

Emails from John jciemnie@caltech.edu

Hey Tara,

Cool to hear you're doing this! I think you'll find more success by reducing the phenazines anaerobically and then transferring them to your microscope setup in sealed slides. I made reduced, anaerobic slide chambers with these (<https://www.sunjinlab.com/product/ispace-3mm/>) spacers and by adding TCEP, a reducing agent, in the anaerobic hood. I only began to see blue color accumulate in the PYO sample outside the anaerobic hood days later so I think those spacers kept a good enough seal against ambient air. It was a bit tricky handling the fragile spacers, cover slips, and slides with the anaerobic hood gloves though: just a heads up.

I'd be wary of using pH to reduce the phenazines because pH almost always has significant effects on fluorescence that would confound your reduced-state measurements. Also possible your mystery signal is partially pyoverdine if it's being produced under your experimental conditions. I've found pyoverdine to be even brighter than reduced phenazines at the excitation wavelengths you're trying.

I hope you guys are successful with FLIM, I would love to know the spatial distribution of redox state throughout a PA biofilm!

John

Hi John,

We've been slowly making progress on lifetime and spectral imaging pyocyanin. But I can't seem to get a stable preparation of the reduced, colorless species of pyocyanin. Except for maybe one instance, all of our imaging data looks like it's the yellow, radical form (I included a nice figure of chemical structure from Nate Glasser's dissertation doc below), even when we do a titration with TCEP and let it sit in the anaerobic chamber for >3 days (some of the titration spectra are below). Do you have any more tips on the prep? What specific concentration of pyocyanin and concentration and pH of TCEP do you use?

One interesting finding so far from our attempts is that the radical, yellow form has a very long lifetime (looks like >10 ns), but I am guessing the radical form isn't something you would find in the natural environment. So not too exciting in the biological world.

thanks so much!

Tara

Hi Tara,

Sounds like a pH problem. Are you buffering and titrating the TCEP solution to pH 7.0 before you add it to the PYO? TCEP solutions are super acidic without that. I think we buffered with MOPS, and for some reason I think I remember you can't buffer with phosphate... might be info on that in sigma's material info for TCEP.

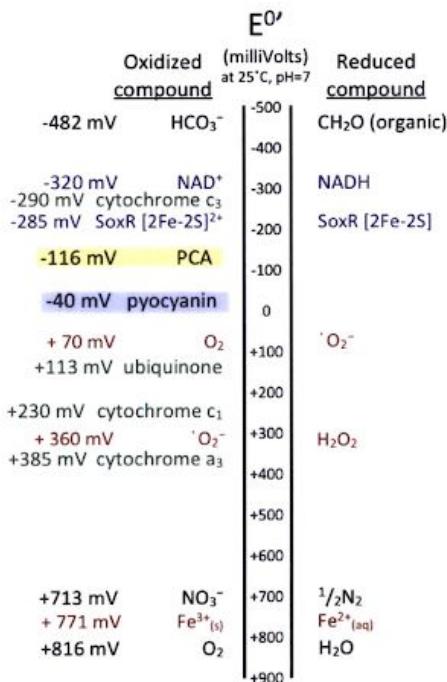
John

Papers / dissertations:

<https://pdfs.semanticscholar.org/3729/d9e1b907f951d940bc2041fac1b83d98ba13.pdf>

<https://pdfs.semanticscholar.org/bf2f/80b8afc167be454d2bb6c88f81783911e479.pdf>
<https://iai.asm.org/content/iai/52/1/263.full.pdf>

<https://pdfs.semanticscholar.org/1c27/6eb525978ab95a0ff0fbcb5f1f0f5a69eb68.pdf>



- Pyocyanin can accept electrons from NADH and iron-sulfur complexes and dump onto Oxygen
- 20 uL of a MOPS-buffered solution of 0.8mM pyocyanin at pH 7.2 added to 180 uL of culture

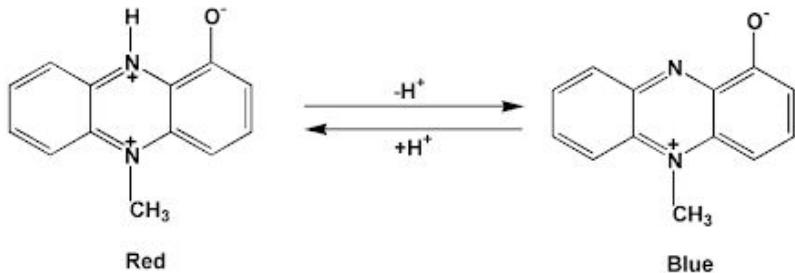


Fig 1.2 (a) Structure of pyocyanin



Fig 1.2. (b) colours of pyocyanin in acid and alkaline solution (c) the intermediary green colour

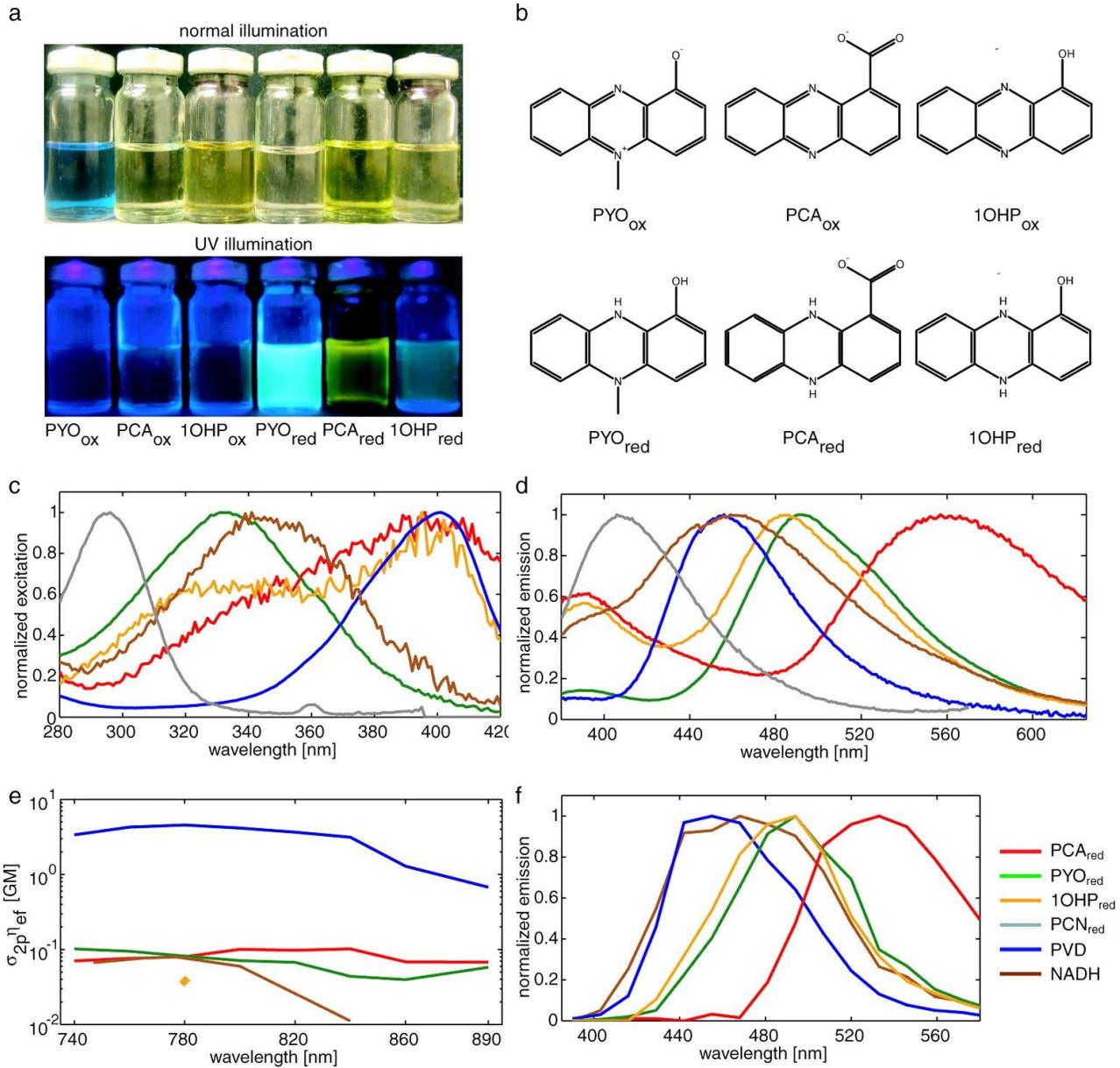
10/01/19 - Imaging anaerobic phenazines and ASM cultures on LSM 880

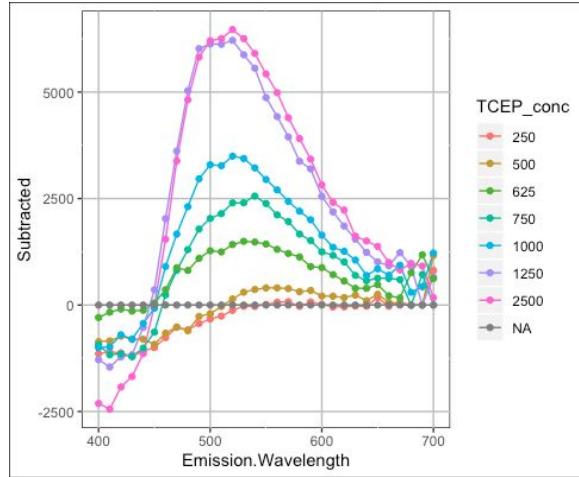
Background:

Fluorescent phenazines from Dianne newman:

- Reduced phenazine-1-carboxylic acid (PCA) = precursor to other phenazines
- Reduced 1-hydroxyphenazine (1OHP)
- Reduced Pyocyanin (PYO)
- Oxidized and reduced Phenazine 1-carboxamide (PCN)

Recommended





<https://pdfs.semanticscholar.org/1c27/6eb525978ab95a0ff0fbcb5f1f0f5a69eb68.pdf>

1-oh-phz: DTT

PCA: TCEP

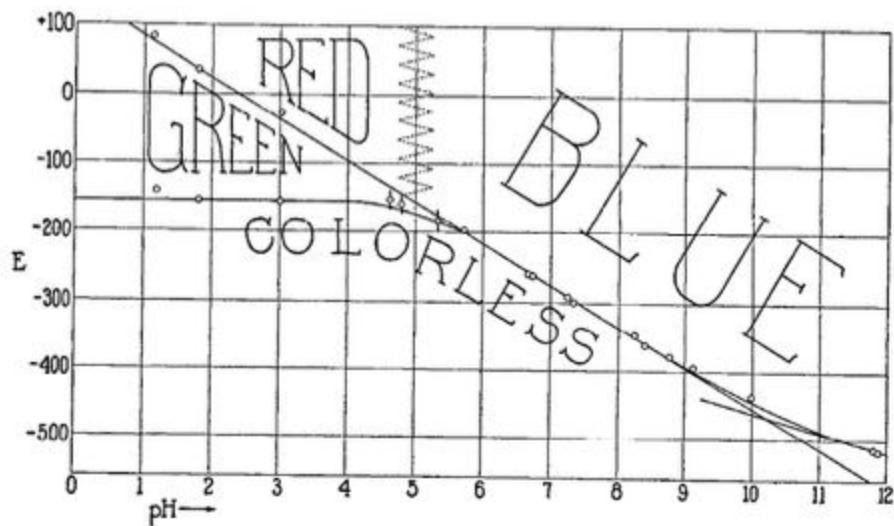


Fig 1.3 The normal potential of pyocyanin at varied pH at 30°c (Friedheim and L. Michaelis Journal of biological chemistry Feb, 1931)

- Pyocyanin or 1-hydroxy-5- methylphenazine is considered as a resonance hybrid of the mesomeric forms of N-methyl-1-hydroxyphenazine (Hillemann, 1938) (15) and is capable of undergoing a two-electron reduction to a colourless product, leukopyocyanin

