

Lecture 5: fluorescent proteins; genetically encoded sensors and actuators

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Review of lecture 2



Outline

- Sensors
 - Bioluminescence vs fluorescence
 - Overview of fluorescent proteins
 - Mechanisms to couple cellular phenomena to changes in fluorescence signal
 - Genetically encoded indicators: calcium, voltage, etc.
- Actuators
 - Optogenetic
 - Photocaged molecules
 - Opsins
 - LOV-Jalpha interactions
 - CALI
 - Chemogenetic
 - Metabotropic: DREADDs
 - Ionotropic: PSAM/PSEM, GluCl

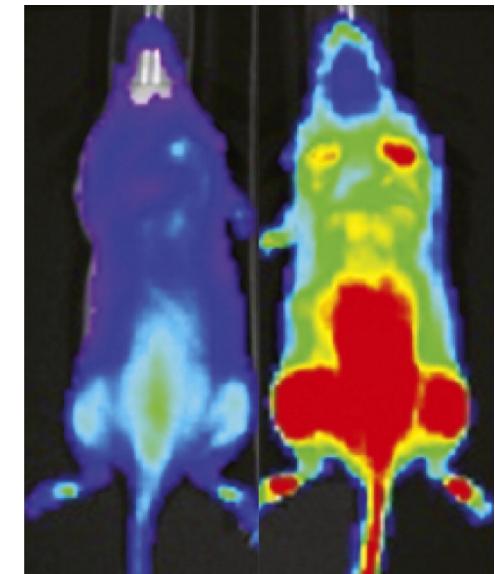
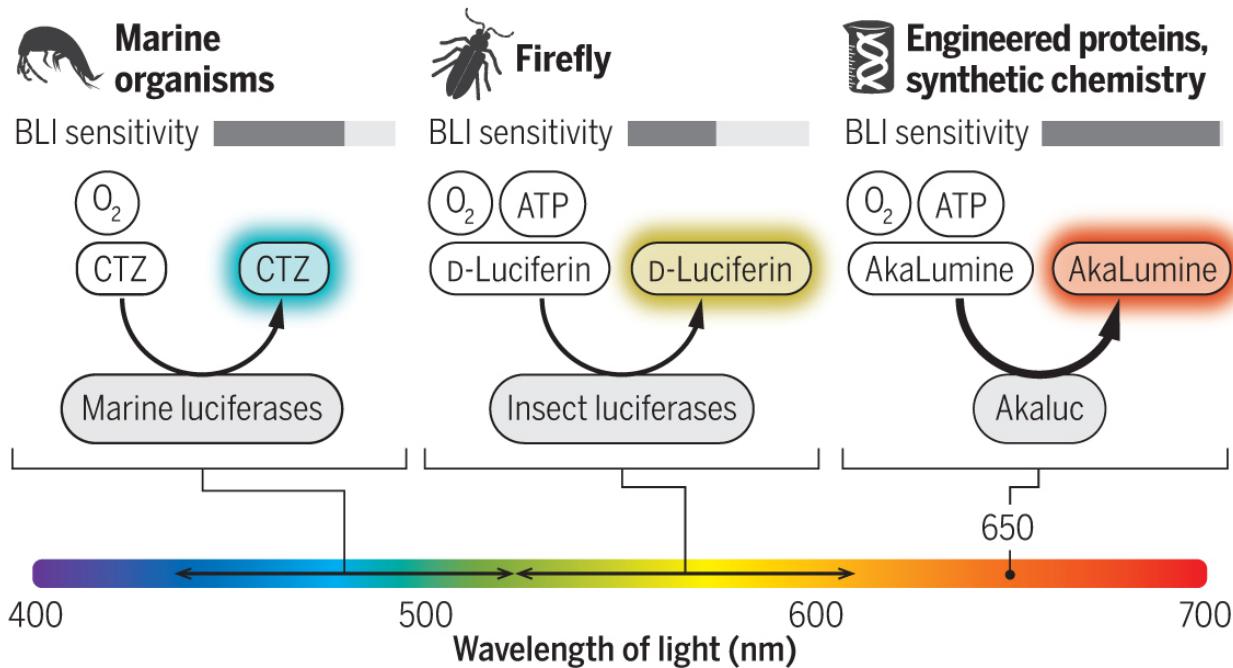
Bioluminescence generates photons



Luciferases oxidize luciferins to generate photons

Luciferase-luciferin bioluminescent pairs

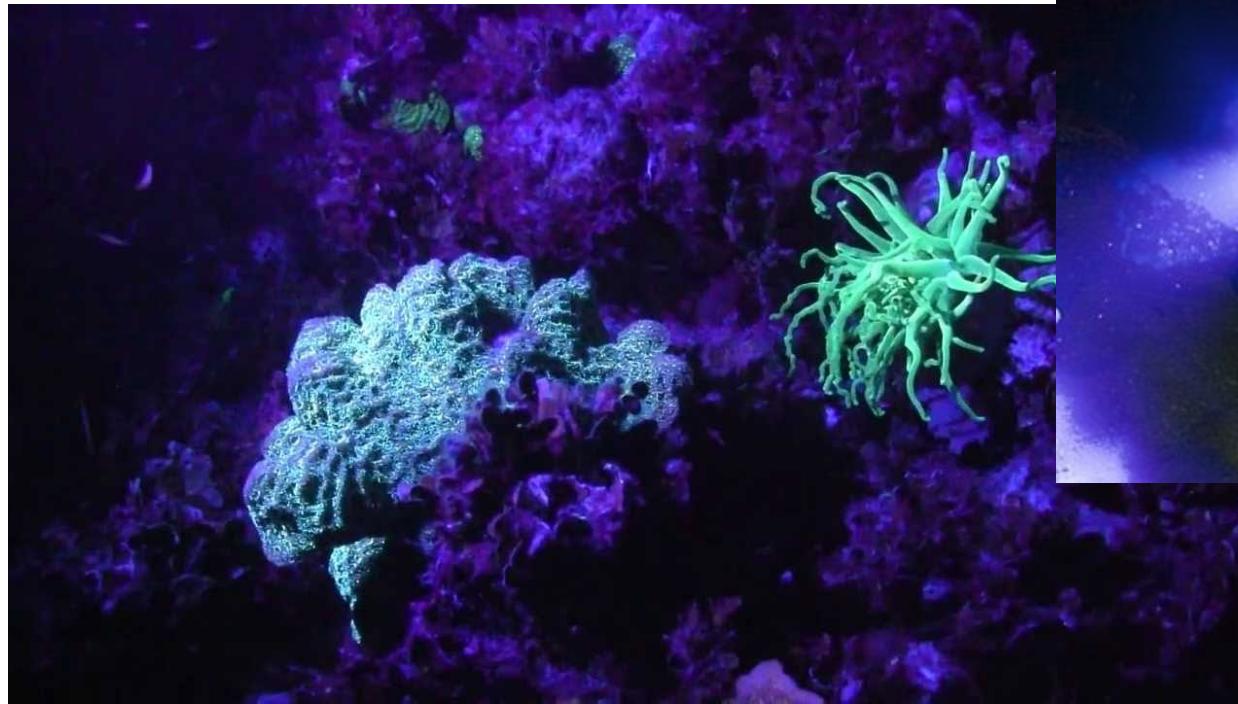
Akaluc, an engineered firefly luciferase, is optimized to use an unnatural luciferin, AkaLumine. The Akaluc system (AkaBLI) enables bioluminescence imaging (BLI) with unprecedented sensitivity *in vivo*, unlike marine luciferases, which are mainly used *in vitro*.



<https://science.sciencemag.org/content/359/6378/868/tab-figures-data>

<https://newscenter.lbl.gov/2011/02/10/firefly-glow-for-hydro-peroxide/>

Fluorescence: excitation by photons and emission of lower-energy photons

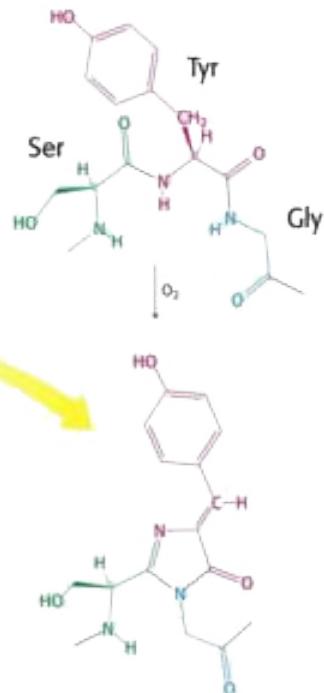
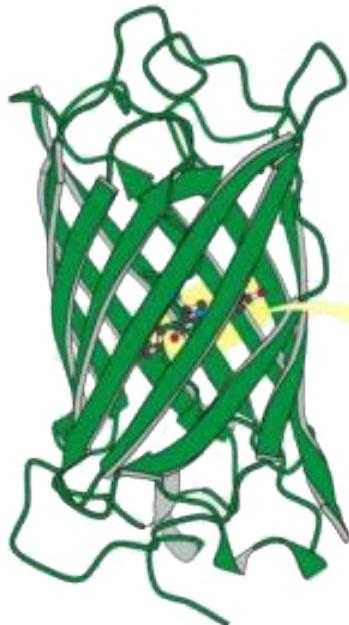


Fluorescence, not floorescence

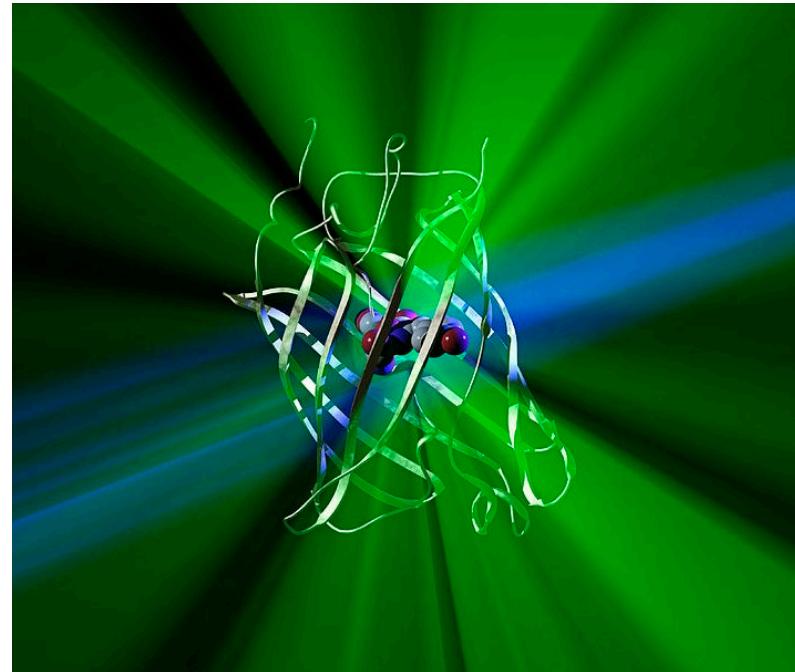
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Side chains inside the GFP β -barrel react to form the chromophore

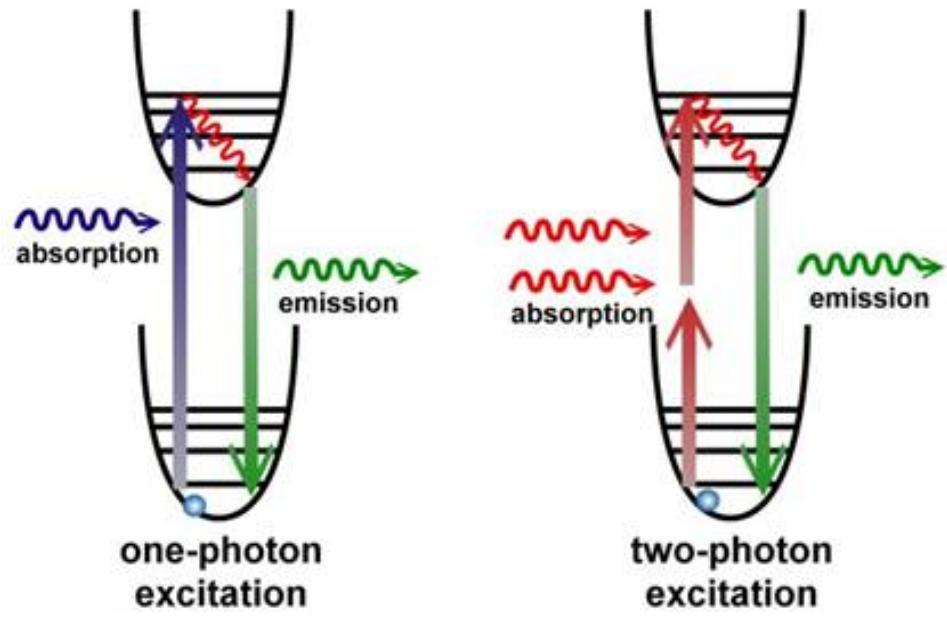
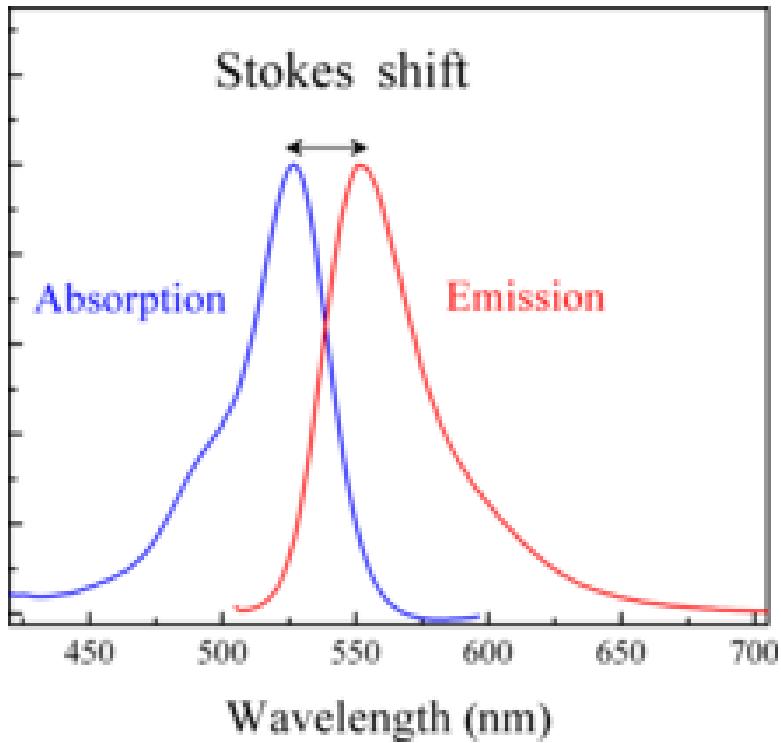


