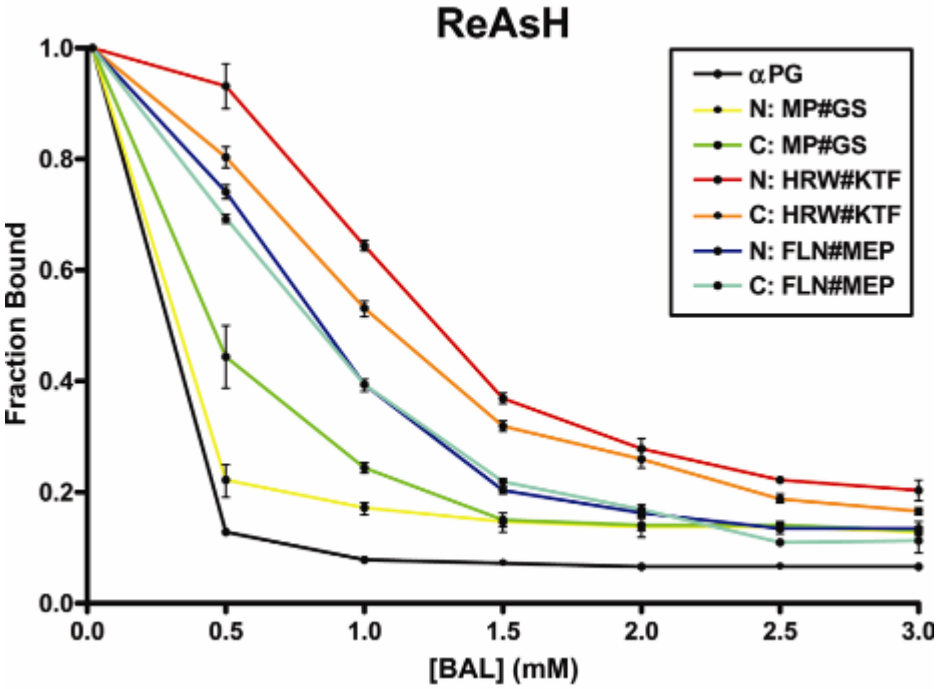
- YRECCPGCCMWR (5N-GFP) precipitates upon ReAsH labeling, explaining high dithiol resistance
- Two remaining peptides show diffuse cytoplasmic and nuclear labeled protein with no precipitation or aggregation

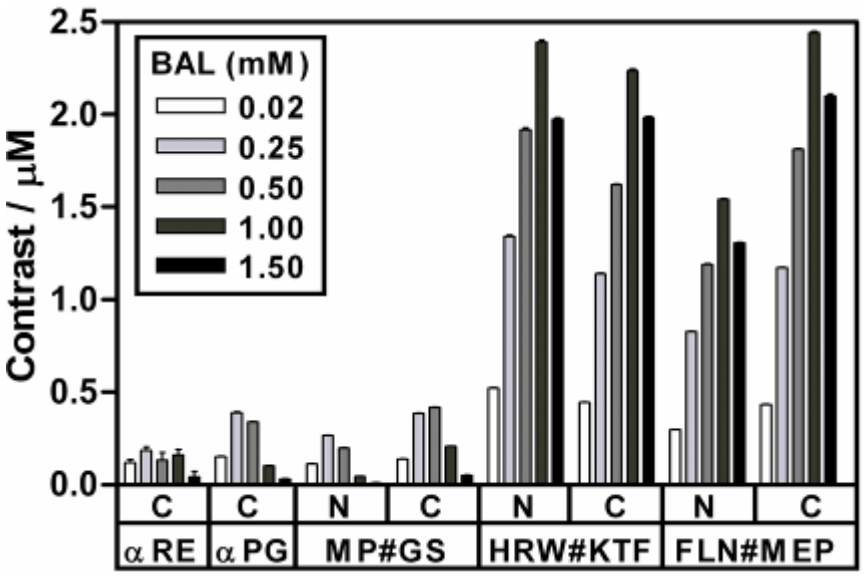
Brent Martin

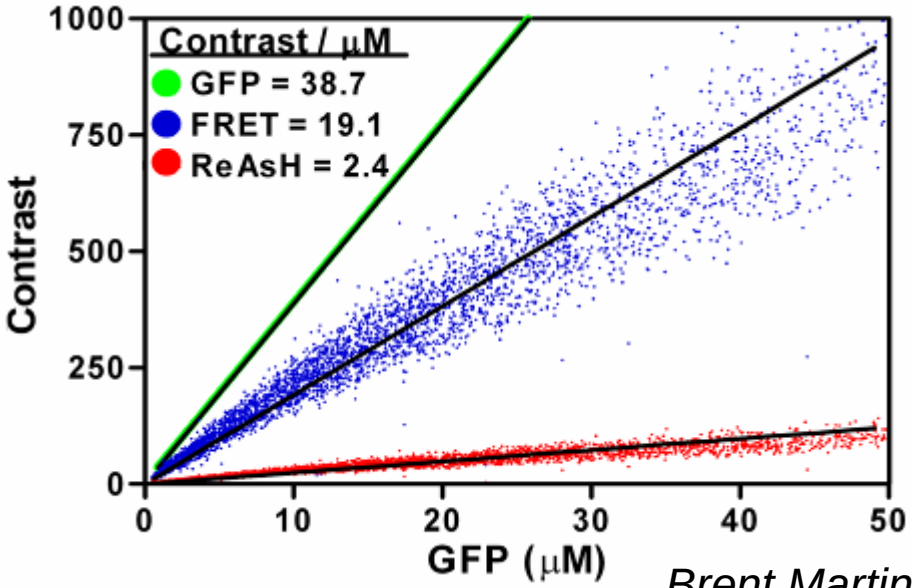
New sequences improve binding to ReAsH (and FlAsH) and give greater detection sensitivity

- GFP is still >15-fold more sensitive
- FRET ~ GFP for sensitivity
- High thiol washes not toxic

- Lipid modification of N-terminal cys_4 can occur but easily prevented





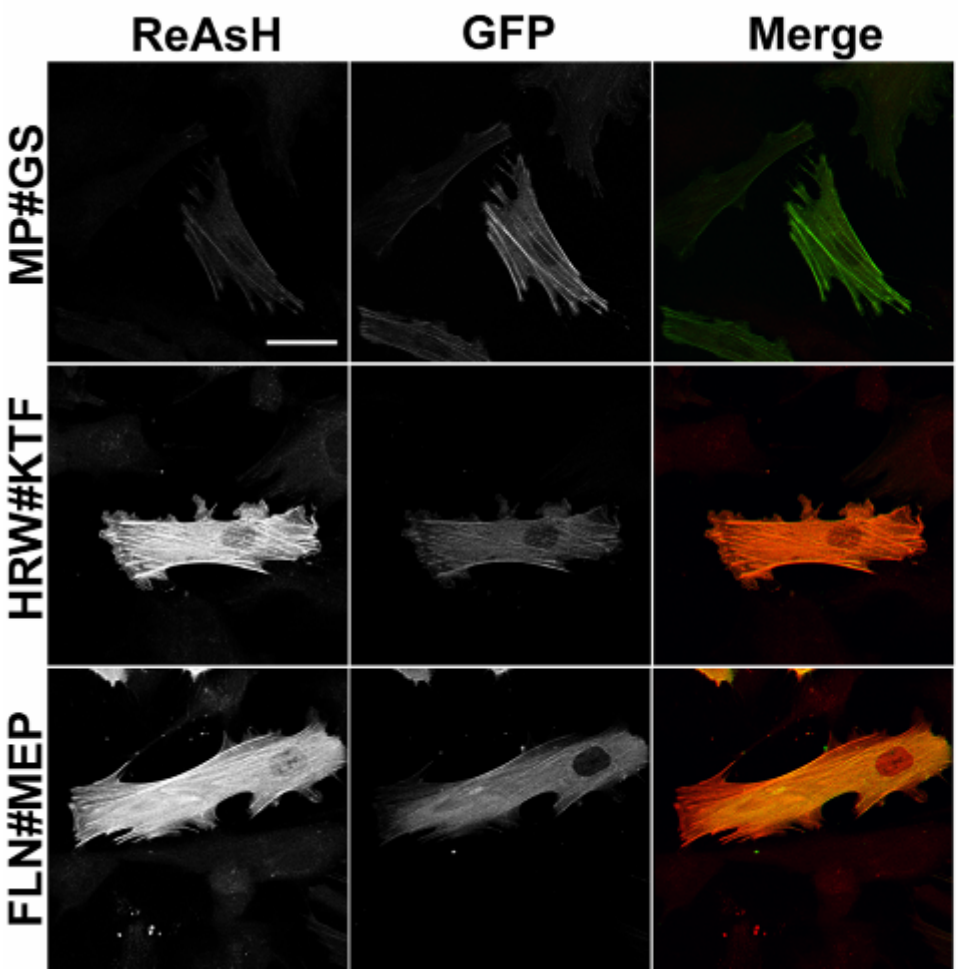


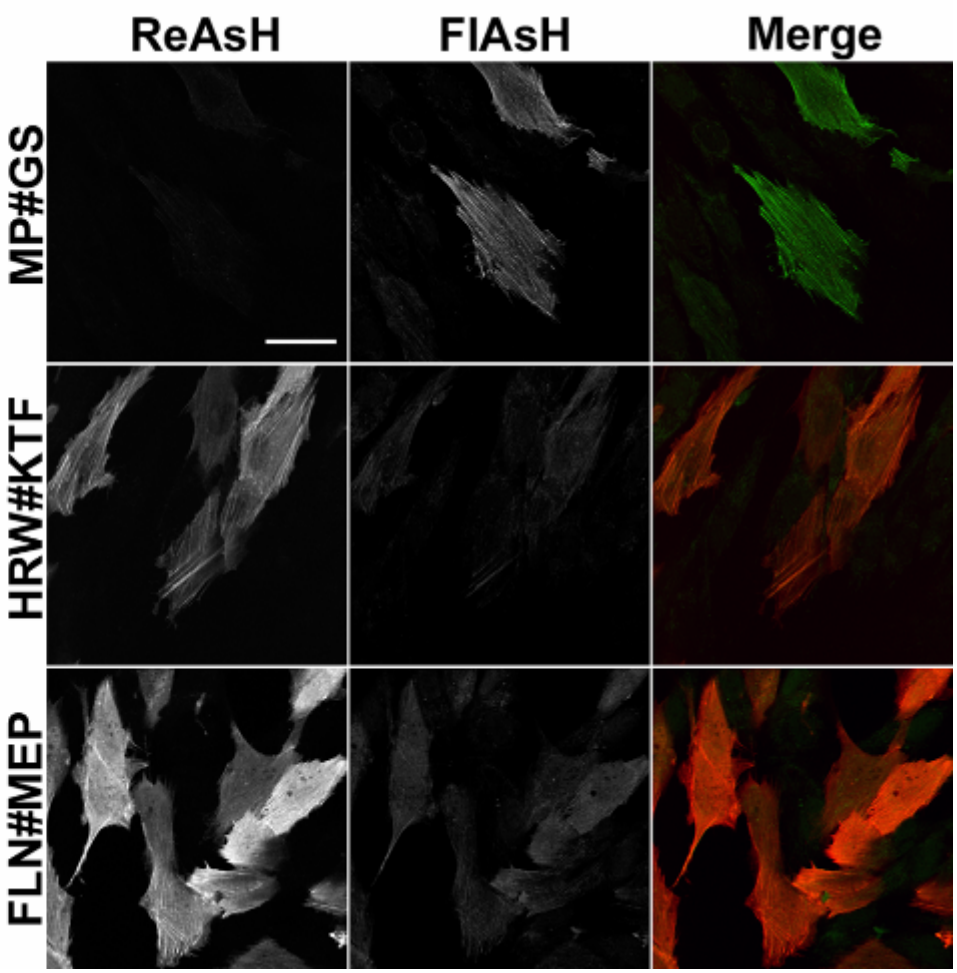
Brent Martin

Improved Sequences improve detection of β-Actin

4N-GFP-β-Actin

4N-β-Actin





wash with 0.75 mM BAL

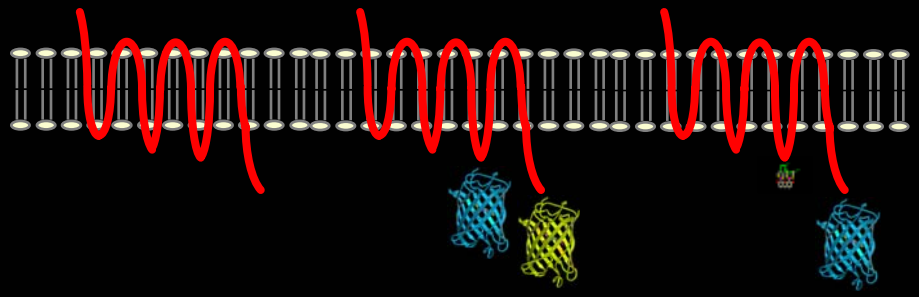
label with ReAsH, wash with 0.75 mM BAL
then label with FlAsH

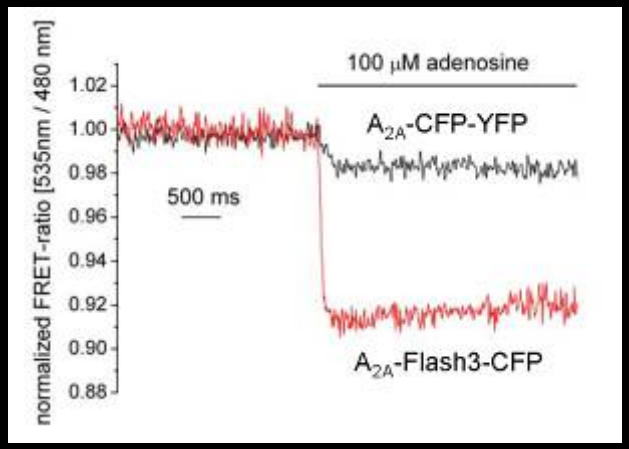
Brent Martin, Ben Giepmans

FlAsH-tetracysteine insertion into GPCR is better than YFP insertion

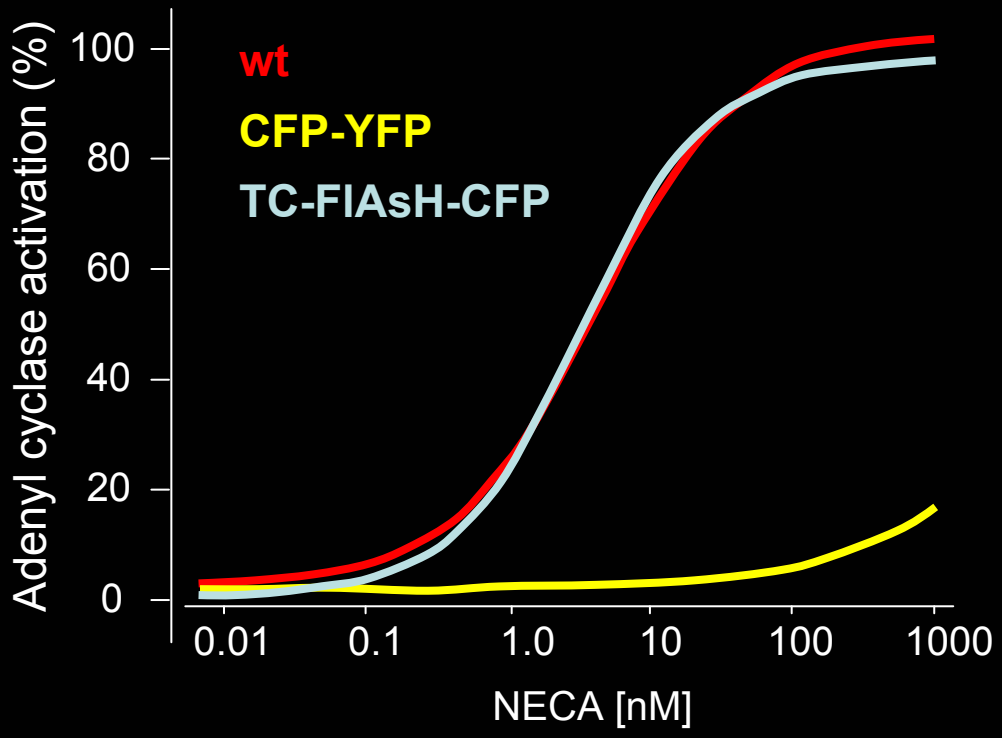
Bigger FRET response, no perturbation of coupling to downstream effector: **Smaller is better!**