- Pyocyanin can exist in either oxidized or reduced form, the later being an unstable form of pyocyanin that reacts rapidly with molecular oxygen (17).
- The pigment is wine-red at acid condition due to the basic property of one of the N atoms and blue at alkaline reaction (Friedheim *et al* 1931).
- When the alkaline blue solution is reduced, say by a trace of colloidal palladium
- ca in a stream of hydrogen, the colour vanishes and is reestablished on exposure to the air. However, when the acid red form of the pigment is reduced there is an intermediary green stage
- The blue pigment pyocyanin is reversibly oxidizable and reducible. In ranges of pH>6 it behaves entirely as a reversible dye of a quinoid structure

<https://pdfs.semanticscholar.org/3729/d9e1b907f951d940bc2041fac1b83d98ba13.pdf>

Levels of nadh in bacteria

https://watermark.silverchair.com/783.pdf?token=AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_9Cf3qfKAc485ysgAAAi0wgglpBqkqhkiG9w0BBwaggglMIICFglBADCCAg8GCSqGSib3DQEHAeBglghkgBZQMEAS4wEQQM8pxVTgAaFoJSiQYAgEQgIIB4ASx6eGsNZ636RUt676c7ON2v9uQYargANQIZ_t_Pzv0h-HEaVIFiNIFa_2nlbv5B9EGQoU5qFXfPr6gvtpcPDBw0kyZQGrpguyfm6BbIQIMpHnb00gEtiuxlz20gLb28CrvBmhb-YhMEKZdSo2LzivyzHf6JWWai-IBFLRhbrTbzyK_cBfNh2WiocfgFL7kRUKY9FTDt0DnL6RLi7UmvRxnLTvyTOhC0LNP_jixSQ7am68wZNU2861DkT26yxB7VW-cKF0XeeLfVR3JtjoS6gsmLOWbfH2CzSLbVnzKQh-G5iTmVlw0wXD7UJZ2yG6fVtli39u0ikU60MeJd0k0wQdnHlelf4YomHI_Jw5uORmkQ4Ks5Qb5K_wNQSGM0Ftn6VhTEG-SdM7zZxgTYCGG7adLtGxVb5GJF1bdDevuUW4k09GF-JDvt5n4u97mT6Kw6eaxQzzAvD_KNJ-0lzyylw0SIHfS6yoKVpPbBfF-X67P6aHDsx3t_AbHlxnsKJqJk_J_Olh4mSnrgFtWYY2xef_toQyMVEUK_RaMW73jwgRsKDBt95YNBqyTDcc3SOp1ZFmewO_OYBk0IQpRH-jLrDDNU9GLaZlaXazRloS-zB1OjlyGTvhemic6rsx3g

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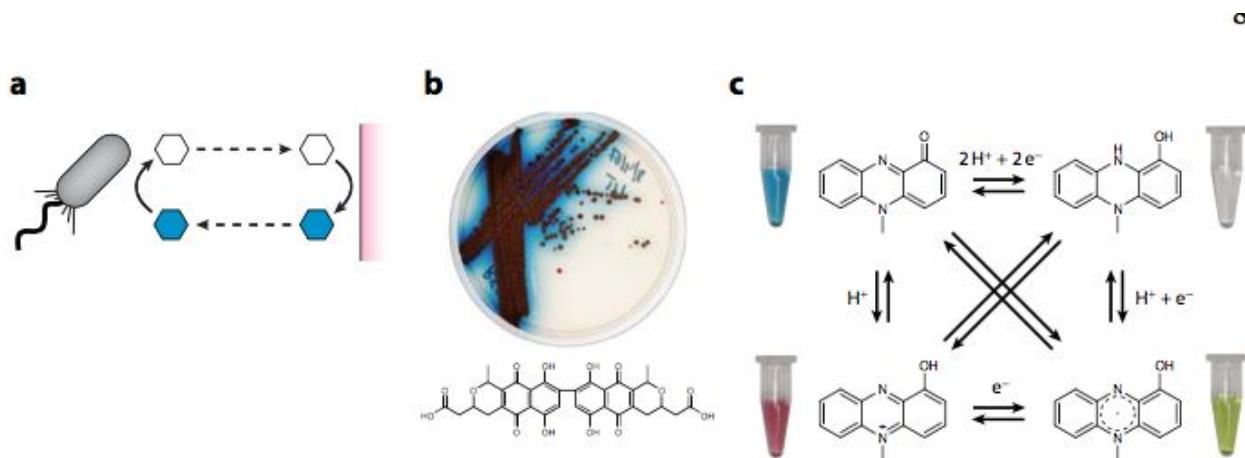


Figure 1. The colorful world of EES. (a) Action at a distance. Cells can perform redox chemistry on small molecules, which then diffuse, or electrically conduct, to extracellular substrates that can be many cell lengths away. (b) The colorful world of microbes as illustrated by *Streptomyces coelicolor*, producing the blue molecule actinorhodin whose structure is shown. (c) One molecule, four colors. The color of pyocyanin depends on both the pH and reduction potential. The tubes pictured each contain approximately 200 μM pyocyanin in water. The radical and fully reduced forms can be prepared by titrating pyocyanin with sodium dithionite, producing to an immediate and stunning color change.

500 μM phenazine + 5 mM reducing agent

1. PYO + TCEP - Very fluorescent today, also Old sample very fluorescent
2. PCA + TCEP - not fluorescent today, Old sample very fluorescent
3. PCN + TCEP- not fluorescent today, Old sample very fluorescent
4. Hydroxy phenazine (OH-Phz) + TCEP- not fluorescent today, Old sample very fluorescent

Samples with DTT are suspiciously similar. Maybe glue fluorescence or DTT is fluorescent??

5. PYO + DTT
6. PCA + DTT
7. PCN + DTT
8. Hydroxy phenazine + DTT

9. PA14 in artificial sputum media (plate) --- didnt look like PA14 made pyocyanin in these conditions!
10. Δphz in artificial sputum media (plate) (no phenazines)
11. Artificial sputum media (non-inoculated plate, background)

12. PYO + Artificial sputum media + reducing agent - not very fluorescent

13. PCA + Artificial sputum media + reducing agent - Seems good, similar to old sample PCA TCEP
14. PCN + Artificial sputum media + reducing agent - not very fluorescent...
15. Hydroxy phenazine (OH-Phz) + Artificial sputum media + reducing agent - not very fluorescent

16. Pyoverdine (non-anaerobic) 500 um
17. Pyoverdine (non-anaerobic) 250um + Artificial sputum media (0.5x)

If extra time:

18. Pycochelin (excite at 690 nm) 100 uM solution in water
19. PCN oxidized (excite at 690 nm) (100 uM solution in water)

Experiment Notes: (Simon)

emission filters:

495 longpass dichroic,

Ch1: 460/80

Ch2: 540/50

Laser power calibration using thorlabs PM100D power meter No objective in place
(Mai Tai at 740nm) (VERY ROUGH ESTIMATES)

Zen AOM= power

0.2% =0.74uW

1% = 1.7mW

5% = 10mW

10% =19mW

15% =29mW

20% = 39mW

30% =57mW

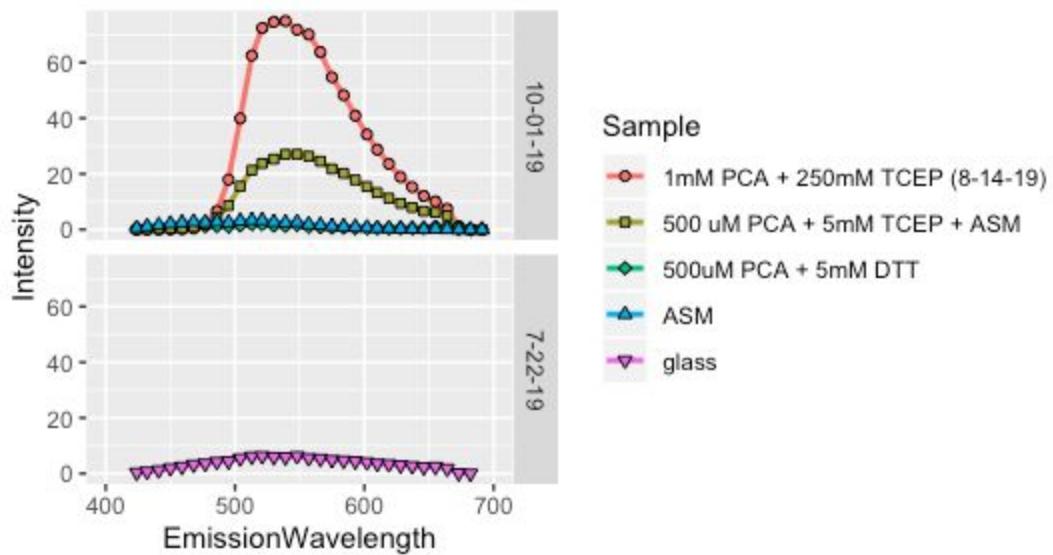
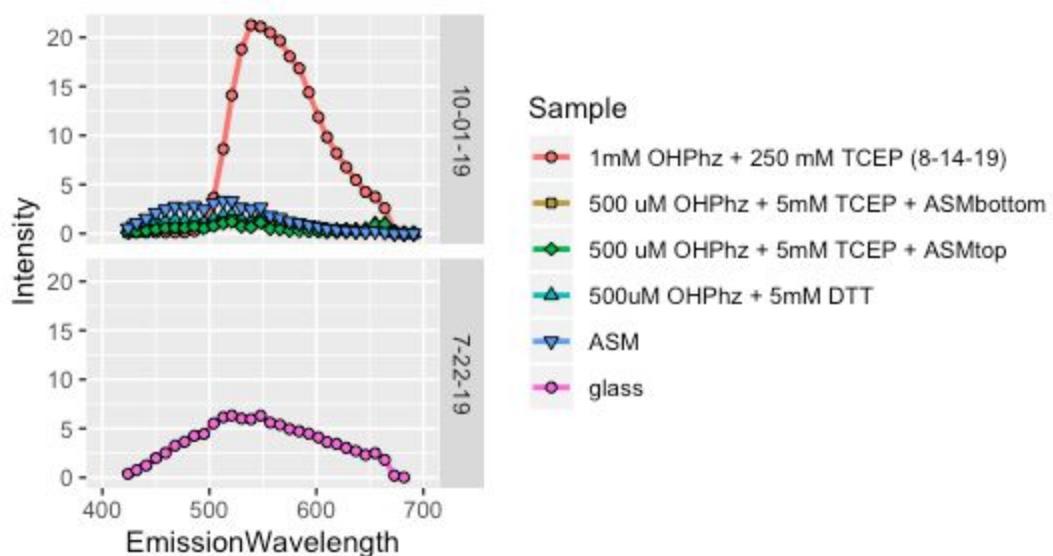
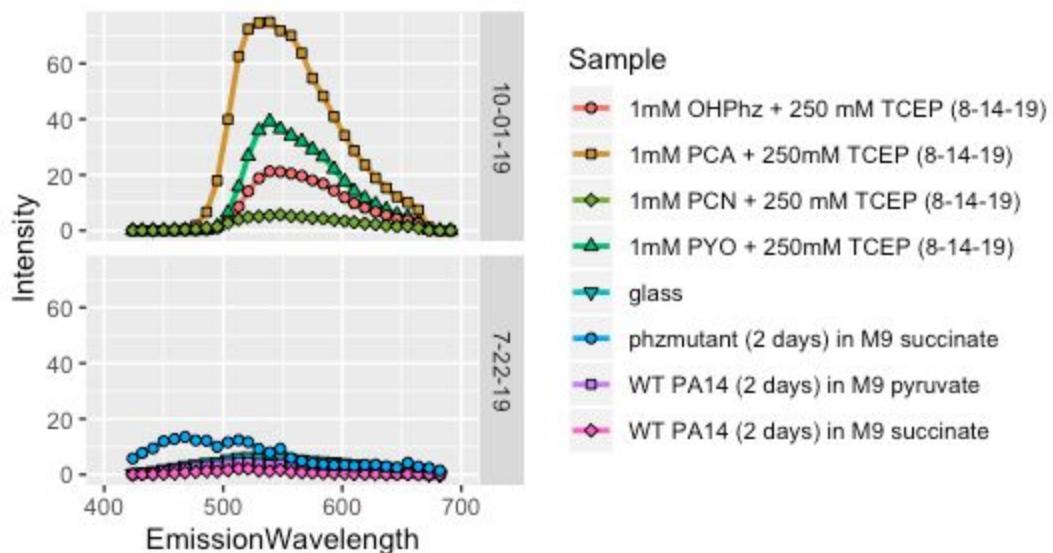
40= out of range