Fluorescence of GFP chromophore by cyclization reaction including rearrangement and oxidation



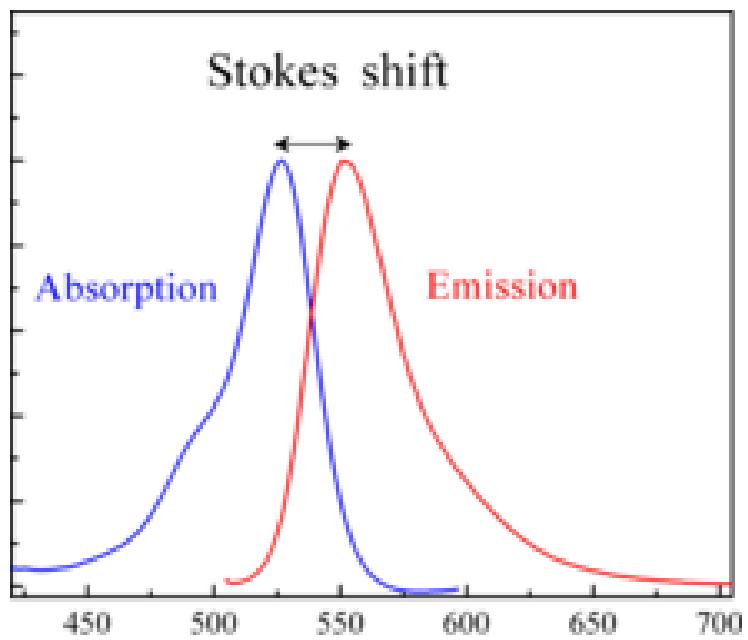
- The β -barrel shields the chromophore
 - FPs photobleach less than most fluorescent dyes

Stokes shift: the difference between excitation and emission wavelengths



Spectra encountered in neuroscience are usually asymmetrical because high and low energy are different

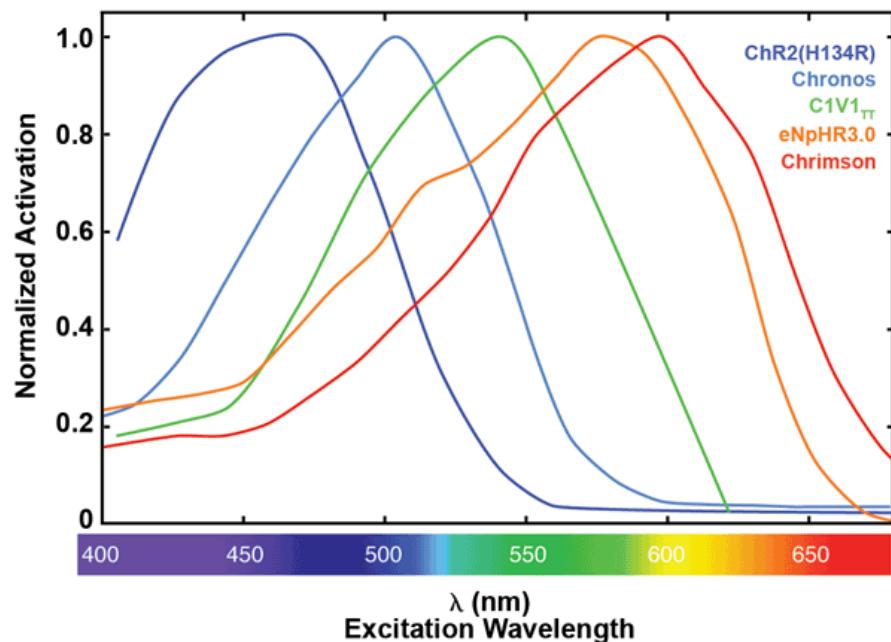
Fluorescence



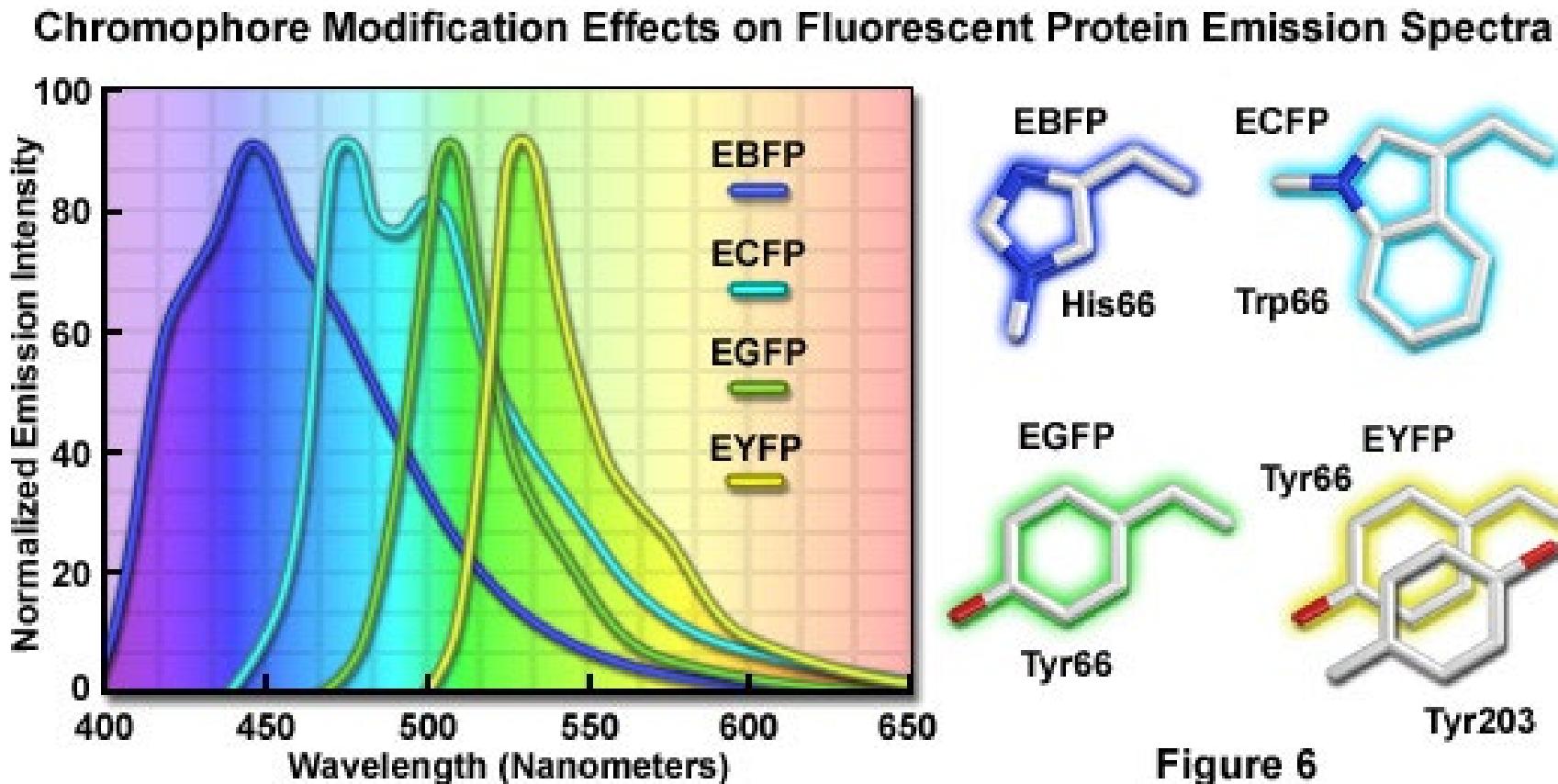
High
energy

Low
energy

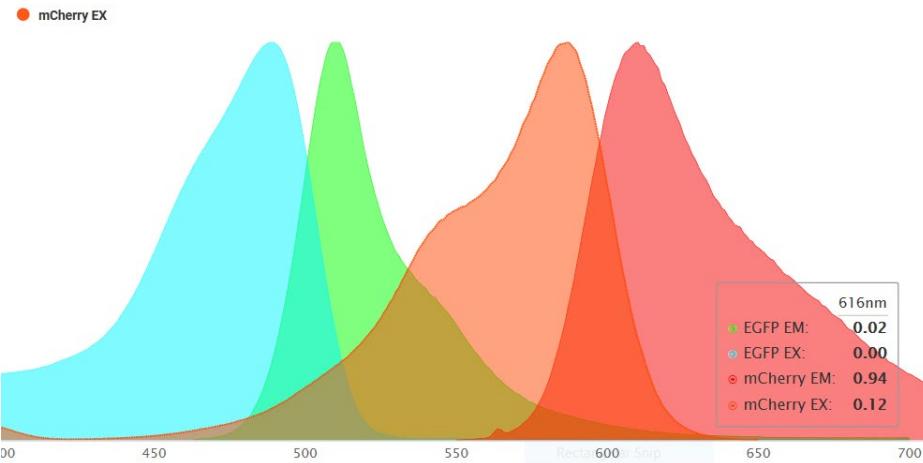
Opsin action spectrum



Mutations enable changes in excitation and emission spectra



EGFP and mCherry represent the two main families of FPs



- *Aequoria victoria* GFP: EGFP, BFPs, CFPs, YFPs, mCitrine
- *Discosoma* DsRed: “RFP”, the mFruits (mCherry, tdTomato, etc.) but not mCitrine
- GFP or DsRed antibodies generally cross-react with all family members

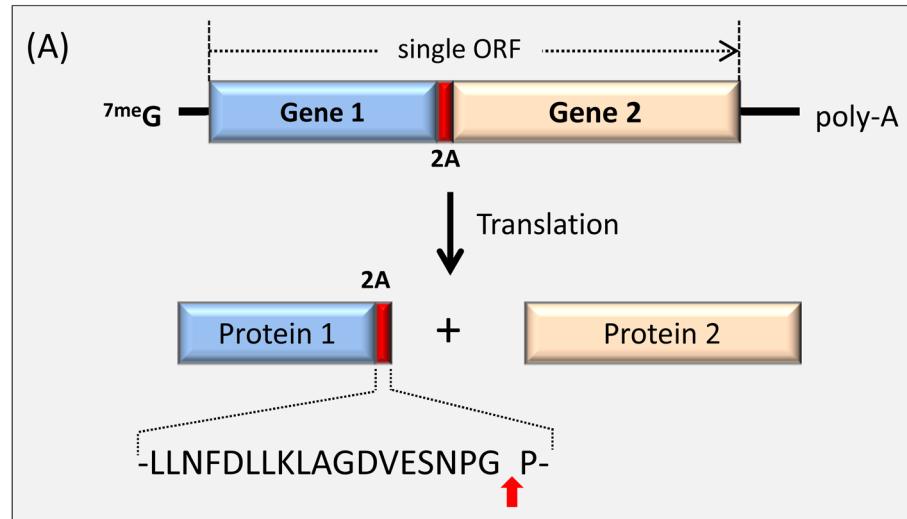
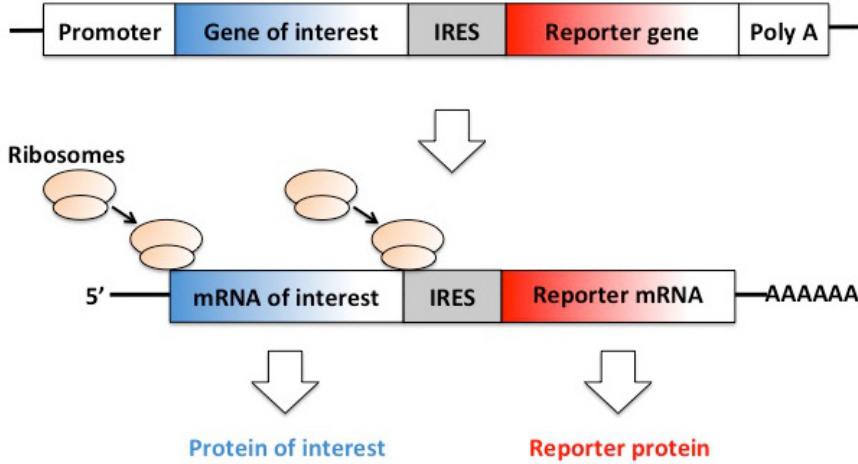
Highly recommended site with database of all fluorescent proteins and dyes: fpbase.org

Cellular localization is important!!

