Fluorescent protein-based tools for neuroscience

An animated primer on biosensor development

Robert E. Campbell
Department of Chemistry

UNIVERSITY OF ALBERTA

東京大学 THE UNIVERSITY OF TOKYO

Imaging Structure & Function in the Nervous System
Cold Spring Harbor, July 31, 2019.

Outline

- [ ] Fluorescent proteins (FPs)
- [ ] Other fluorophore technologies
- [ ] Single FP-based biosensors

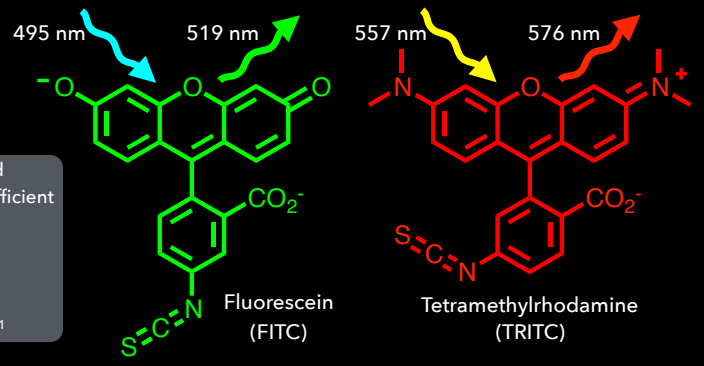
Lots of structural information

No molecular information

Lots of structural information & fluorescent color provides molecular information

(more colors = more information)

Fluorescence microscopy requires fluorophores



ϕ = quantum yield
ε = extinction coefficient
Brightness ~ $\phi * \varepsilon$

i.e., for fluorescein
ϕ = 0.92
ε = 73,000 $M^{-1}cm^{-1}$

Non-natural fluorophores made by chemical synthesis

Non-natural fluorophores for protein labelling

Trends Bioch. Sci., **1984**, 9, 88-91.

Emerging Techniques

Fluorescent analog cytochemistry

D. Lansing Taylor, P. A. Amato, K. Luby-Phelps and P. McNeil

Functional molecules or organelles, covalently labeled with fluorescent probes, can be re-incorporated into living cells where they reveal native molecular activity in a wide variety of cellular processes.

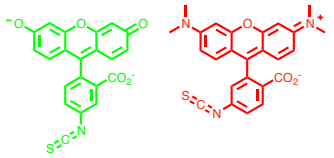
Fluorescent analog cytochemistry is a new approach to elucidating the behavior, interaction and spatial organization of specific cellular components in living cells[1,2]. A fluorescent analog consists of the native cellular component plus one or more covalently attached fluorescent probes, chosen for their spectral properties and/or environmental sensitivities. The technique, previously called molecular cytochemistry[3], takes advantage of the sensitivity of fluorescence detection methods and the specificity of fluorescently-labeled molecules. Cells containing fluorescent analogs can be analysed by qualitative and/or quantitative fluorescence microscopy and by flow cytometry.

Basic principles

Five sequential steps are involved: (1) purification of the molecule or organelle, and fluorescent labeling to produce the fluorescent analog which is selected for by purifying a defined ratio of dye to molecule and an absence of unbound fluorescent dye; (2) comparison of the biochemical, biophysical and physiological properties of the fluorescent analog with those of its unlabeled counterparts *in vitro*; (3) characterization of the spectroscopic properties of the analog *in vitro*, both alone and in combination with other molecular species with which it may interact *in vitro*; (4) incorporation of the analog into living cells followed by testing the functional capability of the analog *in vitro*; and (5) analysis of the cells containing both the fluorescent analog and a soluble control molecule labeled with a distinct fluorophore. Some of the technical and biological aspects of this method as applied to contractile proteins have been reviewed recently[2-4].

A fluorescent analog of the cytoskeletal protein actin has been characterized in detail[5]. Actin labeled at Cys 374 with 5-iodoacetamido-fluorescein (AF-actin) polymerizes into F-actin filaments forms Mg^{2+} paracrystals and activates myosin magnesium ATPase, all to a degree comparable with unlabeled actin controls. The labeled filaments appear normal under the electron microscope and behave as expected in cell extracts capable of gelation, solution

The authors are at the Center for Fluorescence Research in Biomedical Sciences, and the Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, PA 15213, USA.



Proteins of interest

A non-natural fluorophore must be chemically linked to a protein of interest…

Getting non-natural fluorophores into a cell

Trends Bioch. Sci., **1984**, 9, 88-91.

Emerging Techniques

Fluorescent analog cytochemistry

D. Lansing Taylor, P. A. Amato, K. Luby-Phelps and P. McNeil

Functional molecules or organelles, covalently labeled with fluorescent probes, can be re-incorporated into living cells where they reveal native molecular activity in a wide variety of cellular processes.

Fluorescent analog cytochemistry is a new approach to elucidating the behavior, interaction and spatial organization of specific cellular components in living cells[1,2]. A fluorescent analog consists of the native cellular component plus one or more covalently attached fluorescent probes, chosen for their spectral properties and/or environmental sensitivities. The technique, previously called molecular cytochemistry[3], takes advantage of the sensitivity of fluorescence detection methods and the specificity of fluorescently-labeled molecules. Cells containing fluorescent analogs can be analysed by qualitative and/or quantitative fluorescence microscopy and by flow cytometry.

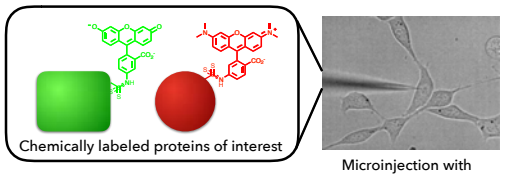
Basic principles

Five sequential steps are involved: (1) purification of the molecule or organelle, and fluorescent labeling to produce the fluorescent analog which is selected for by purifying a defined ratio of dye to molecule and an absence of unbound fluorescent dye; (2) comparison of the biochemical, biophysical and physiological properties of the fluorescent analog with those of its unlabeled counterparts *in vitro*; (3) characterization of the spectroscopic properties of the analog *in vitro*, both alone and in combination with other molecular species with which it may interact *in vitro*; (4) incorporation of the analog into living cells followed by testing the functional capability of the analog *in vitro*; and (5) analysis of the cells containing both the fluorescent analog and a soluble control molecule labeled with a distinct fluorophore. Some of the technical and biological aspects of this method as applied to contractile proteins have been reviewed recently[2-4].

A fluorescent analog of the cytoskeletal protein actin has been characterized in detail[5]. Actin labeled at Cys 374 with 5-iodoacetamido-fluorescein (AF-actin) polymerizes into F-actin filaments forms Mg^{2+} paracrystals and activates myosin magnesium ATPase, all to a degree comparable with unlabeled actin controls. The labeled filaments appear normal under the electron microscope and behave as expected in cell extracts capable of gelation, solution

The authors are at the Center for Fluorescence Research in Biomedical Sciences, and the Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, PA 15213, USA.

…and then manually injected into a cell



Microinjection with micropipet

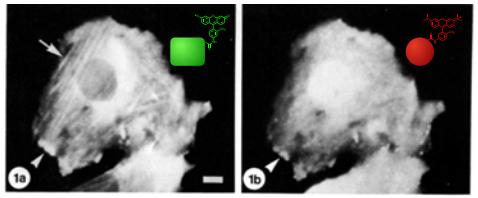
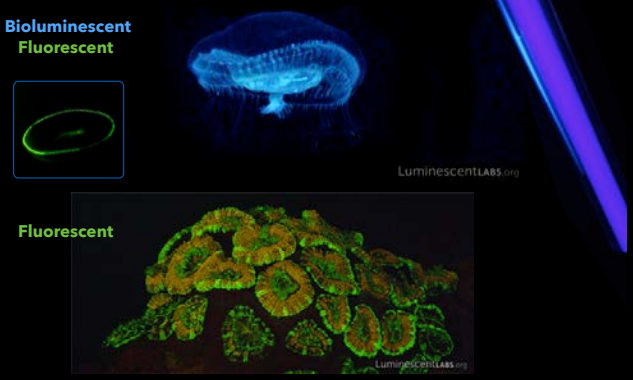


Fig. 1. A spreading 3T3 cell co-injected with (a) fluorescein labeled actin and (b) rhodamine-labeled ovalbumin. The actin fluorescence is most intense in stress fibers (arrows) and in the ruffles at the leading edge of cells (arrowhead). The soluble ovalbumin control distributes throughout the cytoplasm and serves as a control for pathlength and accessible volume. Note the elevated fluorescence intensity of rhodamine-labeled ovalbumin in the ruffles (arrowhead). Bar = 10 μm.