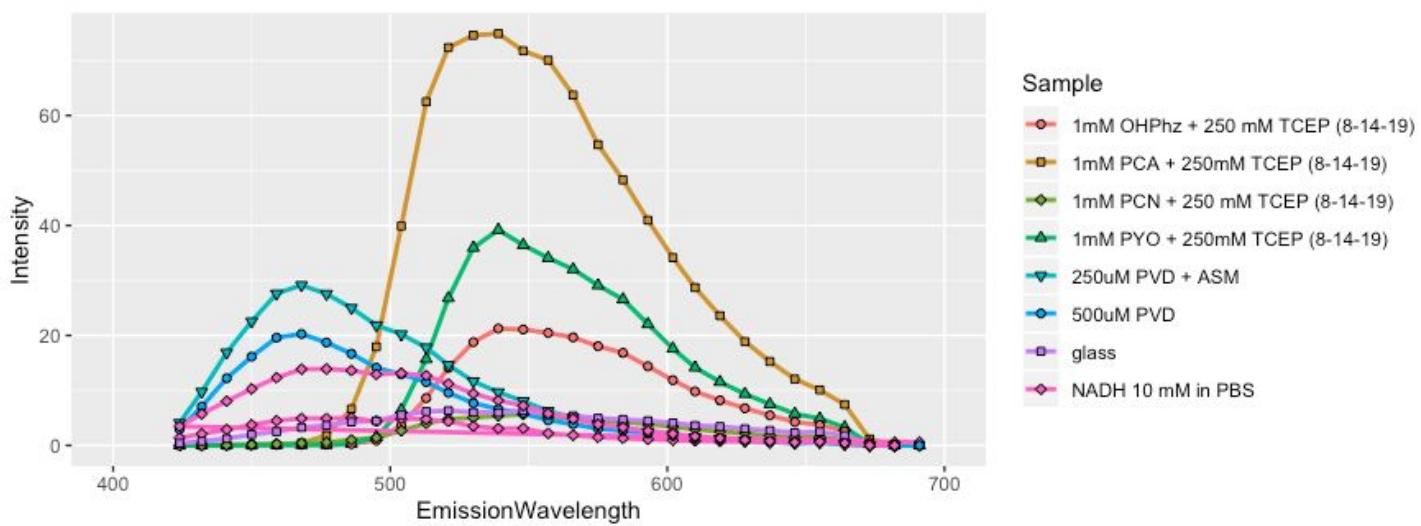
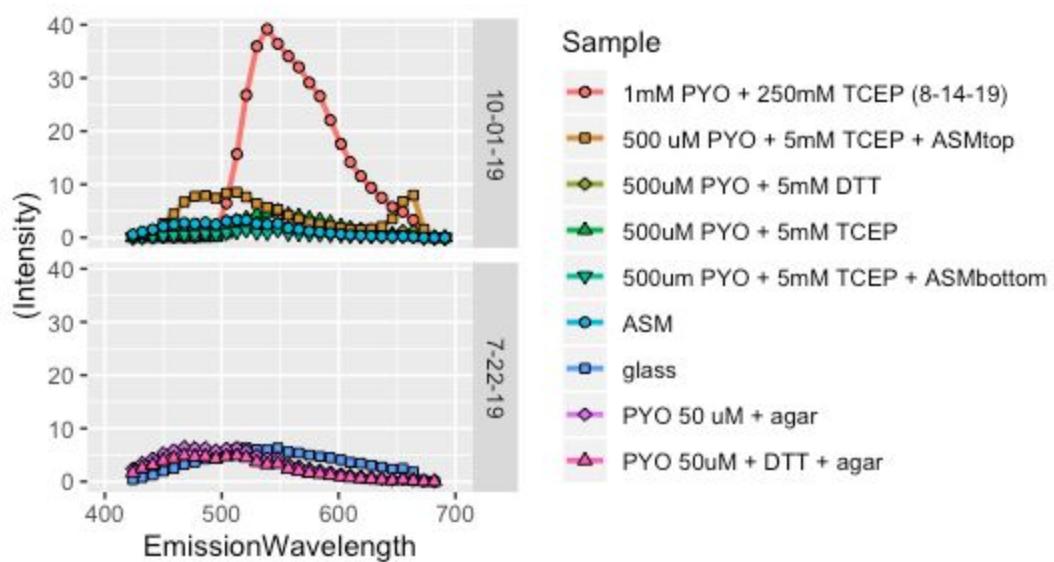
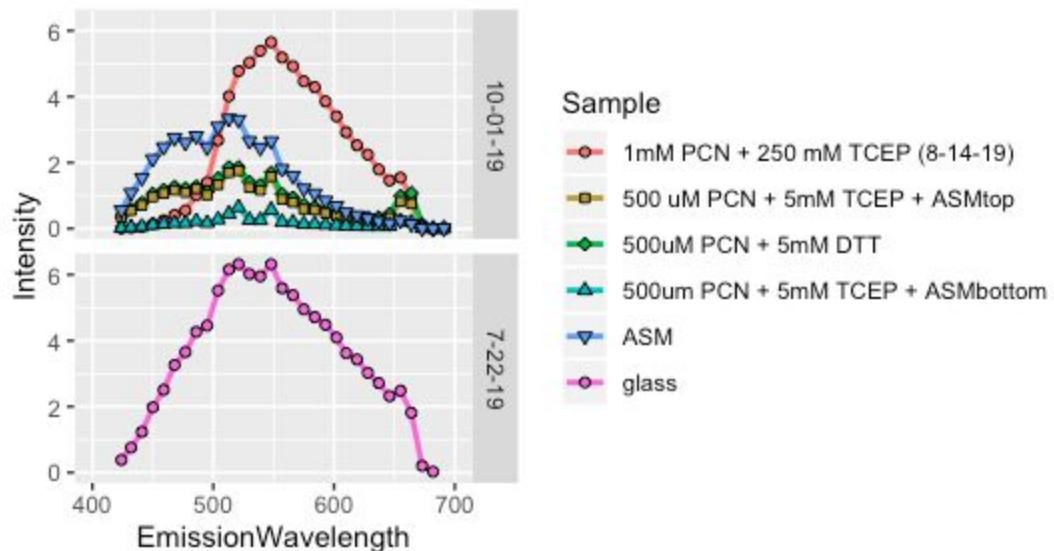
Lifetime Calibration With Coumarin 6 in ethanol 740nm eX,

25X W obj, 0.8NA LD LCI PInApo

Most of the imaging done at 10% power

ASM mucin or other aggregates have high background fluorescence, consider another media type

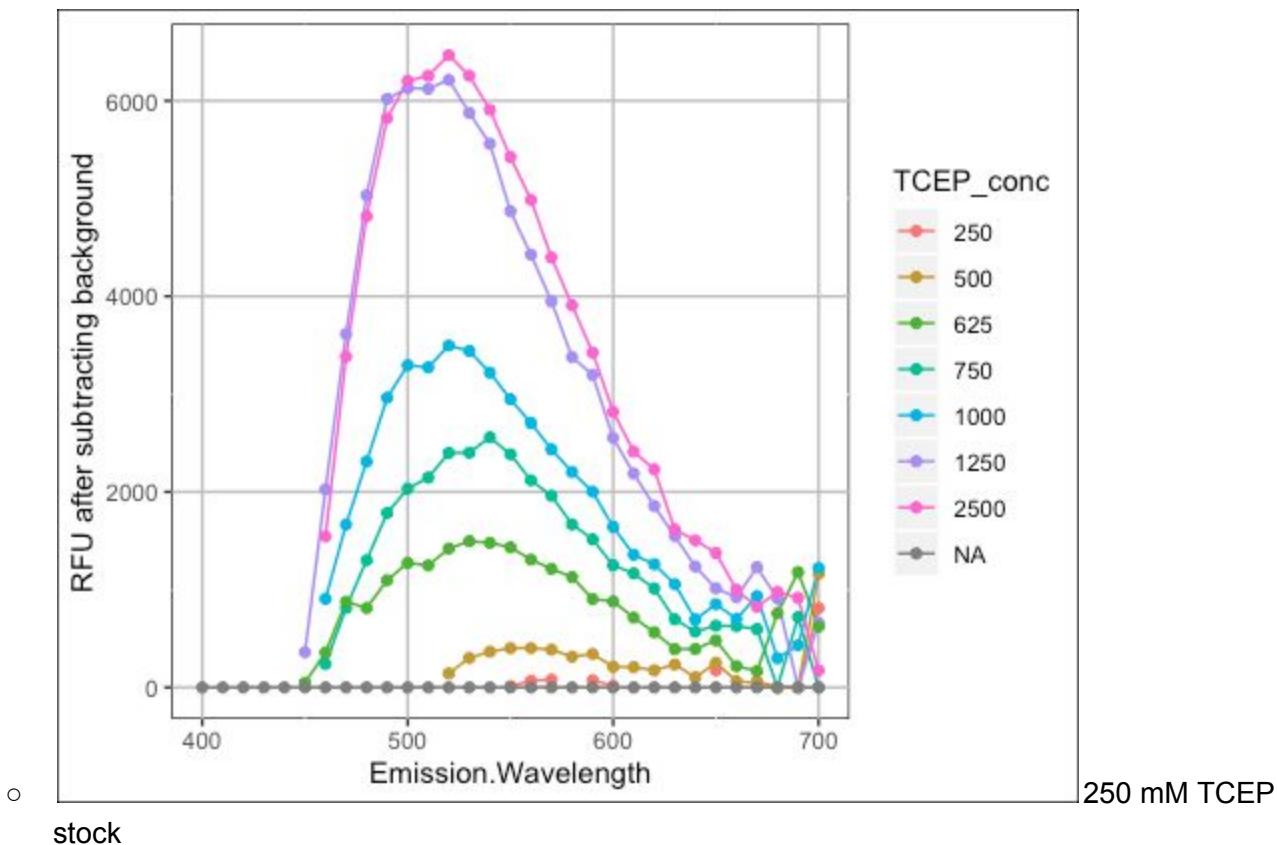




10-02-19 Fluorometer of phenazines in different reducing and pH conditions

10-02-19

- Tara transferred 10 mM stocks of pyocyanin (20% ethanol) and hydroxy phenazine (methanol) and 4.5 mM stock of PCN (chloroform) into the anaerobic chamber in Whiteson lab
- Also transferred the following solvents:
 - pH 5 phosphate buffer
 - pH 9 phosphate buffer
 - 1X PBS

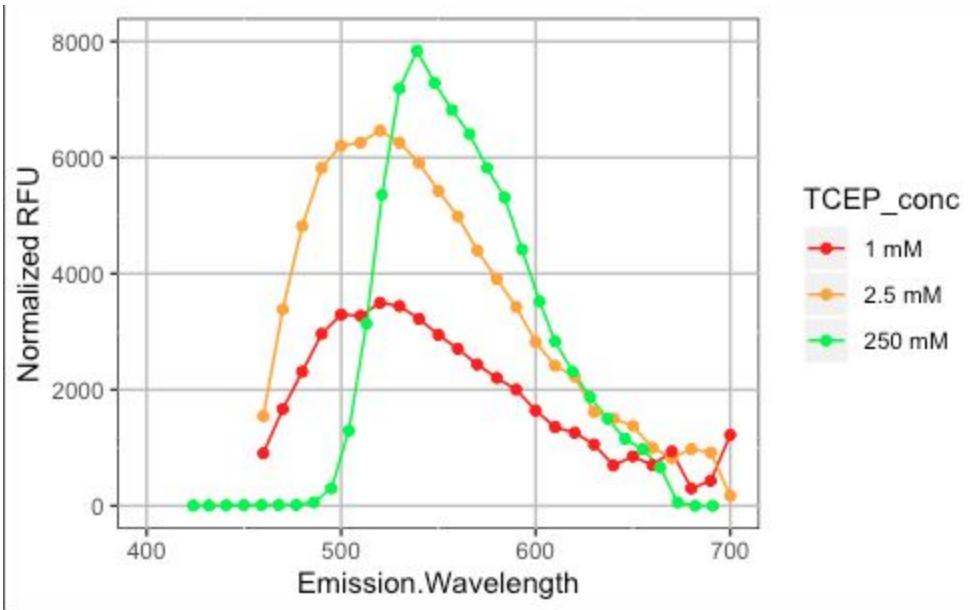


10-06-19 Results:

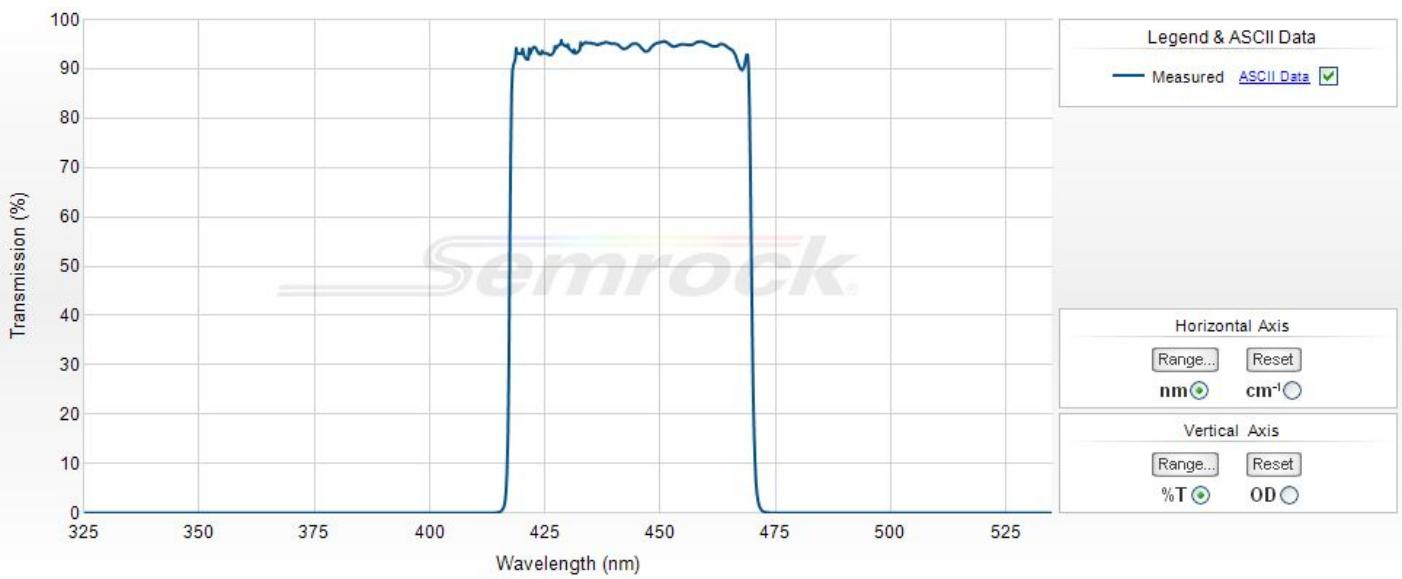
- No colorless pyocyanin, spectra looks similar to LSM-880

10-09-19 Fluorometer of pyocyanin + gradient of TCEP

Combined data from LSM-880 (Green line) and 1-photon fluorometer in German lab (orange, red lines)
1 mM pyocyanin in the anaerobic chamber + gradient of TCEP reducing agent



442/46 nm filter:



October 28, 2019

FLIM filters: ch1 442/46 Brightline clinical
ch2:540/50, +495 longpass dichroic

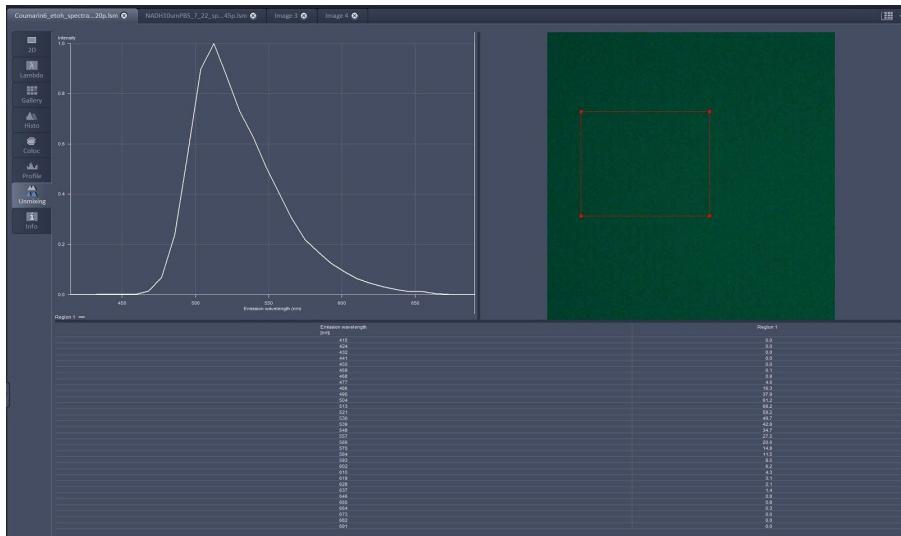
Spectral settings:

410-695 nm
8.9 nm resolution

Coumarin6 in etoh

25x08naW 740ex_20p

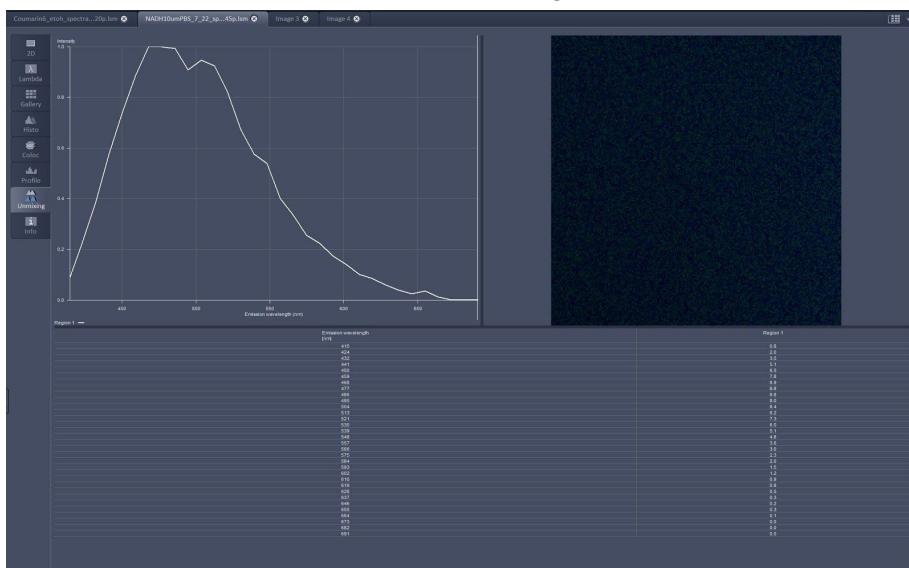
410-695nm, 8.9nm resolution spectral image



NADH 10mm in PBS (solution made 7/22/2019)

25x08naW 740ex_45p

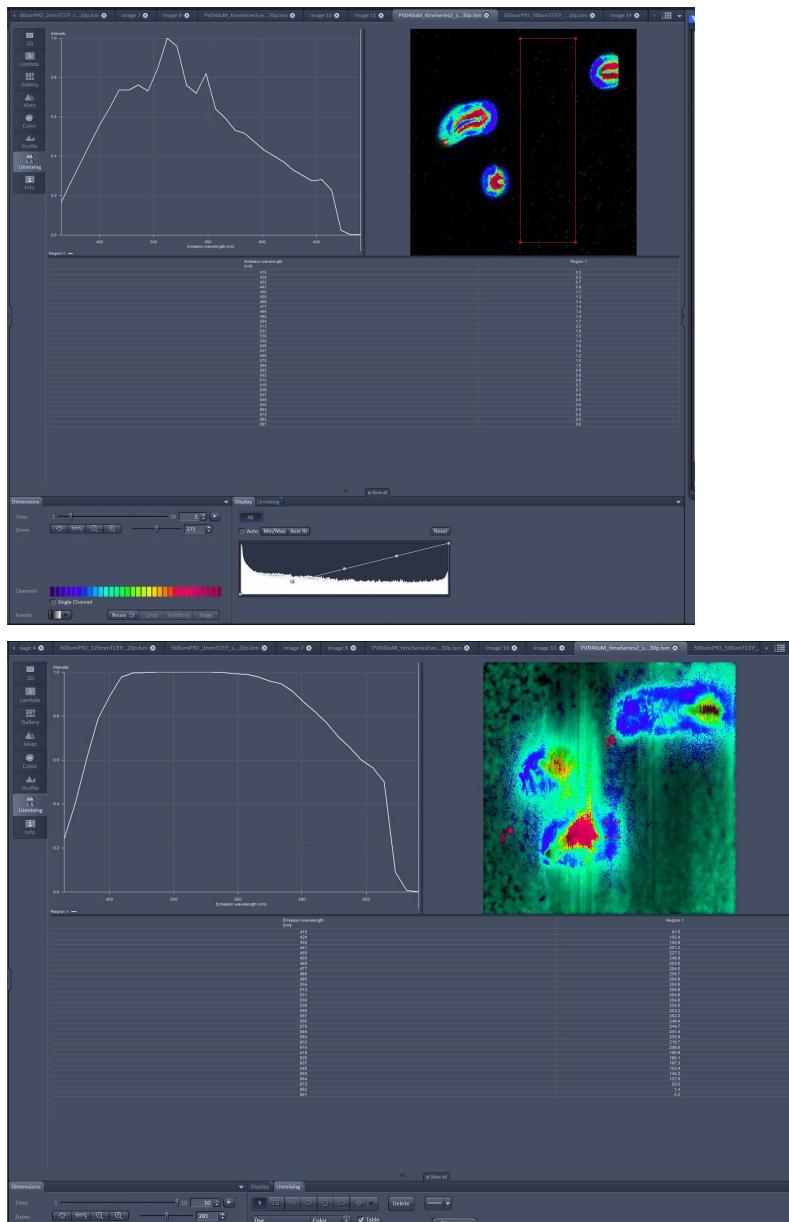
410-695nm, 8.9nm resolution spectral image



Tara preparation of phenazines:

- Except for hydroxy phenazine (which was sitting in chamber since 10-01-19), transferred all phenazines into the chamber on Friday 10-25-19
- TCEP, 1X PBS already sitting in chamber since 10-01-19
- PCA and PCN were dissolved in chloroform, the chloroform evaporated and the dried powder was re-dissolved in anaerobic PBS

PVD 40um



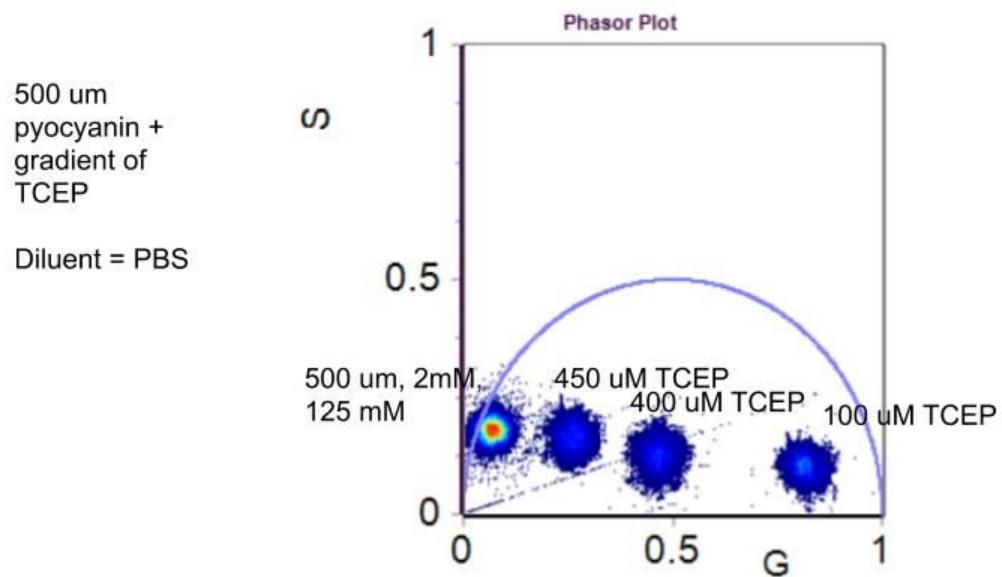
1-OHPhz + 125 mM DTT = not fluorescent

1-OHPhz + 190 mM TCEP = not fluorescent

500 um pyocyanin + 600 um TCEP --- not as fluorescent at 780 nm (NADH emission filter)

CH 1 lifetime phasor of pyocyanin:

CH1 lifetime phasor



I don't think we have coumarin collected for CH2 calibration...