- Plasma membrane (e.g. ChR2-EYFP)
 - Transmembrane proteins expressed at lower levels (so >10X dimmer)
 - Labels processes >> soma
 - Usually impossible to tell which cells are expressing it
- Cytoplasmic (e.g. ChR2 – P2A – EYFP)
 - Labels processes << soma
 - If labeling is dense, may require confocal/two-photon to see which cells are positive
- Nuclear (e.g. ChR2 – P2A – nls-dTomato)
 - “nls” – nuclear localization sequence. At high expression levels it won’t be restricted to the nucleus
 - Concentrating the fluorescence in the nucleus makes it very bright (i.e. sensitive to detect low expression levels)
 - **This is what most people in neuroscience should be using**

IRES and 2A – viral tricks to make separate proteins from one gene

e.g. ChR2-IRES-EGFP or hM4Di-P2A-nls-dTomato

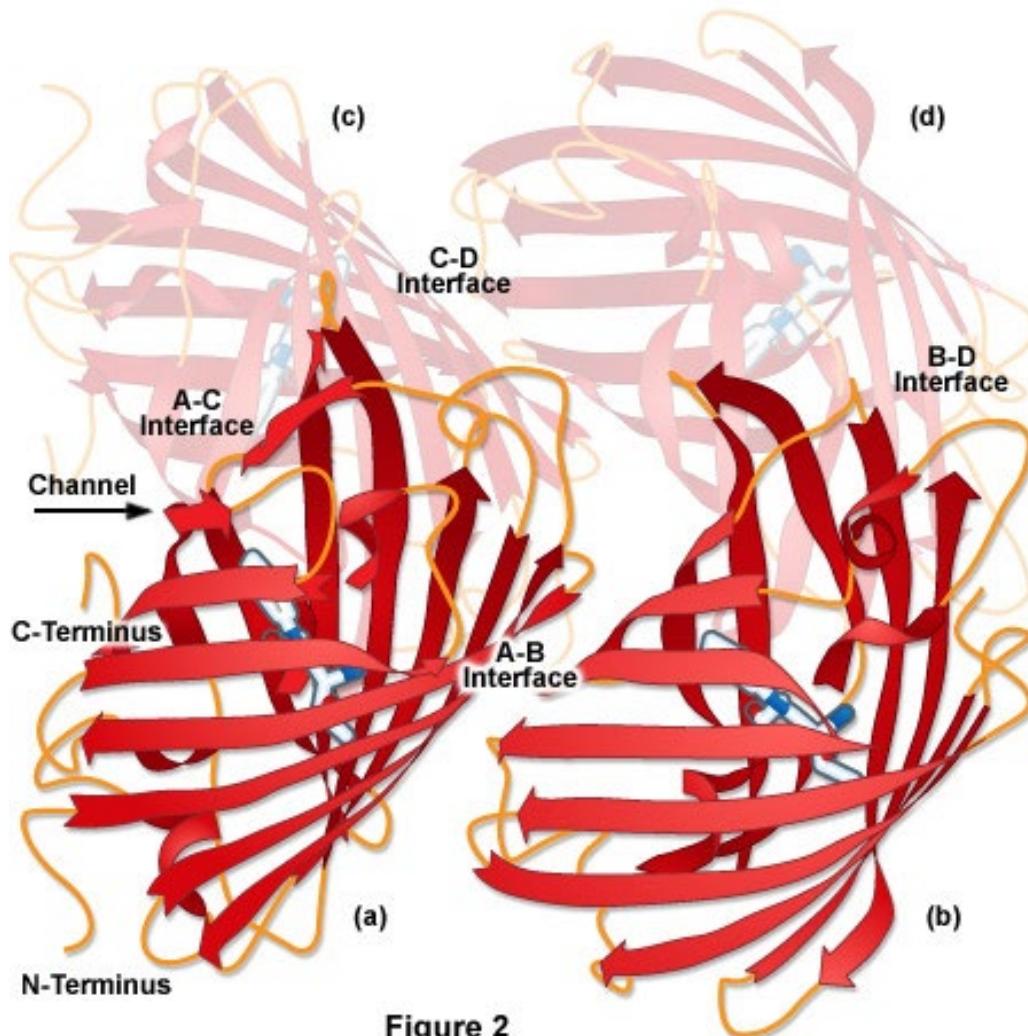


- IRES: internal ribosome entry site
- 2nd translation begins at the IRES
- Both protein sequences unchanged
- Both blue and red proteins are expressed at lower than normal levels
- IRES adds ~1kb length

- 2A: viral “cleavage* sequence”
- Ribosome translates both proteins in one pass without forming peptide bond (*so not actually cleavage)
- Proteins are made in exact 1:1 ratio
- Proteins get a few extra AAs
- Small size fits in viral vectors
- Multiple 2A's (P2A, T2A, etc.) – not all have efficient cleavage

Fluorescent proteins are generally multimeric

Tetrameric Oligomerization in DsRed Fluorescent Protein



- DsRed is tetrameric
- mFruits are monomeric ("m") or tandem dimer ("td") derivatives of DsRed (e.g. mCherry, tdTomato)
- GFP is weakly dimeric (but A206K disrupts)
- This becomes problematic when you fuse FPs to another protein
- mCherry is NOT fully monomeric
- ChR2-mCherry and hM4Di-mCherry fusions are not as good as their EYFP counterparts

For any given FP construct: what do you actually need to know?

- Excitation/emission wavelengths (what color is it?)
- Where is it localized? Membrane vs cytoplasm vs nuclear
- How sensitive is it to fixation or tissue clarification?
- What antibodies to use if it isn't visible in histology?
 - GFP vs DsRed/"RFP"
- Is it monomeric/dimeric/tetrameric?
 - mCherry is NOT fully monomeric! mCherry fusions can be problematic.
- Relevant biophysical parameters:
 - Brightness = extinction coefficient * quantum yield
 - Two photon absorption cross section: not same as extinction coefficient!
 - Tendency to photobleach: unfortunately, no standardized way to measure

Note: there are fluorescent proteins that are not GFP-like

- Here “GFP-like” means having a β -barrel structure shielding the chromophore
- Notable exception 1: Near-infrared fluorescent proteins
 - miRFP670nano, iRFP720 – developed by Vlad Verkhusha’s lab
 - These are spectrally distinct from mCherry/tdTomato and are compatible with the far-red filter set on many fluorescence microscopes
 - We are using these to prevent interference with fiber photometry
- Notable exception 2: rhodopsin-based fluorescent proteins
 - Voltage reporters: Arch, QuasAr
- Notable exception 3: proteins that bind exogenous fluorescent molecules
 - Voltage reporters: Voltron
-

Outline

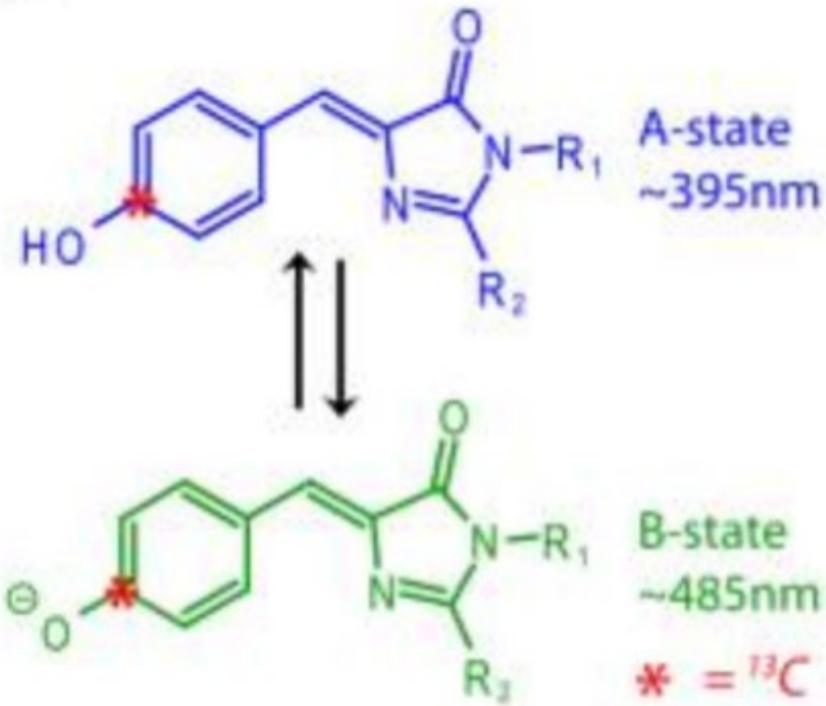
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How do we couple a cellular phenomenon to changes in fluorescence signal?

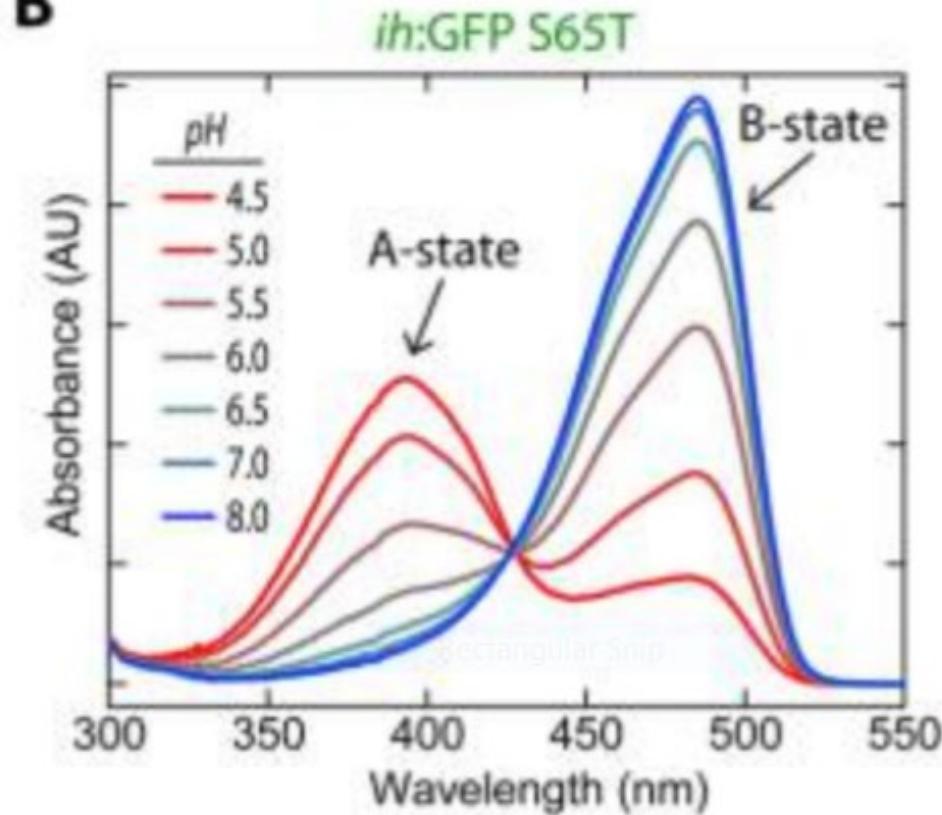
- Chromophore protonation/deprotonation
- Förster Resonance Energy Transfer (FRET)
 - Also called Fluorescence Resonance Energy Transfer

The GFP chromophore is in equilibrium between protonated and deprotonated states

A



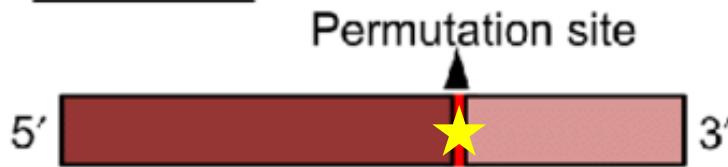
B



Normally, the chromophore in EGFP is in the deprotonated state (B) because the β-barrel shields it from water

Circular permutation of fluorescent proteins

DNA level:

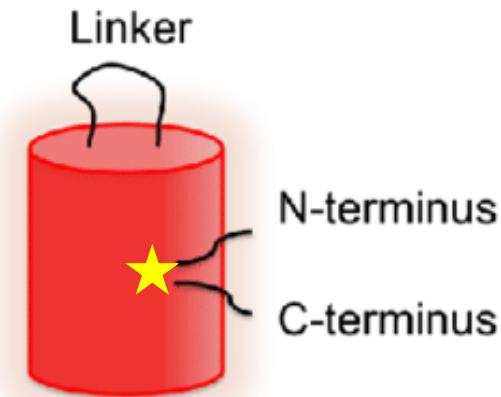


Protein level:

