More biosensors, optical actuators
• Last class
  • Watershed
  • Intro to biosensors

• This class
  • Dye based sensors
  • Protein based sensors
  • Actuators
Biosensors at large

• Anything that changes fluorescence in response to physiological change

• Difficult to optimize across every parameter

• Check pH sensitivity
Measuring signals

- Easy thing to think about is change in fluorescence divided by original fluorescence (dF/F)
- Unfortunately it’s useless
- Signal to noise ratio = signal – background/std
- Smallest feature you can resolve is when SNR = 1
- What is the smallest amount of signal you can resolve in some time measurement

\[
\text{dF/F} = \frac{12-2}{2} = 5
\]

\[
\text{dF/F} = 500\%
\]

Hooray!

\[
\text{dF/F} = \frac{10-.1}{.1} = 99
\]

\[
\text{dF/F} = 9900\%
\]

Hooray?

\[
\text{dF/F} = \frac{12-2}{2} = 5
\]

\[
\text{dF/F} = 500\%
\]

Hooray?
Ratiometric imaging

- Ratiometric imaging is by far the easiest way to get absolute units on a measurement.
- Measuring a single color makes it impossible unless you know the number of the fluorophores present (usually impossible).
- Having two colors at a defined stoichiometry can correct for this issue.
- Doesn’t affect localization based sensors.

\[
\text{Total Fluorescence} = \#\text{Fluorophores} \times QY(\text{env}) \times \text{absorption} + \text{background}
\]

\[
\text{Ratio} = \frac{N_1 \times QY_1 \times \epsilon_1}{N_2 \times QY_2 \times \epsilon_2}
= \frac{QY_1 \times \epsilon_1}{QY_2 \times \epsilon_2}
\]
Calibrating ratiometric sensor

- Use either two excitations, or two emissions (or both)
- First have to map each color at known concentrations of analyte
- Generate, and fit ratio to hill curve, with known concentrations on the x axis
- Use fit as look-up table for future experiments
- You can NOT change laser intensities or alignment after determining fit

\[
F_1 = I_1 \left( N_{Fl} \cdot QY_1 \cdot \varepsilon_1 + QY_2 \cdot N_{Fl} \cdot \varepsilon_2 + QY_{AF1} \cdot \varepsilon_{AF1} \right) + bg
\]
\[
F_2 = I_2 \left( QY_1 \cdot N_{Fl} \cdot \varepsilon_1 + N_{Fl} \cdot QY_2 \cdot \varepsilon_2 + QY_{AF2} \cdot \varepsilon_{AF2} \right) + bg
\]

\[
\text{Ratio} = \frac{QY_1 \cdot \varepsilon_1}{QY_2 \cdot \varepsilon_2}
\]

1. Subtract background
2. Determine laser intensities to use
3. Determine non-expressing autofluorescence from each laser
4. Look at bleed through from each excitation – should be 0
5. Subtract background from non-cell region
6. Subtract bleed through equivalent from each channel
7. Divide to get ratio
Principles of sensor design

- Imagine a FRET pair – highly sensitive to distance between fluorophores
- Find a protein, somewhere in nature, that binds to the ligand you’re interested in measuring
- Attach FPs to either side of protein that undergoes large conformational change
- Try to maximize conformational distance

\[ r = R_0 \left( \frac{1}{E} - 1 \right)^{1/6} \]
Issues with this process

• Nearly every prototype sensor had the same range of 0.25

• Crazy given the large conformational changes

• Remember that proteins are flexible beasts, not static crystal structures

• Even if the FPs are on the same lobe, still see same FRET changes

• Knowledge: Large conformational changes are NOT needed

NMR structure of DNA repair protein
Circularly permuted proteins

- GFP folding is robust, so you can change where the C and N termini live
- Circularly permuted GFP has a very high sensitivity to pH
- Sensors can be made that modulate chromophore stability (pK_a)
Two main schemes for detection

- Find active domain
- Attach FRET pairs
- Attach to cpGFP
- For both schemes, you need a protein domain that will change conformation in the presence of what you want to detect
Dye based sensors
LiveDead stains

- Important thing to test both live and dead stains, hopefully all cells fall into one category
- Kits come with a dye to mark living cells and a second dye that will only stain dead cells
- Can be counted by microscopy or FACS

