Dye Characterization

**Background**

The CyCIF multiplex staining method uses high pH, oxidation chemistry to permanently destroy the fluorescence of cyanide based fluorophores. Currently, a pH 10 and 3% (sometimes 4.5%) H2O2 is the standard.

Gerdes first demoed this technique and subsequently patented it. The Sorger lab has provided most of the refinement and advancement of the technique.

Very little detailed data exists on the bleaching kinetics and characteristics of these dyes. The richest source of info is the Gerdes patent. Its wholly based on absorbance data and lacks in depth analysis on each dye. Additionally, while it goes over a wide range of dyes, it actually only goes over a single dye that is commonly used today. In one instance, it does modulate H2O2 % which can inform our choices. It ranges 0.1%, 0.5%, 1%, 3%, 5%. What stands out in his data is that 0.5% was close to 1% and 1% was almost identical to the higher %s. Why then choose 3%? It very well may be to make a safe bet and it should ‘always’ work. As for pH, Gerdes showed that pH 10 is better than pH 7. No other nuance than that is really present. He also speculated that maybe the high pH broke bonds between dyes and antibodies. He never went on to test this hypothesis though. Which should be easy enough with an HPLC system to see if peaks absorbing the dyes max and say 280nm are wholly in the same peak or if they have a subset where separate peaks exist.

For the Sorger lab’s contribution to this particular area, there is not much. In the 2016 Lin paper, a small kinetic experiment was done with relevant dyes, but it was stained culture (or tissue, I forget) and seeing how the fluorescence dropped over time. It included no modulation.

I given the time cost of this technique, utilizing a 5th channel with Alexa 750 or Cy7 is highly desired and as of now no published works exist characterizing them in any capacity. Although I was told that a lab has unpublished work showing that Alexa 750 may be bleached, but also generates a sub population that has a blue shifted emission max. Cy7 has no data to the best of my knowledge. This shifted spectra would be disastrous for the technique and immediately rule it out. Another dye that may be useful to include is the Biotium CF 750 as they claim that it is a superior dye and doesn’t aggregate like Alexa 750 and Cy7.

A final piece in this puzzle is the use of Hoechst and DAPI dyes. They are used to register cycle positions for alignment. Both of these nuclear dyes show semi reversable photoconversion. It can be due to light and or H2O2. It has been shown that both can be oxidized multiple times which causes them to absorb blue and emit green and sometimes even absorb green and emit red. It was also shown that within an hour, this is partially reverses about as much as it ever will. Obviously, this is an issue with this technique as H2O2 is needed to bleach the dyes. It would be nice to quantify the photoconversion and how the kinetics change with H2O2 %.

**Experimental Overview and Details**

*Modulation Parameters (Listed in Priority Order)*

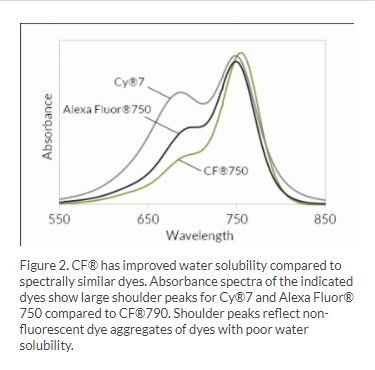
1. H2O2%
2. pH
3. Light
4. Temp

*Selected Dyes (Subject to change)*

1. A488
2. A546
3. A555
4. A594
5. A647
6. A750
7. Cy7
8. CF 750

The use of a **spectrofluorometer** will yield significantly more information about the bleaching process via the emission spectra than previous absorbance measurements have.

*Experimental Considerations*

We are doing the dye testing with PBS suspensions. Ideally, this would mean a perfectly dissolved dye in PBS. No aggregation and a low enough [C] to rule out any self quenching. No self quenching should be easy enough to achieve, but low aggregation could be an issue. Suggestions on how to quantify this or reduce it are encouraged. On Biotium’s website, they list an absorbance shoulder as an indicator. 

We may be able to use that. Next is in all combos to modulate the pH and H2O2 variables, we must keep PBS [C] and dye [C] fixed. Not hard to do, but we must do the calculations to ensure it.

2 Additional modulation variables are listed too (temp and light). These are questions that we don’t need as in depth of info on. We want to answer if temp at all changes the speed, so doing one dye is sufficient. Light is about using light exposure to accelerate bleaching. Gerdes does not use light, but the Sorger lab says it helps and recommends white light. We want to answer two questions:

1. Do wavelengths close to OD max for a given dye change bleach time as compared to white light sources?
2. How much can light accelerate the bleaching process?

Much is still being debated on how to execute the light portion of the experiments. Its quite possible that a spectrofluorometer is not the best instrument for this portion and another experimental setup will be to be established.

We have H2O2 refreshing experiments. This can be done at BCH. The hypothesis here is that H2O2 decomposes rapidly into water, but its unclear just how fast it is. We will monitor absorption of a certain wavelength to measure H2O2 % and establish a half-life of it. Additionally, we will do refreshing experiments where we pump fresh H2O2 solutions onto cells labelled with Phalloidin with some relevant dyes.