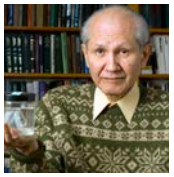
Some sea creatures make natural fluorophores



Some natural fluorophores are genetically encoded proteins and can be transplanted into cells as DNA!

http://www.luminescentlabs.org/

The discovery of a protein fluorophore



Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous Hydromedusan, *Aequorea*[1]

OSAMU SHIMOMURA,[2] FRANK H. JOHNSON AND YO SAIGA
Department of Biology, Princeton University, Princeton, New Jersey, and the Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington

respect from the greenish luminescence of the whole organism or fresh squeezates.[3] The light-emitting potency was calculated to be 42,500 L.U./mg, which is equivalent to the total light of 6.6 ug of *Cypridina* luciferin whose molecular weight is 469 (Hirata, Shimomura and Eguchi, '59).

Ultraviolet absorption spectrum

Except for a slight bulge at 310 mμ, the ultraviolet absorption spectrum (fig. 3) is similar to that of simple proteins, with a peak at 280 mμ. After the luminescent reaction the bulge at 310 mμ disappears and a new absorption maximum shows up at 333 mμ.

Requirement of Ca^{++} for luminescence, and effects of other cations

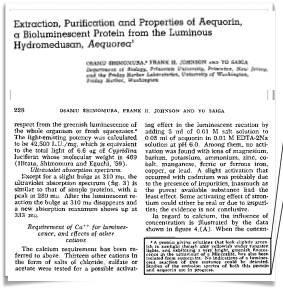
The calcium requirement has been referred to above. Thirteen other cations in the form of salts of chloride, sulfate or acetate were tested for a possible activating effect in the luminescent reaction by adding 5 ml of 0.01 M salt solution to 0.05 ml of aequorin in 0.01 M EDTA-2Na solution at pH 6.0. Among them, no activation was found with ions of magnesium, barium, potassium, ammonium, zinc, cobalt, manganese, ferric or ferrous iron, copper, or lead. A slight activation that occurred with cadmium was probably due to the presence of impurities, inasmuch as the purest available substance had the least effect. Some activating effect of strontium could either be real or due to impurities; the evidence is not conclusive.

In regard to calcium, the influence of concentration is illustrated by the data shown in figure 4.(A). When the concen-

[3] A protein giving solutions that look slightly greenish in sunlight though only yellowish under tungsten lights, and exhibiting a very bright, greenish fluorescence in the ultraviolet of a Mineralite, has also been isolated from squeezates. No indications of a luminescent reaction of this sustance could be detected. Studies of the emission spectra of both this protein and aequorin are in progress.

Shimomura et al., J. Cell. Comp. Physiol. **1962**, 59, 223.

The discovery of a protein fluorophore

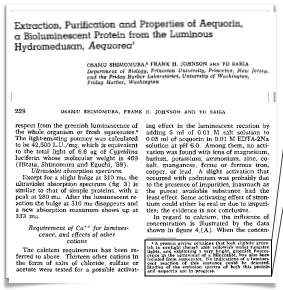


[3] A protein giving solutions that look slightly greenish in sunlight though only yellowish under tungsten lights, and exhibiting a very bright, greenish fluorescence in the ultraviolet of a Mineralite, has also been isolated from squeezates. No indications of a luminescent reaction of this sustance could be detected. Studies of the emission spectra of both this protein and aequorin are in progress.

Shimomura isolated and, over decades, performed extensive studies on this **Green Fluorescent Protein (GFP)**

Shimomura et al., J. Cell. Comp. Physiol. **1962**, 59, 223.

30 years later, the gene for GFP was cloned by Prasher



1962 - 1992

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

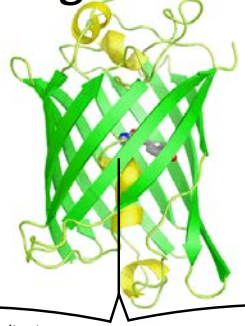
Douglas C. Prasher*, Virginia K. Eckenrode*, William W. Ward†, Frank G. Prendergast‡ and Milton J. Cormier*

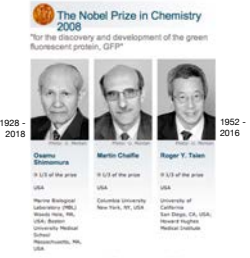
SUMMARY

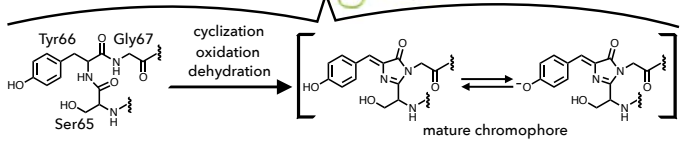
Many coelenterates utilize green-fluorescent proteins (GFPs) as energy-transfer receptors in bioluminescence. GFPs fluoresce in vivo upon receiving energy from either a luciferase-oxyluciferin excited-state complex or a Ca^{2+}-activated photoprotein. These highly fluorescent proteins are unique due 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 236-aa-residue polypeptide with a calculated M_r of 26 DSA. 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 AGFP2 genomic clone is comprised of at least three exons spread over 2.4 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.

Shimomura et al., J. Cell. Comp. Physiol. **1962**, 59, 223; Prasher et al., Gene **1992**, 111, 229.

Aequorea jellyfish green fluorescent protein

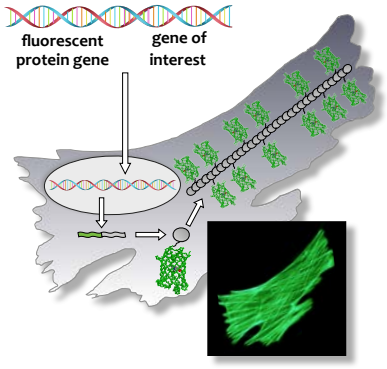


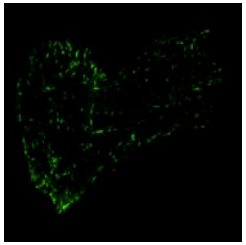


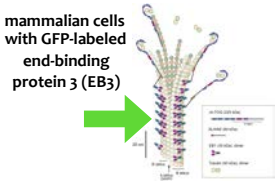


Shimomura, FEBS Lett. **1979**, 104, 220; Ormö et al., Science **1996**, 273, 1392; Tsien, Annu. Rev. Biochem. **1998**, 67, 509; http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2008/

Typical live cell imaging using GFP

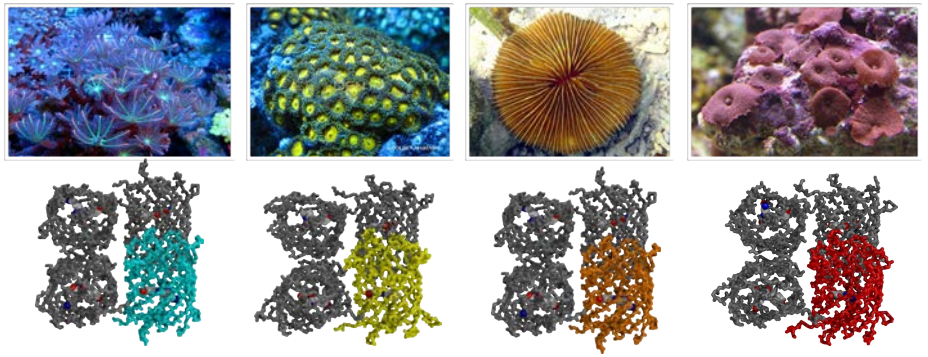






Movie and image from Michael W. Davidson; Microtubule schematic from Nakamura et al., PLoS ONE, **2012**, 7(12), e51442

Natural FP color variants in coral and anemone



cyan FP from *Clavularia* sp.

yellow FP from *Zoanthus* sp.

orange FP from *Fungia concinna*

red FP from *Discosoma* sp.

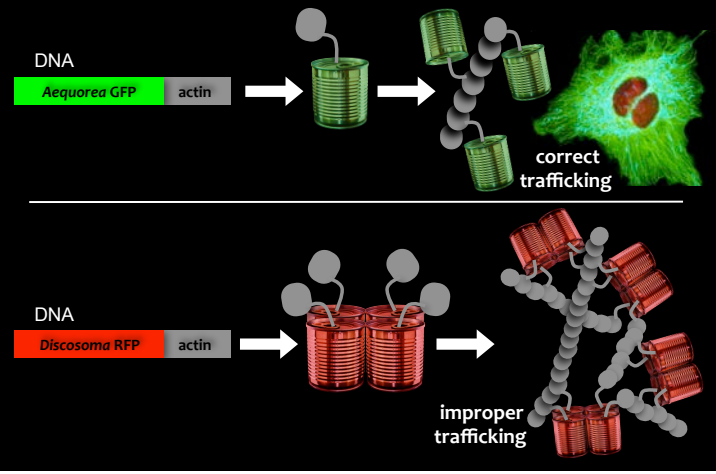
Matz et al., Nat. Biotechnol. **1999**, 17, 969; Henderson & Remington, Proc. Natl. Acad. Sci. U. S. A. **2005**, 102, 12712; Remington et al., Biochemistry **2005**, 44, 202; Kikuchi et al., Biochemistry **2008**, 47, 11573;Yarbrough et al., Proc. Natl. Acad. Sci. U. S. A. **2001**, 98, 462. Images: reefguide.org; goldenmarindo.com; www.seascapestudio.net.

