Point 1: We can determine a cell's redox state from the excitation-emission pattern of a genetically-encoded protein sensor.

Some background on sensors

We work with fluorescent sensors. You shine light of a certain wavelength at them and, depending on that wavelength, the sensor shines light back at different intensities.

The relationship between the wavelength of the of light you shine on a sensor ("excitation wavelength") and the amount of light that it sends back ("emission intensity") is called an absorption spectrum.

Our sensors have an extra feature in that they have two "states", like the "on" and "off" state of a light switch. In one state, the sensor is oxidized and, in the other, the sensor is reduced. These two different states will have different absorption spectra. For our sensor, they look like Figure 1:

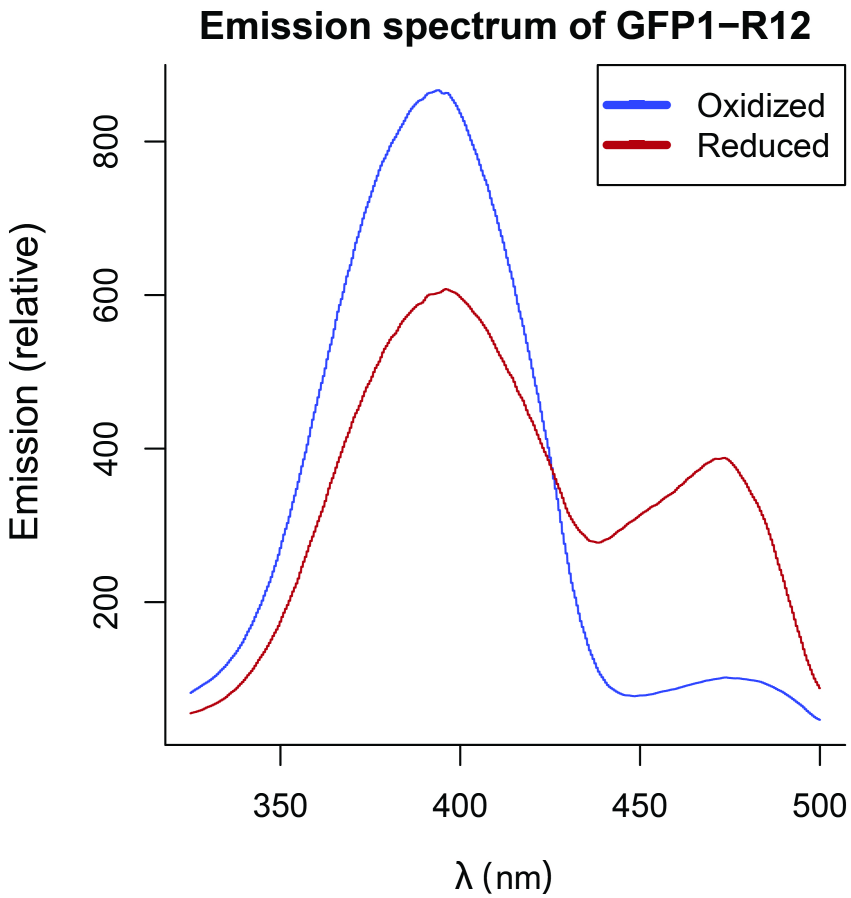

Figure 1. The horizontal (x) axis shows the wavelength λ of light being shined on the sensor. The vertical (y) axis shows the amount of light that the sensor shines back at the microscope.

How do spectra change at different redox states?

If we have a whole population of sensors, some fraction of them will be oxidized (depending on the redox state of the cell). If half of the sensors are oxidized and half are reduced, then the resulting excitation-emission spectrum will be exactly halfway between the "oxidized" and "reduced" spectra. In fact, for any percentage of sensors that are oxidized and reduced, the resulting spectra will be the weighted average between the two extreme spectra (Figure 2).