01-19-2020 Imaging pyocyanin in MOPS+TCEP and hydroxy phenazine

Buffer instructions

Preparation Instructions This product is soluble in water (50 mg/ml), yielding a clear, colorless solution. DTT is also soluble in ethanol, acetone, ethylate, chloroform, and ether.¹ Storage/Stability DTT solutions should be prepared fresh daily. The recorded half-life (hours) of DTT solutions at various pH and temperatures are shown in Table 1 (all are in 0.1 M potassium phosphate buffer).¹³

Table 1. DTT Solution Stability

pH	Temperature	Half Life (hrs)
pH 6.5	20 °C	40
pH 7.5	20 °C	10
pH 8.5	20 °C	1.4
pH 8.5	0 °C	11
pH 8.5	40 °C	0.2
pH 8.5	20 °C (+ 0.1 mM Cu ²⁺)	0.6
pH 8.5	20 °C (+ 0.1 mM EDTA)	4

TCEP is typically very soluble in aqueous buffers at nearly any pH. Therefore, working concentrations and 10X stock solutions may be readily prepared in most aqueous buffers. TCEP is stable in aqueous, acidic, and basic solutions. When TCEP is dissolved directly in water, the resulting pH is approximately 2.5. TCEP is not very stable in phosphate buffers, especially at neutral pH. Therefore, if TCEP is to be used in PBS buffers, prepare the working solution immediately before use.

- Prepared new stock of pyocyanin in 20% ethanol, moved to the anaerobic chamber on 1-13-2020
- The hydroxy phenazine (10 mM), TCEP (250 mM), and DTT (250 mM) were left there since October

Set up titration with TCEP:

Make a 5 mL stock of 20 mM TCEP diluted in MOPS:

- 0.25 mL of TCEP (250 mM stock) + 4.75 mL of MOPS buffer

1-18-2020 day before titration:

- 1 mM pyocyanin + no TCEP (just MOPS buffer)
- 1 mM pyocyanin + 0.5 mM TCEP
- 1 mM pyocyanin + 1 mM TCEP
- 1 mM pyocyanin + 2 mM TCEP
- 1 mM pyocyanin + 10 mM TCEP

Let sit in chamber for ~24h

Also consider making fresh stocks since TCEP might not be stable in MOPS buffer for that long

Imaging day (1/19/2020):

Excitation: 740 nm

FLIM filters:

- ch1 442/46 Brightline clinical
- ch2:540/50, +495 longpass dichroic

Calibration / collect controls and background first:

- Coumarin
- NADH solution
- Glass (negative control)
- MOPS + TCEP (background)
- Unmodulated light

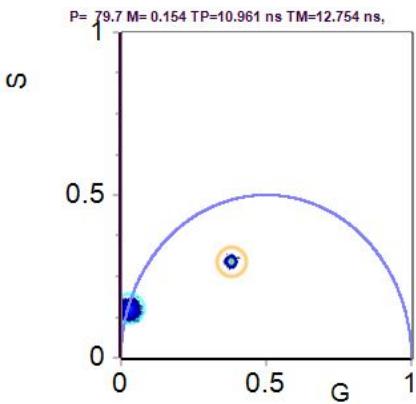
Data from 1-19-2020:

Fluorescence lifetime:

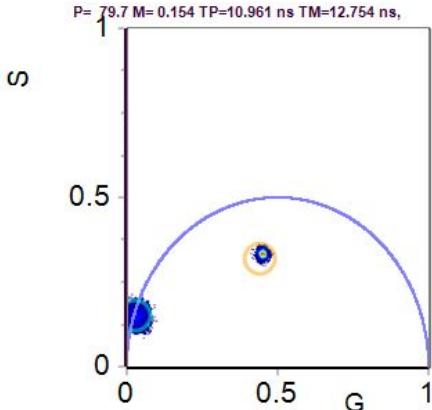
Adjusting the pH of buffer - effect on lifetime (1 mM Pyocyanin + 1.25 mM TCEP)

- CH1 = NADH filter (turquoise)
- CH2 = Green filter

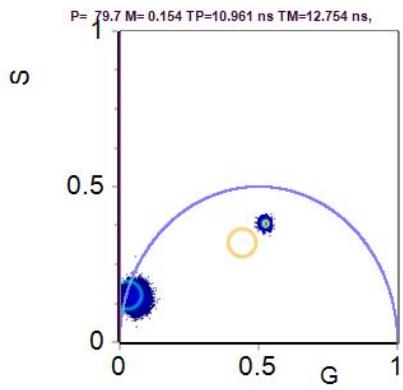
No adjusting (pH ~6.4):



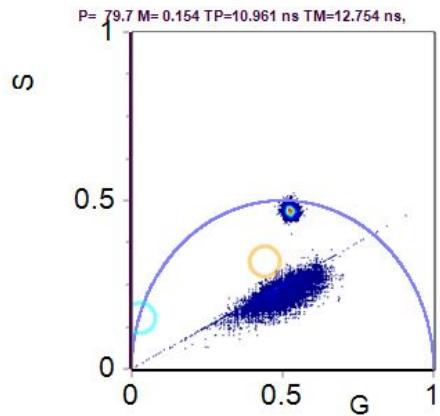
pH ~7:



pH ~7.4:

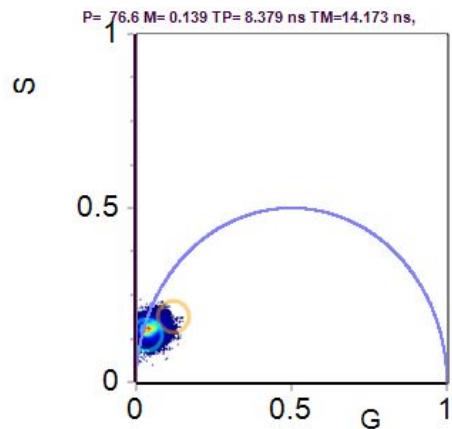


pH ~8:



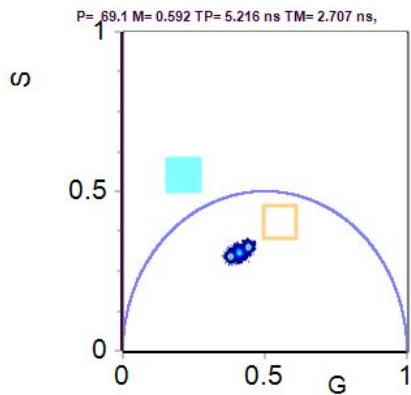
Effect of titrating in TCEP (pH ~6.4)

Doesn't seem to affect lifetime of CH1 "species"



1, 1.25, 2 mM of TCEP + 1 mM pyocyanin, overlapping phasor

Does affect lifetime of CH₂ species



Conclusions:

- Looks like higher pH results in more of the “yellow” species (max peak ~550 nm) based off of spectral and lifetime (ch2)
 - Contradicts what I was guessing, based off of Michaelis plot:

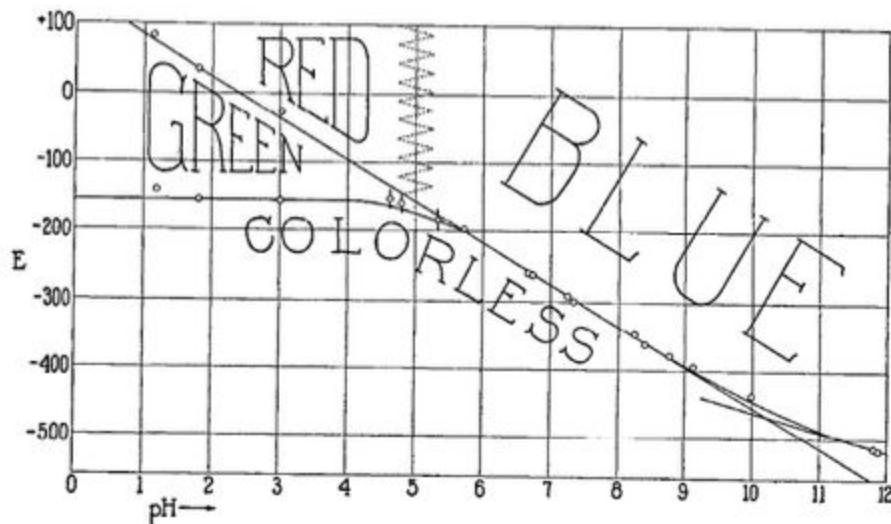


Fig 1.3 The normal potential of pyocyanin at varied pH at 30°c (Friedheim and L. Michaelis Journal of biological chemistry Feb, 1931)

- Haven't tried buffering to a lower pH (based off of pH paper, the pyocyanin + ethanol + TCEP +MOPS had pH ~6.4)
- Titrating sodium dithionite? Titrating DTT?
- NADH but will fluorescence
- From glasser dissertation - 250 uM Phenazine carboxylic acid and 10 mM sodium dithionite

1/24/2020 - set up pH titration in the anaerobic chamber

To make stock of 0.5 mM pyocyanin + 0.5 mM TCEP (50 uL)

Pyocyanin

(2mM) (Vi) = 0.5mM (50 uL)

Vi = 12.5 uL

TCEP:

10 mM (Vi) = 0.5 * 50

Vi = 2.5 uL

Volume of MOPS=35 uL

Chamber conditions = 37C; 5% CO₂

HCL titration

50 uL of 0.5 mM pyocyanin + 0.5 mM TCEP

Volume of 1M HCl (uL) added to tube	pH	Color
0		blue
1		blue
2	2-4 (can't tell with pH paper)	red
3		
4		
5		
6		
7		
8		
9		
10		

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NaOH titration

50 μ L of 0.5 mM pyocyanin + 0.5 mM TCEP

Volume of 1M NaOH (μ L) added to tube	pH	Color
1		blue
2		blue
3		blue
4		blue
5		blue
6		blue
7		blue
8		blue
9		blue
10	pH 7.6	blue

50 μ L of 0.5 mM pyocyanin + 1 mM TCEP

Volume of 1M NaOH (μ L) added to tube	pH	Color
1		blue
2		blue
3		blue

4		blue
5		blue
6		blue
7		blue
8		blue
9		blue
10	pH 7.6	blue
v		

50 μ L of 0.5 mM pyocyanin + 0.5 mM TCEP

Volume of 1M NaOH (μ L) added to tube	pH	Color
1		blue
5		blue
6		Light blue
6.5		Light blue
7		Light blue
7.5		Light blue
8		Light blue
8.5		Light green?
9		Light green?
9.5	7.6	Light green?

For next time, do a titration with NaOH:

50 uL of a pyocyanin stock (0.5 mM pyo + 2 mM TCEP pH 7)

Somewhere between 8-9.5 uL of 1M NaOH should give us colorless solution?