RFP and GFP have similar chromophores

***Aequorea* GFP** ***Discosoma* RFP**

Shimomura, FEBS Lett. **1979**, 104, 220; Gross et al., Proc. Natl. Acad. Sci. U. S. A. **2000**, 97, 11990.

The trouble with tetramers



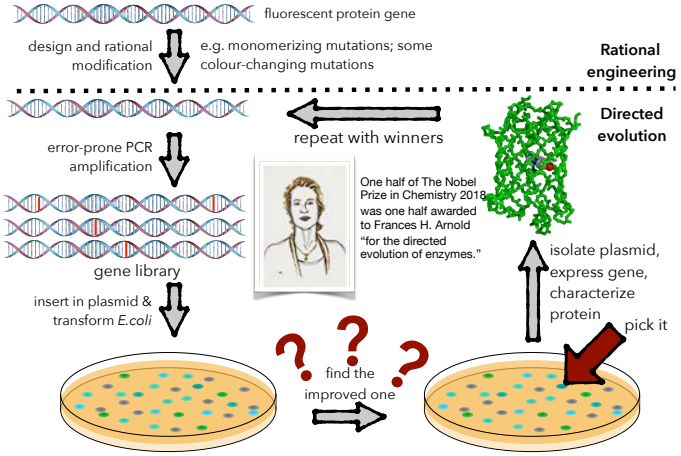
Nature has generously provided us with a variety of genetically encoded fluorophores

However, ideal for jellyfish and coral ≠ ideal for research,
so we must engineer fluorescent proteins to suit our needs!

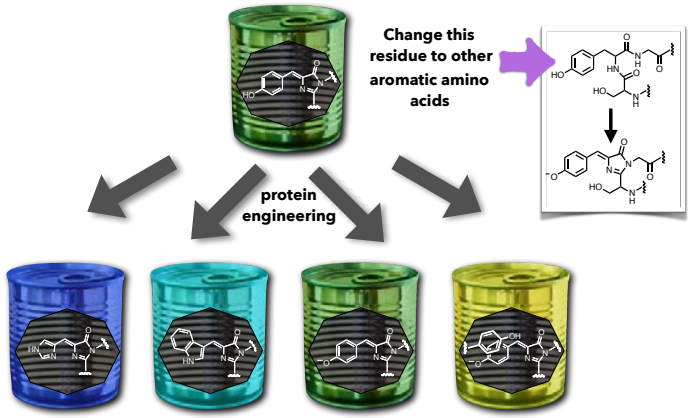
The 'ideal' FP for live cell imaging would...

...be brightly fluorescent (EC * QY),
...be a monomer,
...be red or near-infrared fluorescent,
...be resistant to photobleaching,
...be insensitive to changes in pH,
...be tolerant of fusion to other proteins,
...have fast and efficient folding and maturation,
...have narrow absorption and emission profiles,
...have a single exponential lifetime.

Engineering fluorescent proteins

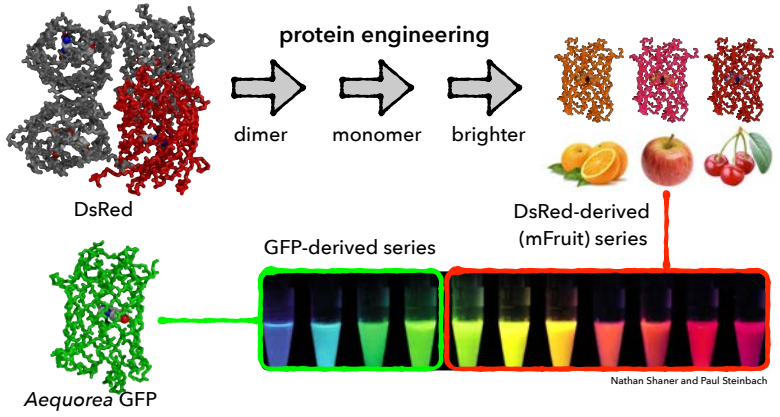


Early examples of engineered GFP variants



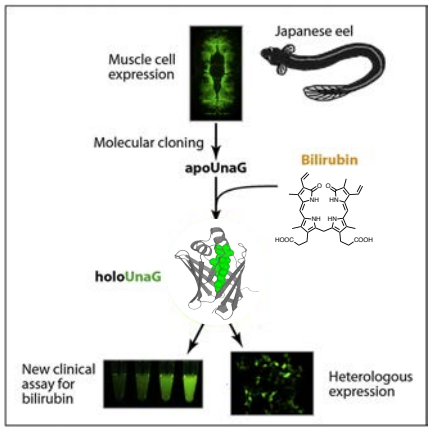
Heim et al., Proc. Natl. Acad. Sci. U. S. A. **1994**, 91, 12501; Heim et al., Nature **1995**, 373, 663; Cubitt et al., Trends Biochem. Sci. **1995**, 20, 448; Heim & Tsien, Curr. Biol. **1996**, 6, 178; Ormö et al., Science **1996**, 273, 1392.

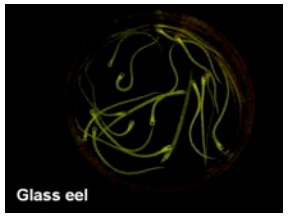
A palette of monomeric fluorescent proteins

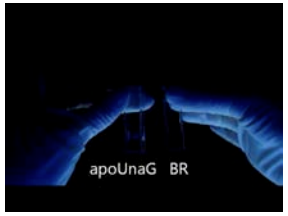


Campbell et al., Proc. Natl. Acad. Sci. U. S. A. **2002**, 99, 7877; Wang et al., Proc. Natl. Acad. Sci. U. S. A. **2004**, 101, 16745; Shaner et al., Nat. Biotechnol. **2004**, 22, 1567; Shu et al., Biochemistry **2006**, 45, 9639; Shaner et al., Nat. Methods **2008**, 5, 545.

Fluorescent proteins that are not homologs of GFP

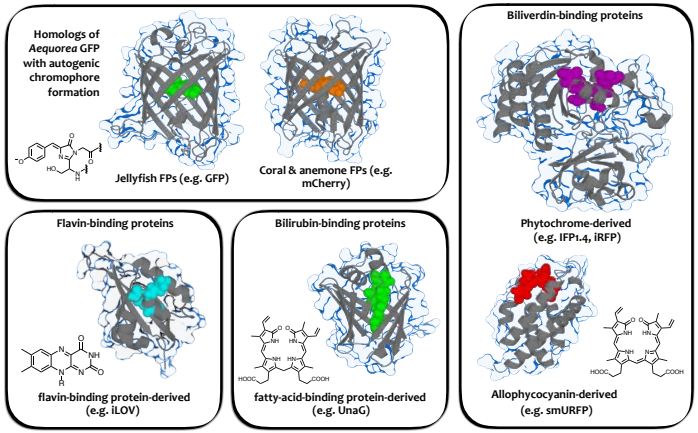






Kumagai et al., Cell **2013**, 153, 1602 (UnaG)