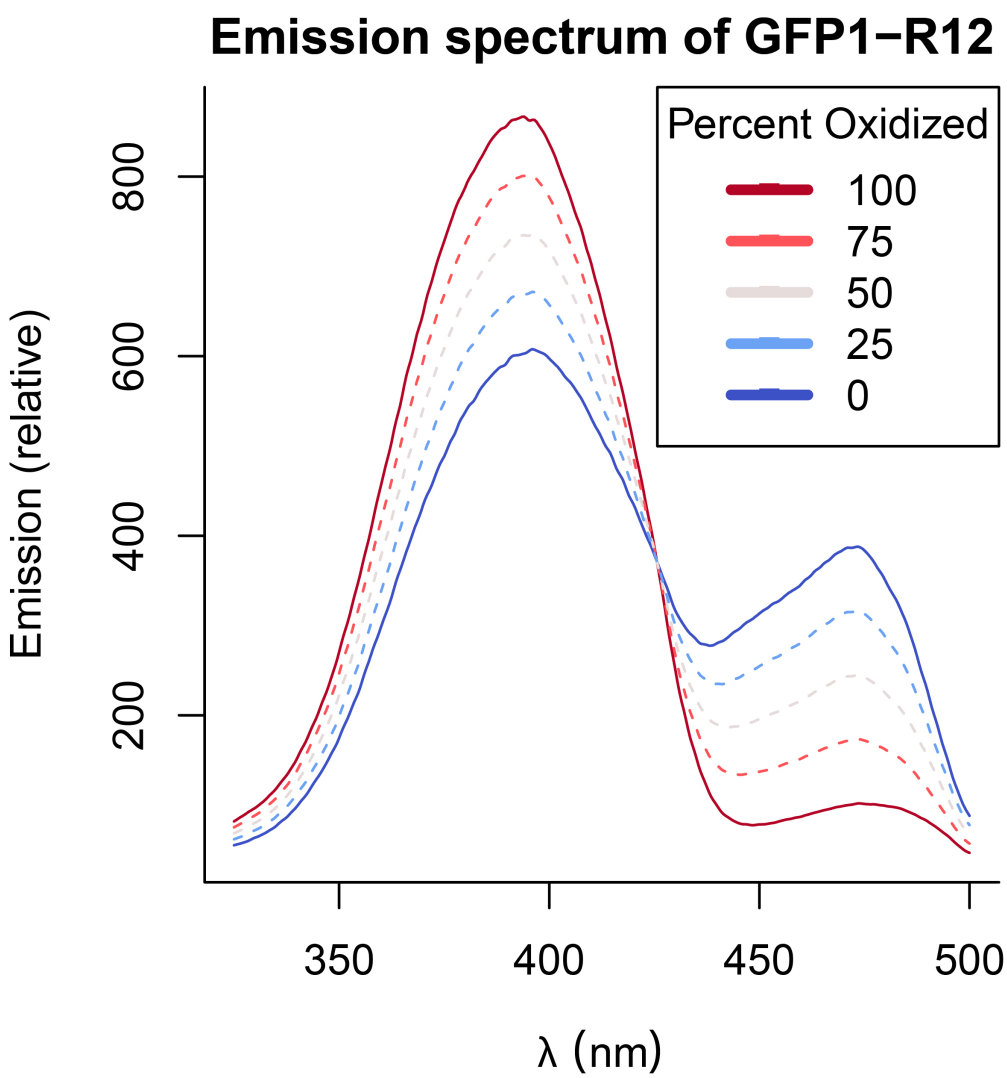

Figure 2. The horizontal axis shows the wavelength λ of light being shined on the vertical axis shows the amount of light that the sensor shines back at the microscope. The solid blue line shows the "extreme" case in which all sensors are reduced, and the solid red line shows the "extreme"case where all the sensors are oxidized. For cases where the sensors are not fully oxidized or fully reduced, the emission is a weighted average of the two extremes.

Some background on why the "percent oxidized" is useful

Our goal with **point 1** is to "determine a cell's redox state", right? So what does the "percent oxidized" have to do with redox state?

Everything, it turns out. When a redox-sensitive GFP is designed, physical chemists generally measure that protein's *midpoint potential*, which is the redox potential at which exactly half of the sensors are oxidized and half are reduced.

If we know the midpoint potential of a redox-sensitive GFP and the fraction of sensors that are oxidized, we can use the Nernst equation to figure out the redox potential of the environment where the redox-sensitive GFP lives.

The Nernst equation can be written like this: $E = E^\circ - \frac{RT}{2F} * ln(\frac{Reduced}{Oxidized})$. In that equation, E is the redox potential and E° is the midpoint potential. R, T, and F are the gas constant, the temperature, and the faraday constant, respectively. These are all constants and, under normal lab conditions, $\frac{RT}{2F}$ equals approximately 12.71.