1/27/2020 Prep pyocyanin for fluoreimeter

- Moved a new stock of 2 mM pyocyanin in 20% ethanol from the freezer to the anaerobic chamber on Friday (incubated 3 days)
- Try to get the colorless, reduced form of pyocyanin by doing the following:
 - Make 50 uL stocks of 0.5 mM pyocyanin with 2 mM TCEP and NaOH:
 - 12.5 uL of the 2mM pyocyanin
 - 10 uL of 10mM TCEP stock
 - 17.5 - 19.5 uL of MOPS buffer and 8 - 10 uL of MOPS buffer

Determine effect of titrating NaOH with 2mM of TCEP

0.5 mM Pyocyanin (50 uL)

***Prep samples this way for 710:

Volume of 10 mM TCEP (uL)	Volume of 1X MOPS buffer to add (uL)	Volume of pyocyanin (2mM) to add	Volume of 1M NaOH to add (uL)	Concentra- tion of TCEP (mM)	Eye Color ~30 minutes	UV emission color
10	17.5	12.5	8	2	colorless	green
10	18	12.5	8.5	2	colorless	green
10	18.5	12.5	9	2	colorless	green
10	19	12.5	9.5	2	colorless	green

Determine effect of titrating TCEP with _8_ uL of NaOH

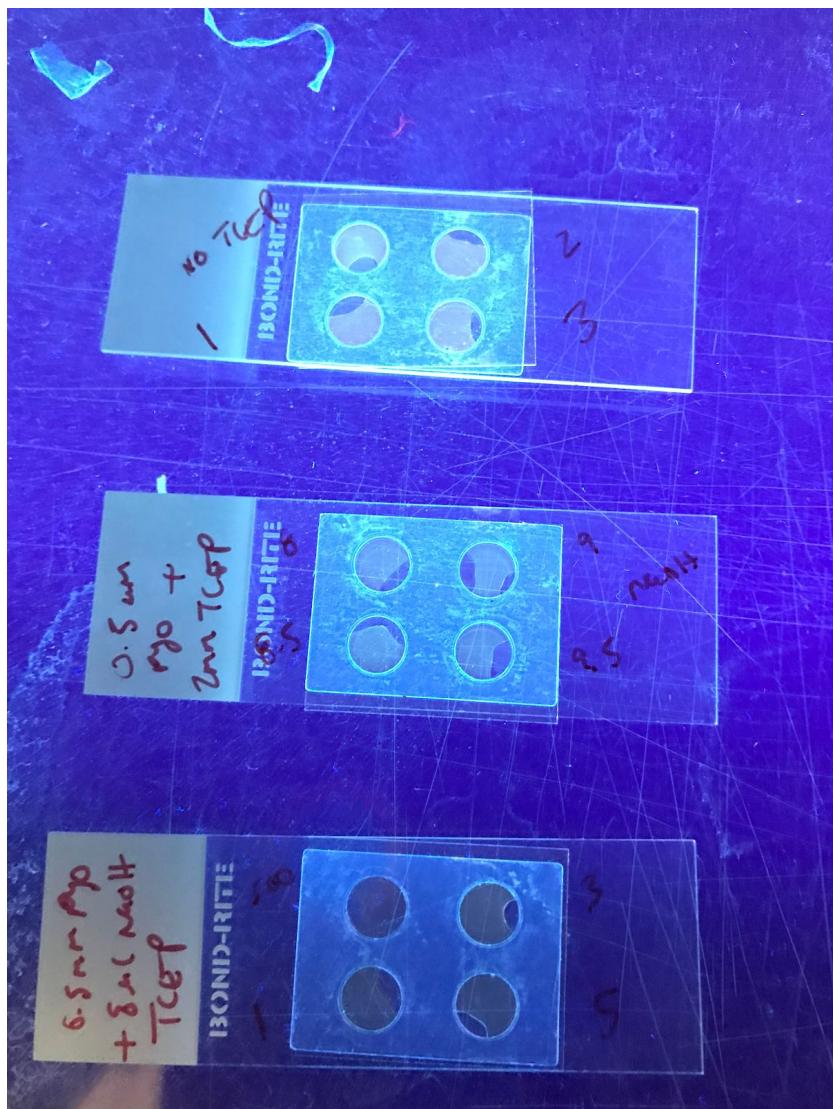
0.5 mM Pyocyanin (25 uL)

Volume of 20 mM TCEP (uL)	Volume of 1X MOPS buffer to add (uL)	Volume of pyocyanin (2mM) to add	Volume of 1M NaOH to add (uL)	Concentra- tion of TCEP (mM)	Eye Color ~30 minutes	UV emission color

** 1.25 of 10 mM ***	9.5	6.25	8	0.5	blueish	none
1.25	9.5	6.25	8	1	Clear / yellowish	yellow-green
2.5	8.25	6.25	8	2	Yellowish (but should be colorless since same as last table???)	yellow-green
3.75	7	6.25	8	3	yellowish	yellow-green
5	5.75	6.25	8	4	yellowish	
6.25	4.5	6.25	8	5	yellowish	yellow-green

10 mM TCEP + 0.5 mM of pyocyanin from last week = yellow by eye, greenish color for fluorescence emission

Picture of sealed slides with the dye titrations on our UV dock:



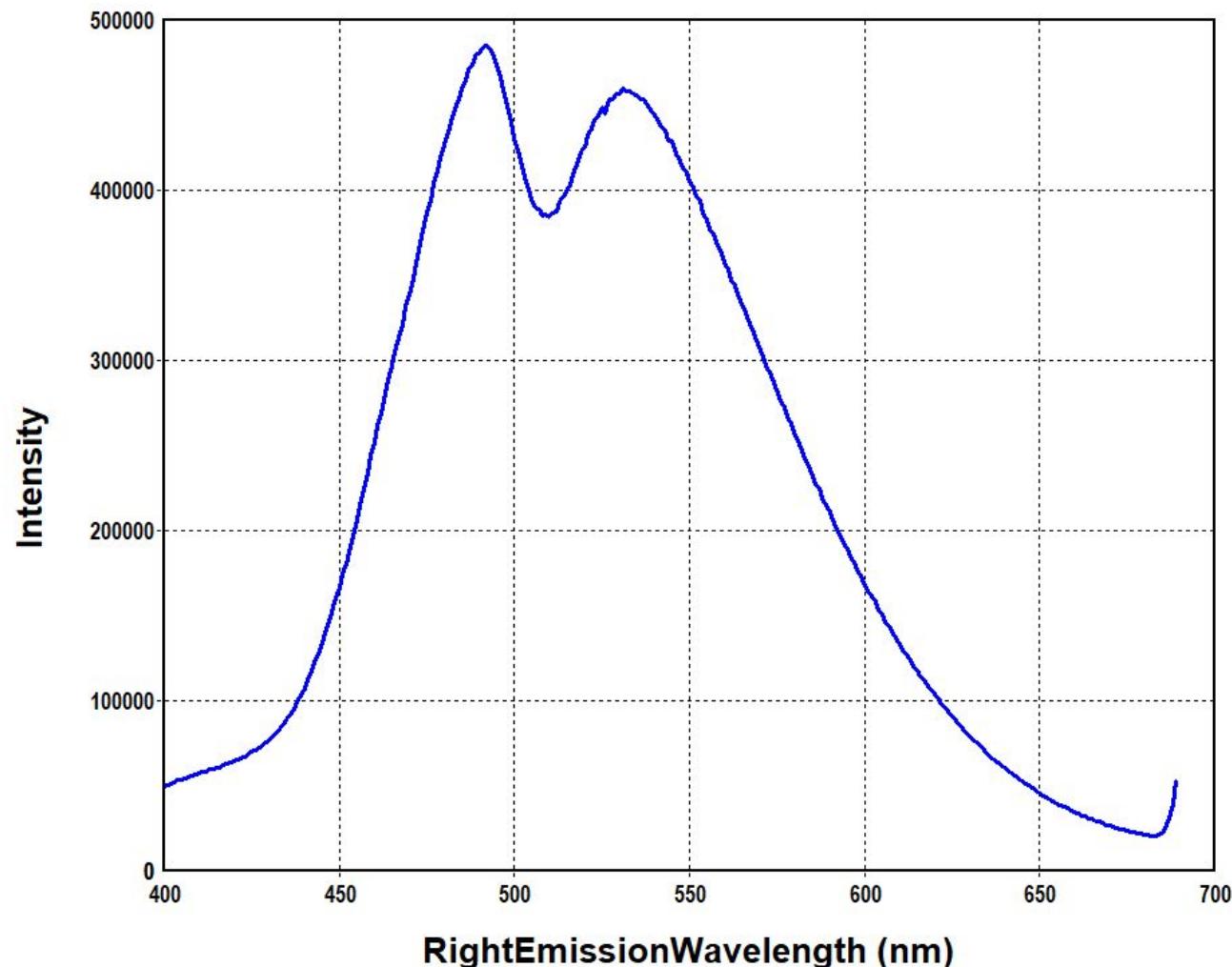
1/28/2020 Prep pyocyanin for fluoreimeter

0.5 mM pyocyanin + 1 mM TCEP + 8 uL 1M NaOH, sat for 30 minutes in chamber

- Still see yellow bump
- Next time, try:
 - 0.5 mM pyocyanin + 0.5 mM TCEP + 8 uL 1M NaOH in chamber
 - 0.5 mM pyocyanin + 1 mM TCEP + 6 uL of 1M NaOH