Adenosine A_{2A} receptor chimeras

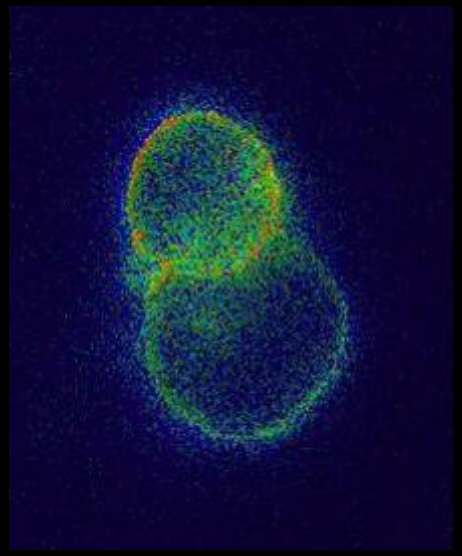




The FlAsH approach results in a much larger agonist-induced change in FRET



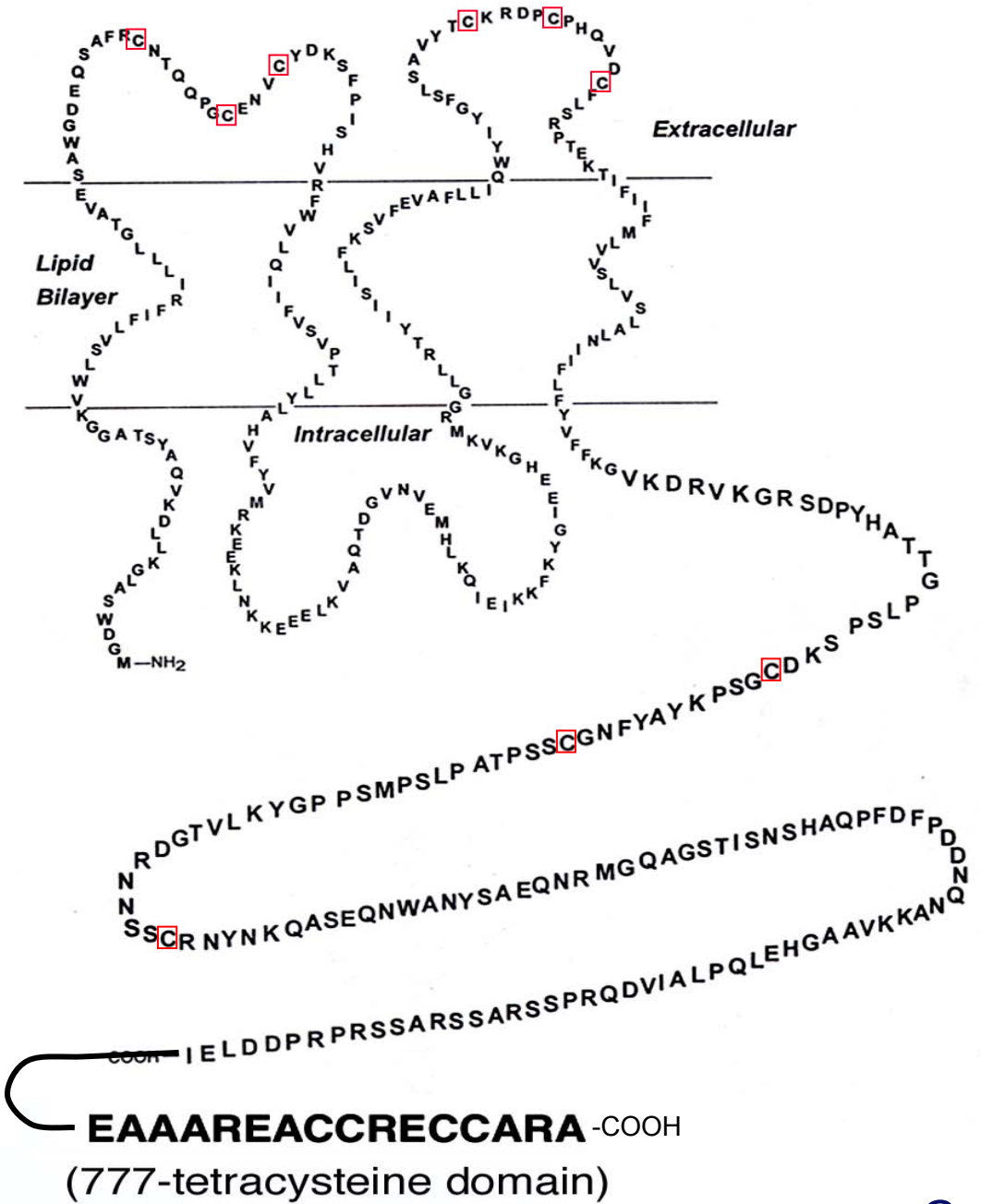
Receptor kinetics are not significantly different from the wt receptor



Carsten Hoffman, Martin Lohse (Univ. Wuerzburg)

Hoffmann et al. (2005) Nat Meth

Connexin43-tetracysteine



- Connexin43, a constituent of gap junctions, was fused at its C terminus to EAAAREACCRECCARA
- Expression in cultured HeLa cells (which do not normally express Cx43) gives cellular localization to Golgi apparatus and gap junctions
- No obvious toxicity from transient or stable protein expression
- Endogenous cysteines (C) do not interfere

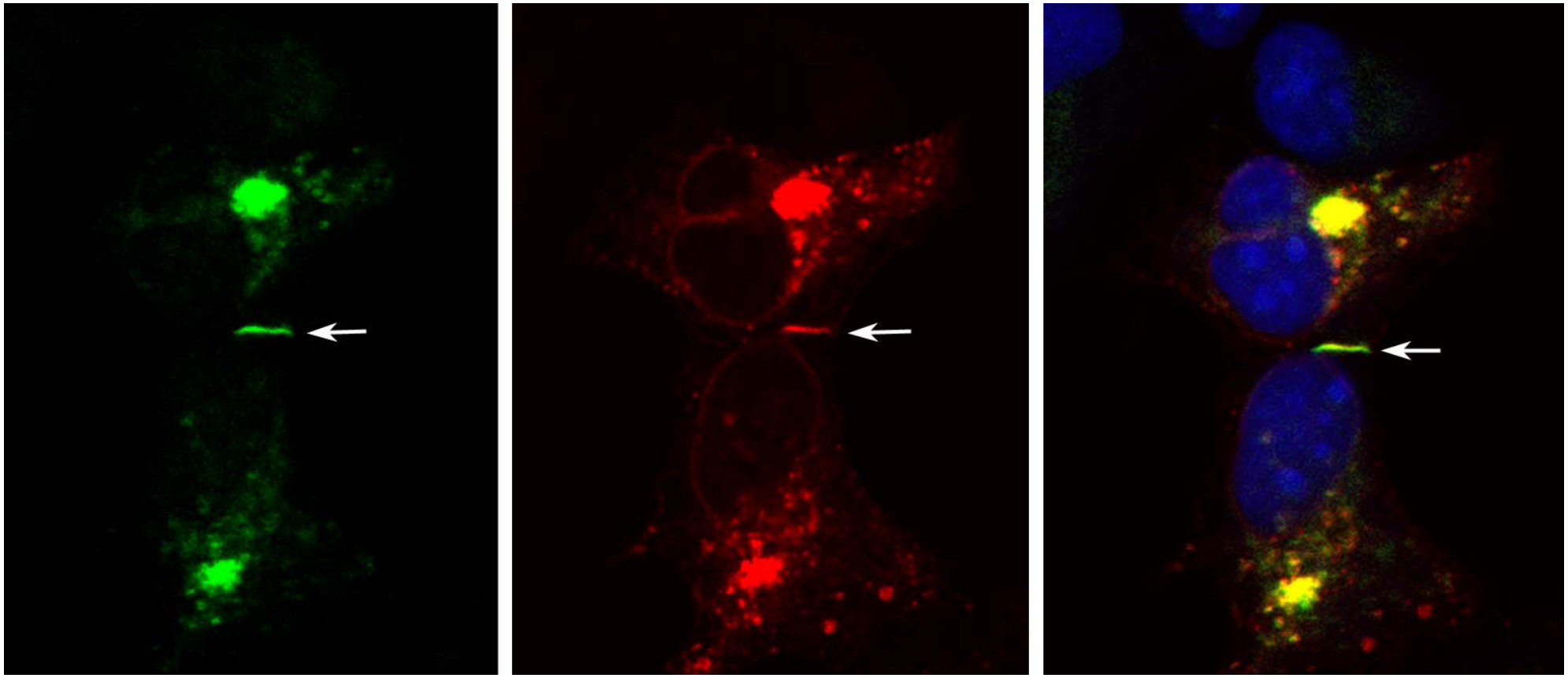
Gaietta *et al* (2002) *Science* 296: 503-507

Double Labeling of Cx43-cys$_4$ by FlAsH and a Cx43 Specific Antibody

FlAsH labeling

Anti-Cx43 antibody-rhodamine

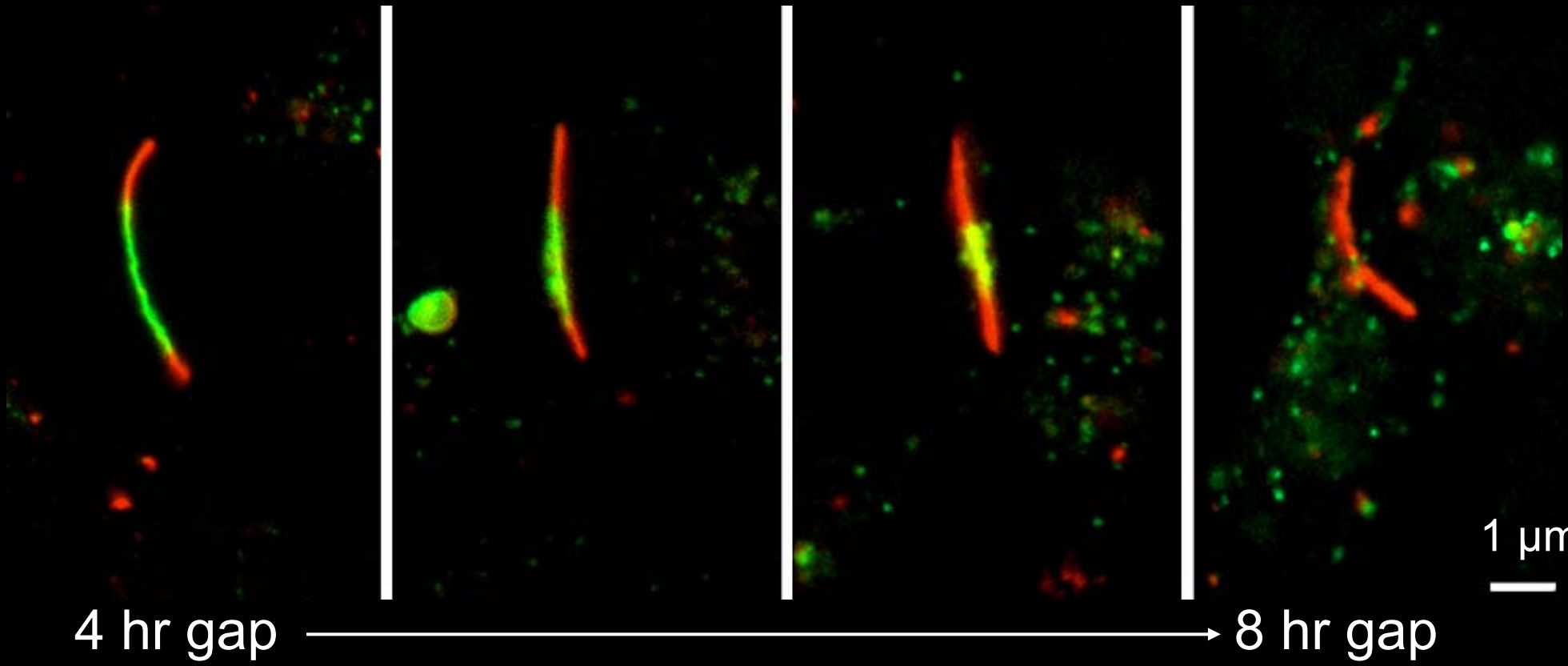
Combined with nuclear stain



Guido Gaietta, Tom Deerinck

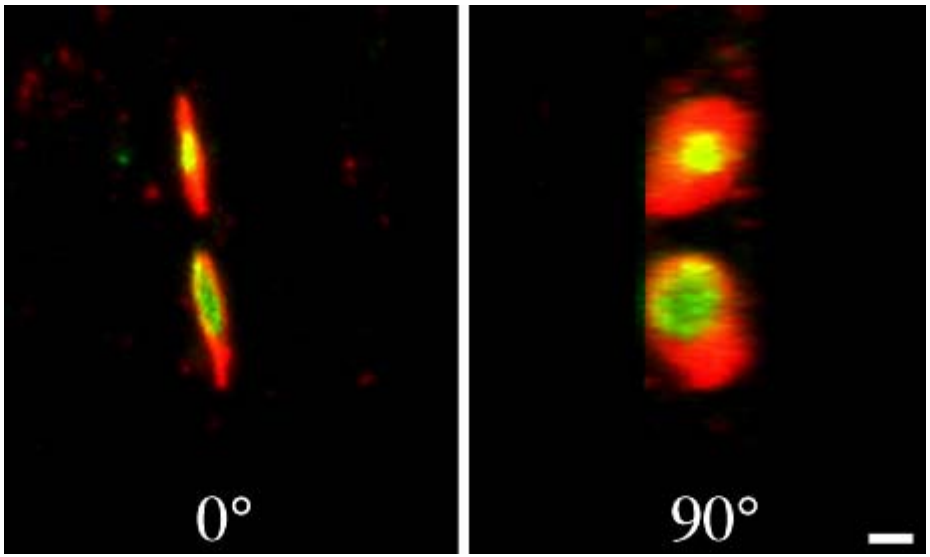
Sequential labeling with two colors distinguishes old from new protein molecules