If this doesn't quite make sense yet, let's do an example. Let's figure out what the redox potential of the cell must be if either 30%, 50%, or 70% of our sensors are oxidized. Let's say that our midpoint potential is $-270mV$. Also remember that 30% oxidized means that 0.3 of the sensors are oxidized while $1 - 0.3 = 0.7$ of the sensors are reduced.

- 30%: $E = -270mV - 12.71 * ln(\frac{0.7}{0.3}) = -280mV$
- 50%: $E = -270mV - 12.71 * ln(\frac{0.5}{0.5}) = -270mV$ *At 50% oxidized, the potential is the midpoint
- 70%: $E = -270mV - 12.71 * ln(\frac{0.3}{0.7}) = -259mV$

So we can see that, if we can figure out the percentage of sensors that are oxidized in a cell, we can also figure out the redox potential.

So, we're done, right?

So now let's try to set up an experiment. First, you record the emission spectra of an animal in a fully oxidized environment, and then an animal in a fully reduced environment so that you obtain your "extreme" cases of fully oxidized and fully reduced sensors. Then, you measure an animal with an unknown redox potential, and see how closely that spectrum falls towards the oxidized or reduced extreme. From there, you should be able to at least estimate the percentage of sensors that are oxidized.

Why we're not done yet.

The big problem with the above approach is that it's difficult and would take a long time to measure the entire emission-excitation spectra for each experimental case.

So what if, instead of measuring the *whole* spectra, we just use one wavelength? For example, at around $400nm$ in Figure 1, a reduced cell should emit much more light than an oxidized cell, right? The problem with using just one wavelength is that it depends on the concentration of sensors. For example, let's say that you have two cells, Cell A and Cell B. Cell A is fully oxidized, and has 1,000 sensors all emitting light at a relative emission of 500 units, for a total emission of $500 * 1000 = 500,000$ units. Cell B is fully reduced, but only has 500 units. Even though each reduced sensor emits 1000 units of light--twice that of an oxidized sensor--the total emission is $1000 * 500 = 500,000$ units of light, indistinguishable from the oxidized cell.

The solution is to instead use two wavelengths, and take the ratio. Let's take the same case of Cell A and Cell B from the paragraph before. Let's assume that, at wavelength 1, oxidized and reduced sensors emit 500 and 1000 units, respectively and, at wavelength 2, oxidized and reduced sensors emit 150 and 400 units, respectively. If, as before, Cell A is fully oxidized, it's **ratio** emission is $\frac{500*1000}{150*100} = 6.67$ ratio units, whereas

Cell B is fully reduced with a ratio emission of $\frac{1000*500}{400*500} = 2.5$ ratio units. Since ratio is independent of concentration, it can be used to determine the redox potential.

Optional detour: Say that again, but this time with math.

If all the sensors are in an oxidized or reduced state, the emission intensity that we record at any wavelength λ is equal to the emission of one sensor at that wavelength, multiplied by the total number of sensors:

$I_{\lambda,Oxidized,Total} = N_{Total} * I_{\lambda,Oxidized}$

$I_{\lambda,Reduced,Total} = N_{Total} * I_{\lambda,Reduced}$

In a mixed population, the emission intensity is a weighted average of the two extremes:

$$I_{\lambda,Mixed,Total} = \frac{N_{Oxidized}}{N_{Reduced}}(I_{\lambda,Oxidized,Total} = N_{Total} * I_{\lambda,Oxidized}) + (1 - \frac{N_{Oxidized}}{N_{Reduced}})(I_{\lambda,Reduced,Total} = N_{Total} * I_{\lambda,Reduced})$$

By taking the ratio between the intensity at two wavelengths (λ_1 and λ_2), the total number of molecules N_{Total} cancels:

$$R = \frac{I_{\lambda_1,Mixed,Total}}{I_{\lambda_2,Mixed,Total}} = \frac{I_{\lambda_1,Mixed,Total}=\frac{N_{Oxidized}}{N_{Reduced}}(I_{\lambda_1,Oxidized,Total}=N_{Total}*I_{\lambda_1,Oxidized})+(1-\frac{N_{Oxidized}}{N_{Reduced}})(I_{\lambda_1,Reduced,Total}=N_{Total}*I_{\lambda_1,Reduced})}{I_{\lambda_2,Mixed,Total}=\frac{N_{Oxidized}}{N_{Reduced}}(I_{\lambda_2,Oxidized,Total}=N_{Total}*I_{\lambda_2,Oxidized})+(1-\frac{N_{Oxidized}}{N_{Reduced}})(I_{\lambda_2,Reduced,Total}=N_{Total}*I_{\lambda_2,Reduced})} =$$