N-terminus C-terminus



**Circular
permutation**

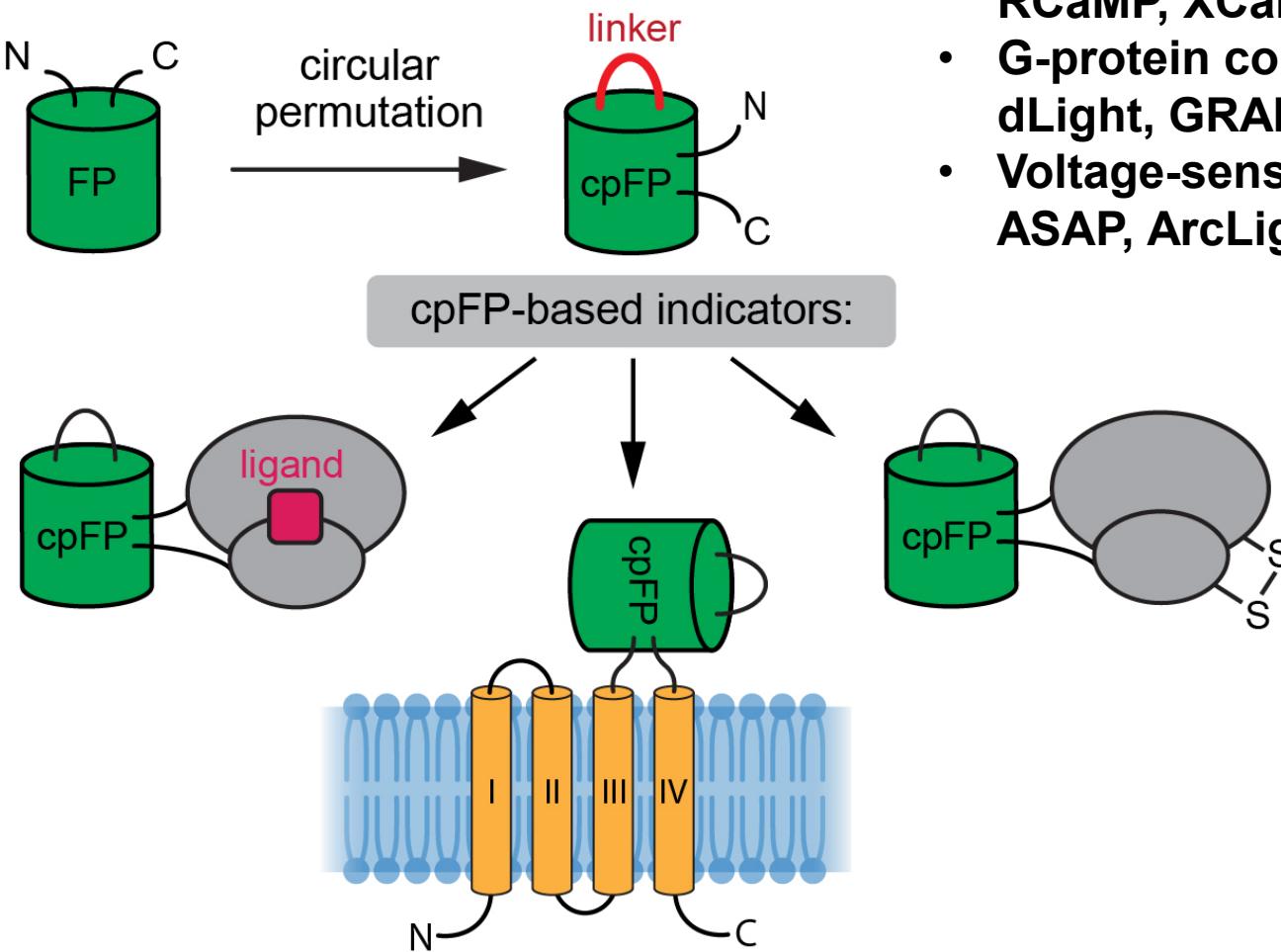


- The original N- and C-termini are far away from the chromophore
- The new N- and C-termini are right next to the chromophore

Circular permutation opens the β -barrel and exposes the chromophore to water, protonating the chromophore

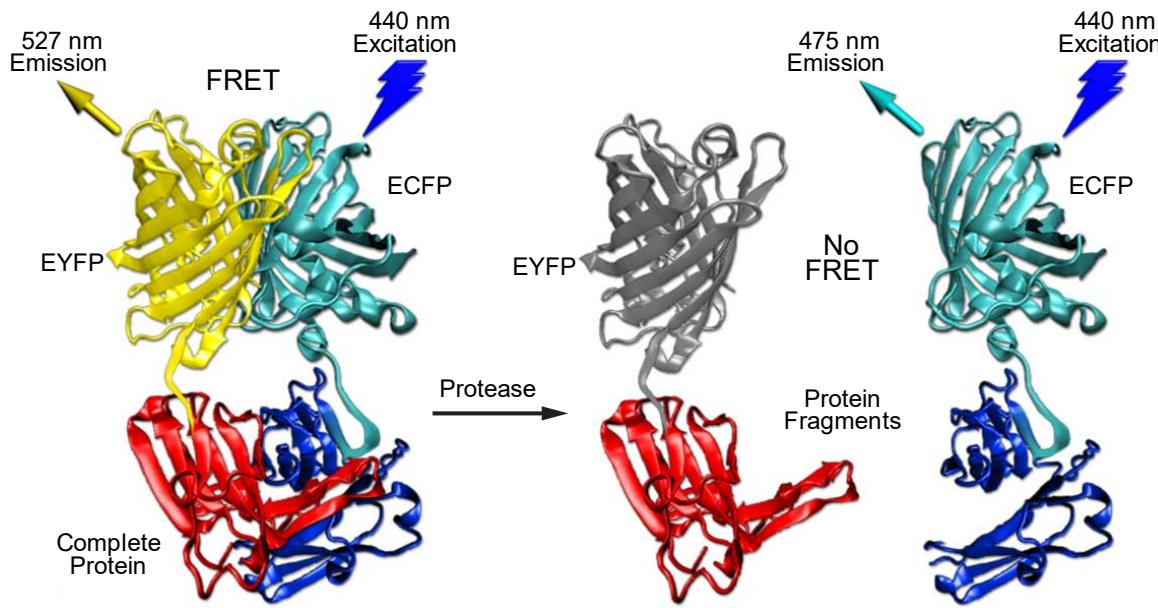
Open: protonated, low fluorescence

Closed: deprotonated, high fluorescence



- Calcium binding: GCaMP, RCaMP, XCaMPs, etc.
- G-protein coupled receptors: dLight, GRABDA, etc
- Voltage-sensitive proteins: ASAP, ArcLight

Förster Resonance Energy Transfer (FRET)



$$Q = \frac{1}{1 + \frac{R^6}{r^6}}$$

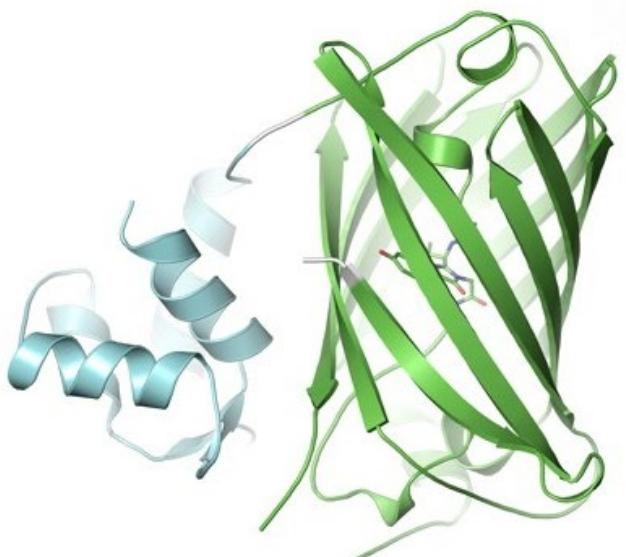
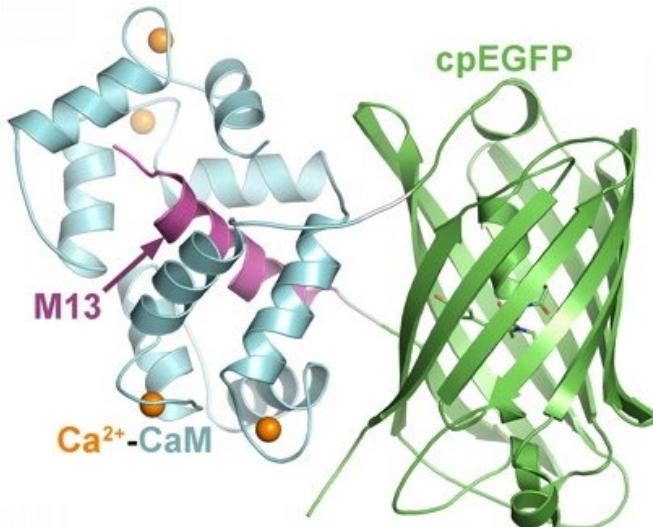
r⁶, not r²!!

- Also called Fluorescence Resonance Energy Transfer
- Conceptually: ECFP emits blue photons that excite EYFP
 - If far apart, exciting ECFP yields a blue photon
 - If close together, EYFP absorbs the blue photon, and exciting ECFP yields only a yellow photon
- The actual mechanism is more complicated than that

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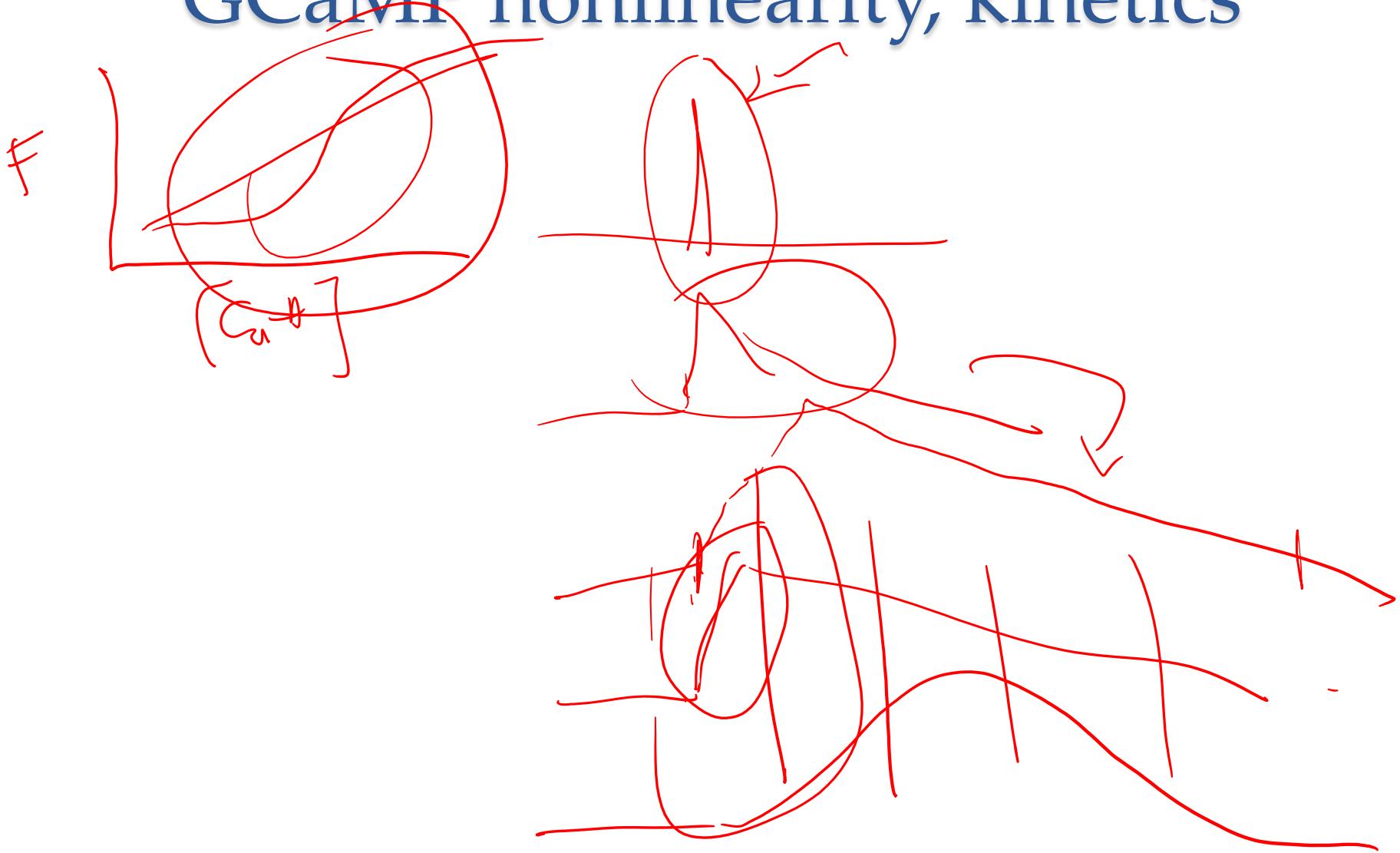
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GCaMP (and related GECIs)