Pulse first with FlAsH, wash out unbound dye, wait 4-8 hr for cells to make new protein, chase with ReAsH.
Old connexins stain green, new proteins stain red

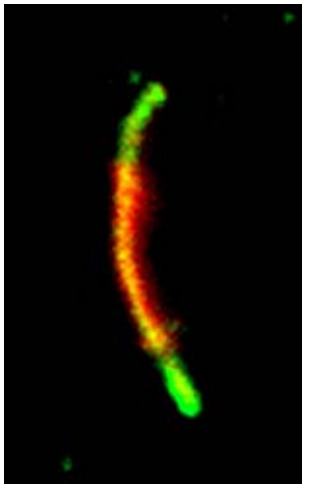


Gaietta *et al* (2002) *Science* 296: 503-507

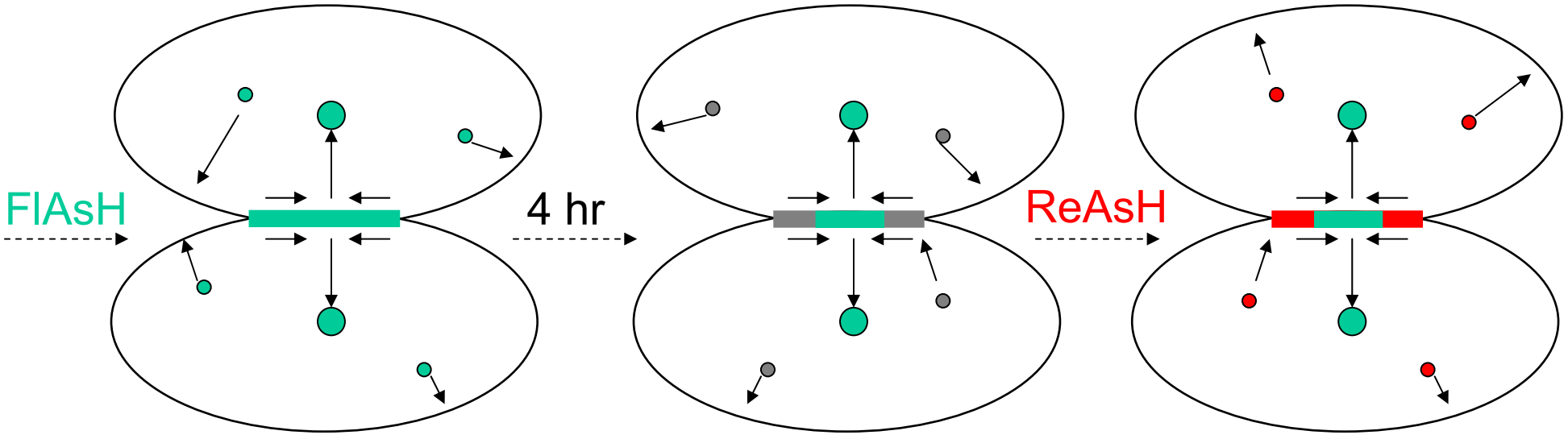
3-D reconstruction shows concentric zones



Color pattern reflects order of staining, e.g. ReAsH before FlAsH gives:

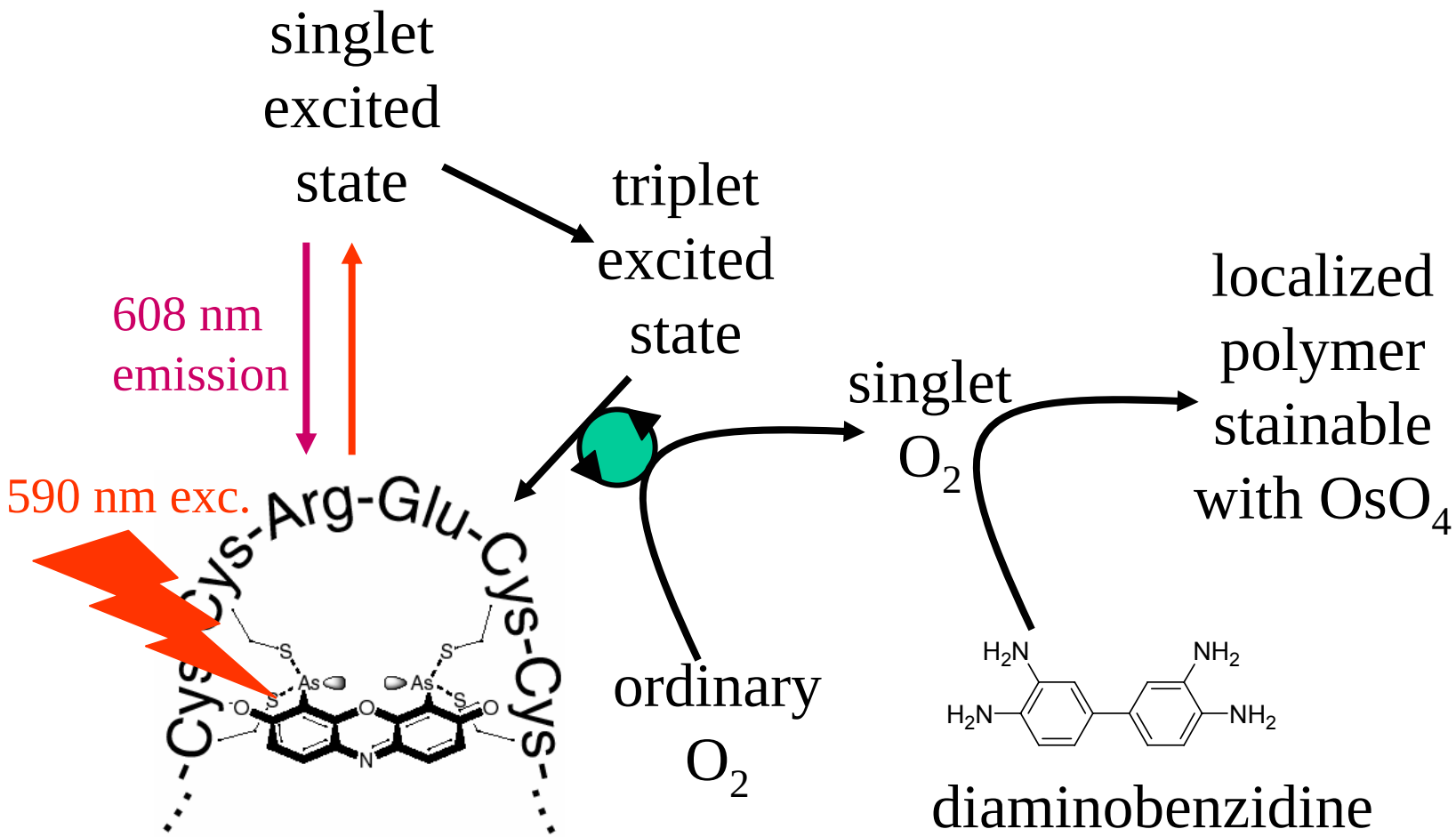


Interpretation:



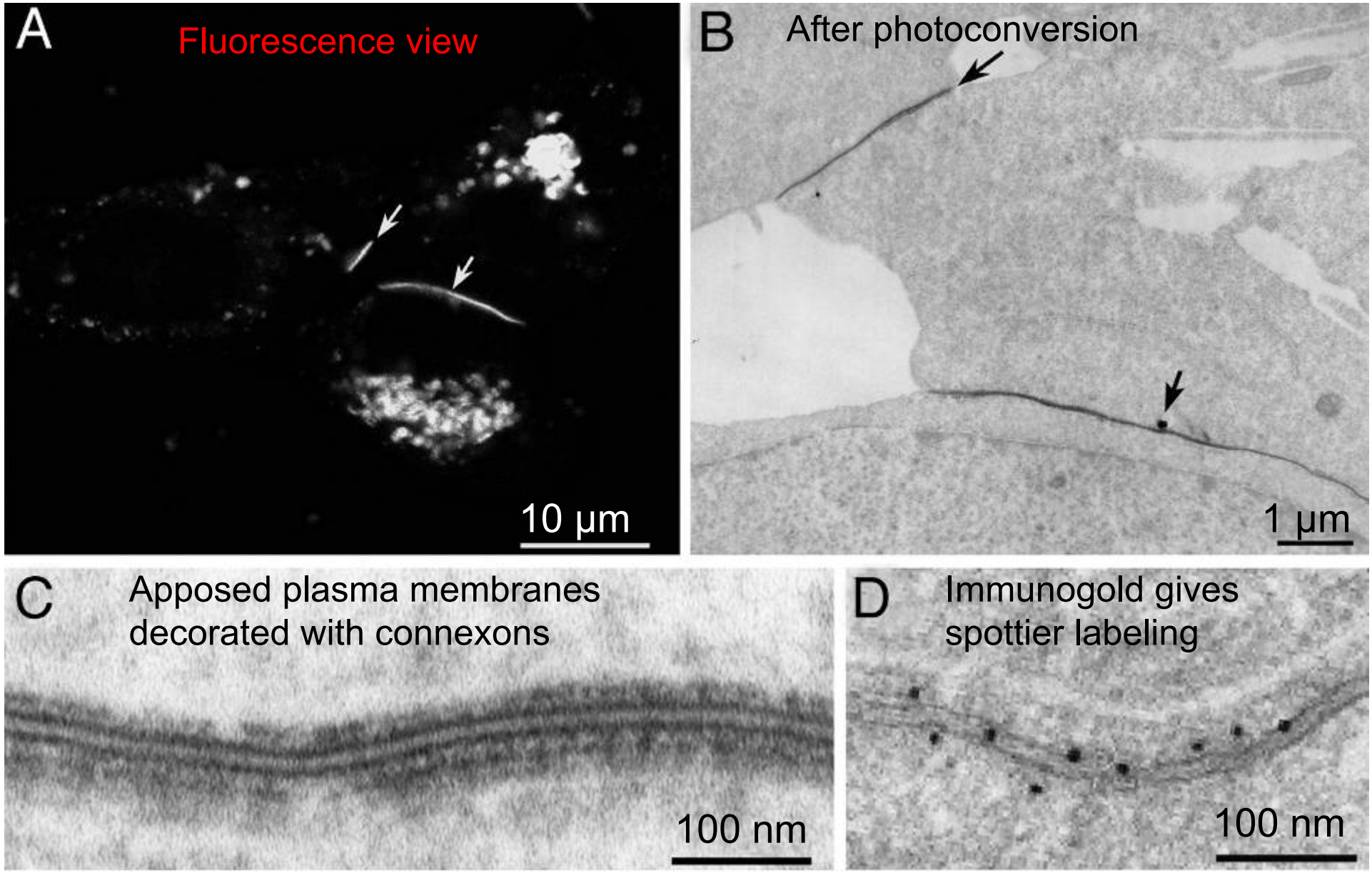
Gaietta *et al* (2002) *Science* 296: 503-507

Fluorescence photooxidation to correlate light and electron microscopy



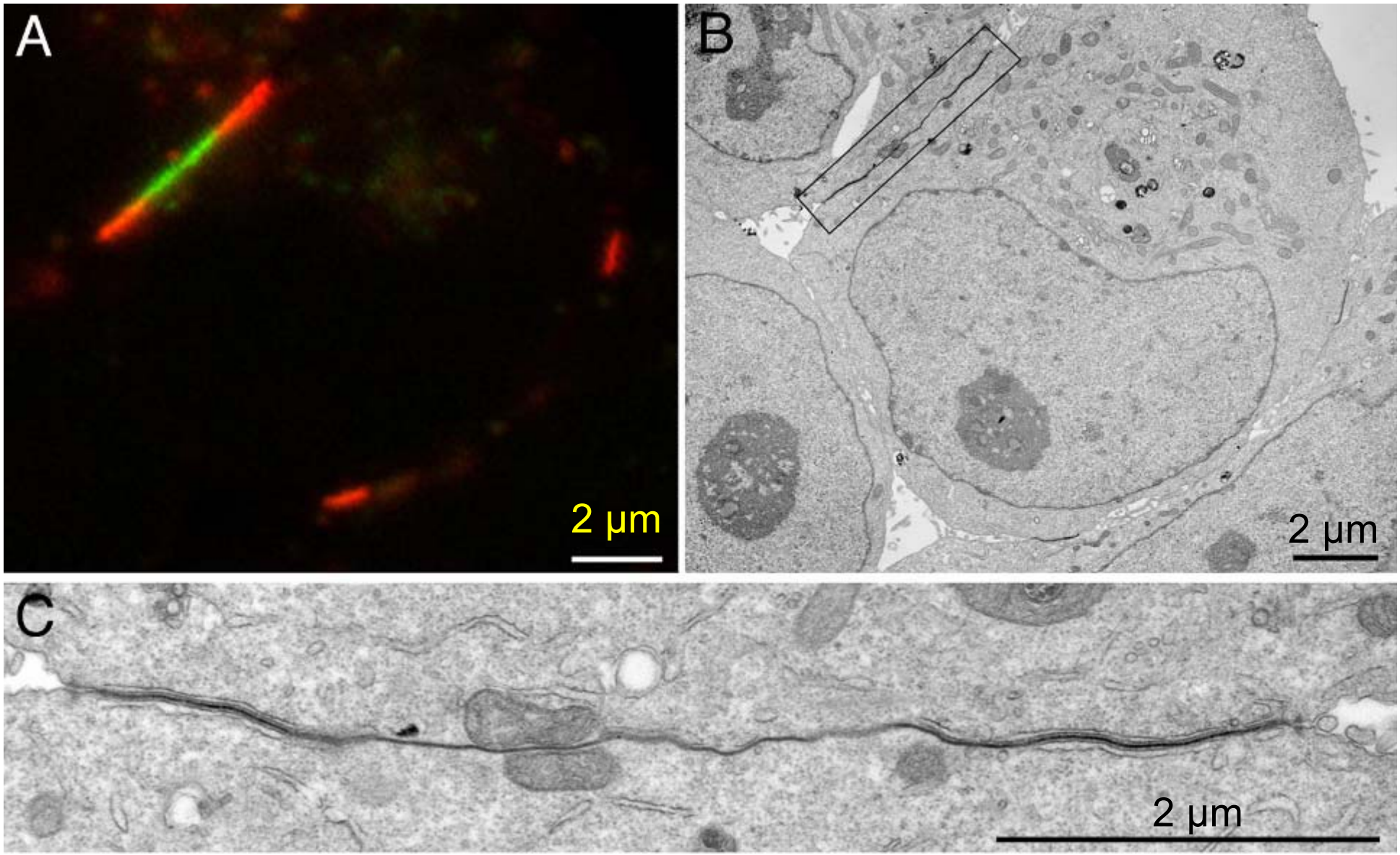
Protein introduced by transfection; all other reagents are small molecules