$$\frac{\frac{N_{Oxidized}}{N_{Total}}*I_{\lambda_1,Oxidized}+(1-\frac{N_{Oxidized}}{N_{Total}})*I_{\lambda_1,Reduced}}{\frac{N_{Oxidized}}{N_{Total}}*I_{\lambda_2,Oxidized}+(1-\frac{N_{Oxidized}}{N_{Total}})*I_{\lambda_2,Reduced}}$$

Main points, thus far

- Ratiometric sensors are like normal fluorescent sensors in that, if you excite them with a certain wavelength of light, they emit light at a certain intensity.
- To record a concentration-independent measure of redox state, we excite ratiometric sensors at *two* wavelengths, and we record the ratio of the emissions at those two different wavelengths.

How do we find the fraction oxidized from the ratio emission R?

In short, we use a function that takes an **R** value and returns a fraction oxidized, or **OxD**. That function has three constant values that vary depending on each sensor and each microscope. The first two values are the maximum and minimum values of R, R_{max} and R_{min}, which correspond to the R values where all of the sensors are oxidized or all reduced. The third constant value can be a bit more confusing: it is the ratio between the oxidized and reduced emissions at *one* wavelength, specifically the second wavelength in the ratio (λ_2 of $R = \frac{I_{\lambda_1}}{I_{\lambda_2}}$). We call that third parameter δ_{λ_2}.

Once we have those three parameters, which we can determine experimentally, we can find the fraction oxidized (**OxD**) by the following equation:

$$OxD = \frac{R_{min}-R}{(R_{min}-R)+\delta_{\lambda_2}(R-R_{max})}$$

And from there, the redox potential can be found from the fraction oxidized, as we showed above.

Optional detour: How'd you get that equation?

Where did that equation for the fraction oxidized come from?

Assume a fully reduced state. Then, the intensities observed at a wavelength λ are equal to the product of N_T, the total number of roGFP molecules, and $I_{\lambda,R}$, the intensity of each roGFP molecule at a given wavelength in the reduced state.

$$I_{\lambda,R} = N_T * I_{\lambda,R}$$
$$I_{\lambda,Ox} = N_T * I_{\lambda,Ox}$$

At a redox state between maximally reduced and maximally oxidized, the intensity at a given wavelength is a weighted sum of the molecules found at either discretely oxidized or reduced state. We therefore can rewrite any state in in terms of the previous two equations (as we showed in the last math detour)

$$I_\lambda = \frac{N_{Ox}}{N_T} * I_{\lambda,Ox} + \frac{N_{Red}}{N_T} * I_{\lambda,Red}$$

Consider the intensity ratio:

$$\frac{I_{\lambda_1}}{I_{\lambda_2}} = \frac{\frac{N_{Ox}}{N_T} * I_{\lambda_1,Ox} + (1 - \frac{N_{Ox}}{N_T}) * I_{\lambda_1,Red}}{\frac{N_{Ox}}{N_T} * I_{\lambda_2,Ox} + (1 - \frac{N_{Ox}}{N_T}) * I_{\lambda_2,Red}} =$$

For brevity, let $OxD = \frac{N_{Ox}}{N_T}$. Then cross-multiply:

$$I_{\lambda_1} * OxD * (I_{\lambda_2,Ox} + (1 - OxD) * I_{\lambda_2,Red}) =$$

$$I_{\lambda_2} * OxD * (I_{\lambda_1,Ox} + (1 - OxD) * I_{\lambda_1,Red})$$

Simplify and express OxD in terms of known quantities:

$$OxD = \frac{I_{\lambda_2} I_{\lambda_1,Red} - I_{\lambda_1} I_{\lambda_2,Red}}{I_{\lambda_1} I_{\lambda_2,Ox} - I_{\lambda_1} I_{\lambda_2,Red} - I_{\lambda_2} I_{\lambda_1,Ox} + I_{\lambda_2} I_{\lambda_1,Red}}$$

To simplify, let:

$$R_{Red} = \frac{I_{\lambda_1,R}}{I_{\lambda_2,R}}$$

$$R_{Ox} = \frac{I_{\lambda_1,Ox}}{I_{\lambda_2,Ox}}$$

$$\frac{I_{\lambda_1}}{I_{\lambda_2}} = \frac{I_{\lambda_1}}{I_{\lambda_2}}$$

$$\delta_{\lambda_2} = \frac{I_{\lambda_2,Ox}}{I_{\lambda_2,Red}}$$

We can now re-derive the definition of OxD in terms of ratio values.