Calcein AM – used to detect functional esterases
Ethidium Homodimer – Loss of membrane integrity

FUN1 – Starts green, live cells convert it to red
Counterstain all cells with Calcofluor White (blue)
pH sensors

- pH changes everything, so it’s very easy to generate pH sensitive fluorophores
- Numerous dyes and FPs, variety of colors and sensitivities
- Want to worry about color, photostability, solubility, $pK_a$
New red pH sensor

Semisynthetic fluorescent pH sensors for imaging exocytosis and endocytosis


Received: 23 March 2017
Accepted: 12 October 2017
Published online: 10 November 2017
Calcium sensors

**Fluo3**

- **Structure**
- **Indicator**
  - K_d(Ca^{2+})
  - R^2
  - R^2'
  - R^5
  - R^6
- **Concentrations**
  - Fluo-3: 0.39 μM
  - Fluo-4: 0.35 μM
  - Fluo-5F: 2.3 μM
  - Fluo-5N: 90 μM
  - Fluo-4FF: 9.7 μM

**Oregon Green BAPTA**

- **Fluorescence emission**
  - Ex = 488 nm
  - 39.9 μM free Ca^{2+}

**Fura Red**

- **Fluorescence emission**
  - Ex = 488 nm

**Fluo-4 AM Acetoxyethylster**

- **Images**
  - A: 10 μm
  - B: [Ca^{2+}] (nM)
  - C: Time duration (ms)

**Diagram**

- Extracellular medium
- Cell membrane
- Cytoplasm
- Esterases
- Ca^{2+}
- Reaction:
  - OOCO
  - COO^-
Voltage sensors

- Two types, those that localize to charged region of space (slow), and those that live in the membrane and sense the local electric field (can be fast)
- Dianepps is the most common. Phototoxic, low signal to noise ratio
- New dye by Tsien group is the best, Fluuovolt
Sodium an potassium ions

- No exceptional fluorescent dyes (or FPs)
- Usually extremely sensitive to changes in pH
- Hard to get dyes that are selective for one ion over another

SFBI – Na sensitive salt
Additional dye based sensors

• Other metals (Zn, Mg) – Similar to Ca sensitive domains – come in a variety of colors
• Reactive oxygen species – One or more reactive species modifies the dye to become fluorescent
• Na, K, Cl – Crown ether chelators
• Endocytosis – OxyBURST, oxidated endosomes turn on fluorescence, fluorescent growth factors
• Ion channels – Fluorescently labeled toxins
Fluorescent protein sensors
FP based sensors

- GFP is pH sensitive
- pH directly modifies chromophore
- pHluorin, ratiometric pHluorin, and super ecliptic pHluorin are all mutations of GFP that move the pKₐ into the physiological range
- Measuring the emission at 488 is a measure of cellular pH
• http://biosensor.dpb.carnegiescience.edu/
Calcium FP sensors

- First one was based on FRET Cameleon
- Calmodulin M13 domain
- Emerged from Tsien lab in 2000
Newer Ca$^{++}$ sensors

- GCaMP, Twitch, Pericams, CatchER

Calcium sensors are EXTREMELY good. Researchers have optimized all the properties – brightness, sensitivity, kinetics, folding.

Still lacking a really good red single color calcium sensor.

Each of the main sensors (GCaMP6, TWITCH, etc.) have > 1000 citations. Good to go.
HyPer – FP to detect H$_2$O$_2$

- Fuse cpYFP to hydrogen peroxide sensing domain from prokaryotes, OxyR
- Increased H$_2$O$_2$ will cause an increase in fluorescence
- Based on modification of cysteine (sulfur) groups
- Bright enough to get sub-cellular resolution

HeLa mitochondria undergoing apoptosis

Original paper has > 500 citations, probably OK to use or an updated version
Perceval – ATP sensing

- Start with GlnK domain from *E. coli*.
- Attach a circularly permuted Venus.
- PercevalHR – sensor of ATP:ADP ratio.
- Senses in the range of mammalian cytoplasmic ATP levels.
- Ratiometric sensor, dual excitation.
- Needs pHRed correction, have to monitor changes in pH.
On to Matlab...