Photoconversion of Connexin43-CCXXCC stained with ReAsH



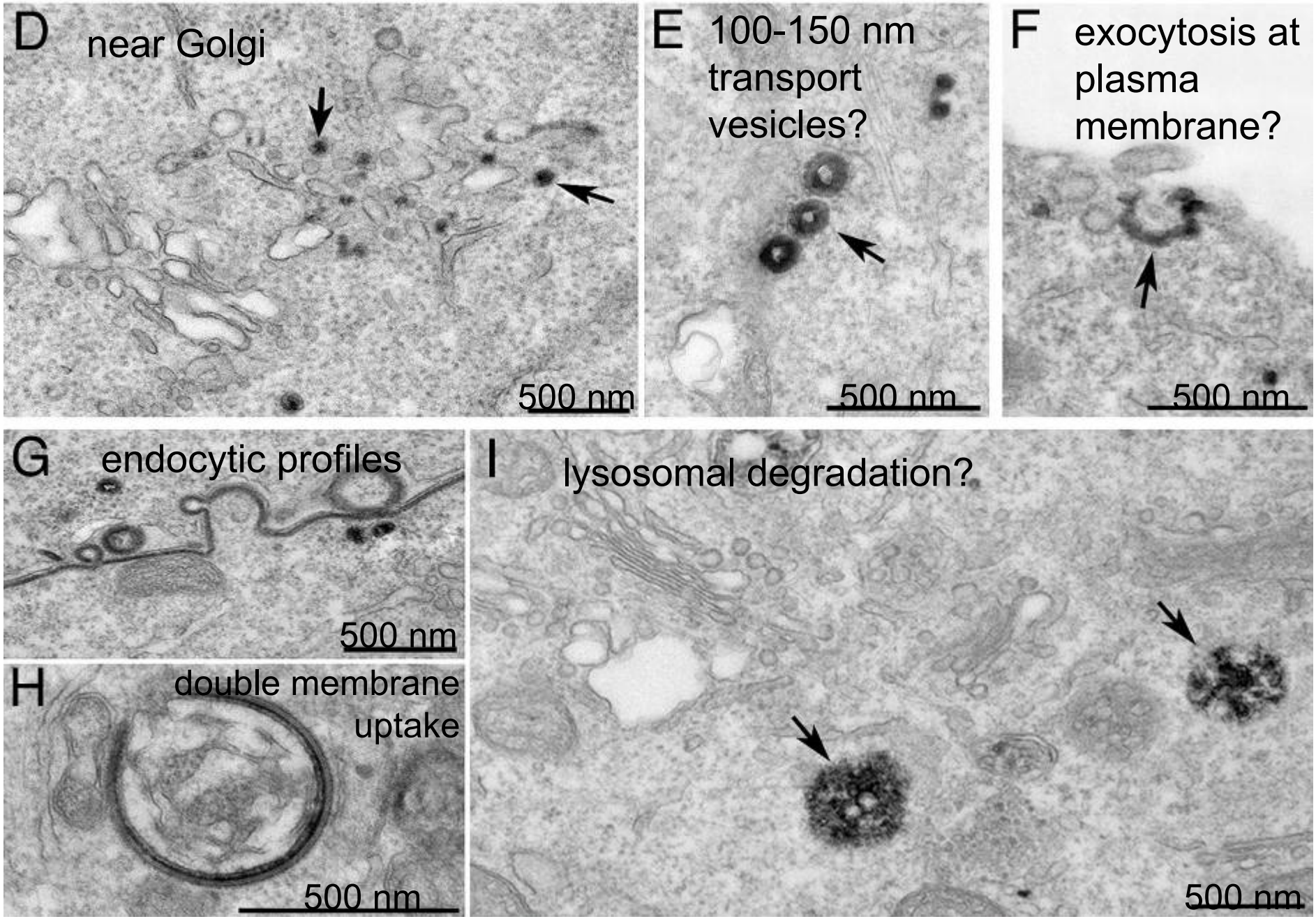
Gaietta *et al* (2002) *Science* 296: 503-507

Photoconversion of pulse-labeled ReAsH



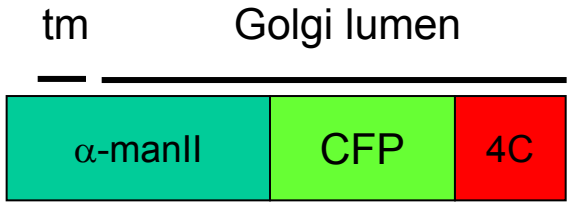
Gaietta *et al* (2002) *Science* 296: 503-507

Exo- and endocytic trafficking



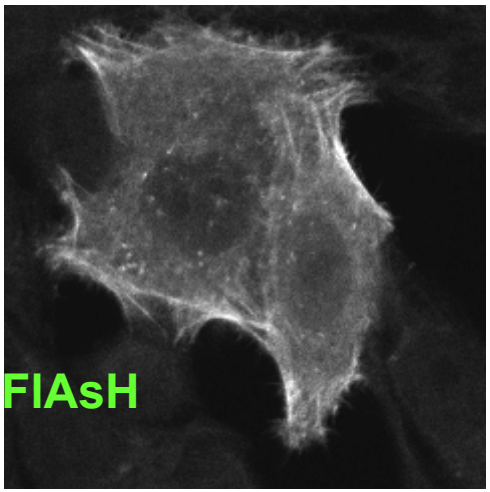
Gaietta *et al* (2002) *Science* 296: 503-507

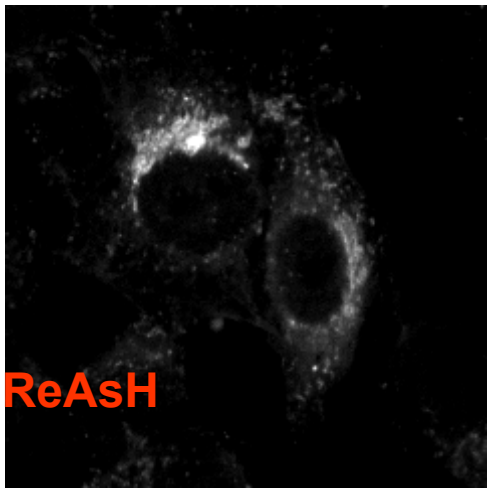
Correlated Live-Cell Imaging and EM using tetracysteine tags in oxidizing compartments- the Golgi

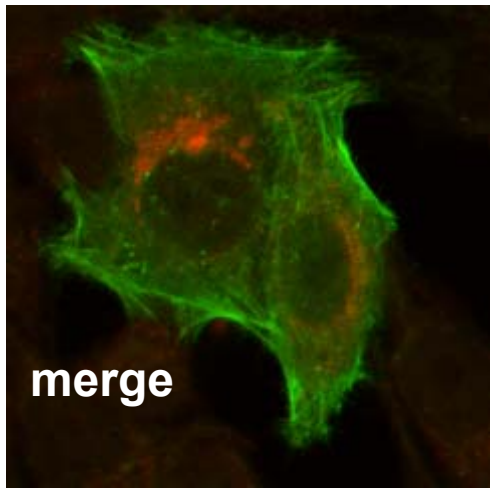


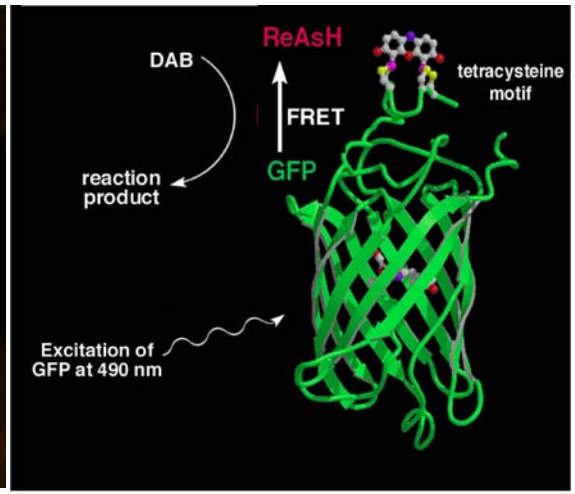
TBP (tributylphosphine) treatment allows ReAsH labeling of α-mannosidase II CFP-4cys

The luminal tetracysteine tag only labels in reducing conditions









Actin-4C stained first with FlAsH

before reduction

Man-CFP-TC stained with ReAsH with TBP reduction

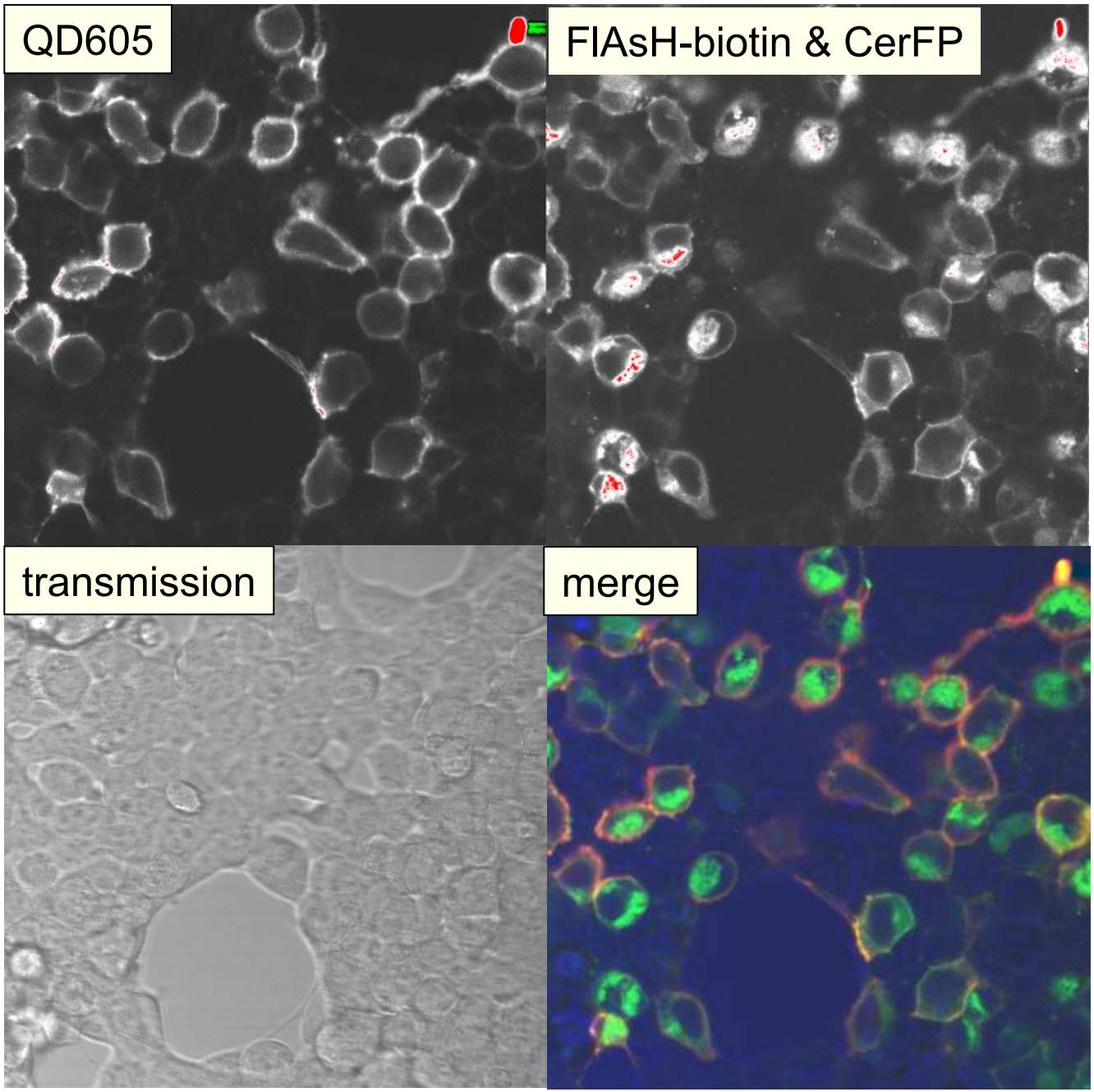
after reduction

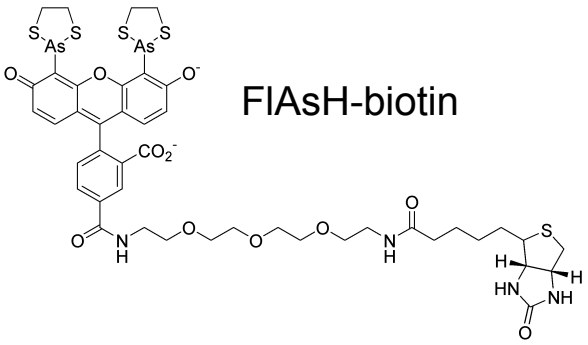
after reduction

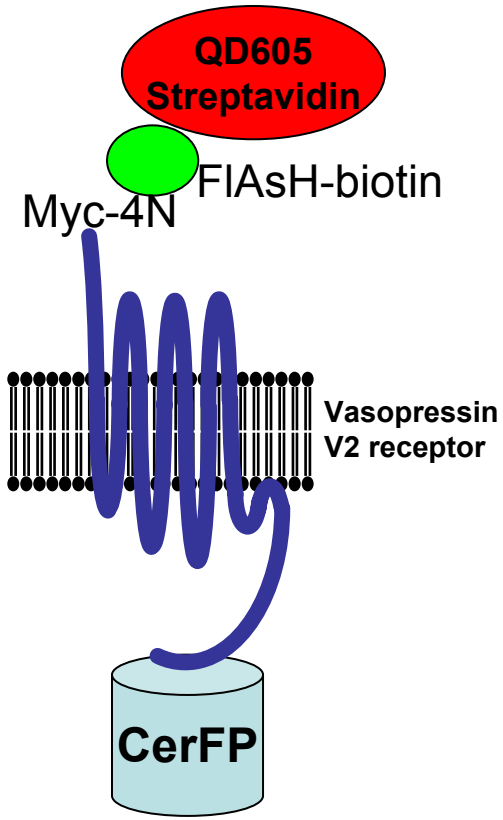
Use GFP-ReAsH FRET photoconversion for higher specificity