- GECI: genetically encoded calcium indicator
- The M13 peptide is toxic to cells, especially during development
 - Ca++ buffering is not the mechanism of GCaMP toxicity
 - Less toxic variants now exist (GCaMP-X and the XCaMPs)
 - Test serial dilutions of each new AAV batch
- Different GCaMP variants have different baseline fluorescence
- GCaMP has multiple Ca++ binding sites with cooperative binding
 - Nonlinear, limited dynamic range
- Different GCaMP variants exist with different on and off kinetics

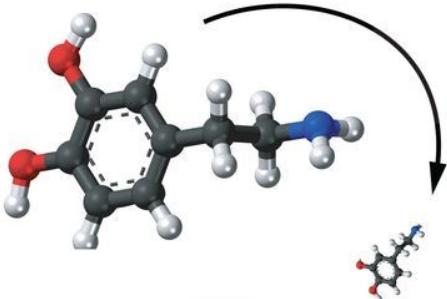
GCaMP nonlinearity, kinetics



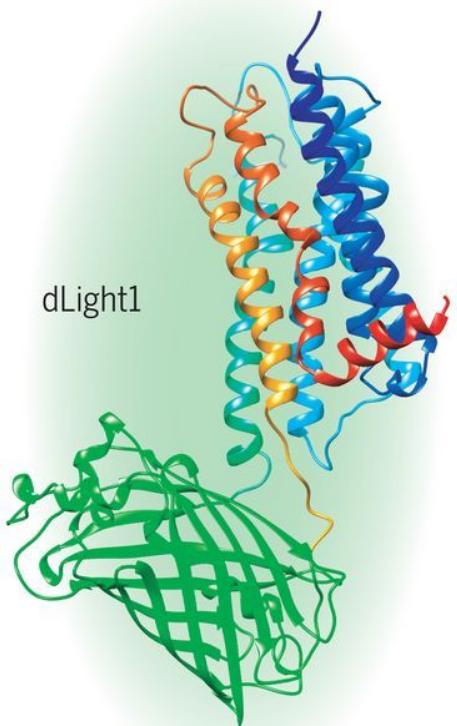
GCaMP nonlinearity, kinetics

GPCR-based reporters

Dopamine



dLight1



- GPCR: G-protein coupled receptor
- dLight: D1 dopamine receptor
- GRABDA: D2 dopamine receptor
 - Now available in red versions
- Sensors available for serotonin, norepinephrine, opioids, etc.
- ~400 non-olfactory GPCRs in genome

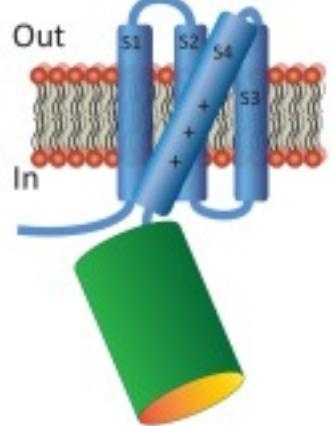
Genetically encoded voltage indicators (GEVIs)

- Making GEVIs is much harder than GECIs
 - Voltage only detectable at the membrane, not in the cytoplasm
 - Voltage sensing is a lot more complicated than calcium binding
 - Voltage sensors are more likely to perturb normal cellular function
 - Harder to screen GEVI candidates in vitro
- Imaging GEVIs is much harder than GECIs
 - Fluorophore is membrane-localized, not cytoplasmic
 - Signal comes mostly from processes, not soma. Each pixel contains membrane from many cells
 - Signals are much shorter in duration – requires high imaging speeds and very strong signal
 - Membrane potential is more strongly correlated between different cells than spikes are – hard to tell which cell the signal is from

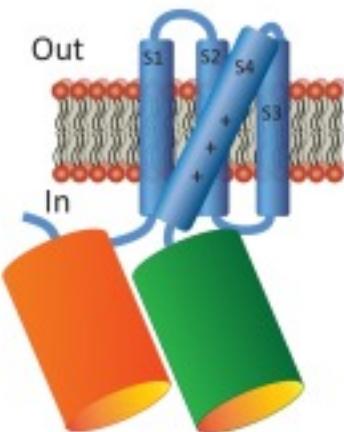
Schematic structures of three types of GEVIs

(A) Type 1: Voltage sensitive phosphatase based mosaic sensors

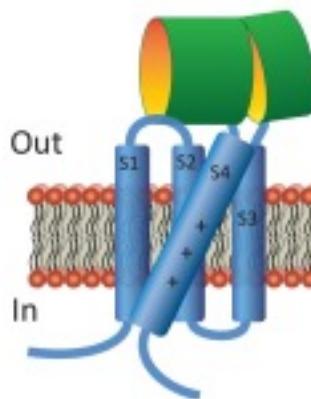
Single FP



Butterfly FRET pair

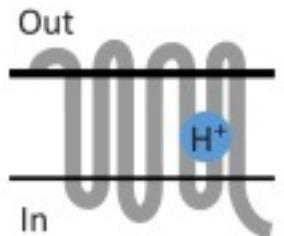


Circularly permuted

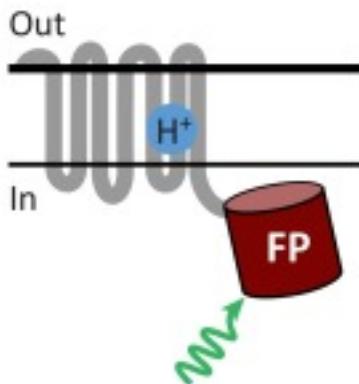


(B) Type 2: Microbial rhodopsin based sensors

Single chromophore

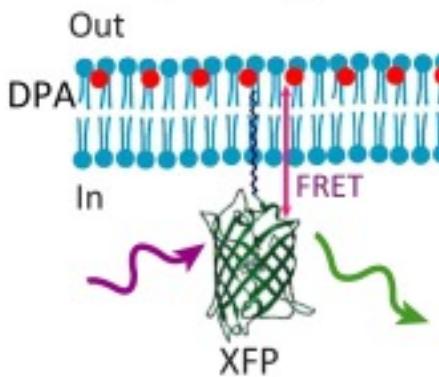


FRET quenching



(C) Type 3: Dual component sensors

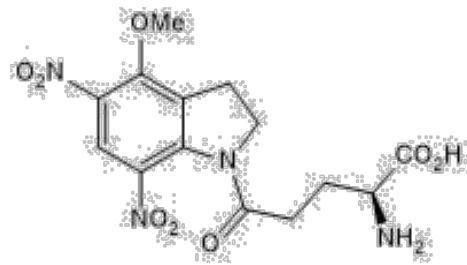
FRET quenching



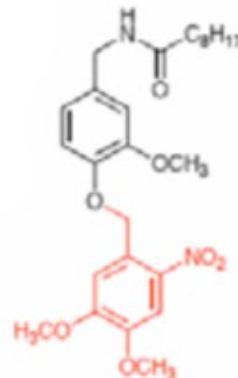
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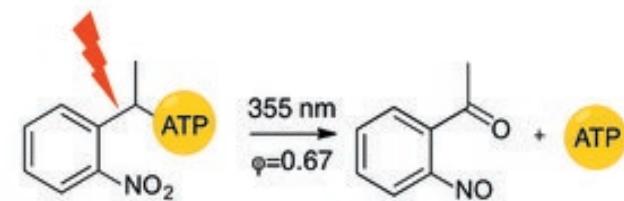
Photocaged molecules



Caged glutamate +
endogenous receptors

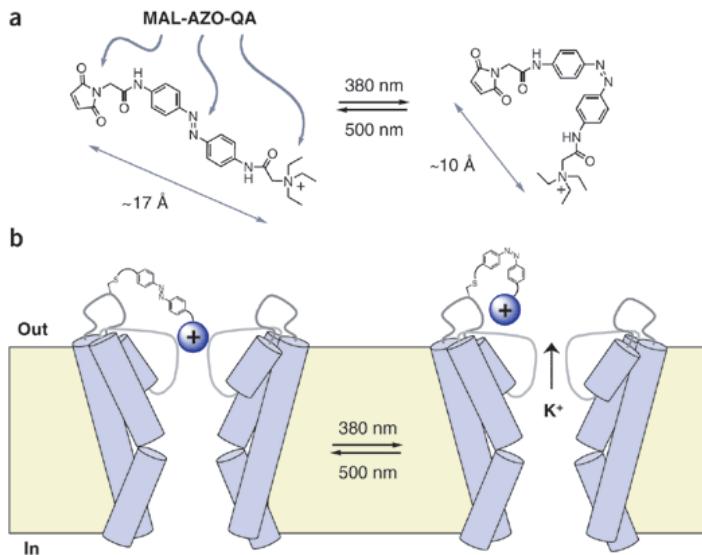


Caged capsaicin +
TRPV1 receptor



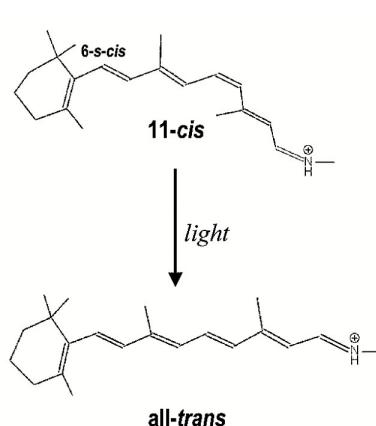
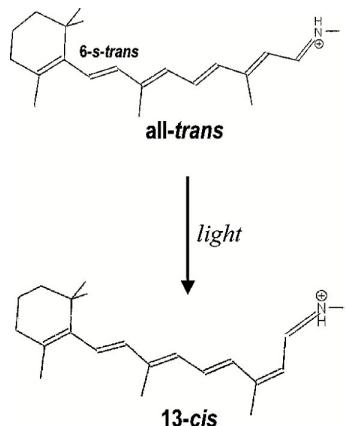
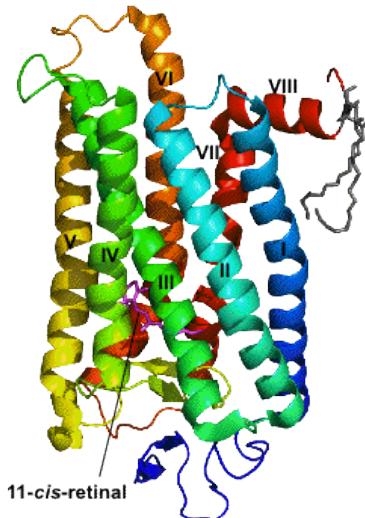
Caged ATP +
P2X₂ receptor

MAL-AZO-QA +
modified K⁺ channel

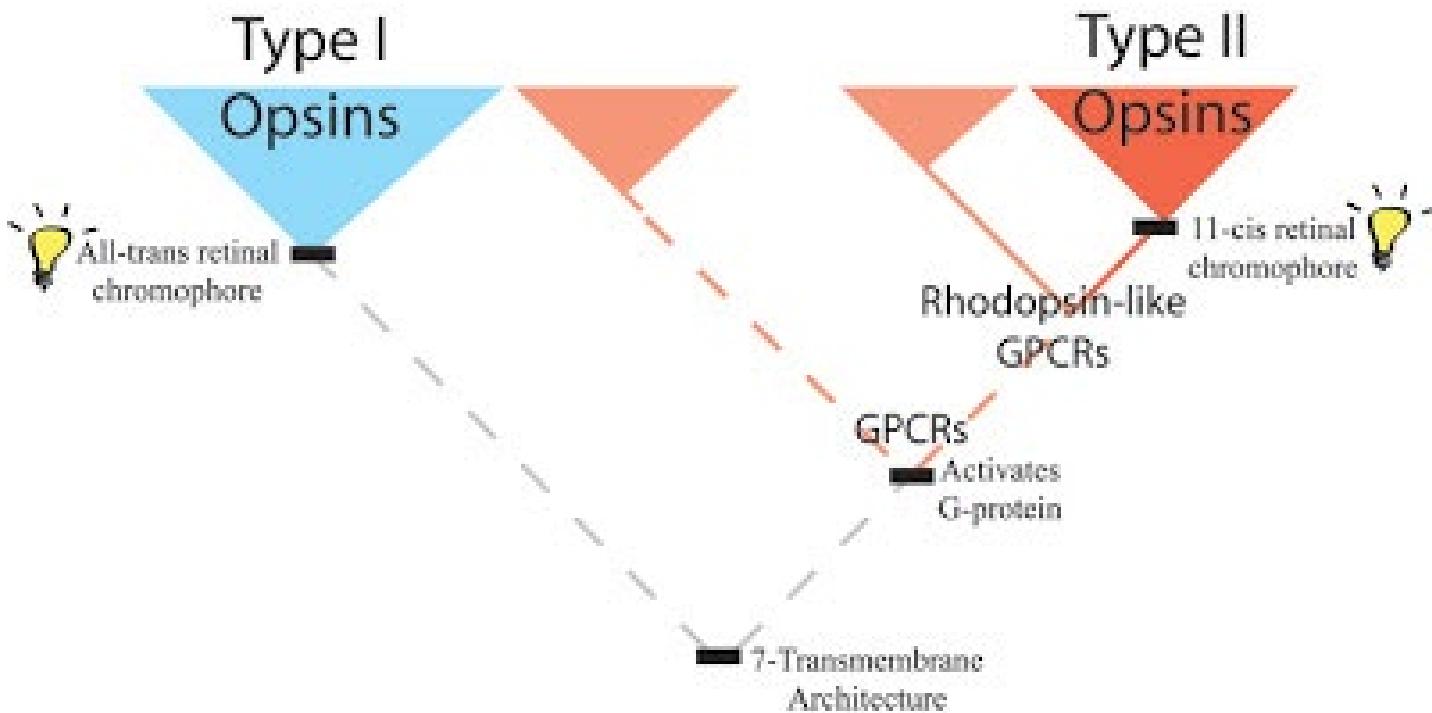


The key idea: proteins
can't sense light, so an
exogenous light-sensitive
cofactor is required

Opsins: addition of cofactor is not (always) required



But how do you isomerize it back?