0.5 mM pyocyanin + 1 mM TCEP + 8 uL 1M NaOH, sat for 30 minutes in chamber

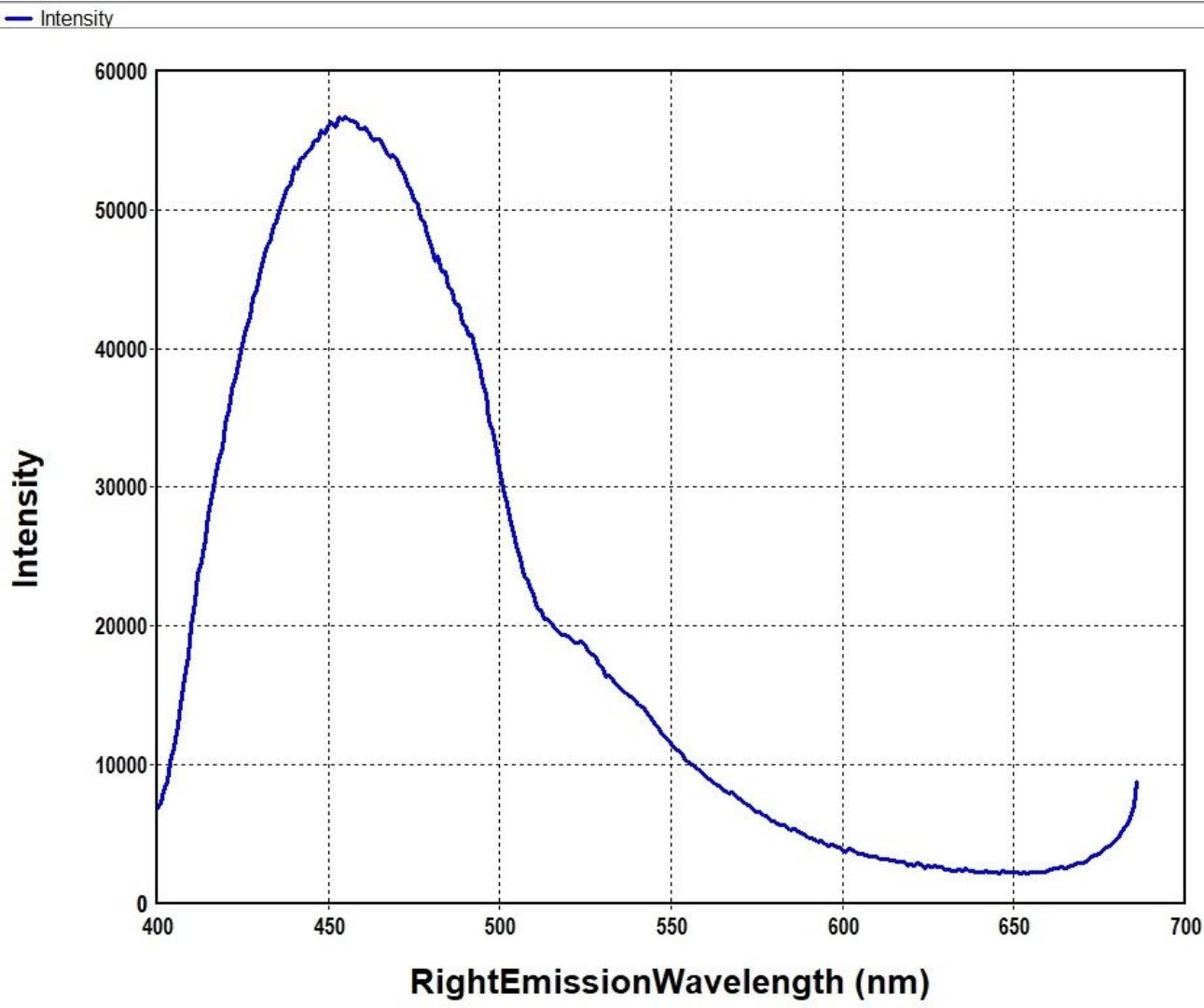
— Intensity



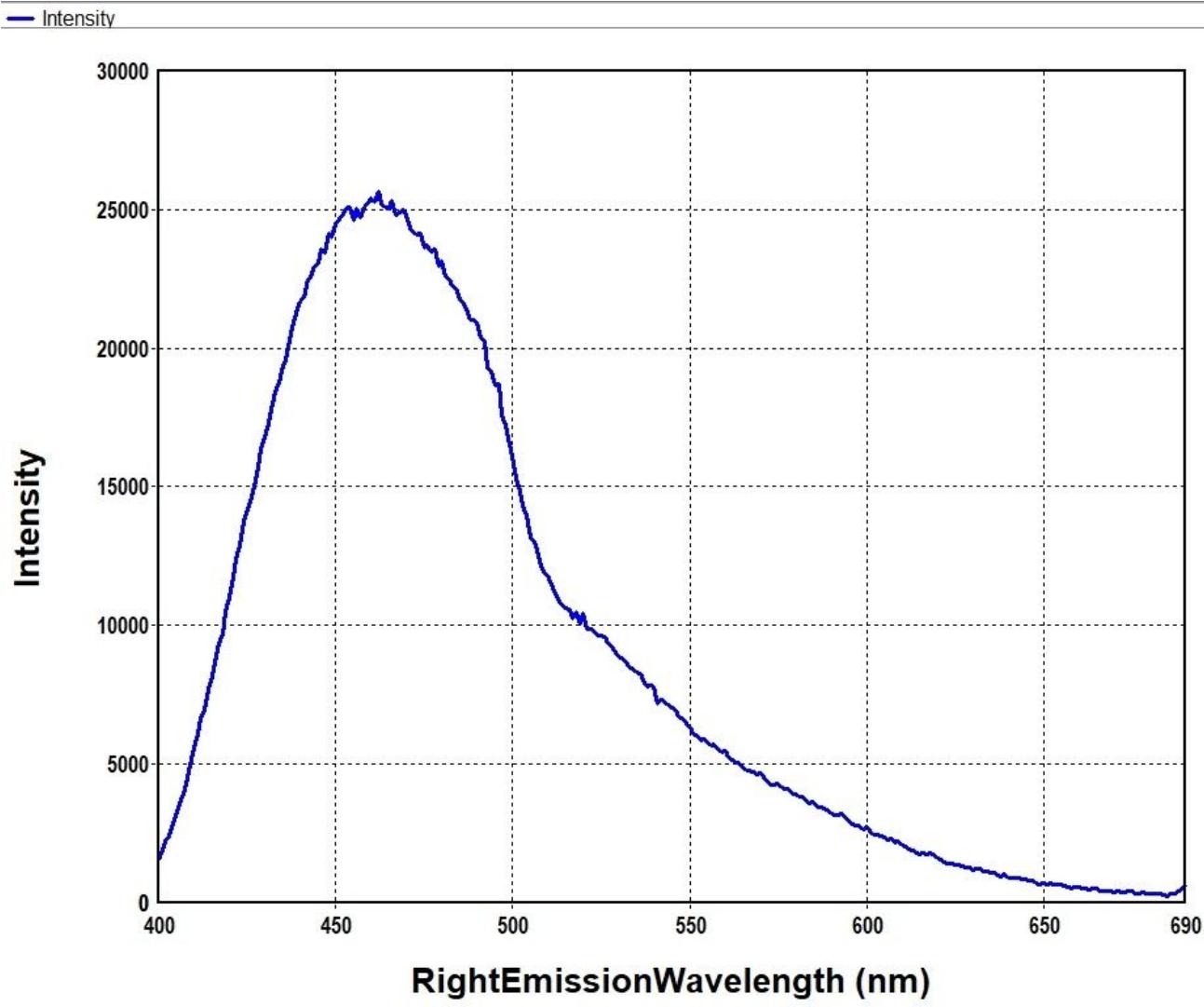
1/30/2020 Prep pyocyanin for fluorimeter

Volume needed for medium sized cuvette in fluorimeter? 300 ul looks fine

- 300 uL of 10 mM NADH in DPBS:



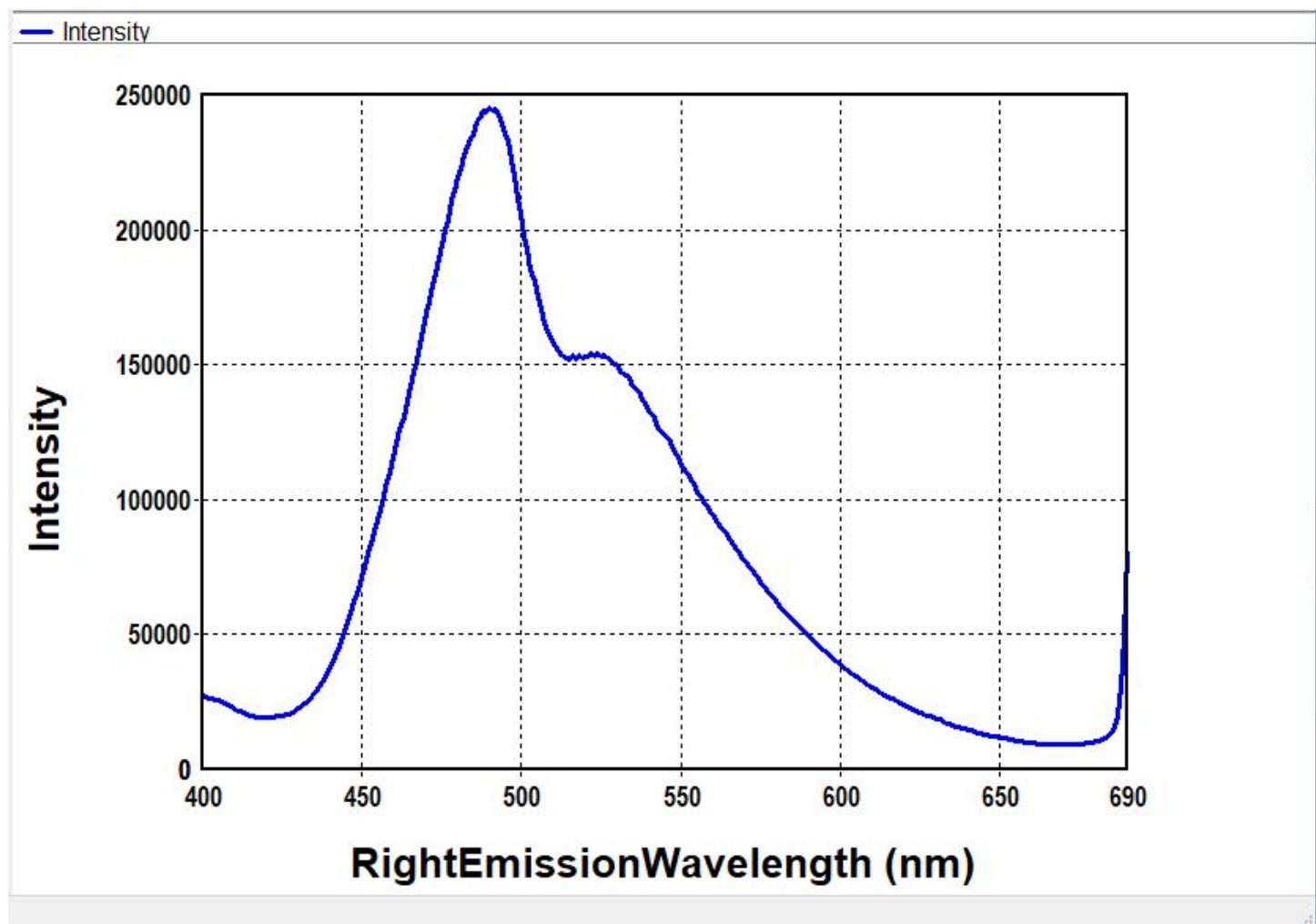
- Versus 1 ml of 10 mM NADH:



Note - a stock from 1/24 experiments looks colorless: 50 ul stock of 0.5 mM PYO + 1mM TCEP + 8 ul of NaOH

Need to let it incubate longer.

Spectra of freshly-made (~30 minute incubation) 0.5 mM pyocyanin + 0.75 mM TCEP + 8 μ l of 1M NaOH (per 50 μ l of solution):



1/30/2020 Prep pyocyanin w/ NaOH + TCEP gradient for imaging next week

600 μ L stocks of 0.5 mM pyocyanin with 96 μ L of NaOH and gradient of TCEP reducing agent:

Concentration of TCEP	μ L of TCEP (10 mM stock)	μ L of PYO (2 mM stock)	μ L of NaOH (1M)	μ L of MOPS buffer
0.75	45	150	96	310
1	60	150	96	294

2/03 = Note, still blue, something not right

2/03/2020 Imaging PYO, PA14, phz knockout on LSM 880

- One of the PYO samples looked like a mix of reduced and radical and maybe something else (from 1/30 = 0.5 mM PYO + 0.75 mM TCEP + 8 uL of NaOH per 50 uL solution)
- The PA14 cultures were making pyocyanin, but didnt see long lifetime signal
 - ASM is a problem. Need to image in M9 media next time.
- Also didnt see long lifetime signal (as expected) for phz ko
- Set up new stocks for next time:

Prepared 2-03-2020

Checked fluorescence w/ UV handlight on 2-07-2020 (Friday) and 2-09-2020 (Sunday)

With 0 uL of NaOH

Concentration of TCEP	uL of TCEP (10 mM stock)	uL of PYO (2 mM stock)	uL of NaOH (1M)	uL of MOPS buffer	Measured pH (pH strips)	Fluorescence appearance	Eye color
0.4	2	12.5	0	35.5		Y? on 2/07	B on 2/07
0.5	2.5	12.5	0	35		not	B on 2/07
0.6	3	12.5	0	34.5		not	G on 2/07
0.7	3.5	12.5	0	34		Green (yellow-green?) on 2/07	Y on 2/07

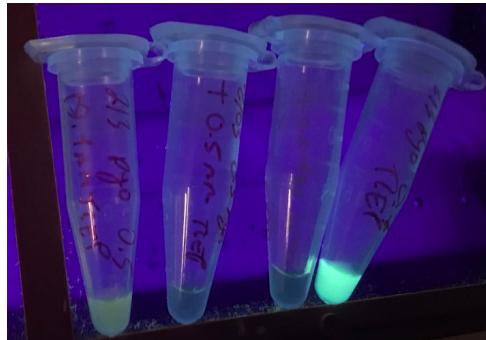


Photo taken after 4 days of incubation (2-07-2020)

Ordered in increasing concentration of TCEP

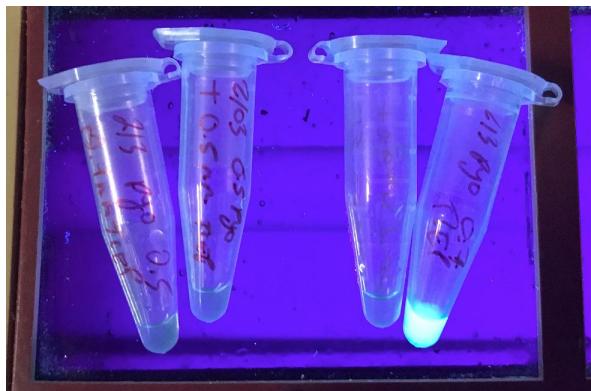


Photo taken after 6 days of incubation (2-09-2020)