Four classes of fluorescent protein

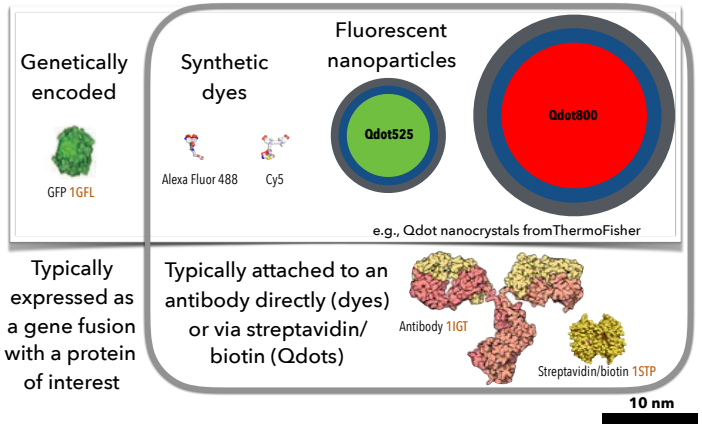


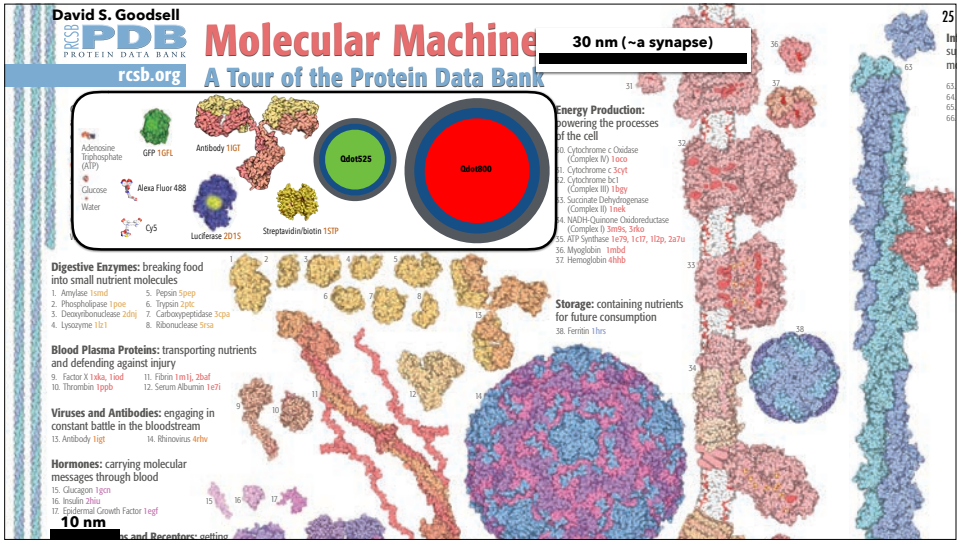
Shu et al., Science **2009**, 324, 804 (IFP1.4); Filonov et al., Nat. Biotechnol. **2011**, 29, 757 (iRFP); Yu et al., Nat. Methods **2015**, 12, 763 (miFP); Rodriguez et al. Nat. Methods **2016**, 13, 763 (smURFP); Kumagai et al., Cell **2013**, 153, 1602 (UnaG); Buckley et al., Curr. Opin. Chem. Biol. **2015**, 27, 39 (iLOV).

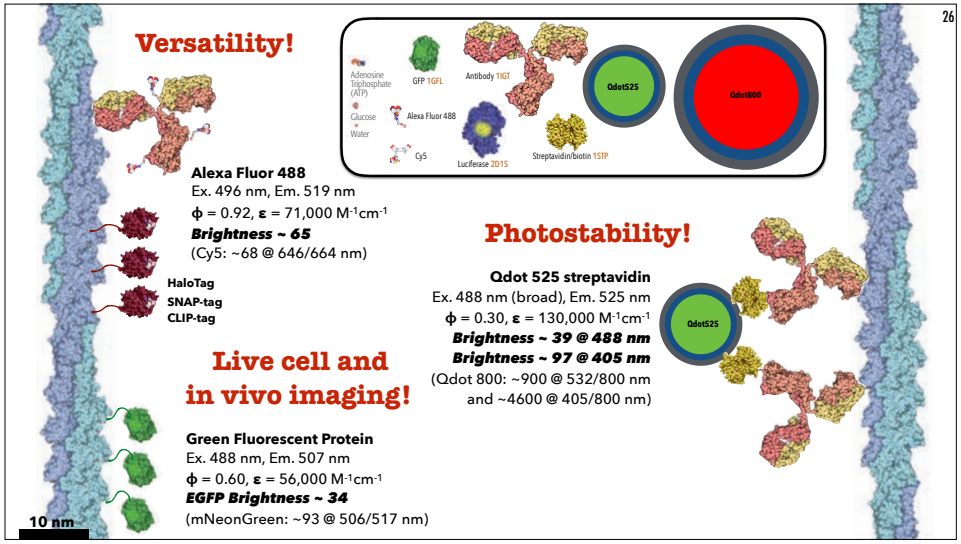
Outline

- [x] Fluorescent proteins (FPs)
- [ ] Other fluorophore technologies
- [ ] Single FP-based biosensors

Types of fluorophores

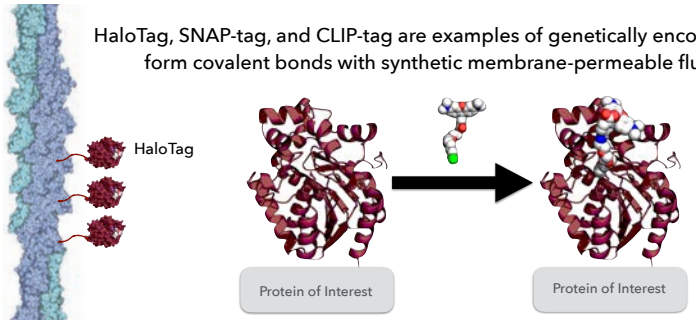






Semi-genetically encoded fluorescent proteins

HaloTag, SNAP-tag, and CLIP-tag are examples of genetically encoded proteins that form covalent bonds with synthetic membrane-permeable fluorophores.



- Halotag is a mutant of a bacterial haloalkane dehydrogenase
- SNAP- and CLIP-tag are engineered variants of the DNA repair protein O6-alkylguanine-DNA alkyltransferase

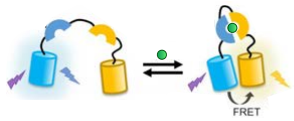
Keppler et al. Nat. Biotechnol., **2003**, 21, 86 (SNAP-tag); Gautier et al. **2008**, 15, 128 (CLIP-tag); Los et al. ACS Chem. Biol., **2008**, 3, 373 (HaloTag).

Outline

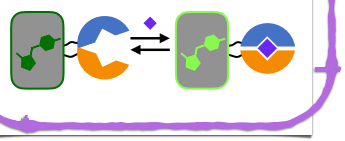
- [x] Fluorescent proteins (FPs)
- [x] Other fluorophore technologies
- [ ] Single FP-based biosensors

Designs of fluorescent protein-based biosensors

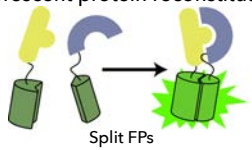
Förster resonance energy transfer



Single fluorescent protein



Fluorescent protein reconstitution



Split FPs

Dimerization-dependent FPs

CHEMICAL REVIEWS >1000 works cited!

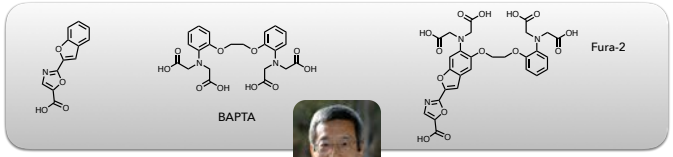
Genetically Encoded Fluorescent Biosensors Illuminate the Spatiotemporal Regulation of Signaling Networks

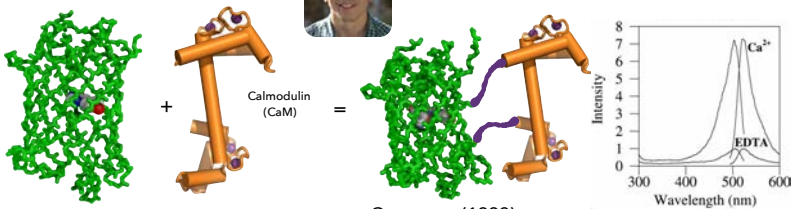
Eric C. Greenwald,[†] Sohum Mehta,[+,†] and Jin Zhang[*,†]

Miyawaki et al., Proc. Natl. Acad. Sci. U. S. A. **1999**, 96, 2135; Ghosh et al., J. Am. Chem. Soc. **2000**, 122, 5658; Kerppola, Chem. Soc. Rev. **2009**, 38, 2876; Nagai et al., Proc. Natl. Acad. Sci. U. S. A. **2001**, 98, 3197; Nakai et al., Nat. Biotechnol. **2001**, 19, 137; Alford et al., Chem. Biol. **2012**, 19, 353; Ding et al., Nat Methods **2015**, 12, 195.

Roger Tsien's Ca2+ biosensor equation

fluorophore + Ca^{2+} binding motif = Ca^{2+} biosensor

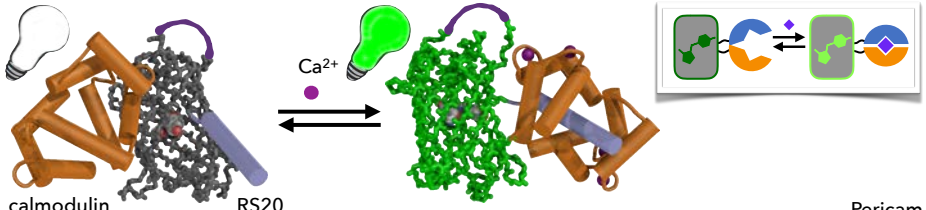




Camgaroo (1999)

Grynkiewicz, Poenie, and Tsien, J Biol Chem. **1985**, 260, 3440; Baird et al. Proc. Natl. Acad. Sci. U.S.A. **1999**, 96, 11241.