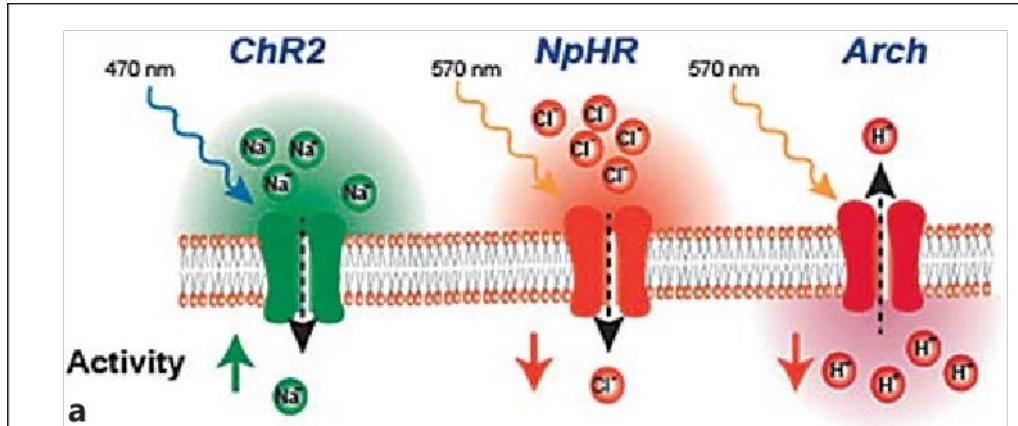
Different opsins re-isomerize their chromophore differently

- Metazoan opsins
 - **Gi-coupled family:** vertebrate visual opsins
 - Chromophore dissociates from protein after absorbing photon. Is re-isomerized by a special biochemical pathway only in the retina
 - **Gq-coupled family:** invertebrate visual opsins, mammalian melanopsin
 - Chromophore does not dissociate. Re-isomerizes after absorption of second photon at different wavelength
- Microbial opsins
 - **Ion pumps and channels:** bacteriorhodopsin, halorhodopsin, channelrhodopsin, etc.
 - Mostly used for photosynthesis
 - Chromophore does not dissociate from protein. Re-isomerizes in dark

Metazoan opsins

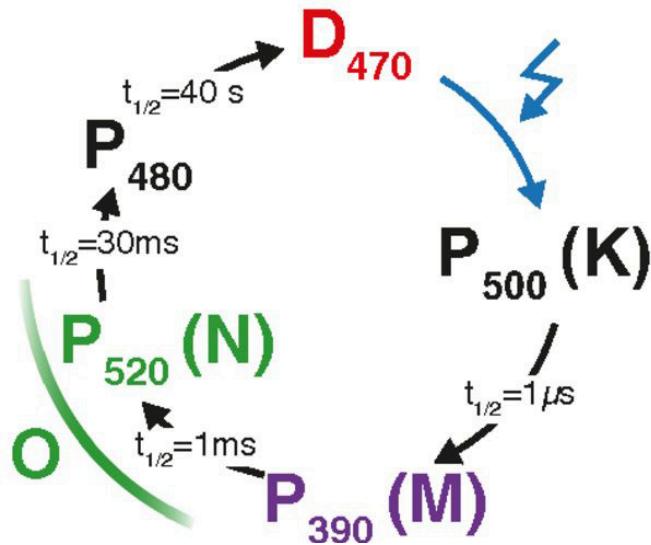
- “OptoXRs” – mutant rhodopsins available in Gs-, Gi-, and Gq-coupling versions
- Wild-type rhodopsin (Gi-coupled) does suppress presynaptic vesicle release
- Have not been used much
 - Chromophore does not regenerate, so runs down over time
 - Anecdotal: too light sensitive, activated by room light
- **Lamprey parapinopsin: a bistable Gi-coupled opsin for silencing presynaptic terminals**
 - Blue light activates it (suppressing vesicle release)
 - Green light inactivates it
 - Bruchas lab had a poster at SfN two years ago...

Microbial opsins

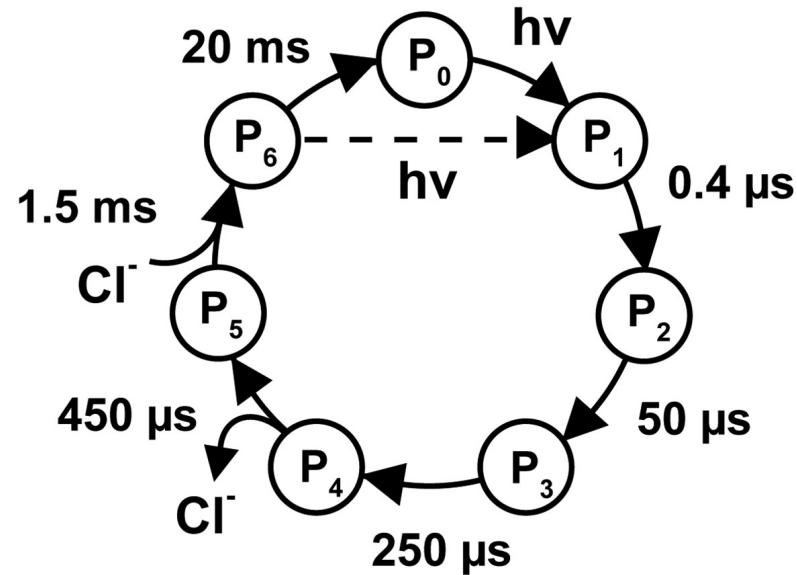


- Channels
 - Cation (excitatory): ChR2, ReaChR and ChrimsonR (red-shifted)
 - Low light sensitivity, small unitary conductance
 - Anion (inhibitory):
 - Artificial: iChloC, iC++, SwiChRca, etc. – low conductance
 - Natural: GtACRs - high conductance
- Pumps
 - Chloride (inhibitory): Halo (aka NpHR), Jaws (red-shifted)
 - Proton (inhibitory): Arch
- Great resource: <https://www.addgene.org/guides/optogenetics/>

Opsin photocycles



ChR2 (cation channel)



Halo (Cl⁻ pump)

Kinetics

Channels: fast on, slow off

Pumps: fast on, fast off

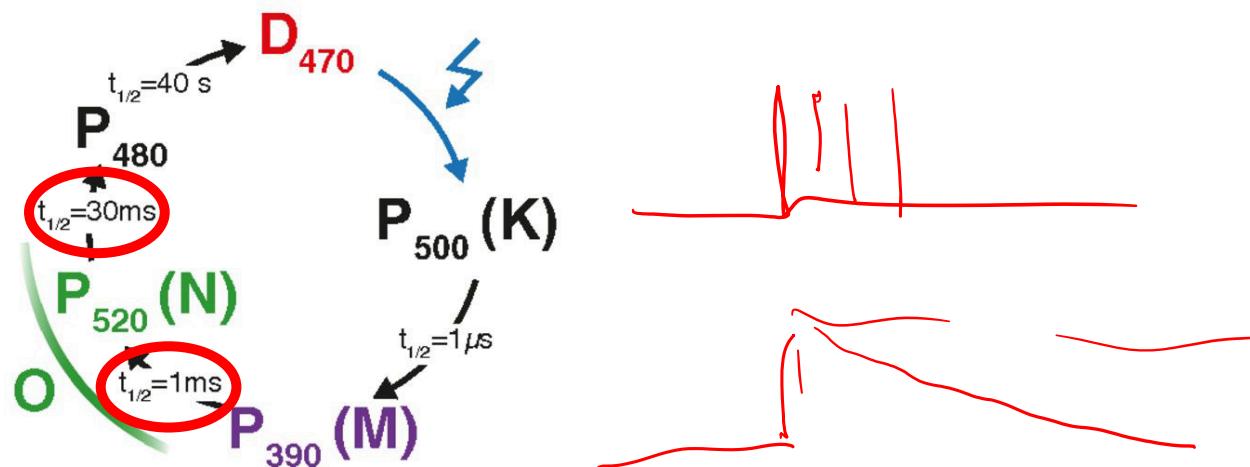
Light sensitivity

Channels: one photon = many ions

Pumps: one photon = one ion

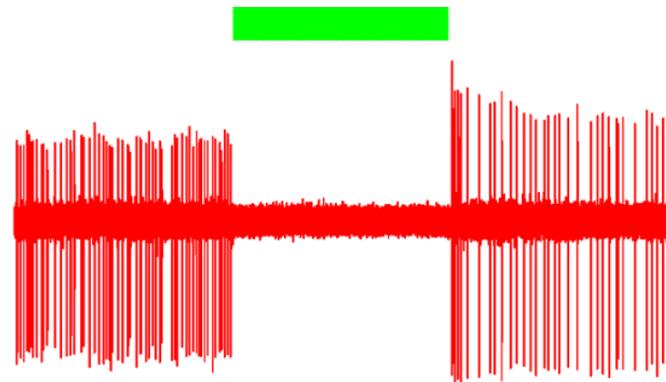
There is a tradeoff: faster off-kinetics means fewer ions per photon

Opsin currents are filtered light waveforms

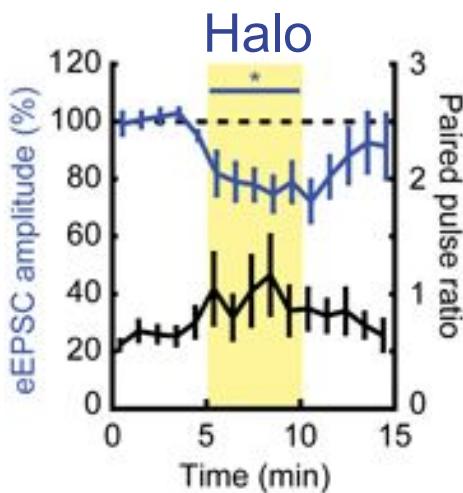
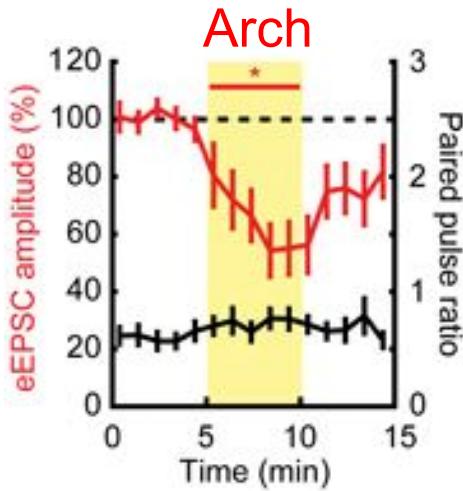


Exciting cells is easier than inhibiting them

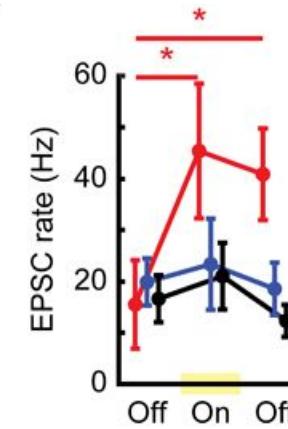
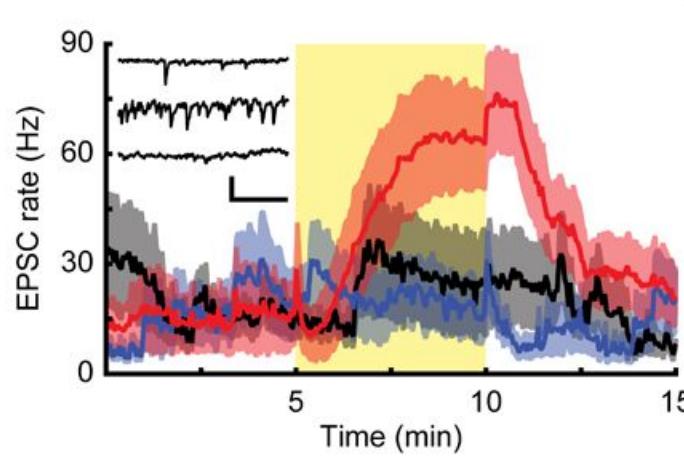
- Inhibition requires more light
 - Excitation: brief pulses
Inhibition: constant illumination
 - Channels: many ions/photon
Pumps: few ions/photon
 - Tissue heating becomes a factor
 - Inhibition is often incomplete
- Post-inhibitory rebound
 - Worse with Halo (Cl^-) than Arch (H^+)
- Pumps alter ion concentrations
 - Halo increases intracellular [Cl^-]: GABA becomes excitatory
 - Arch increases intracellular pH: causes spontaneous vesicle release
- GtACRs (high-conductance Cl^- channels) are the most promising



Inhibiting axon terminals with microbial opsins works, sort of



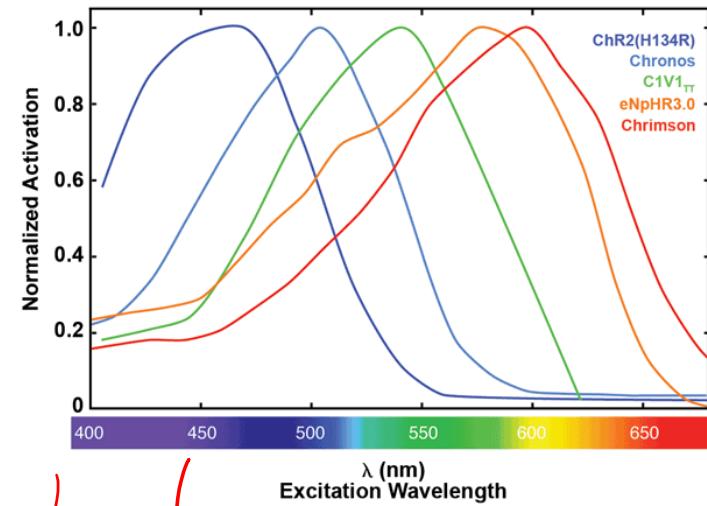
Mahn et al, Nat Neurosci 2016



- Neither Arch nor Halo blocks release completely
- Arch causes increased rate of spontaneous release (of unclear significance)
- GtACRs don't suppress vesicle release at all
- GtACRs suppress somatic APs but induce a single axonal AP on light initiation
- Lamprey parapinopsin may be our savior...? ● 43

Tips/caveats for opsins

- All opsins are stimulated by short-wavelength light
- Pulsed light can lead to entrainment
- To inhibit part of cortex, stimulating its inhibitory neurons is more effective than inhibiting its excitatory neurons
- Stimulation of axon terminals will cause retrograde spikes to propagate to the soma and everywhere else that cell projects
- Optotagging is not trivial and can leads to lots of false positives!