Guido Gaietta, Tom Deerinck
Ben Giepmans

Labeling Tetracysteines with Quantum dots



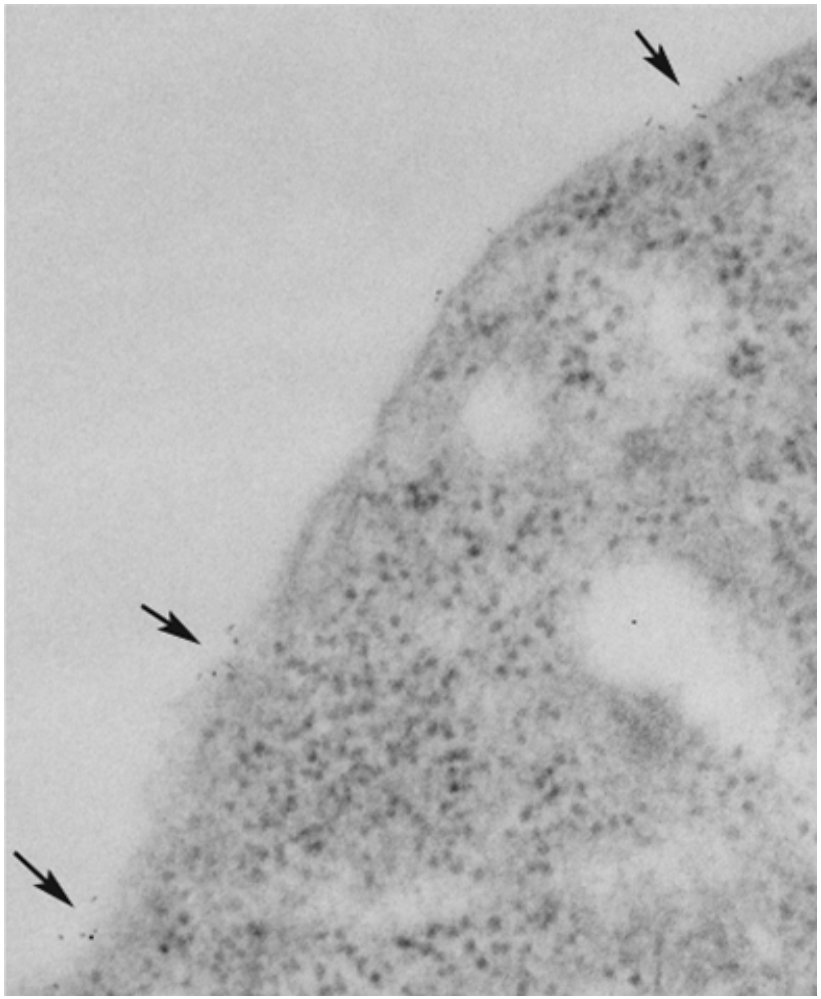


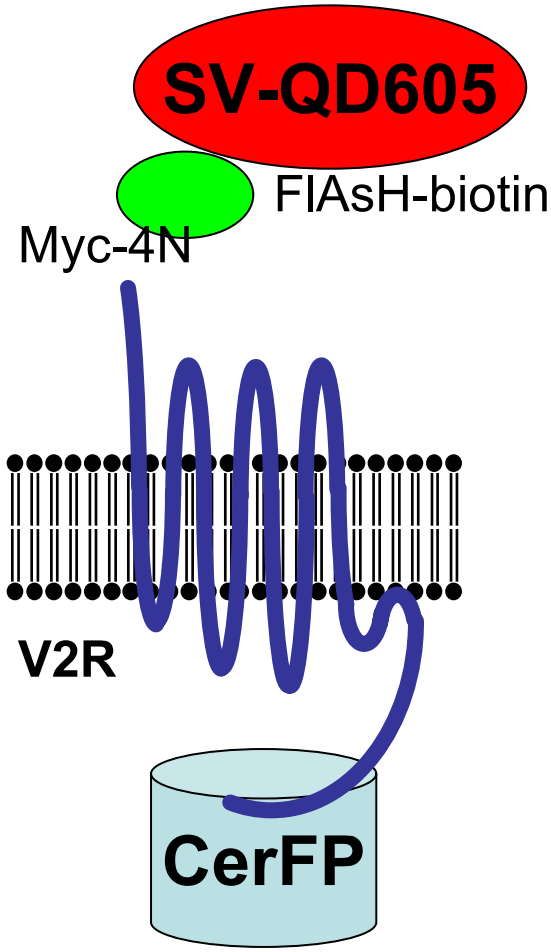


4N-Myc-V2R-receptor-CerFP

Sonia Terrillon, Ben Giepmans

FlAsH-Biotin QDs: Alternative for ReAsH-mediated photoconversion



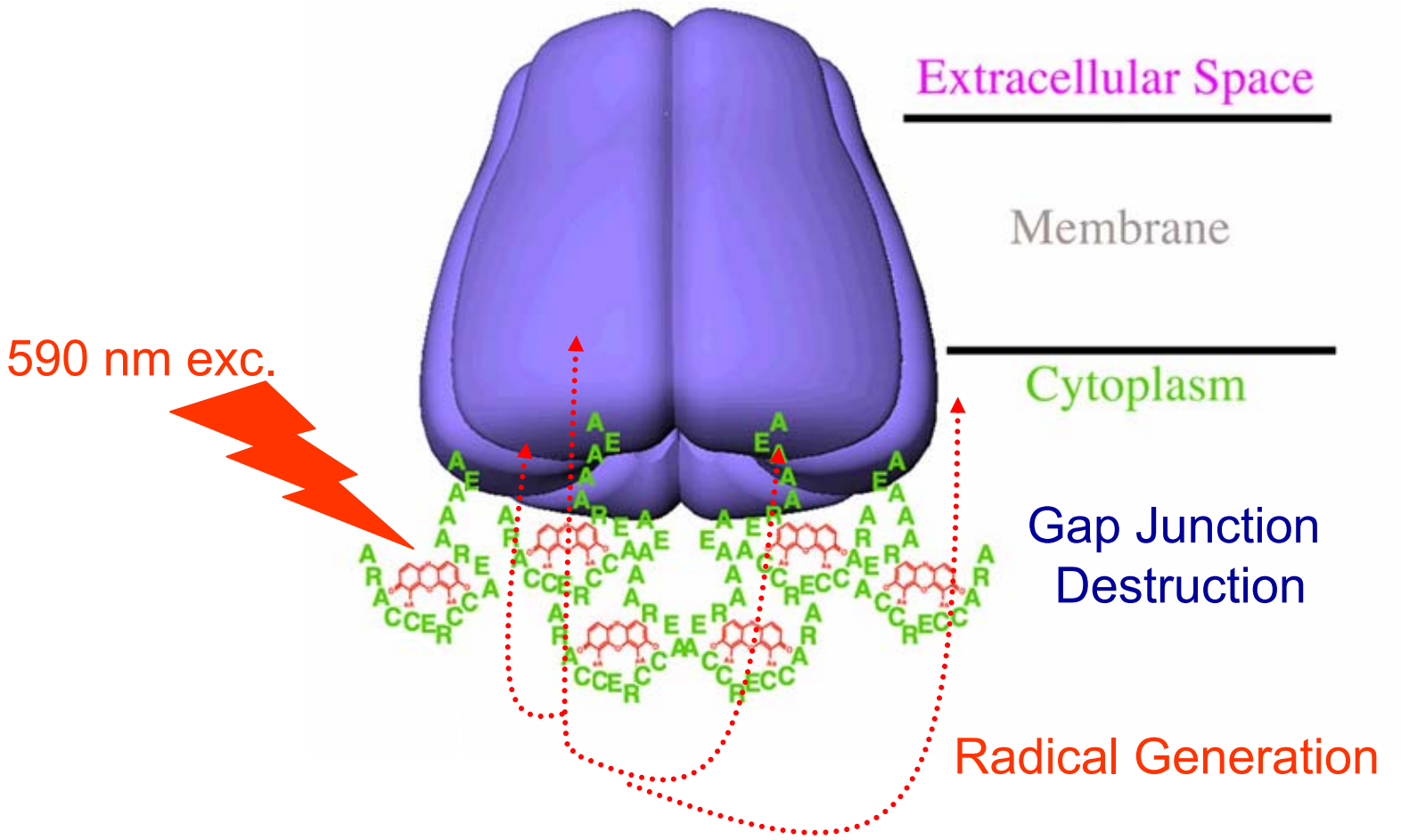


4N-Myc-V2R-receptor-CerFP

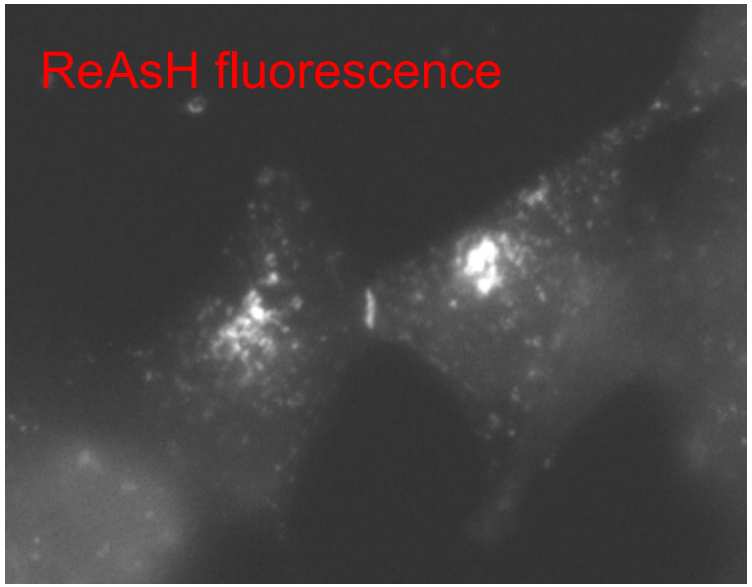
- Single-molecule detection
- Intracellular tetracysteines will require permeabilization (dead cells) or an efficient method for QD uptake in living cells such as cell penetrating peptides

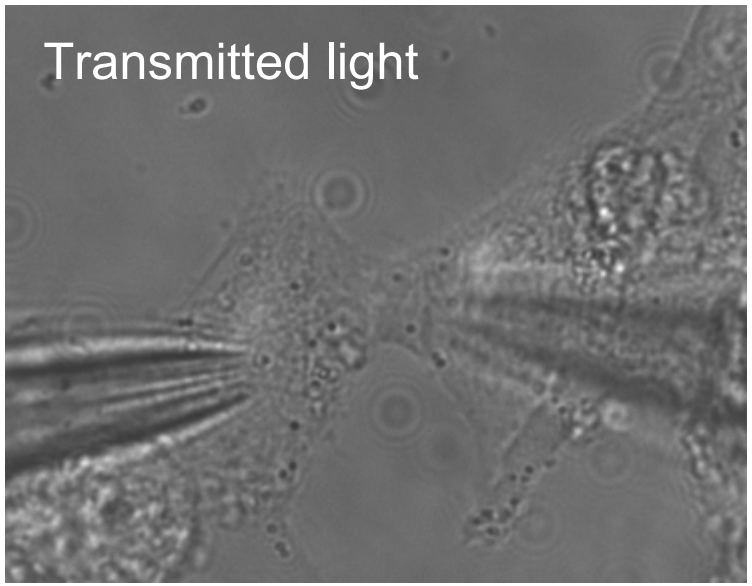
Sonia Terrillon, Ben Giepmans

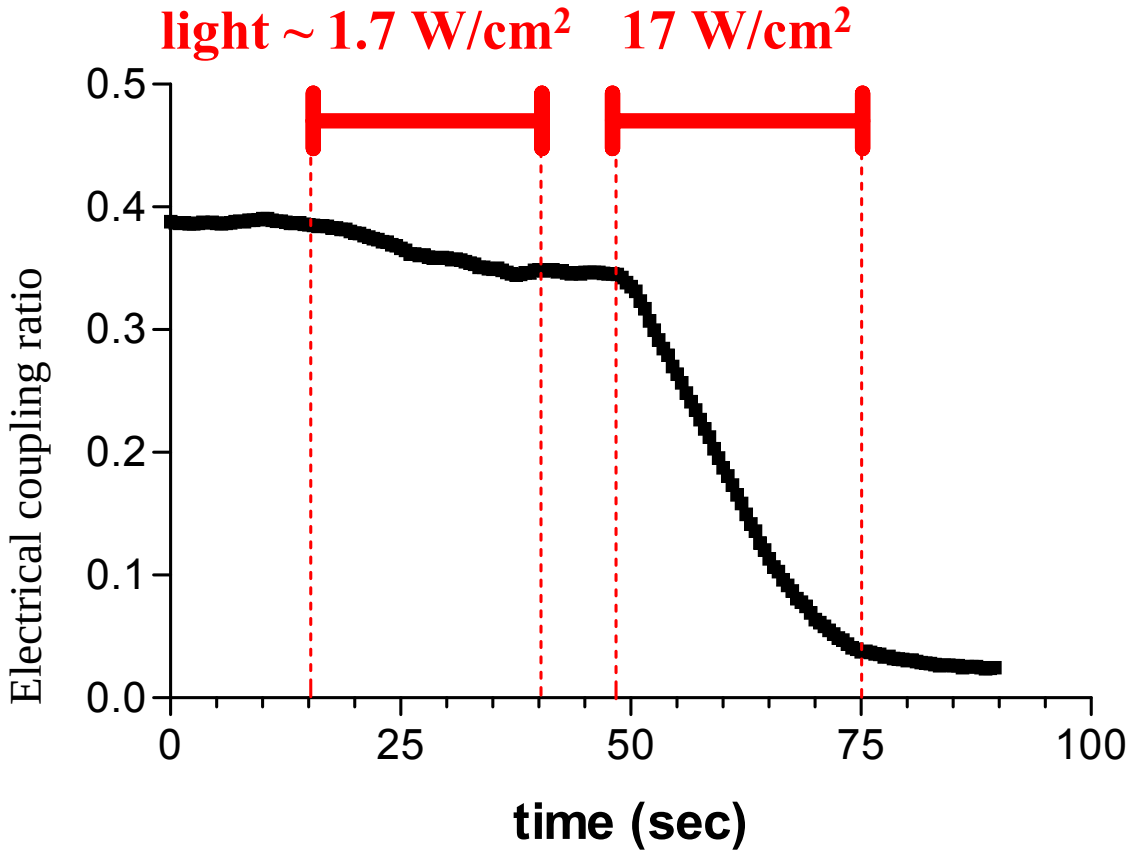
Chromophore-Assisted Light Inactivation (CALI) Of Gap Junctions using ReAsH



Strong illumination can inactivate ReAsH-stained connexins (= genetically targeted, chromophore-assisted light inactivation)





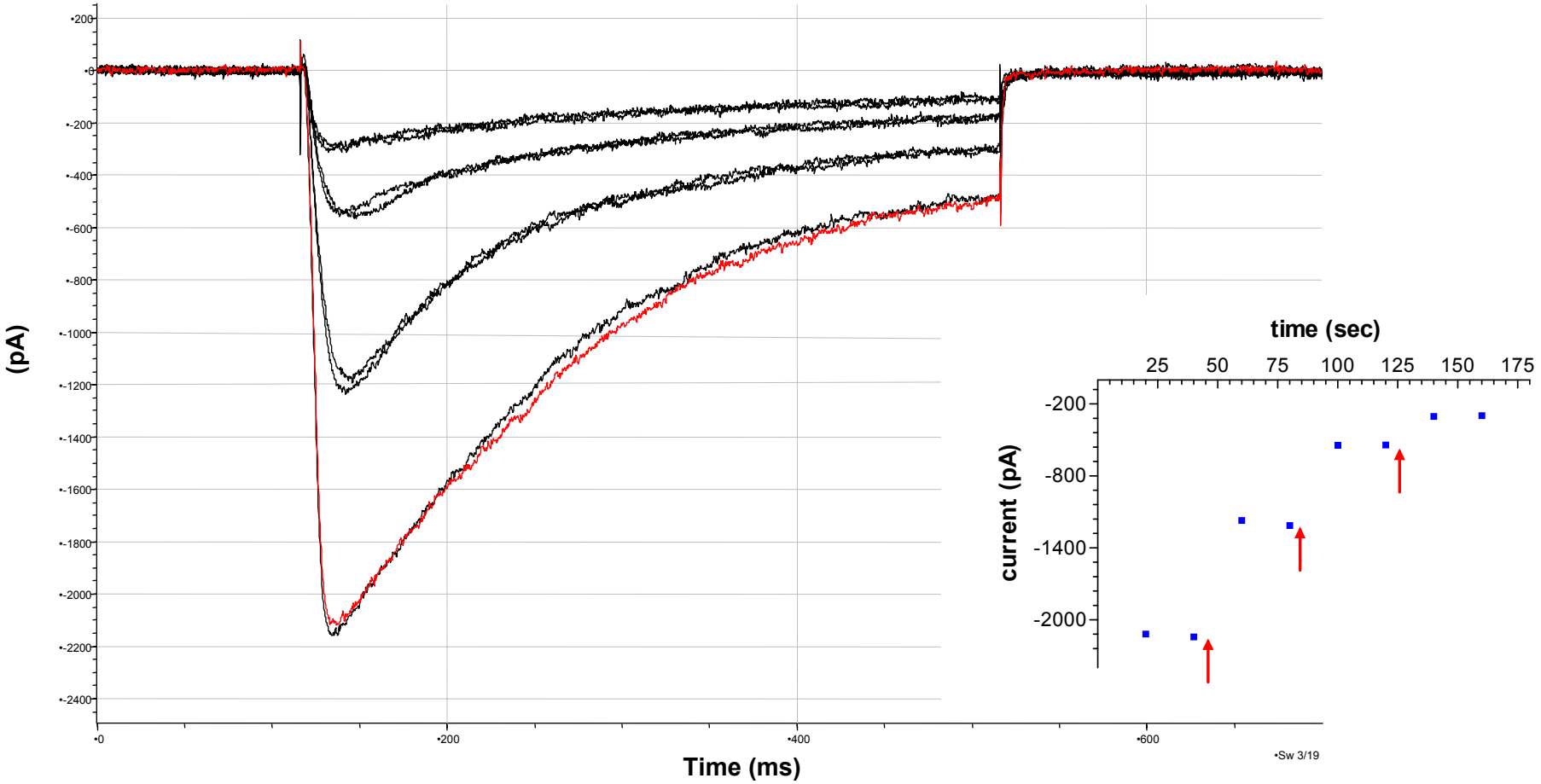


Oded Tour

Tour et al (2003) *Nature Biotech.* **21**: 1505-1508

ReAsH-mediated photoinactivation of L-type Ca^{2+} channels

3 repeats of 10 second excitation



Oded Tour

1. 86% CALI in 30 seconds
2. Good voltage clamp and stable recordings (2 to 4 sweeps between exposures).