With 4 uL of NaOH

Concentration of TCEP	uL of TCEP (10 mM stock)	uL of PYO (2 mM stock)	uL of NaOH (1M)	uL of MOPS buffer	Measured pH (pH strips)	Fluorescence appearance	Eye color
0.2	1	12.5	4	32.5		Y on 2/07	B on 2/07
0.4	2	12.5	4	31.5		Y on 2/07	B on 2/07
0.5	2.5	12.5	4	31		Y on 2/07	B on 2/07
0.6	3	12.5	4	30.5		Y on 2/07	Y on 2/07
0.7	3.5	12.5	4	30		Y on 2/07	Y on 2.07



Photo taken after 4 days of incubation (2-07-2020)

Ordered by increasing concentration of TCEP



Photo taken after 6 days of incubation (2-09-2020)

With 8 uL of NaOH

Concentration of TCEP	uL of TCEP (10 mM stock)	uL of PYO (2 mM stock)	uL of NaOH (1M)	uL of MOPS buffer	Measured pH (pH strips)	Fluorescence appearance	Eye color
0.4	2	12.5	8	27.5		Y on 2/07	Y on 2/07
0.5	2.5	12.5	8	27		Y on 2/07	B on 2/07
0.6	3	12.5	8	26.5		Y on 2/07	B on 2/07
0.7	3.5	12.5	8	26		Y on 2/07	Y on 2/07



Photo taken after 6 days of incubation (2-09-2020)

Prep new stocks on 2/07/2020 (Fri)

With 0 uL of NaOH

Concentration of TCEP	uL of TCEP (10 mM stock)	uL of PYO (2 mM stock)	uL of NaOH (1M)	uL of MOPS buffer	Measured pH (pH strips)	Fluorescence appearance	Eye color
0.4	2	12.5	0	35.5	6.4	Y-g on 2/07	
0.5	2.5	12.5	0	35		Y-g on 2/07	
0.55	2.75	12.5	0	34.75		Y-g on 2/07	
0.6	3	12.5	0	34.5		Y-g on 2/07	
0.62	3.1	12.5	0	34.4		Y-g on 2/07	

0.64	3.2	12.5	0	34.3		Y-g on 2/07	
0.66	3.3	12.5	0	34.2		Y-g on 2/07	
0.68	3.4	12.5	0	34.1		Y-g on 2/07	
0.7	3.5	12.5	0	34		Y-g on 2/07	
0.8	4	12.5	0	33.5		Y-g on 2/07	



Photo taken after 2 days of incubation (2-09-2020)

With 2 uL of NaOH

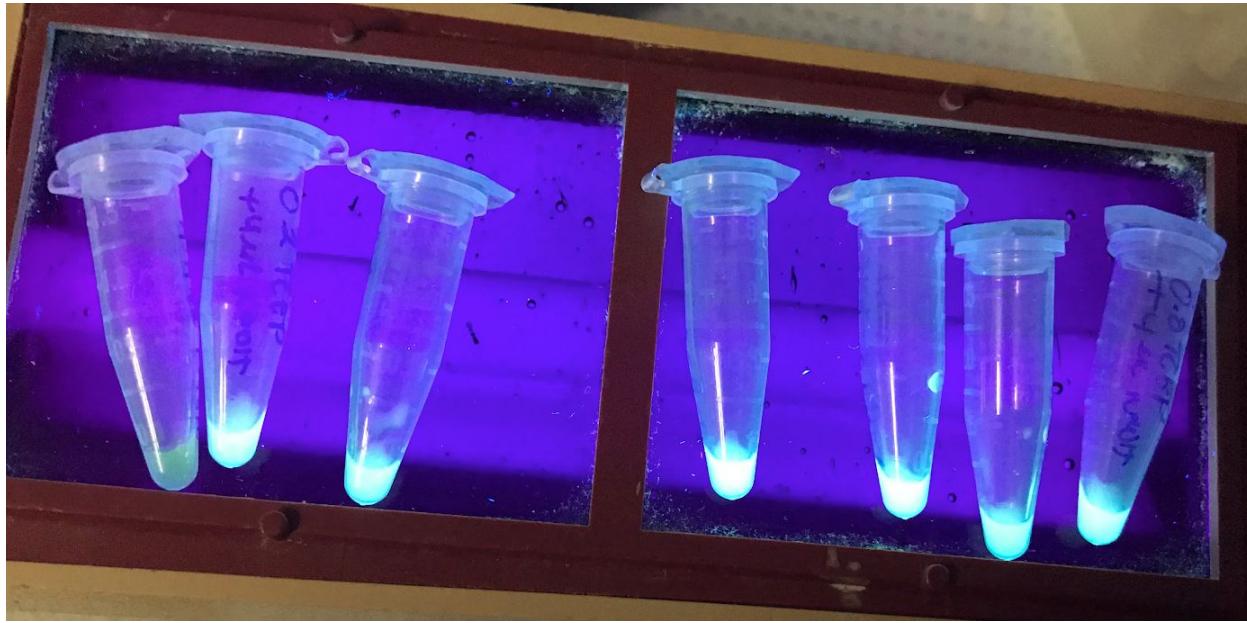
Concentration of TCEP	uL of TCEP (10 mM stock)	uL of PYO (2 mM stock)	uL of NaOH (1M)	uL of MOPS buffer	Measured pH (pH strips)	Fluorescence appearance	Eye color
0.1	0.5	12.5	2	35	7.2		
0.2	1	12.5	2	33.5			
0.4	2	12.5	2	32.5			
0.5	2.5	12.5	2	33			
0.6	3	12.5	2	32.5			
0.7	3.5	12.5	2	32			
0.8	4	12.5	2	21.5			



Photo taken after 2d of incubation (2-09-2020)

With 4 uL of NaOH

Concentration of TCEP	uL of TCEP (10 mM stock)	uL of PYO (2 mM stock)	uL of NaOH (1M)	uL of MOPS buffer	Measured pH (pH strips)	Fluorescence appearance	Eye color
0.1	0.5	12.5	4	33	7.6		
0.2	1	12.5	4	32.5			
0.4	2	12.5	4	31.5			
0.5	2.5	12.5	4	31			
0.6	3	12.5	4	30.5		Y-g on 2/07	
0.7	3.5	12.5	4	30		Y-g on 2/07	
0.8	4	12.5	4	29.5		Y-g on 2/07	



2-13-2020 prepping samples for imaging on the 880 on 2/17 (Mon) and 2/18 (Tues)

- Grow PA14 and phz ko in ASM (liquid) and M9 succinate (liquid)
- 2 dishes of each:
 - Transfer 1 dish into anaerobic chamber 24h before
 - Add 50 uL of the pyocyanin stock to some of the cells

Plate grown aerobically, stays aerobic throughout the experiment

PA14 in ASM	PA14 in ASM	PA14 in ASM	PA14 in M9s	PA14 in M9s	PA14 in M9s
PA14 in ASM	PA14 in ASM	PA14 in ASM	PA14 in M9s	PA14 in M9s	PA14 in M9s
Phz ko in ASM	Phz ko in ASM	Phz ko in ASM	Phz ko in M9s	Phz ko in M9s	Phz ko in M9s
Phz koin ASM	Phz ko in ASM	Phz ko in ASM	Phz ko in M9s	Phz ko in M9s	Phz ko in M9s

Plate grown aerobically, move to anaerobic chamber on Monday for 24h. Image on Tuesday

PA14 in ASM	PA14 in ASM	PA14 in ASM	PA14 in M9s	PA14 in M9s	PA14 in M9s
PA14 in ASM	PA14 in ASM	PA14 in ASM	PA14 in M9s	PA14 in M9s	PA14 in M9s
Phz ko in ASM	Phz ko in ASM	Phz ko in ASM	Phz ko in M9s	Phz ko in M9s	Phz ko in M9s
Phz koin ASM	Phz ko in ASM	Phz ko in ASM	Phz ko in M9s	Phz ko in M9s	Phz ko in M9s

Monday:

- Move anaerobic chamber plate to the chamber
- Add 50 uL of Pyocyanin to 3 replicates
- Image on Monday:
 - Aerobic PA14 in ASM
 - Aerobic phz ko in ASM
 - Aerobic PA14 in ASM + pyocyanin (~1h incubation)
 - Aerobic phz ko in ASM + pyocyanin (~1h incubation)
 - Anaerobic PA14 in ASM
 - Anaerobic phz in ASM
 - Anaerobic PA14 in ASM + pyo (~1h incubation)
 - Anaerobic PA14 in ASM + pyo

Repeat for M9 succinate

03-12-2020 electrode reduction of pyocyanin with Adam in Allon's lab

First attempt following this protocol: <https://pubs.acs.org/doi/pdf/10.1021/es702290a>

- 15 mL of 821 μ M of pyocyanin in 0.1 M KCl buffered with 10 mM CH₃C(O)ONH₄-MOPS at pH 7.
- ~16h with voltage -0.345 V
- Ag AgCl₂ reference
- Working electrode: glassy carbon
- Counter electrode: platinum wire
- Color changed from blue to green
- With stirbar

03-13-2020 LSM-880 and DIVER imaging

Tara on 880:

- The green pyocyanin from Adam
- PA14 on ASM plate (grown for 5 days) "aerobic" - transferred chunk to a dish with coverslip on the bottom

- Δ phzA-G1/2 on ASM plate (grown for 5 days) “aerobic” - transferred chunk to a dish with coverslip on the bottom
- PA14 on ASM plate (grown for 5 days) “aerobic” - transferred chunk to a dish with coverslip on the bottom --smooshed between coverslips for an hour notable color change from green to brown
- 2 mM stock of pyocyanin sitting in the chamber since 1-31-2020 --- fluorescent, has mix of radical and reduced

Simon on the DIVER:

- 1st set of z-stacks: CH1 = DIVER; CH2=460/80 nm filter
- Later z-stacks:
 - No coverslip of PA14 D5 - z stack
 - Coverslip of PA14 D5 (color change)
 - Coverslip of phz ko D5 (x 2 plates)
 - Coverslip of phz ko D1 (dim??)
 - No coverslip of PA14 D1 z stack
 - Coverslip of PA14 D1 z stack
 - No coverslip of phz ko (D5)
 -
-

03-17-2020

Make 1L of M9 succinate plates

	ml of stock
M9 5x	200
1M MgSo ₄	1
1M CaCl ₂	0.1
Trace Metals (1000x)	0.1
Vitamin BME (100x)	1
Succinate (1M)	100
UPW	586
Agar (2% stock)	112

Sputum imaging on Friday (3-20-20)

P. aeruginosa PA14 mCherry from Albert Siryaporn

Grown to stationary phase

Sputum samples from Michigan:

- S7524
- S8704
- S8734

Mat Tek P35G-1.5-14C 35 mm dish, 14 mm microwell no 1.5 glass

350 uL of sputum + 10 uL of stationary PA14

Put coverslip on top and sealed plate

DIVER imaging (spartan 6) on 6-18-20

Transfer samples on 6-11-20 to the anaerobic chamber (no CO₂, RT):

- Dilute 10mM pyocyanin to 2 mM pyocyanin with MOPS, put in chamber
- Prep 1-hydroxyphenazine: make 10 mM stock in methanol. Dilute to make 2mM stock with MOPS.
- Transfer TCEP to chamber

6-15-20:

- Inoculate ASM and M9 succinate plates with 1 colony of PA14 (normal sized petri dishes)
- Incubate aerobic 37C

6-18-20:

- Imaging on the diver with spartan 6 flimbox
- Coverslips on for 30-60 minutes before actually imaging
- Imaging parameters:
 - CH1 = DIVER NADH filter
 - excitation= 740 nm
 - fov=114 um
 - Image the radial center of the plate, axial: every 100 um from 0-1mm deep
 - 32 us dwell time
 - Objective = 0.4 NA 20X Air
- Laser power for ASM plates:
 - Top (0) = 25%
 - Bottom (1mm deep) =66%
- Laser powers for M9 plates:
 - Top = 15% =
 - Bottom= 40% =
- Bacterial culture samples:
 - 3X WT PA14 in ASM (center)
 - 3X Δphz in ASM (center)
 - 3X WT PA14 in m9 succinate (outer region, bacterial growth)
 - 3X Δphz in m9 succinate (outer region, bacterial growth)
- Laser power:
 - 0% = 3.6 mW
 - 15% = 4.9 mW
 - 25% = 7.94 mW
 - 40% = 18.2 mW

- **66% = 108 mW**
- **70% = 141 mW**