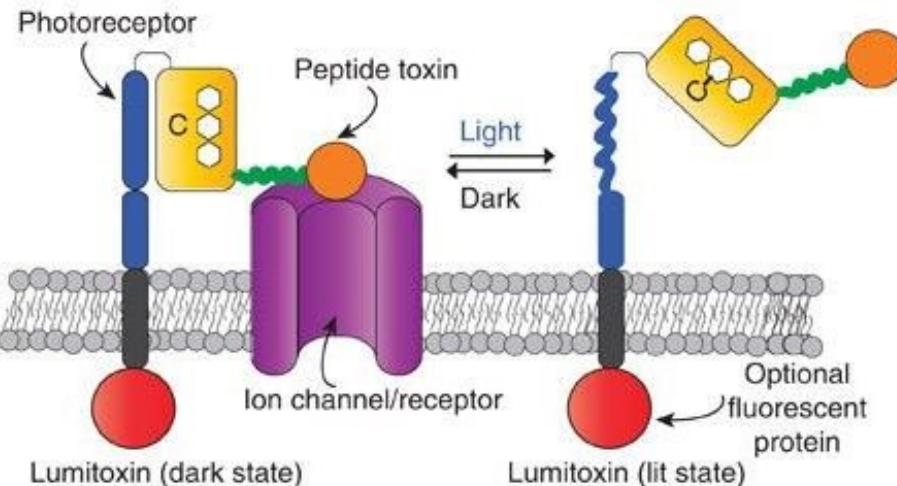


Stujenske and Gordon paper with matlab toolbox for light propagation and heating calculations:

<https://www.ncbi.nlm.nih.gov/pubmed/26166563>

STUJENSKE
J GORDON

LOV-Jalpha: versatile light-sensitive protein domains

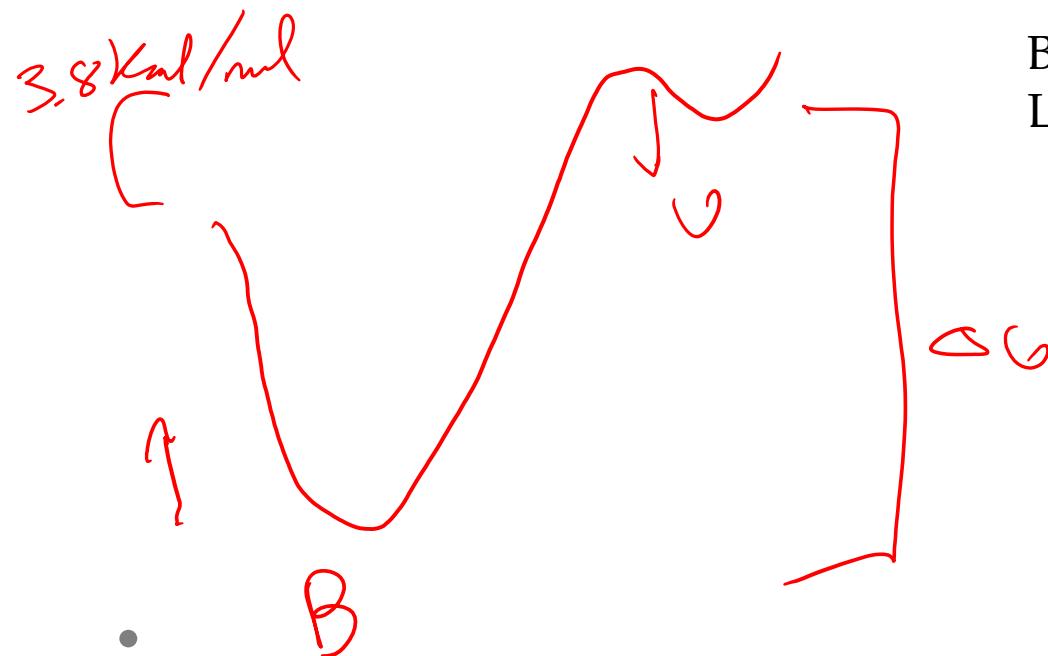


LOV-Jalpha used for many applications

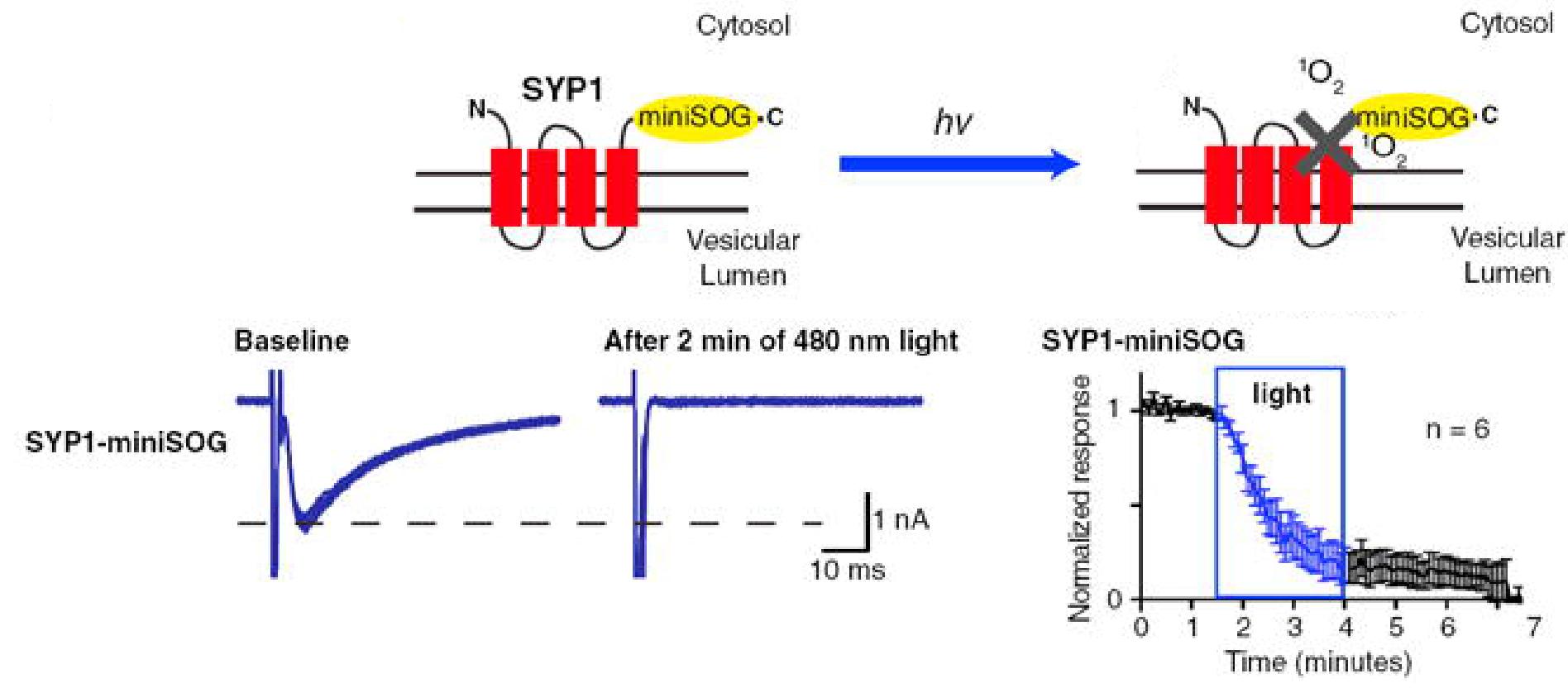
Turns on in ~1 second, off kinetics variable

Lumitoxins: not widely used

Blue photon energy: 64 kcal/mol
LOV-Jalpha $\Delta\Delta G = 3.8 \text{ kcal/mol}$



CALI: chromophore-assisted light inactivation

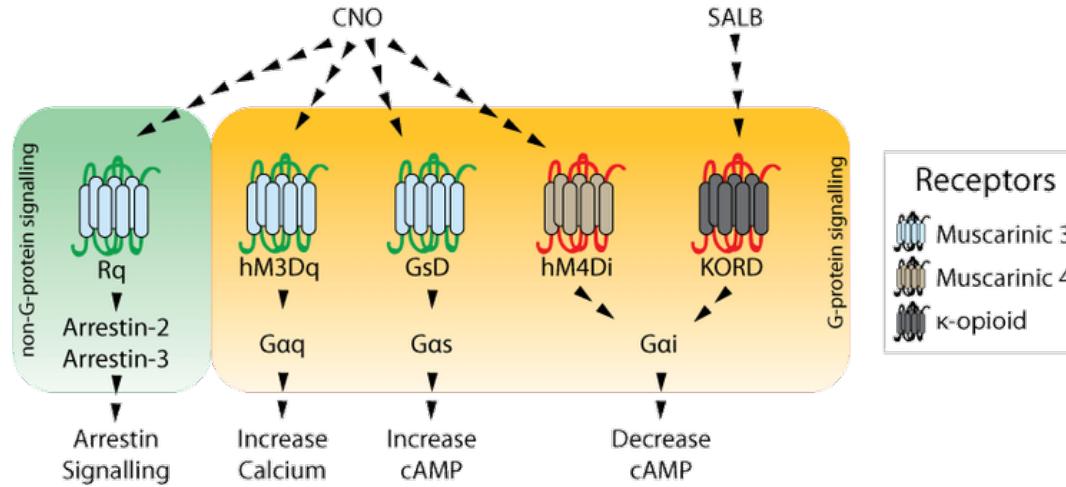


Opinion: CALI is probably underused in neuroscience

Outline

- Sensors
 - Bioluminescence vs fluorescence
 - Overview of fluorescent proteins
 - Mechanisms to couple cellular phenomena to changes in fluorescence signal
 - Genetically encoded indicators: calcium, voltage, etc.
- Actuators
 - Optogenetic
 - Photocaged molecules
 - Opsins
 - LOV-Jalpha interactions
 - CALI
 - **Chemogenetic**
 - Metabotropic: DREADDs
 - Ionotropic: PSAM/PSEM, GluCl

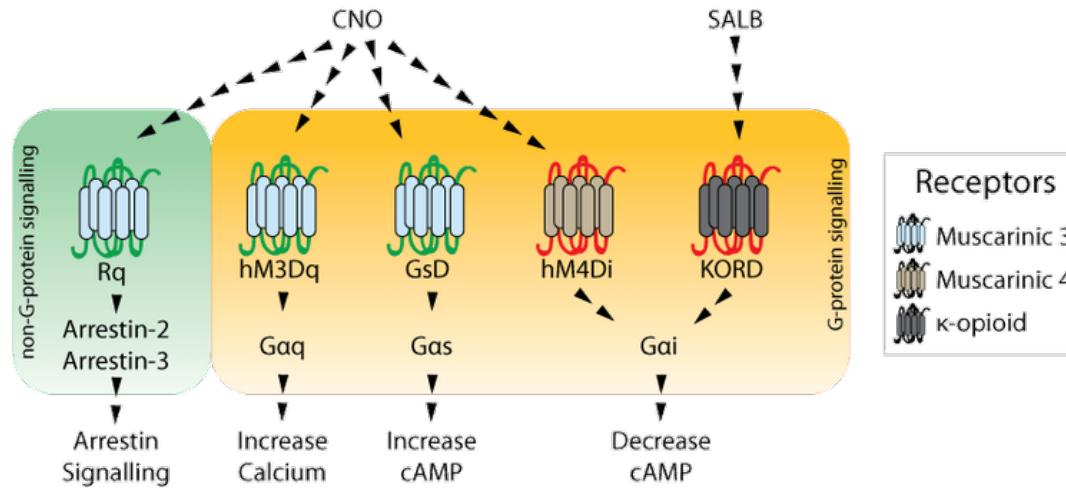
DREADDs: Designer Receptors Exclusively Activated by Designer Drugs