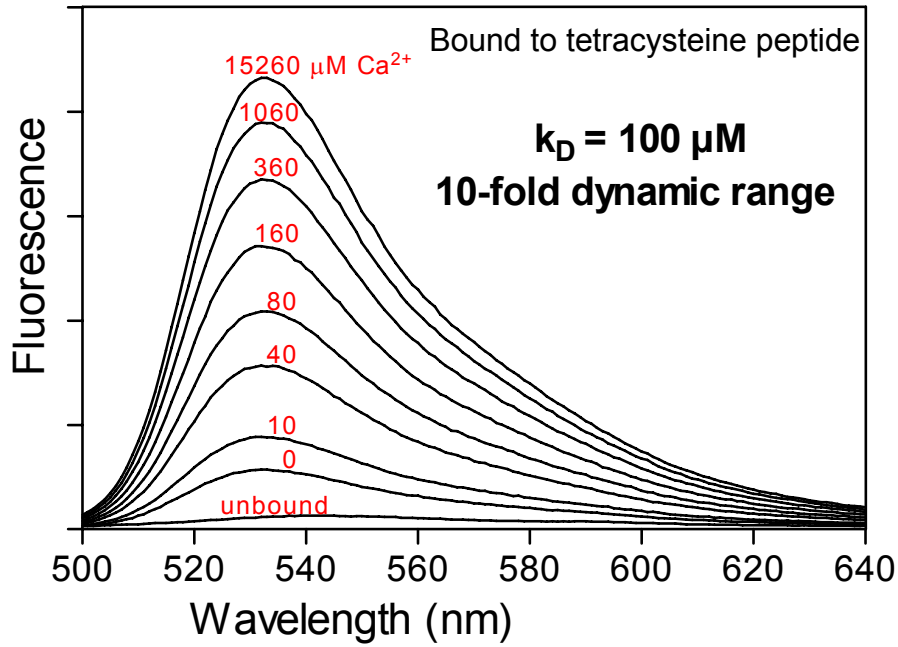
Tour et al (2003) *Nature Biotech.* **21**: 1505-1508; channel cDNA and cell line from R.W. Tsien

Tetracysteine-biarsenical CALI

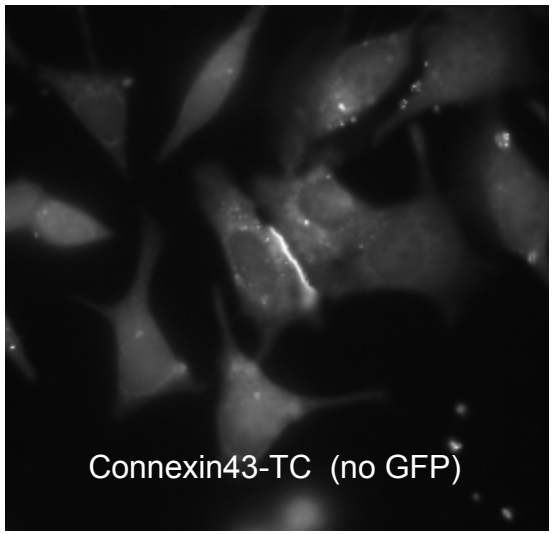
- Compared to traditional CALI, eliminates need to raise innocuous Abs, label with dye, microinject just the right amount
- Compared with noncovalent small molecule inhibitors, avoids need for custom drug development/med chem, allows isoform specificity
- Compared with gene knockout/RNA_i: much higher temporal/spatial resolution, less chance for compensation or avalanche of effects
- But only eliminates exogenous tagged copies. Ultimately, one would knock out endogenous copies, replace by tagged copies, show function is normal until CALI suddenly initiated
- Recently some FPs have been reported to undergo CALI

  Relative efficiency: ReAsH > FIAsH ~ fluorescein ~Killer Red >> malachite green ~ GFP.

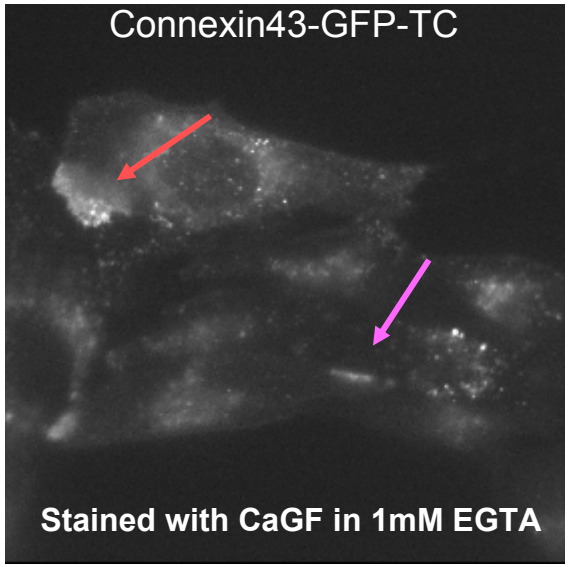
Measuring highly localized Ca^{2+} with a new biarsenical dye, Calcium Green FlAsH-EDT_2 (CaGF)

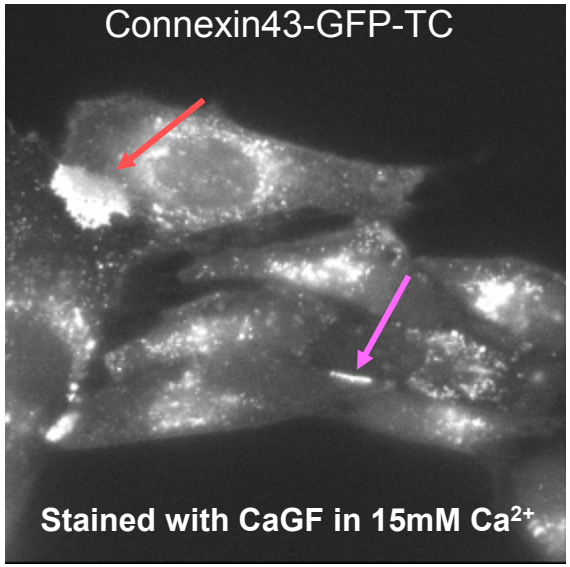


- Low affinity, intensiometric
- Fast on-off rates for Ca^{2+}
- High selectivity over Mg^{2+}
- Loaded as AM ester



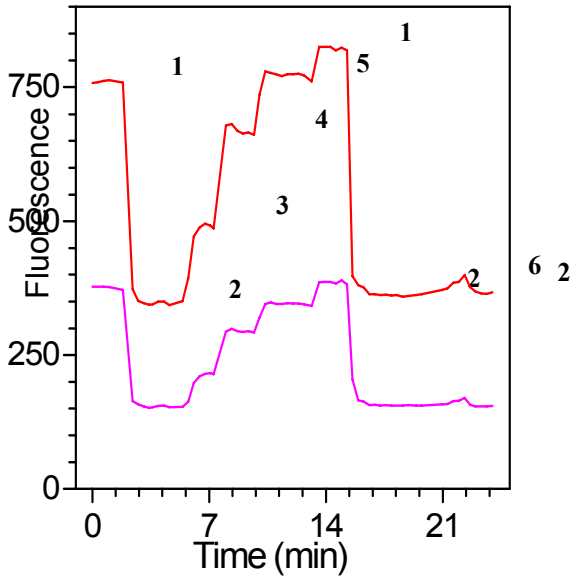
Measuring localized Ca^{2+} at gap junctions with CaGreen FlAsH

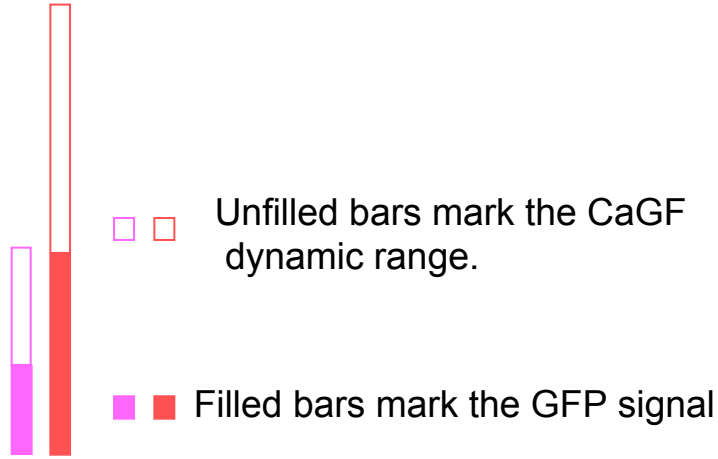


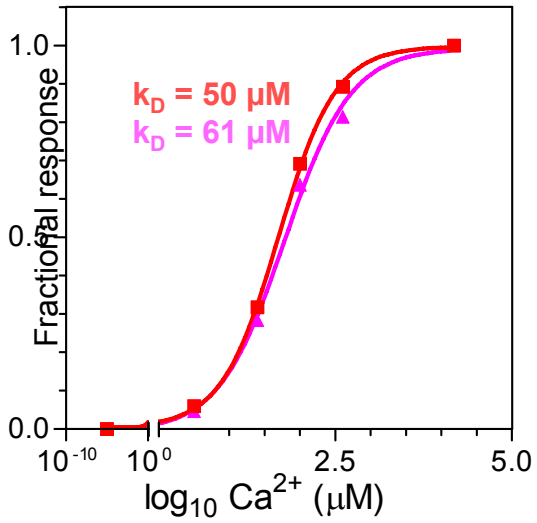


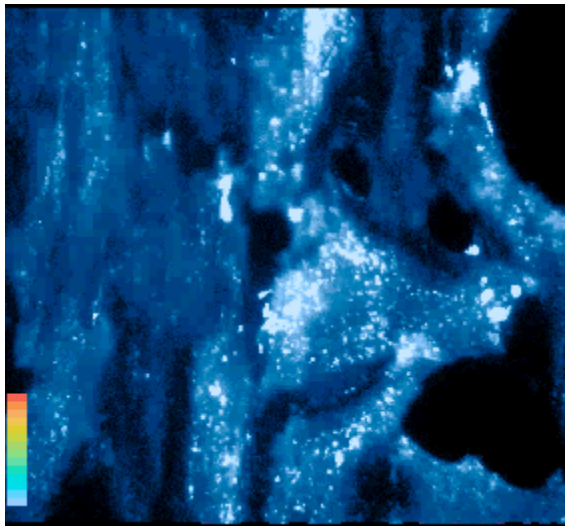
Oded Tour

In situ calibration of bound CaGF



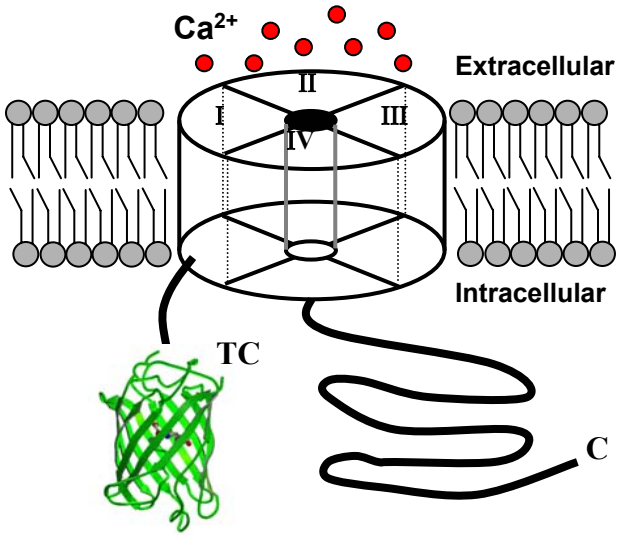


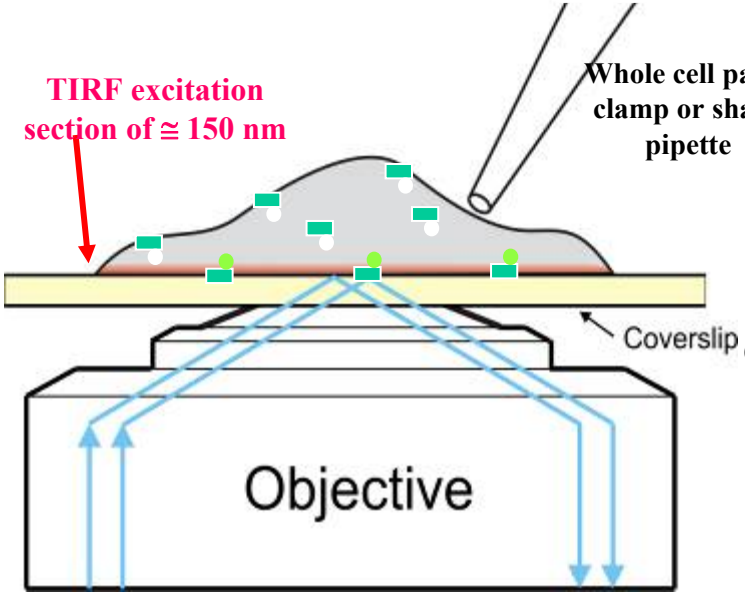




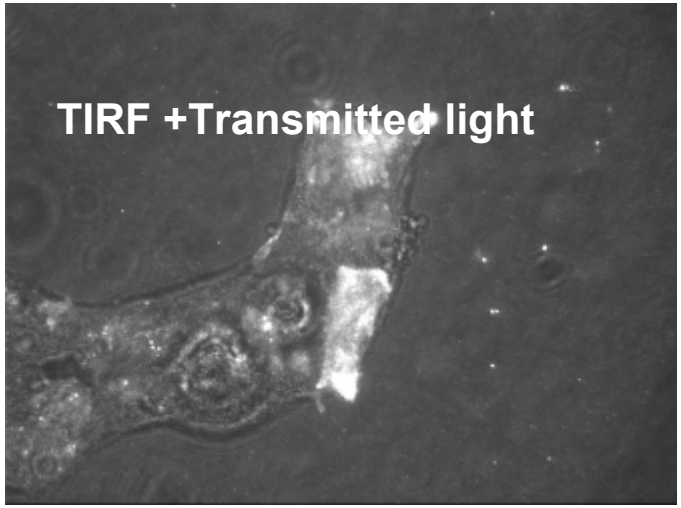
Ca^{2+} waves in HeLa cells
Poking cells with a sharp pipet