Step: Re-arrange terms, multiply by $\frac{-1}{-1}$:

$$OxD = \frac{I_{\lambda_1} I_{\lambda_2,R} - I_{\lambda_2} I_{\lambda_1,R}}{I_{\lambda_1} I_{\lambda_2,R} - I_{\lambda_2} I_{\lambda_1,R} + I_{\lambda_2} I_{\lambda_1,Ox} - I_{\lambda_2,Ox} I_{\lambda_1}}$$

Step: Work to factor out $I_{470,R} I_{470}$ from the numerator and denominator write some in terms of ratio values:

$$OxD = \frac{I_{\lambda_2,R} I_{\lambda_2} (\frac{I_{\lambda_1}}{I_{\lambda_2}} - R_{Red})}{I_{\lambda_2,R} I_{\lambda_2} (\frac{I_{\lambda_1}}{I_{\lambda_2}} - R_{Red} + \delta_{\lambda_2} (R_{Ox} - \frac{I_{\lambda_1}}{I_{\lambda_2}}))}$$

And simplify:

$$OxD = \frac{\frac{I_{\lambda_1}}{I_{\lambda_2}} - R_{Red}}{\frac{I_{\lambda_1}}{I_{\lambda_2}} - R_{Red} + \delta_{\lambda_2} (R_{Ox} - \frac{I_{\lambda_1}}{I_{\lambda_2}})} =$$

$$\frac{R - R_{Min}}{R - R_{Min} + \delta_{\lambda_2} (R_{Max} - R)}$$

Understanding the map from R to OxD

In the last section, we found the equation for the map between *R* and *OxD*. But what would a graph of that equation look like?

Well, it depends on (1) the properties of the redox sensor and (2) the wavelengths you choose to measure your ratio emissions.

For the case of roGFP1-R12 and the ratio $\frac{410}{470}$nm, the graph looks like Figure 3.

