* Fluorophores bound to Secondaries are around 20-50uM.
* Avg 4 Fluorophores per antibody
* Use SNARF work I did as concentration benchmark for measurements.
* SNARF exct = 47k, QY = 0.05 around and [C] used was 1uM
* Exct\*QY = relative brightness
* (SNARF relative brightness)/(dye relative brightness)\*1000 = nm needed for [C]

A goal is to use the same concentration of dye for each sample (is this important though?). We thus must set it based on the dye with the weakest relative brightness. I did a calculation based on a previous experiment on the spectrofluorometer. This in no way means it’s the theoretical lowest. Its merely a starting point. This was determined to be Alexa 555. Its needed [C] to be **150nM**.

* We strongly need unaggregated dyes as we are aiming to measure fluorophore spectras and not any aggregate affects. This means pushing the [C] as low as we can and possibly using a co solvent with PBS such as DMF or UREA. Input in this regard would be helpful .

Using a program called curtipot for pH simulation work below. Appears that H2O2 in the concentrations we use will make a significantly pH impact and we need to compensate for it.

* Its possible that a diluted stock solution of dyes will be employed, diluted in PBS 2x. Its just hard to pipette 0.5uL accurately.

This study is a prelim and 2 phases

**Prelim**

Drive A555 [C] as low as possible while getting good statistics. Initial guess is 150nM, but its possible that it can be driven even lower.

**Phase one**

Calculate time for intensity to reach 50% of initial (bleaching half life)

1. By varying H2O2 and fixing pH
2. Vary pH fix H2O2 %
3. Vary Temp (20C vs 37C) \*should we try this?
4. Modulate light exposure

*Note:*

* 1-3 will be conducted with A647 as it responds the most rapidly to bleaching of any known dye.
* 4 will be conducted with A488 as it bleaches, but it takes well over 2x that of A647. This gives a larger region for light to modulate the bleaching rate.
* Not sure how to conduct light modulation. Ideas are since recommendation is bench top light exposure, take lid off and place a run of the mill light source above it and remove and reclose lid when taking measurement. Another idea is while it is more intense and centered on absorbance maximum, just use exposure light more. Say increase exposure time 2x, 3x and so on. We can then do power calculations on it to see what makes it around what an equivalent exposure from a bench top LED source would be.

**Phase 2**

Calculate time for intensity to reach 50% of initial (bleaching half life) at conditions optimized in Phase 1 with the following dyes

1. A488
2. A555
3. A594
4. A647
5. A750
6. DY755
7. CY7

*Note:*

Do doubles or triplicates of all dyes?

**Assumptions**

1. where I = intensity, Bhl is bleaching half-life. So, I assume each dye varies its half life, but obeys an exponential decay.
2. Following straight from assumption 1, if I optimize A647, I then optimize all other dyes.
3. pH is not significantly impacted by presence of dyes.
4. One of the modulate light experiment possibilities assumes that even with a white light source, the only light that does any bleaching contribution are the wavelengths in the dyes absorption band.
5. Dye behavior attached to a secondary antibody is not appreciably different from that of a dye attached to a secondary/primary complex on a tissue.