GCaMP: the prototypical Ca2+ biosensor (2000)



GCaMP1+
Junichi Nakai
(Saitama)

GCaMP3-7
Loren Looger &
GENIE (Janelia)

Atsushi Miyawaki
(RIKEN)

Pericam

Takeharu Nagai
(Osaka)

Nagai et al., Nat. Biotechnol. **2000**, 18, 313; Nakai et al., Nat. Biotechnol. **2001**, 19, 137; Tallini et al., Proc. Natl. Acad. Sci. U. S. A. **2006**, 103, 4753; Wang et al., Structure **2008**, 16, 1817; Akerboom et al., J. Biol. Chem. **2009**, 284, 6455; Tian et al., Nat. Methods **2009**, 6, 875; Akerboom et al., J. Neurosci. **2012**, 32, 13819; Chen et al., Nature **2013**, 499, 295; Dana et al., Nat. Methods **2019**, 16, 649.

The growing toolbox of (non-Ca²⁺) single GFP-based biosensors

Loren Looger (LL)

Lin Tian (LT)

Tetsuya Kitaguchi (TK)

Yulong Li (YL)

Jin Zhang (JZ)

Michael Lin (ML)

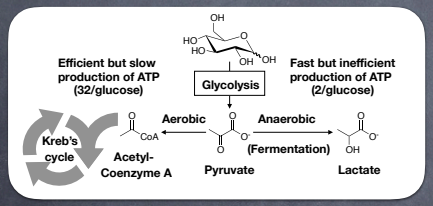
Maltose
MBP.PPYF (LL)
Glutamate
iGluSnFR (LL)
SF-iGluSnFR (LL)
GABA
iGABASnFr (LL)
Nicotine
iNicSnFr (LL)
Extracellular ATP
iATPSnFr (LL)

Dopamine
dLight1 (LT)
$GRAB_{DA}$(YL)
Glucose
Green Glifons (TK)
cAMP
Flamindo2 (TK)
cGMP
Green cGull (TK)
Intracellular ATP
MaLionG (TK)

Protein Kinases (JZ)
ExRai-AKAR
ExRai-CKAR
ExRai-AktAR
Voltage
ASAP3 (ML)
Among many others…

…and from the the Campbell lab:
GINKO (K+)
iLACCO (lactate)
Citrate biosensors

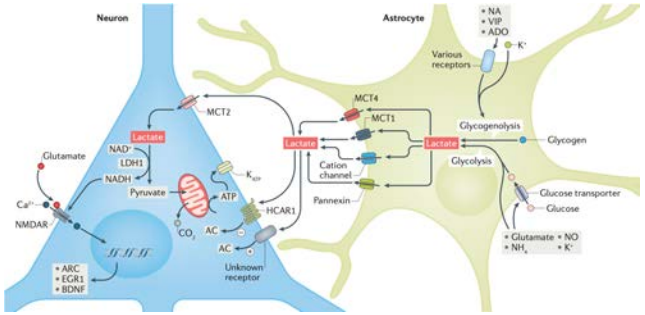
iLACCO:
A single FP-based L-lactate biosensor



There is growing recognition that lactate is a key player in a surprising variety of biological process (including neural activity).

Why L-lactate?

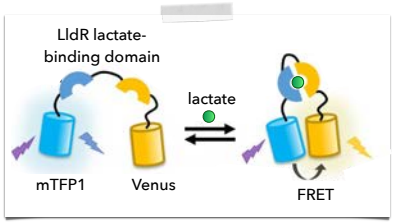
Astrocyte-to-Neuron Lactate Shuttle (ANLS) process

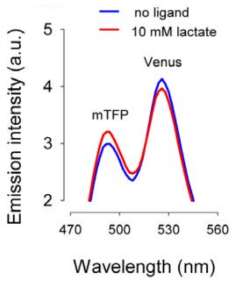


"Lactate is transferred from astrocytes to neurons to match the neuronal energetic needs, and to provide signals that modulate neuronal functions, including excitability, plasticity and memory consolidation."
-Magistretti and Allaman

Magistretti and Allaman, Nature Reviews Neuroscience, **2018**, 19, 235.

Laconic is a currently available FRET-based biosensor for L-lactate

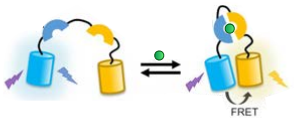




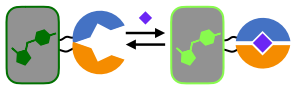
Martin et al. PLoS ONE, **2013**, 8, e57712.

Designs of fluorescent protein-based biosensors

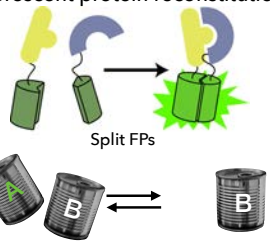
Förster resonance energy transfer



Single fluorescent protein



Fluorescent protein reconstitution

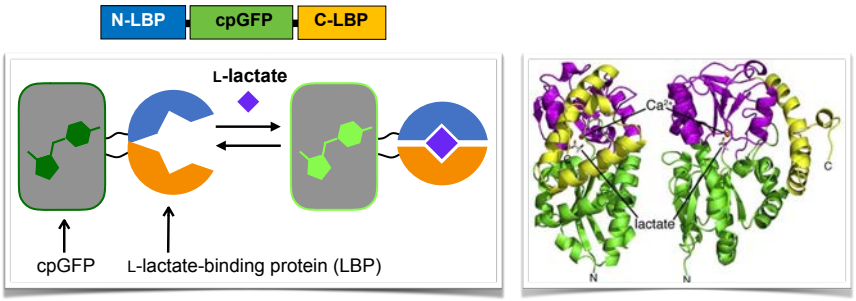


CHEMICAL REVIEWS >1000 works cited!

Genetically Encoded Fluorescent Biosensors Illuminate the Spatiotemporal Regulation of Signaling Networks
Eric C. Greenwald, Sohum Mehta, and Jin Zhang

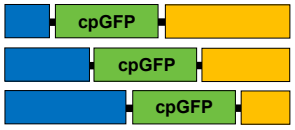
Miyawaki et al., Proc. Natl. Acad. Sci. U. S. A. **1999**, 96, 2135; Ghosh et al., J. Am. Chem. Soc. **2000**, 122, 5658; Kerppola, Chem. Soc. Rev. **2009**, 38, 2876; Nagai et al., Proc. Natl. Acad. Sci. U. S. A. **2001**, 98, 3197; Nakai et al., Nat. Biotechnol. **2001**, 19, 137; Alford et al., Chem. Biol. **2012**, 19, 353; Ding et al., Nat Methods **2015**, 12, 195.

Our goal: an intensiometric lactate biosensor

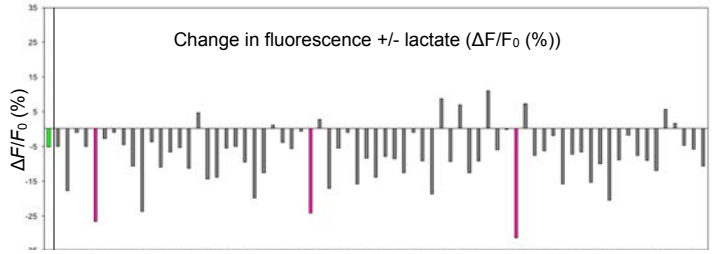


LBP = Periplasmic Calcium L-Lactate-Binding Protein (TTHA0766) from *Thermus thermophilus* HB8

Step 1: Screen insertion sites

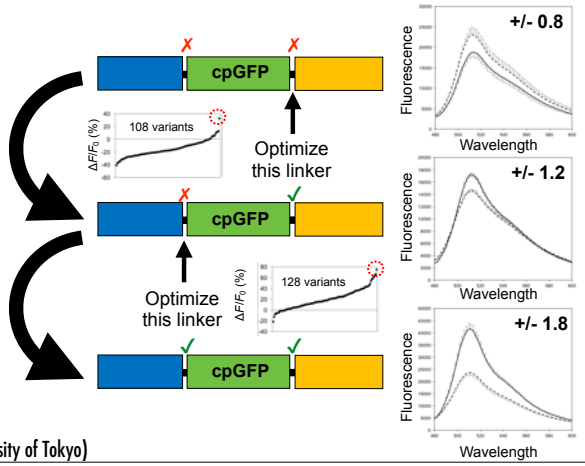


Screening of 70 different cpGFP insertion sites in loops
N-LBP = residues 1 to X
C-LBP = residues X+1 to 361



Insertion site (X)