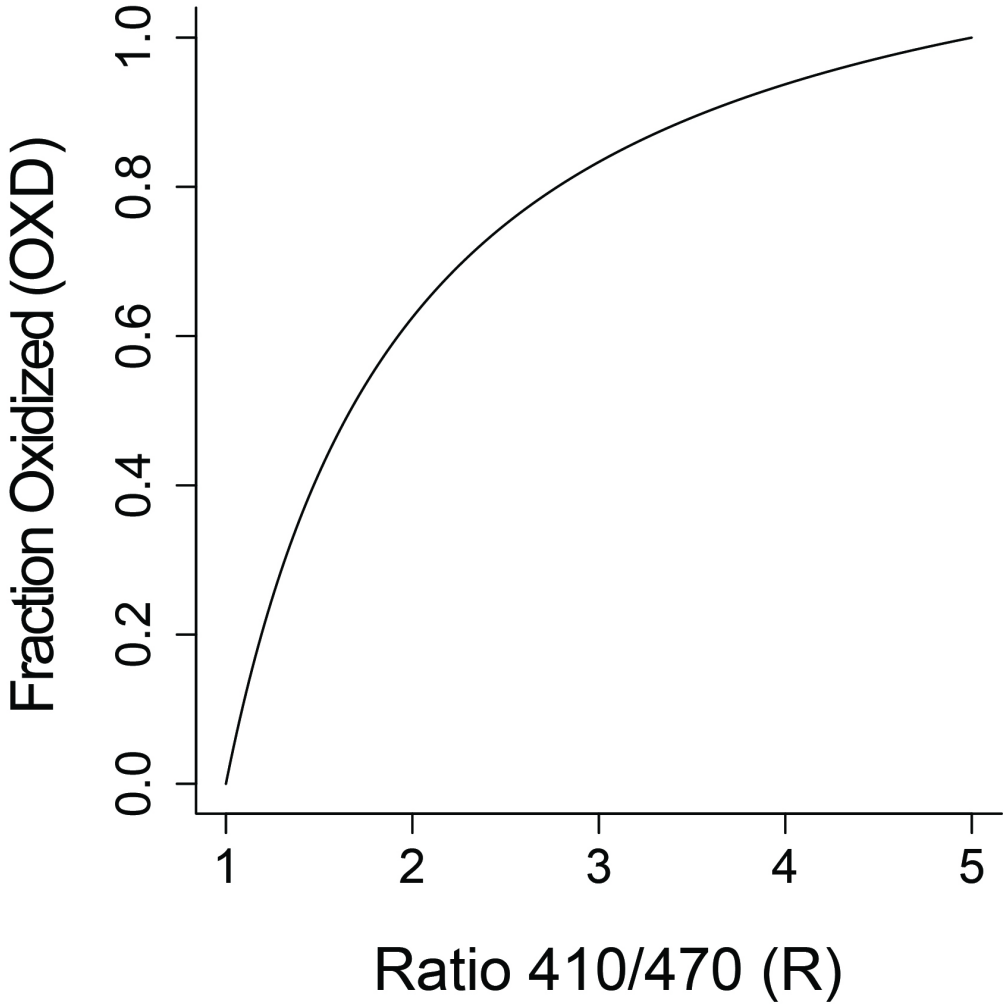

Figure 3. Map from ratio emission (horizontal axis) to fraction oxidized (vertical axis), for the ratio of $\frac{410}{470}$ of the roGFP1-R12 sensor.

In general, all graphs between R and OxD will range from R_{min} to R_{max}. However, sensors with a δ_{λ_2} value closer to 1 will have graphs that are more linear. Sensors with a δ_{λ_2} higher than 1 will curve upwards, whereas those with a value lower than 1 will curve downwards (Figure 4).

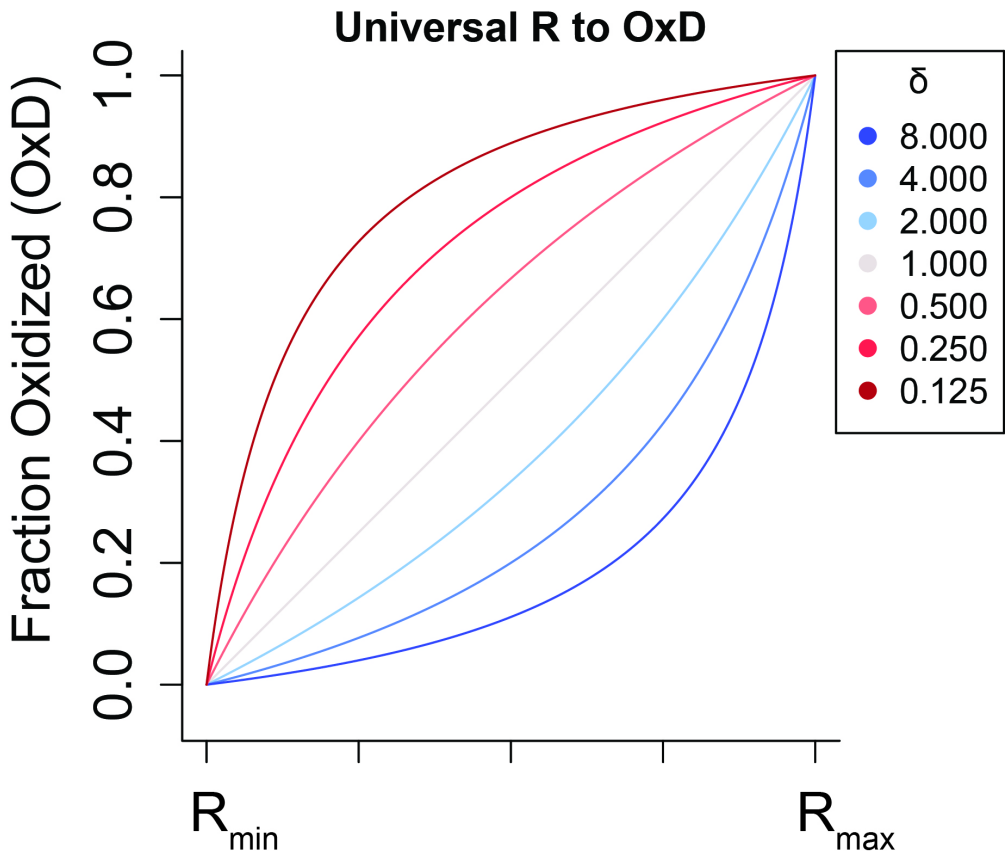

Figure 4. Map from ratio emission (horizontal axis) to fraction oxidized (vertical axis), a general case for showing the trend for any sensor.