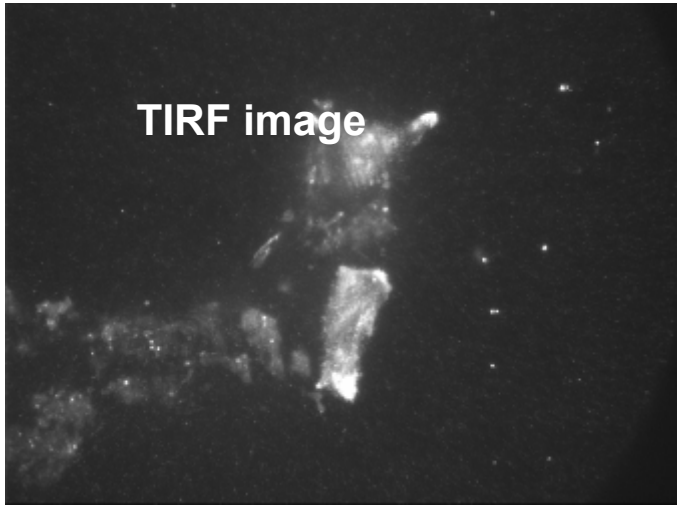
Tagging of a_{1C} calcium channel to measure local [Ca^{2+}] domains near the channel mouths





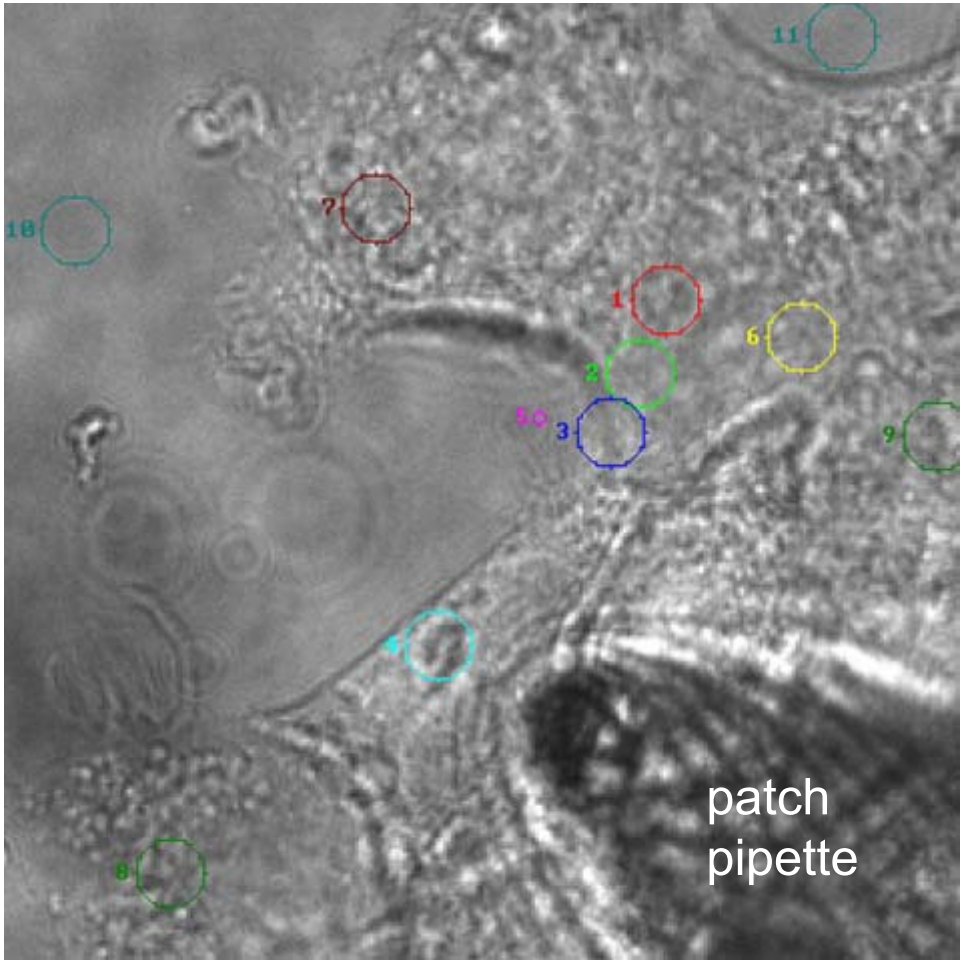
confines the observation to the plasma membrane and excludes intracellular staining



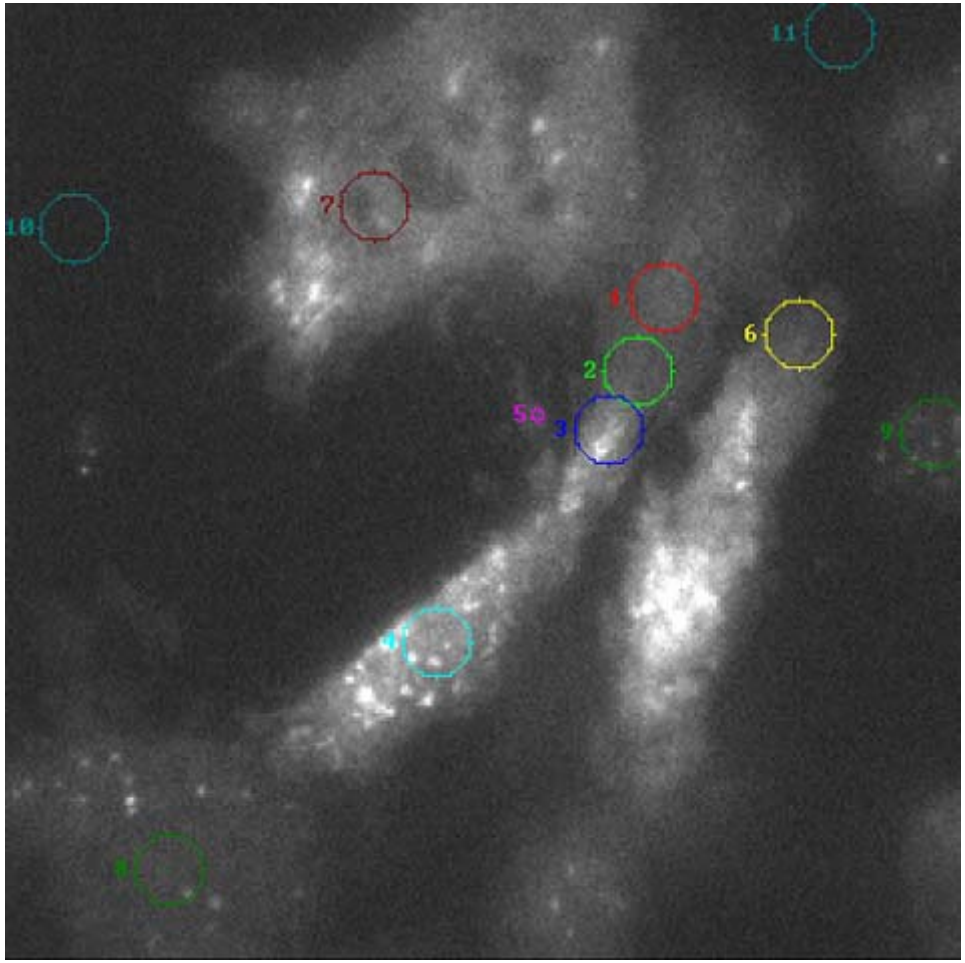


Oded Tour, channel cDNA and cell line from R.W. Tsien

Three transfected cells surrounded by untransfected cells



Transmitted light

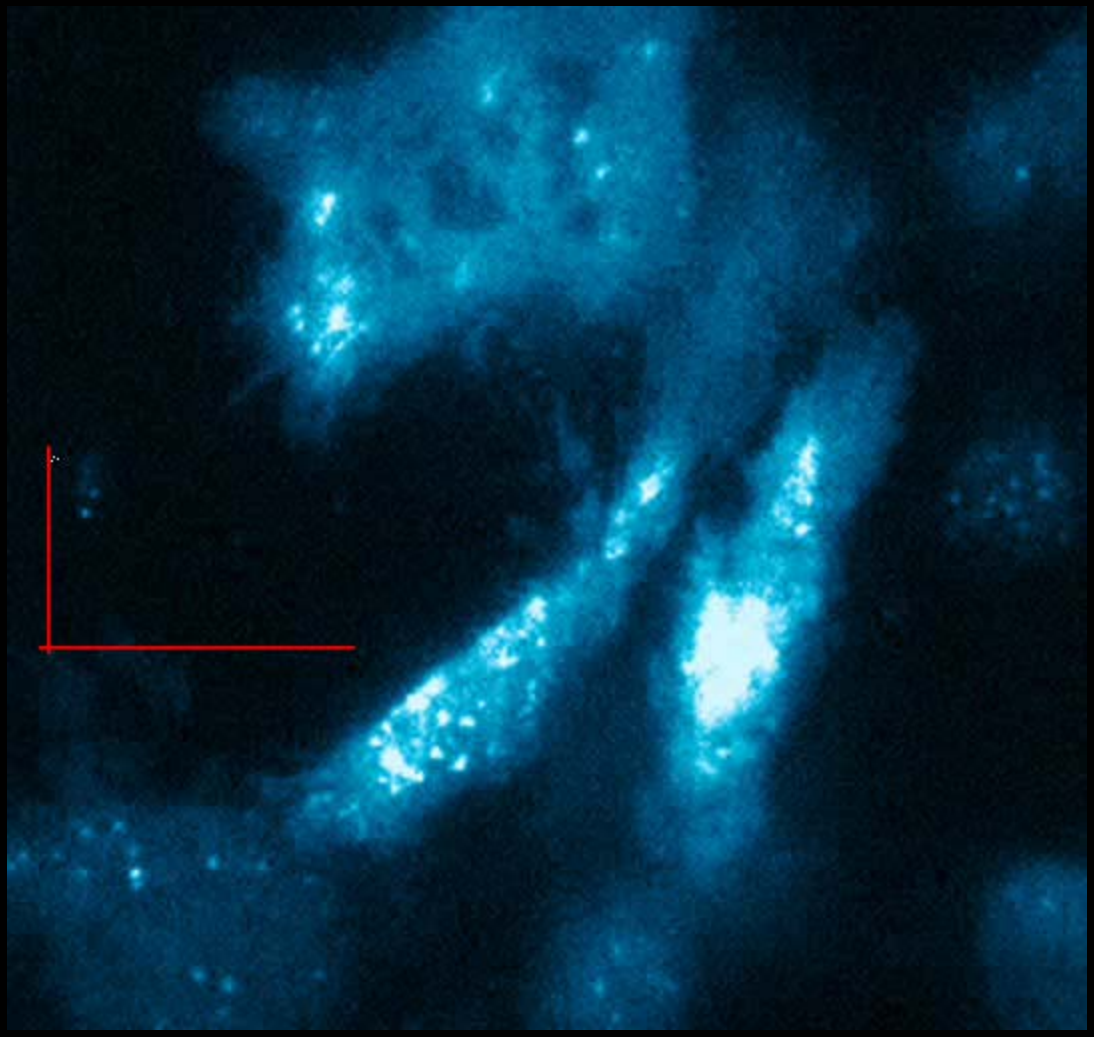


TIRF image

- HEK293 transiently-transfected with 2TC-α_{1C} Ca^{2+} channels and β_4
- Labeled with 1 μM CaGF
- Whole cell patch clamp to depolarize cell

Oded Tour, channel cDNA and cell line from R.W. Tsien

Watching Ca^{2+} channels open with Calcium Green-FlAsH



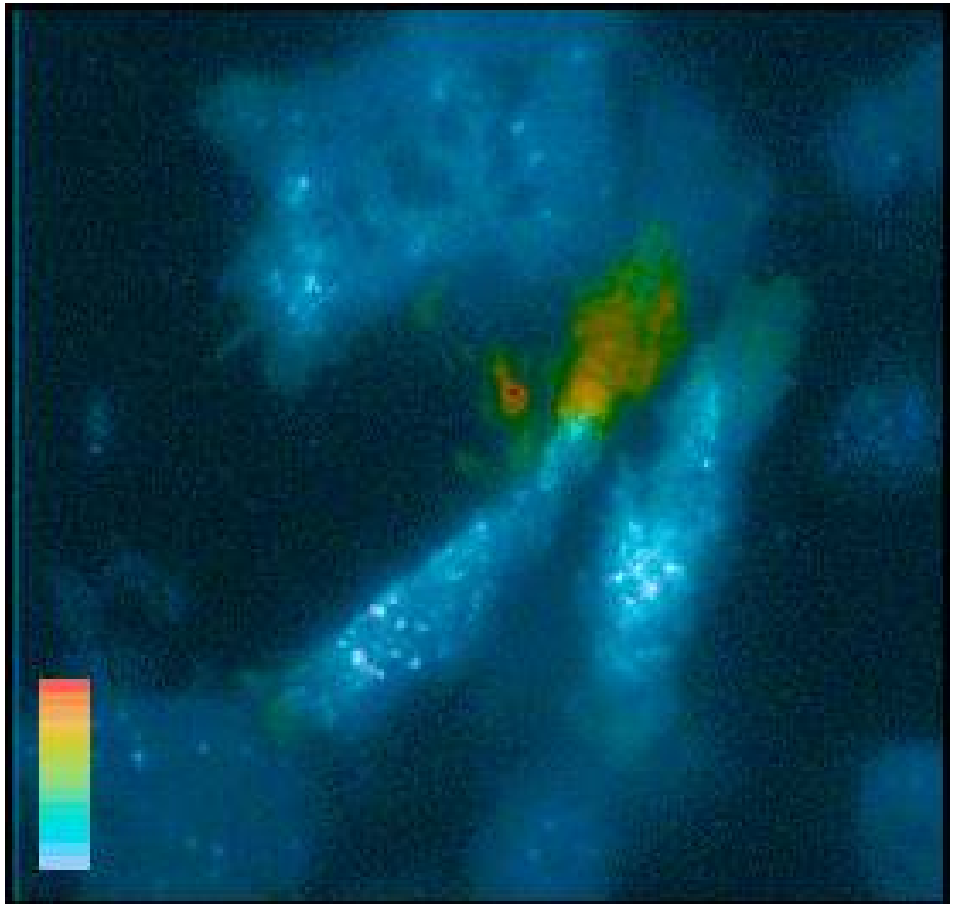
Oded Tour

CaG-FlAsH responds to ionomycin in cell regions where it does not respond to depolarization