- The theory: CNO is a pharmacologically inert compound that activates DREADDs and nothing else
- The reality: the liver converts CNO to clozapine, which activates the DREADDs. Clozapine binds many receptors, but the dose required to activate DREADDs is very small
- Mike Michaelides's group has developed next-generation DREADD ligands that apparently are truly DREADD-selective at useful concentrations
- Salvinorin-B: appears to be pharmacologically inert, has extremely short half-life
 - Anecdotes: some sources of salvinorin-B are contaminated with salvinorin-A, a hallucinogen

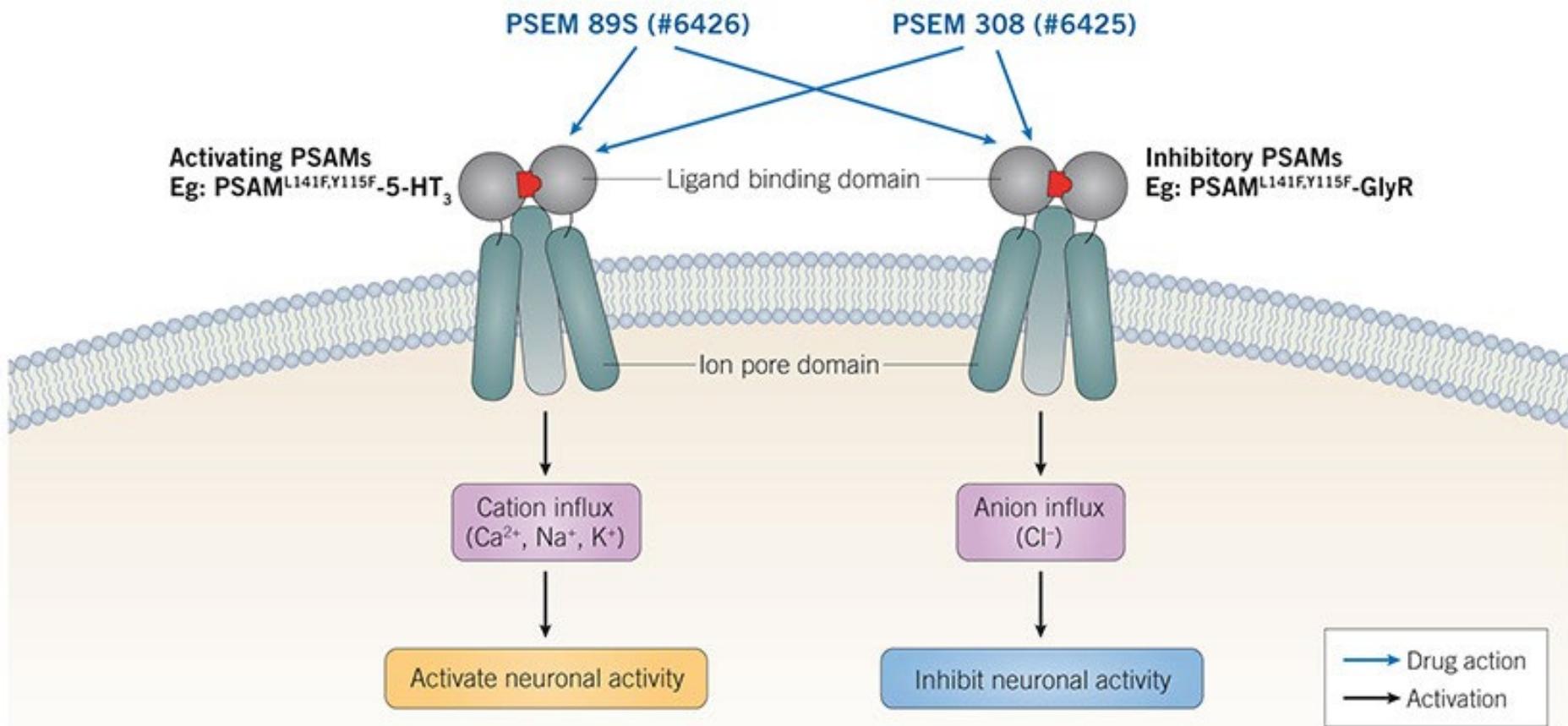
RUN CONTROLS!!

DREADDs: Designer Receptors Exclusively Activated by Designer Drugs



- KORD and hM4Di: activate Gi signaling, decrease cAMP
 - Activates GIRK K⁺ channels (in some cells), hyperpolarizing membrane
 - Inhibits presynaptic vesicle release: inhibits Ca⁺⁺ channels and SNAREs
 - This is the main mechanism underlying behavioral effects
- hM3Dq: activates Gq pathway, increases firing rates. Shown in grid cells to increase firing rates without disrupting grid structure.

Ion Channel Chemogenetics: PSAMs/PSEM



- Ionotropic, available in both excitatory and inhibitory versions
- First generation PSEM/PSAMs had low affinity, and PSEMs were not commercially available
- New generation: activated by microdoses of varenicline or tropisetron, which are approved for use in humans

When to use optogenetics vs chemogenetics?

Optogenetics

Good for short periods, bad for >5-10 seconds at a time*

More equipment, animal requires tether

Best for small brain regions

Precise manipulations, at specific locations, task phases, etc.

Within-session manipulations enable within-subject expt design (e.g. compare activity +/- opto)

Broad generalization: takes longer to set up but can yield data that's easier to interpret

Chemogenetics

Low temporal resolution, good for entire behavioral sessions

Less equipment, animals move freely, more animals simultaneously

Equally good for large or small

Less precision but activity patterns are not grossly non-physiological

Negative control animals necessitate between-subject expt designs

Broad generalization: faster to set up but usually requires more animals, analysis can be more difficult

Alternatives to opto/chemo-genetics

- Both are bad on long timescales. hM4Di has been shown to desensitize and stop working after ~24-48 hours if CNO is put in drinking water
 - Lesions, AAV-tetanus toxin, diphtheria toxin
 - NaChBac (excitatory) or Kir2.1 (inhibitory) are leaky channels that can be expressed under doxycycline regulation and used to increase or decrease excitability of cells for periods of days-weeks
- DREADDs should not be conceptualized as excitatory and inhibitory. They have many complex effects

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Bonus topic: glue

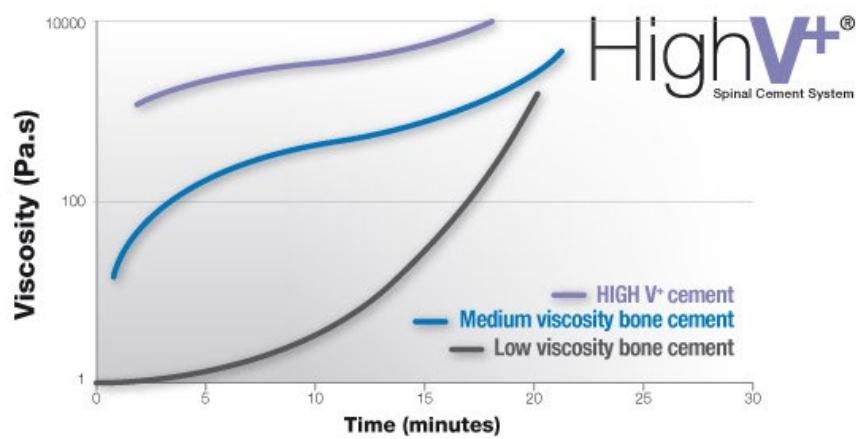
- Cyanoacrylates: super glue, Krazy glue, Vetbond
 - High adhesive strength, non-space-filling. Adheres reasonably well to ceramic, good for repairs
 - Cures rapidly
 - Refrigerate for longer shelf life
- Epoxy resins: two tubes, many different types
 - High adhesive and cohesive strength. Space filling
 - Adheres reasonably well to ceramic and metal, different formulations for different surfaces
 - Different formulations with different curing times
 - Initial cure is rapid. Requires overnight for full strength. Can be sped up by heating
 - Epoxy putty also available
 - Recommend: Bob Smith Industries
- Hot glue gun: useful for temporary attachment
- Solvent welding: attach two pieces of plastic together by putting solvent in the joint
 - Good joint will be full strength
 - Good for laser-cut acrylic
 - Usually non-space-filling, but you can buy space-filling versions
 - Has to be matched to your specific type of plastic

Old-school dental acrylic, aka “grip cement”



- Recommend Unifast Trad
 - Hardens into plastic that is very strong. Moderate adhesive strength, high cohesive strength. Rapid curing to full strength. Usually translucent but opaque available.
 - Mix powder and liquid (add with disposable dropper) in silicone cup. With practice, can mix different ratios for thick/thin. If mixed thick, cures more rapidly. To extend working time, intentionally mix too thin and keep stirring until viscosity is in correct range. Thin mixing slightly worsens polymerization shrinkage.
 - Can be removed by soaking in chloroform with water layered on top (to prevent chloroform evaporation). Can be melted and reshaped with soldering iron.
 - Cons: liquid contains methylmethacrylate monomer. Strong odor before it has cured, toxic if applied directly to brain tissue.

Original application of dental acrylic: “bone cement”



Visible Light Curing (VLC): blue light, ~470 nm



- Blue light curing vs. UV curing: UV curing no longer used in dentistry
- UV curing: still used for optical applications, e.g. coupling optical fibers to a laser diode (Thorlabs Norland cement)
- Unifast LC: light-curable dental acrylic. Similar properties to other powder/liquid dental acrylic but stickier and a little harder to work with

Recommended light-curable dental acrylic: Triad Gel



- Used to make/repair dental appliances (e.g. retainers)
- Easier to work with than traditional dental acrylic: premixed at perfect thickness, minimal odor, nontoxic, near-infinite working time under normal room light, cures in ~5 seconds under blue light wand
- High cohesive strength, lower adhesive strength than traditional acrylic, slightly more brittle
- Transparent, comes in several colors
-

LC composite resins: dental filling material

- Flowable: Flow-it ALC
 - Comes in convenient syringe applicator. Very easy to use
 - Can substitute for Triad Gel
- Non-flowable: Filtek Z250
 - More putty-like texture

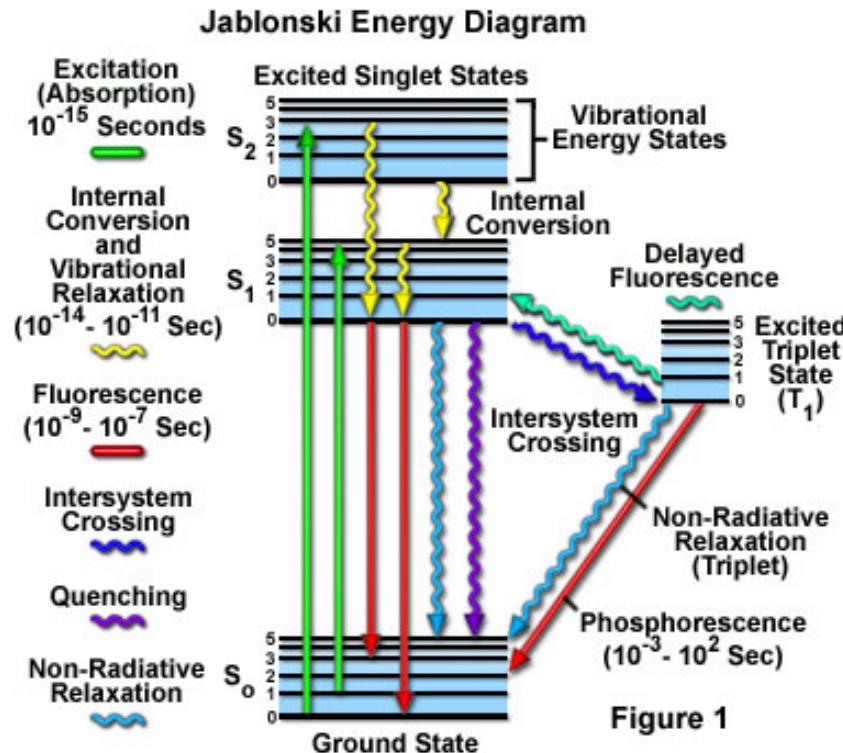


For attachment to bone: dental luting cement



- Luting cement: designed to act as a layer between two dissimilar materials to bond them together (e.g. tooth and filling material). Adheres strongly to skull, providing a base layer on which to apply other adhesives
- Recommend OptiBond Universal: cures on exposure to blue light
- Very thin, use two layers on skull (paint, cure, paint, cure)
- Many labs still use C&B Metabond, but this is faster and easier
-

Biophysics of fluorescence and photobleaching



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mer/java/jablonski/jabintro/jabl
onskijavafigure1.jpg](https://micro.magnet.fsu.edu/pri mer/java/jablonski/jabintro/jablonskijavafigure1.jpg)