Recall from the previous sections that the δ_{λ_2} depends on the ratio between the emission of an oxidized and reduced sensor at the *second* wavelength in the ratio emission. If that's the case, then choosing different second wavelengths with different ratios between oxidized and reduced emissions should change the linearity of the map between R and OxD (Figure 5).

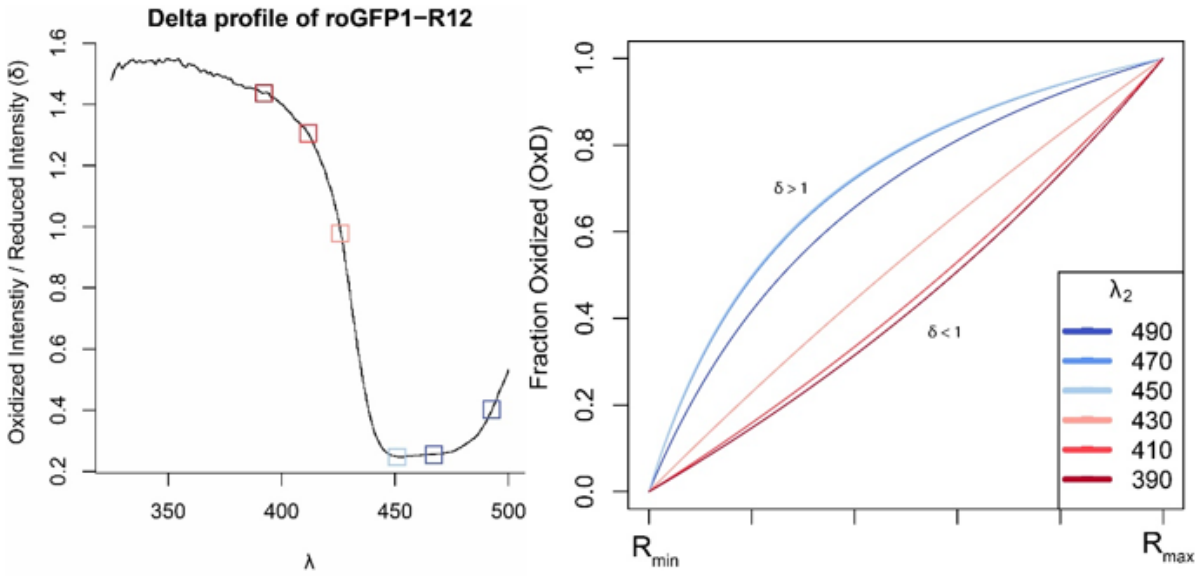

Figure 5 Left: the δ value (vertical axis) of the roGFP1-R12 sensor at different wavelengths (horizontal axis). Boxes correspond to wavelengths shown in the right panel. Right: The map between R and OxD for roGFP1-R12 when different second wavelengths are chosen for the emission ratio.

Understanding the map between R and E

It's been a few sections since we worked with the Nernst equation, so recall that it's $E = E^{\circ} - \frac{RT}{2F} * ln(\frac{Reduced}{Oxidized})$, where E° is the midpoint potential and $\frac{RT}{2F}$ is a constant. We can rewrite this equation in terms of only fraction oxidized by writing: $E = E^{\circ} - \frac{RT}{2F} * ln(\frac{1-Oxidized}{Oxidized})$. If we want to write this equation in terms of R, instead of the fraction oxidized, we can just plug in $\frac{R-R_{Min}}{R-R_{Min}+\delta_{\lambda_2}(R_{Max}-R)}$ in the two spots where "Oxidized" occurs. After we do so and simplify, we get the following:

$$E(R) = E^{\circ} - \frac{RT}{2F} * ln(\delta_{\lambda_2}) + ln(\frac{R_{max} - R}{R - R_{min}})$$

Point 2: Our ratiometric measurements have some noise, or level of precision. If we know that level of precision, we can estimate how accurately we can measure the redox state.

Point 3: If we know how accurately we can measure the redox state at different redox potentials, we can predict a range of values that any given sensor is well-suited to measure.

Point 4: If we can predict a values that any sensor is well-suited to measure, we can compare how well different sensors can measure redox values that we care about.