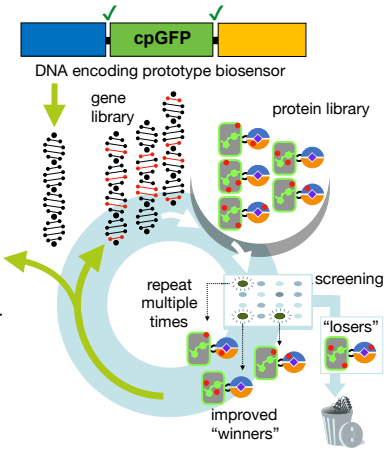
Step 2: Optimize linkers



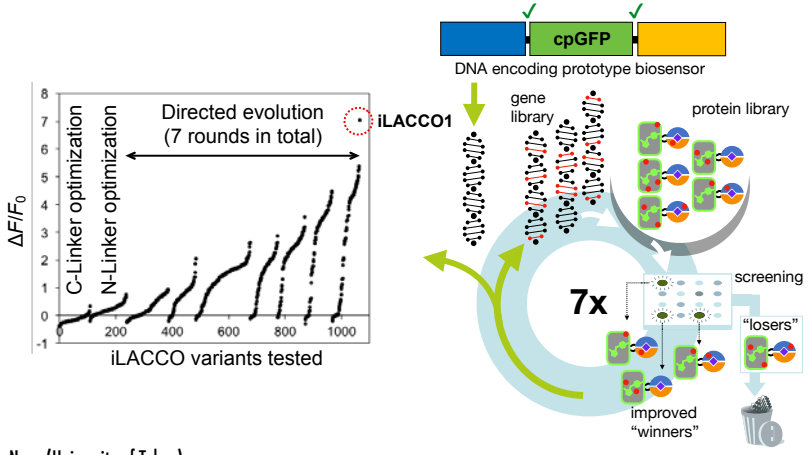
Step 3: Directed evolution

The Nobel Prize in Chemistry 2018 was one half awarded to Frances H. Arnold "for the directed evolution of enzymes."

Optimized, higher-performance biosensor
- Larger fluorescence changes
- High folding efficiency
- Brighter fluorescence
- Optimized ligand affinity

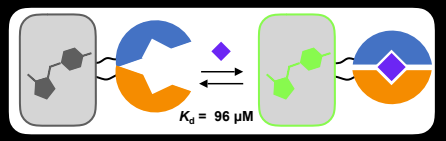


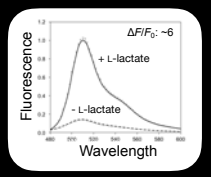
Step 3: Directed evolution



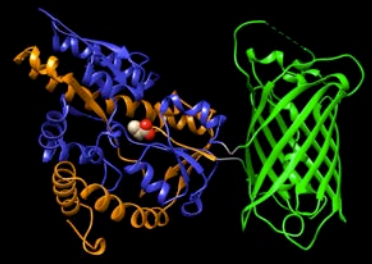
Yusuke Nasu (University of Tokyo)

iLACCO1 crystal structure





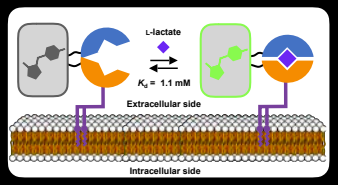
X-ray crystal structure of iLACCO1



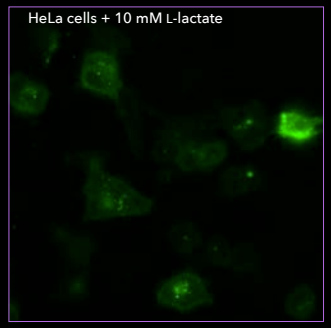
Unfortunately, L-lactate binding is $[Ca^{2+}]$-dependent, so iLACCO1 does not work intracellularly. Fortunately, our aim was to image L-lactate extracellularly, where $[Ca^{2+}]$ is high.

Yusuke Nasu with Shuce Zhang and Dr. Yurong Wen in lab of Dr. Joanne Lemieux (Alberta)

iLACCO1.1 (optimized for extracellular L-lactate)



- ✔Tuned affinity to respond to extracellular concentration range
- ✔Glycophosphatidylinositol (GPI) anchor for extracellular display
- ✔Optimized linker between GPI anchor and biosensor
- ✔Non-lactate binding control version



Yusuke Nasu with Qi Dong (University of Tokyo)

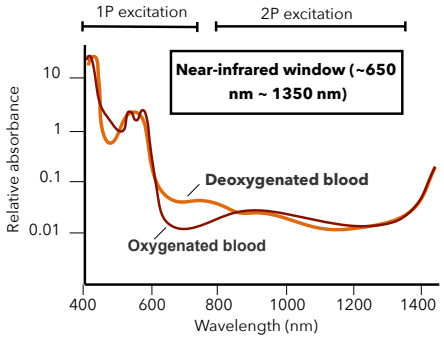
Redder is Better!

Advantages of red-shifted biosensors

- **Imaging deeper in non-transparent animals**
- **Less phototoxicity for long-term observations**
- **Multicolour, multi-parameter imaging**
- **Use with optogenetic stimulation (e.g., ChR2)**

45

Redder is better (for imaging deeper into tissue)



The **near-infrared window** is the range of wavelengths in which tissue is most transparent to light.

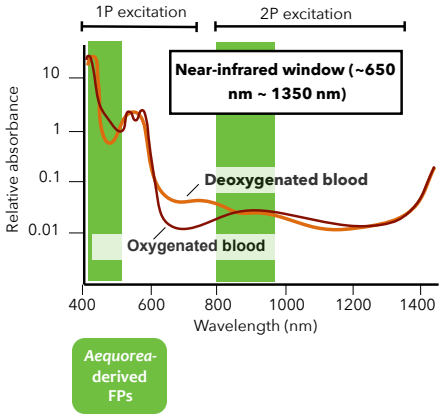
Scattering of light by tissue decreases with increasing wavelength.

Ideally, neural activity biosensors (Ca^{2+}, voltage, etc.) would absorb and fluoresce in this region

Adapted from omlc.org

46

Redder is better (for imaging deeper into tissue)



The **near-infrared window** is the range of wavelengths in which tissue is most transparent to light.

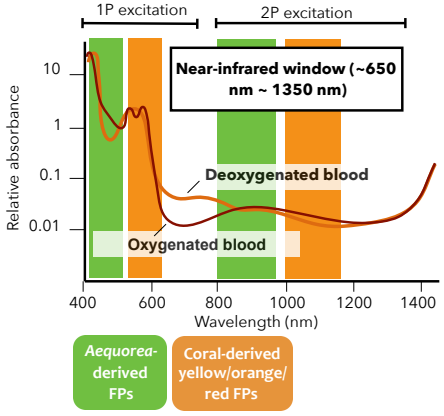
Scattering of light by tissue decreases with increasing wavelength.

Ideally, neural activity biosensors (Ca^{2+}, voltage, etc.) would absorb and fluoresce in this region

Adapted from omlc.org

47

Redder is better (for imaging deeper into tissue)



The **near-infrared window** is the range of wavelengths in which tissue is most transparent to light.

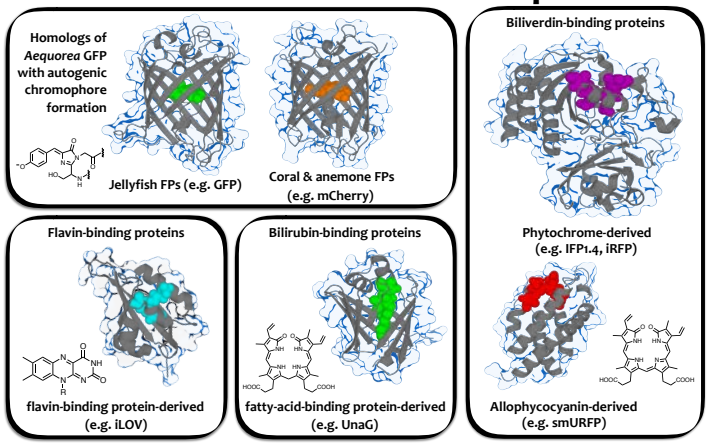
Scattering of light by tissue decreases with increasing wavelength.

Ideally, neural activity biosensors (Ca^{2+}, voltage, etc.) would absorb and fluoresce in this region

Adapted from omlc.org

48

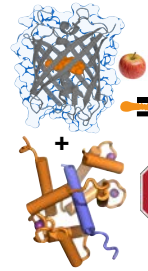
Four classes of fluorescent protein

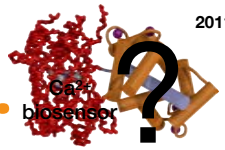


Shu et al., Science **2009**, 324, 804 (IFP1.4); Filonov et al., Nat. Biotechnol. **2011**, 29, 757 (iRFP); Yu et al., Nat. Methods **2015**, 12, 763 (miRFP); Rodriguez et al. Nat. Methods **2016**, 13, 763 (smURFP); Kumagai et al., Cell **2013**, 153, 1602 (UnaG); Buckley et al., Curr. Opin. Chem. Biol. **2015**, 27, 39 (iLOV).

Engineering of red Ca2+ biosensors

Rational engineering & directed evolution





R-GECO1
Ca²⁺ biosensor

2011 - 2019

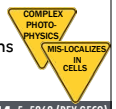
Improved performance
R-CaMP2
jRGECO1a
XCaMP-R

New colors
O-GECO
CAR-GECO
REX-GECO

Affinity tuning for ER and mito
LAR-GECO
R-CEPIA1*er*

and others...