color ratio scale: 0.82 –1.65

CaGF signal due to channel openings

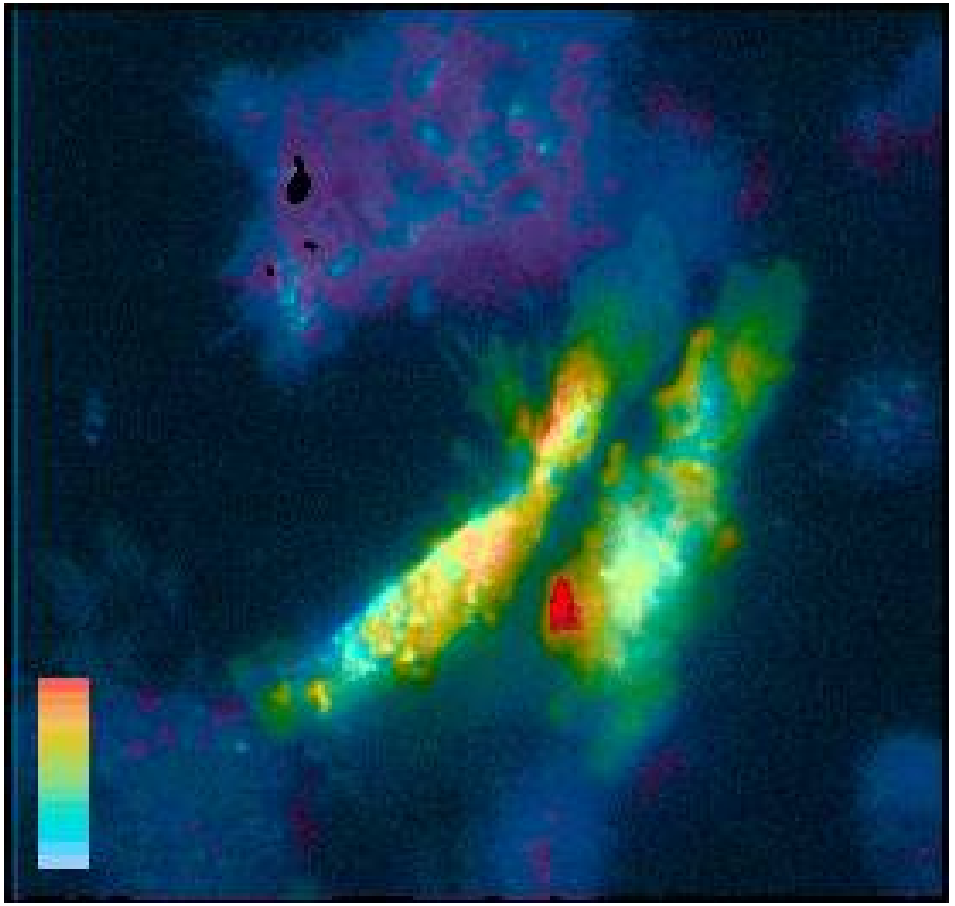
3rd picture after beginning of depolarization



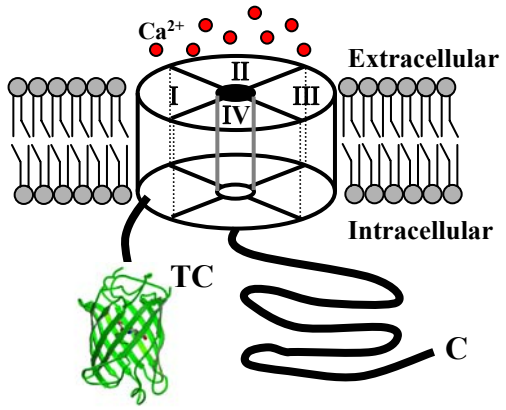
color ratio scale: 0.6 –2.7

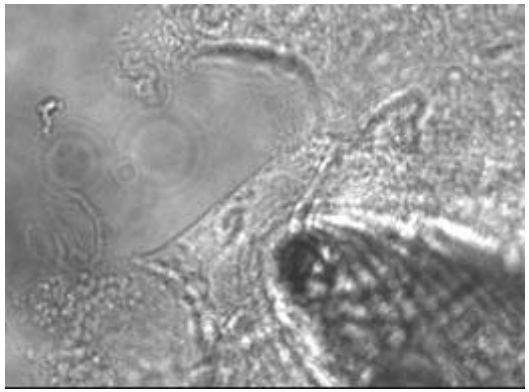
CaGF signal due to 1μM ionomycin

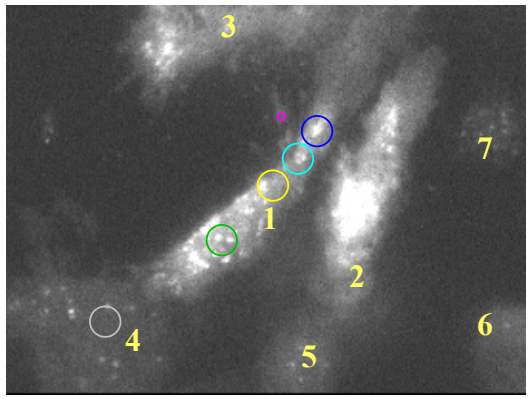
Picture #35 – peak of ionomycin response

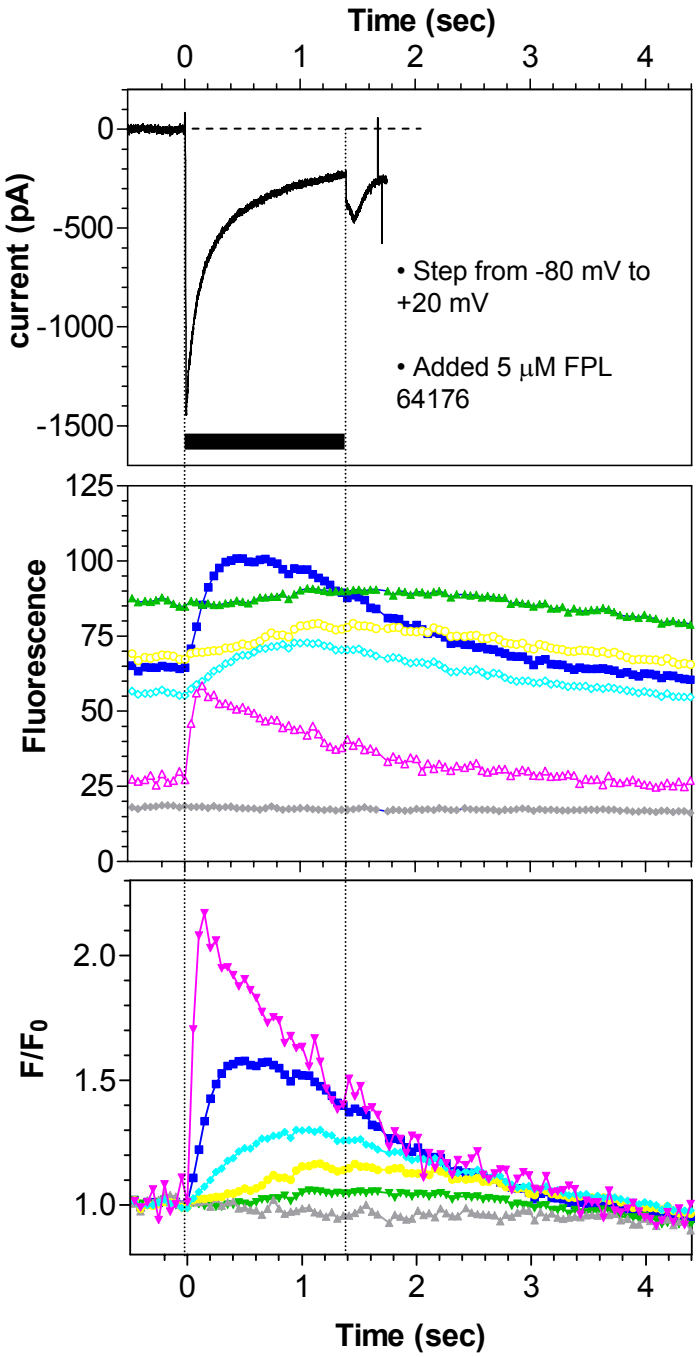


Spatial and temporal analysis of Ca^{2+} transients







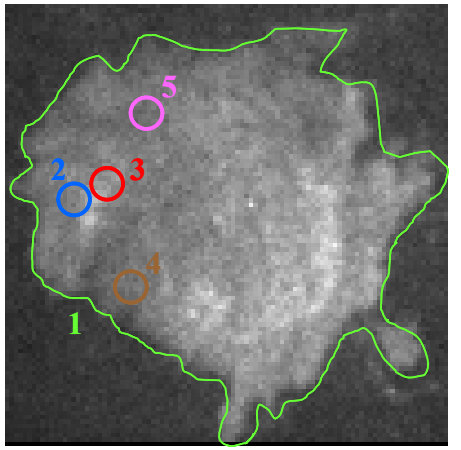


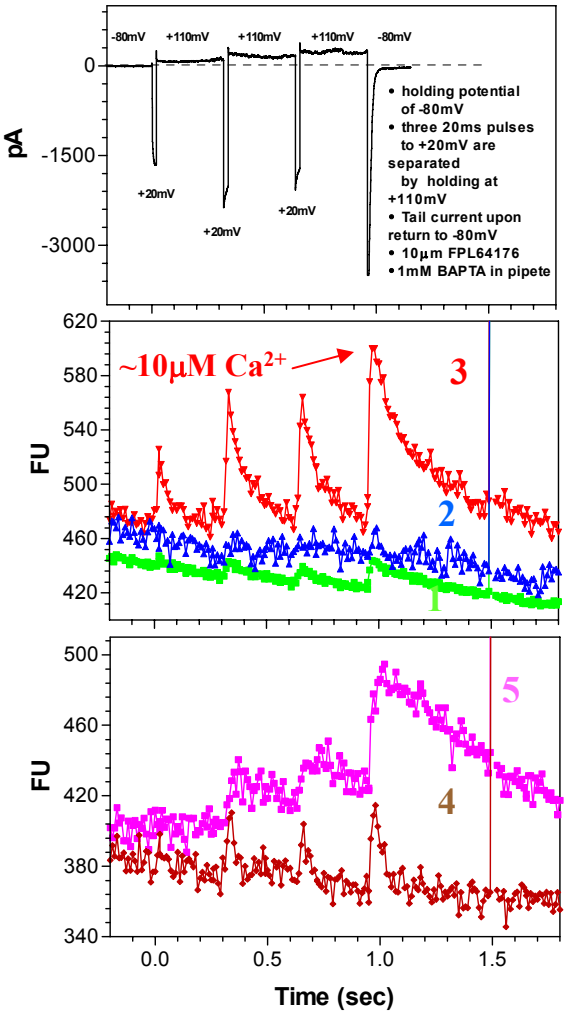
1.2 sec depolarization
With 5μM FPL 64176

- Highly localized and reproducible "hotspots" often at cell periphery with rise times <20ms (frame rate)
- Further from "hotspot" signal is slower and more complex
- Calibration at peak of "hotspots" gives 50-300 μM Ca^{2+}

Oded Tour

Imaging fast localized Ca^{2+} responses at open Ca^{2+} channels

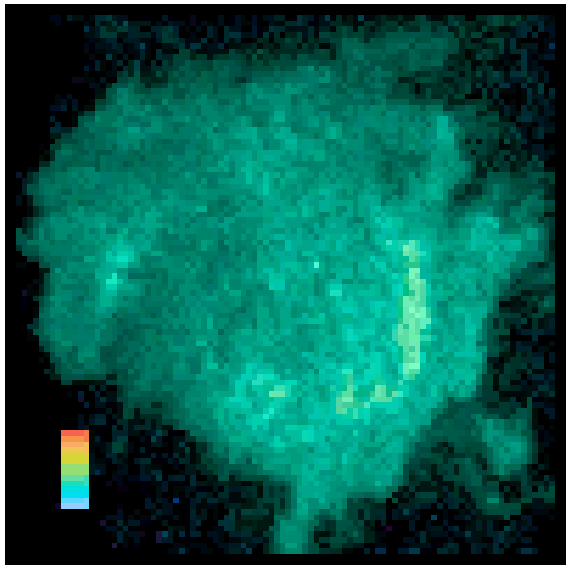




HEK293 transiently-transfected with 5N-GFP-α1C and β4

Train of 20 ms depolarizations separated by holding at +110 mV

Image every 10 ms



- Localized hotspots with different rise/fall kinetics

region 1: whole cell

region 2: area adjacent to hotspot

region 3: hotspot with peaks at ~10 µM Ca^{2+} with biphasic decay

region 4: rapid transients with decays that more closely follow current

region 5: rapid rise time but slow decay gives residual Ca^{2+}

- **Modeling of decay kinetics (Rex Kerr in Sejnowski lab) best fits localized buffering by immobile Ca^{2+} buffer**

Oded Tour

Summary: The tetracysteine-biarsenical method for labeling proteins

Applications and comparisons with Fluorescent Proteins:

- **Alternative to fluorescent proteins (FPs)** when not tolerated by protein of interest but lower sensitivity (higher background) and harder to use. Toxicity not an issue.
- **Protein turnover** by two-color biarsenical pulse chase e.g. connexin43 and GluR. Photoactivatable FPs are an alternative
- **Correlated light and electron microscopy**. Sensitivity less than for fluorescent staining but GFP-ReAsH FRET increases sensitivity
- Can differentially label different tetracysteines tags if in separate compartments such as cytoplasm, Golgi and extracellular by transient reduction
- Can label extracellular tetracysteines with streptavidin QDs using FlAsH-biotin for single molecule experiments and correlated EM
- **Protein inactivation** with high spatial and temporal resolution using CALI
- Reversible protein sequestration possible with 4N-GFP tag and ReAsH or CHoXAsH
- A low affinity **Calcium Green FlAsH** can be used to measure **localized Ca^{2+}**
- Useful for targeting other imaging agents e.g. luminescent, QDs, nanogold, MRI?
- **Protein purification** with immobilized FlAsH
- Monitor protein conformation by **fluorescence anisotropy** as rigid complex

- Successfully used in mammalian and insect cells, yeast, bacteria and virus

Acknowledgements

Albert Griffin
Larry Gross
Rob Campbell
Oded Tour *
Brent Martin *
Rene Meijer
Grant Walkup
Juan Llopis
Yong Yao

Guido Gaietta *
Tom Deerinck *
Ben Giepmans *
Gina Sosinsky
Mark Ellisman (UCSD)

Roger Tsien

References

References available on lab website

www.tsienlab.ucsd.edu

click on Publications

Fluorescent Protein Requests

www.tsienlab.ucsd.edu

click on Samples