However, we have found some problems with this particular red FP domain...



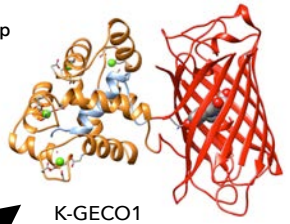
Zhao et al., Science **2011**, 333, 1888 (R-GECO1); Wu et al., ACS Chem. Neurosci. **2013**, 4, 963 (O-GECO, R-GECO1.2); Wu et al., Nat Commun **2014**, 5, 5262 (REX-GECO); Wu et al., Biochem. J. **2014**, 464, 13 (LAR-GECO); Ohkura et al., PLoS ONE **2012**, 7, e39933 (R-CaMP1.07); Inoue et al., Nat Methods **2015**, 12, 64 (R-CaMP2); Suzuki et al., Nat Commun **2014**, 5, 4153 (R-CEPIAer); Dana et al. eLife **2016**, 5, e12727 (jRCaMP1ab, jRGECO1a); Inoue et al., Cell **2019**, 177, 1346 (XCaMP-R).

K-GECO1: an improved red Ca²⁺ biosensor

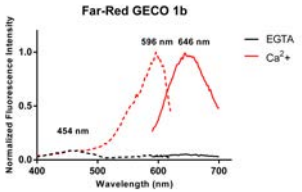
... which led us to develop a new biosensor based on an improved fluorescent protein

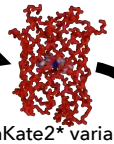
...and recently we've made a highly red shifted variant

Bubble-tip anemone



K-GECO1





mKate2* variant (FusionRed)

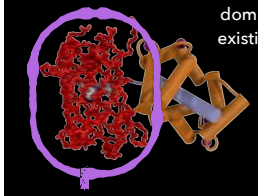
mNeptune → mCardinal → mKelly

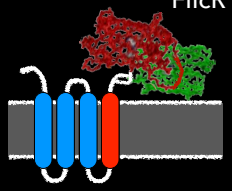
Yi Shen

Shemiakina et al., Nat. Commun. **2012**, 3, 1204 (mKate2 variant FusionRed); Shen et al., BMC Biology **2018**, 16, 9 (K-GECO1).

Converting other biosensors from green to red

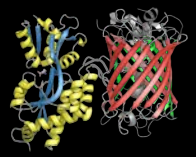
"Transplanting" red FP domain of R-GECO1 into existing green biosensors





FlicR1

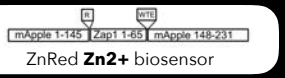
voltage



R-iGluSnFR1

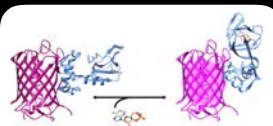
glutamate

Plus a growing selection of red FP biosensors from other labs...



ZnRed **Zn2+** biosensor

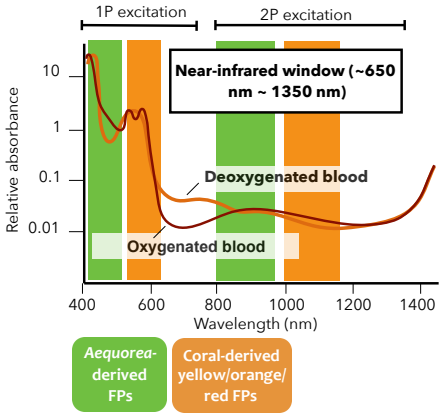
MaLionR **ATP** biosensor



Pink Flamindo **cAMP** biosensor

Jin et al., Neuron **2012**, 75, 779 (Arclight); Abdelfattah et al., J. Neurosci. **2016**, 36, 2458 (FlicR1); Marvin et al., Nat. Methods **2013**, 10, 162 (iGluSnFr); Wu et al., ACS Chem. Biol., **2018**, Article ASAP (R-iGluSnFR1); Chen and Ai, Anal. Chem. **2016,** 88, 9029 (ZnRed); Harada et al., Sci. Rep. **2017**, 7, 7351 (Pink Flamindo); Arai et al., Angew. Chem. IE **2018**, 57, 10873 (MaLionR).

Redder is better (for imaging deeper into tissue)



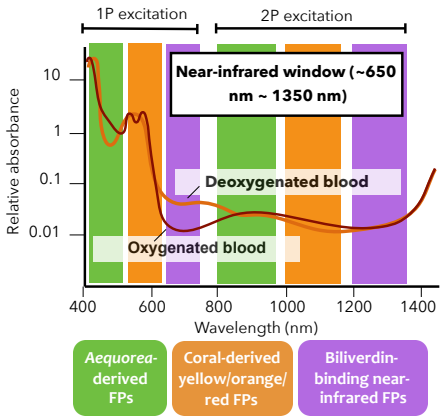
The **near-infrared window** is the range of wavelengths in which tissue is most transparent to light.

Scattering of light by tissue decreases with increasing wavelength.

Ideally, neural activity biosensors (Ca^{2+}, voltage, etc.) would absorb and fluoresce in this region

Adapted from omlc.org

Redder is better (for imaging deeper into tissue)



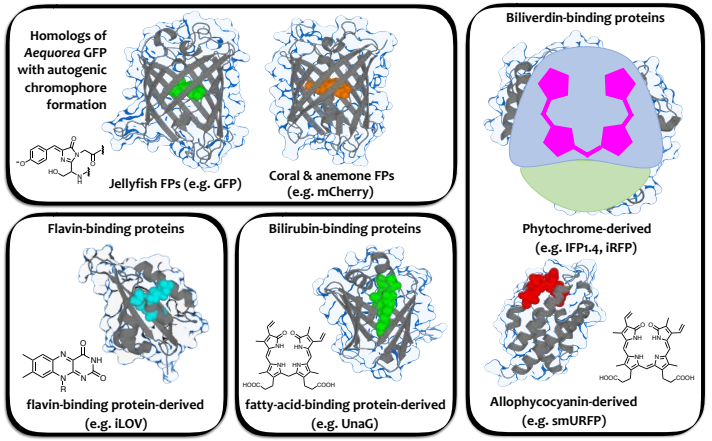
The **near-infrared window** is the range of wavelengths in which tissue is most transparent to light.

Scattering of light by tissue decreases with increasing wavelength.

Ideally, neural activity biosensors (Ca^{2+}, voltage, etc.) would absorb and fluoresce in this region

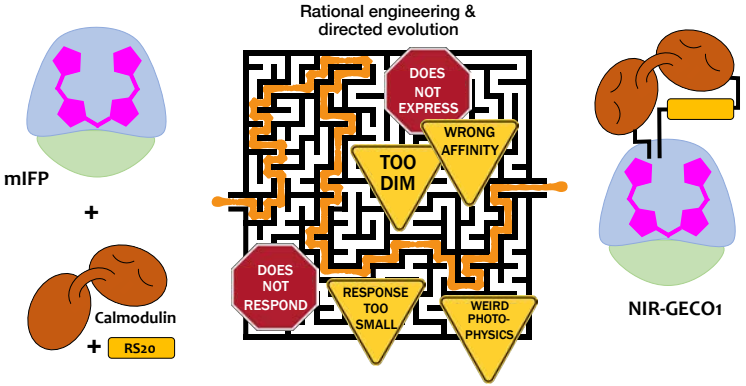
Adapted from omlc.org

Four classes of fluorescent protein



Shu et al., Science **2009**, 324, 804 (IFP1.4); Filonov et al., Nat. Biotechnol. **2011**, 29, 757 (iRFP); Yu et al., Nat. Methods **2015**, 12, 763 (mIFP); Rodriguez et al. Nat. Methods **2016**, 13, 763 (smURFP); Kumagai et al., Cell **2013**, 153, 1602 (UnaG); Buckley et al., Curr. Opin. Chem. Biol. **2015**, 27, 39 (iLOV).

Engineering a NIR Ca2+ biosensor

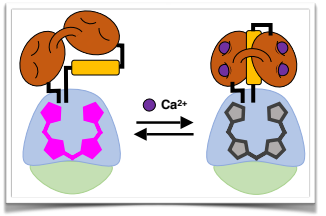


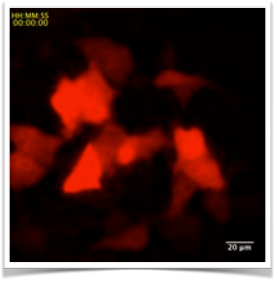
Yong Qian

Yu et al., Nat. Methods **2015**, 12, 763 (mIFP); Qian et al., Nat. Methods, **2019**, 16, 171 (NIR-GECO1).

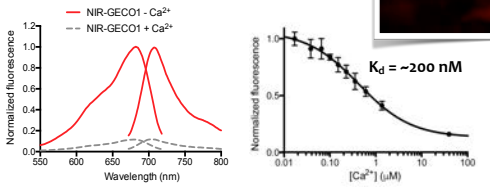
mIFP-derived NIR-GECO1

Absorbance and emission peaks at 678 nm and 704 nm, respectively





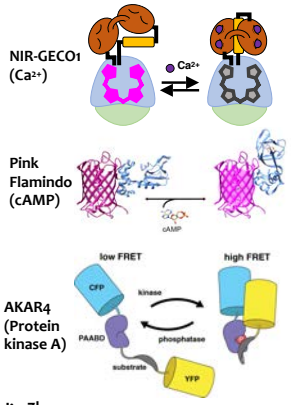
~10-fold decrease in fluorescence intensity upon binding Ca^{2+}

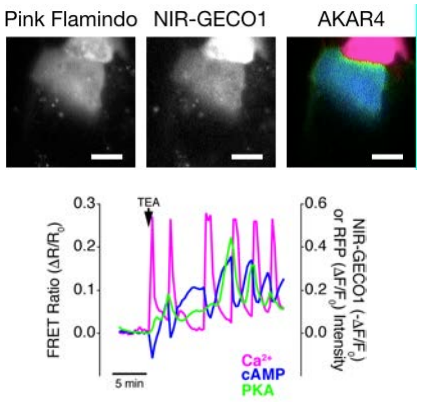


Yong Qian

Yu et al., Nat. Methods **2015**, 12, 763 (mIFP); Qian et al., Nat. Methods, **2019**, 16, 171 (NIR-GECO1).

Multi-colour & multi-parameter imaging

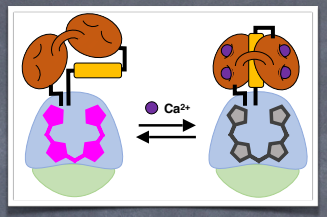




Sohum Mehta and Jin Zhang

Yu et al., Nat. Methods **2015**, 12, 763 (mIFP); Qian et al., Nat. Methods, **2019**, 16, 171 (NIR-GECO1).

Redder is Better!

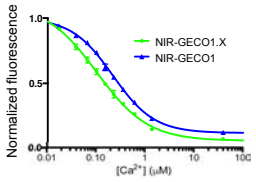


Advantages of NIR-GECO1

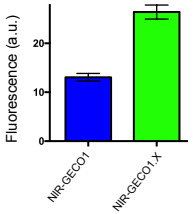
- ☐ Imaging deeper into tissue
- ☑ Multicolour imaging
- ☑ Use with optogenetic actuators

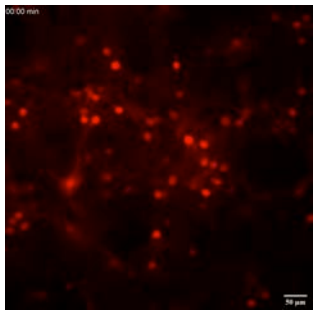
Progress towards NIR-GECO2

Higher affinity ~100 nM



Brighter ~2x





Spontaneous neuronal spiking in dissociated neurons expressing NIR-GECO1.X

Works great in worms!

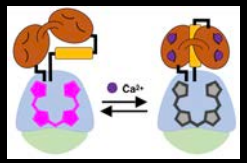
-Ed Boyden, Kiryl Piatkevich

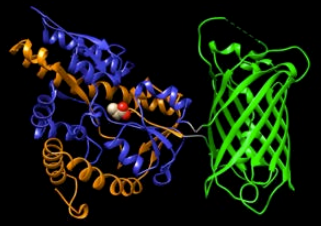
Yong Qian and Kiryl Piatkevich

Summary of single FP-based biosensors

"Redder is better"

- Growing selection of RFP-derived biosensor.
- mIFP-derived NIR-GECO1 is 1st generation NIR Ca2+ biosensor
- Future: 2nd generation NIR-GECO1+ with improved sensitivity for in vivo imaging.





iLACCO1.1 lactate biosensor

- iLACCO1.1 is a 1st generation single FP-based biosensor for extracellular lactate
- Future: Imaging of lactate released by astrocytes and new biosensors for intracellular lactate imaging.

✺ Genes from www.addgene.org/Robert_Campbell/

✺ Contact: campbell@chem.s.u-tokyo.ac.jp or robert.e.campbell@ualberta.ca

Summary

- ☑ Fluorescent proteins (FPs)
- ☑ Other fluorophore technologies
- ☑ Single FP-based biosensors

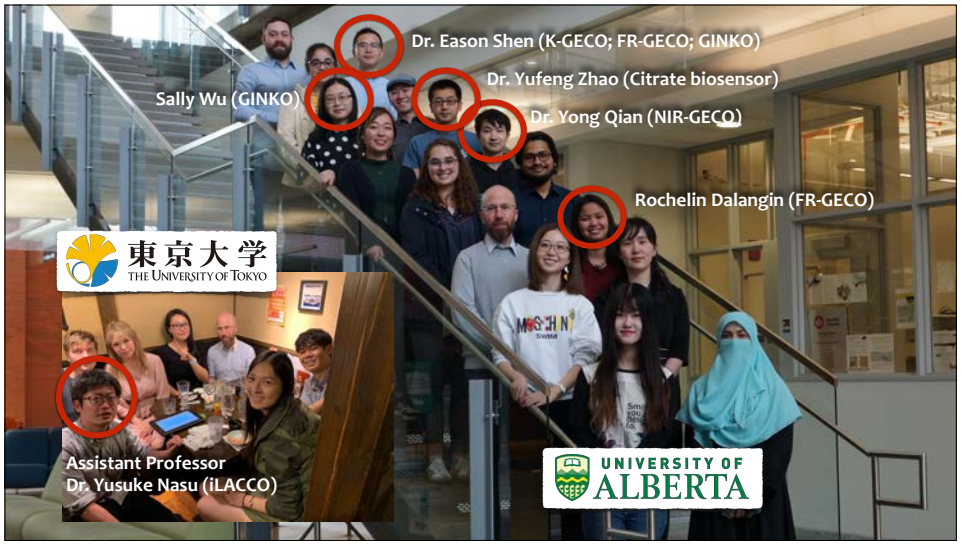
Take-home messages:

1. Despite "optimization" no one FP or biosensor is ideal by all criteria, and it is typically impossible to predict which one will work best in a new application.

2. I recommend trying 2-3 different FPs or biosensors (from different species), and determining which one is best under your experimental conditions.

3. All other factors being the same, redder is better.

4. There is a growing selection of intensiometric single FP-based biosensors, and methods for developing new ones are becoming increasingly well-established.



Acknowledgments

UNIVERSITY OF ALBERTA

Dr. Yong Qian (NIR-GECO1+); Sheng-Yi (Sally) Wu; Shuce Zhang; Rochelin Dalangin; Xiaocen Lu; Dr. Yi Shen; Dr. Yufeng Zhao; Dr. Fang Zheng; Dr. Landon Zarowny; Yan Li

東京大学 THE UNIVERSITY OF TOKYO

Assistant Professor Dr. Yusuke Nasu (lactate); Rina Hashizume; Peter Wojnicki; Hayato Kadoya; *and undergraduate summer interns.*

Currently looking to hire a Project Assistant Professor in Tokyo. Applications welcome!

Alumni

Landon Zarowny, Ph.D.; Wei Zhang, Ph.D.; Matthew Wiens, Ph.D.; Ahmed Abdelfattah, Ph.D.; Tiffany Yan Lai, M.Sc.; Jhon Ralph Enterina, M.Sc.; Yi Shen, Ph.D.; Jiahui Wu, Ph.D.; Nazanin Assempour, M.Sc.; Yan Li, M.Sc.; Yongxin Zhao, Ph.D.; Yidan Ding, Ph.D.; Ahmed Belal, Ph.D.; Haley Carlson, Ph.D.; Hiofan Hoi, Ph.D.; Ritesh Saini, M.Sc.; Spencer Alford, Ph.D.; Andreas Ibraheem, M.Sc.; Zihao Cheng, Ph.D.; Hui-wang Ai, Ph.D.; Carine Lafaille, M.Sc.; Yankun Li, M.Sc.; Dr. Tam Tran; Dr. Yingche Chen; Dr. Cory Beshara; Dr. Hongkin Yap; Dr. Monika Johar; Aillette Sierra Mulet, *and many undergraduates.*

Collaborators for work shown

Kiryl Piatkevich (NIR-GECO1) & Ed Boyden (MIT)
Sohum Mehta (NIR-GECO1) and Jin Zhang (UCSD)
Yurong Wen (X-ray structure) & Joanne Lemieux (Alberta)

Support for this work

- NIH (BRAIN Initiative with JB Pierce and MSU)
- Brain Canada (latform and NIH matching)
- NSERC (Discovery grant and scholarships)
- CIHR (Foundation)
- Alberta Innovates (Scholarships)
- University of Alberta
- University of Tokyo and GSC program
- JSPS Kakenhi (S) 2019-2023