Mike's Lab Talk

10am Monday 6th February 2006

Outline of Talk

- First lab talk so hope I reach my audience!
  - PhD students, postdocs, staff
- Purpose of this lab talk:
  - Describe on-going work
  - Describe progress (+ show data)
  - Describe work to do in the short term
- Stuff for the future

On-going Work

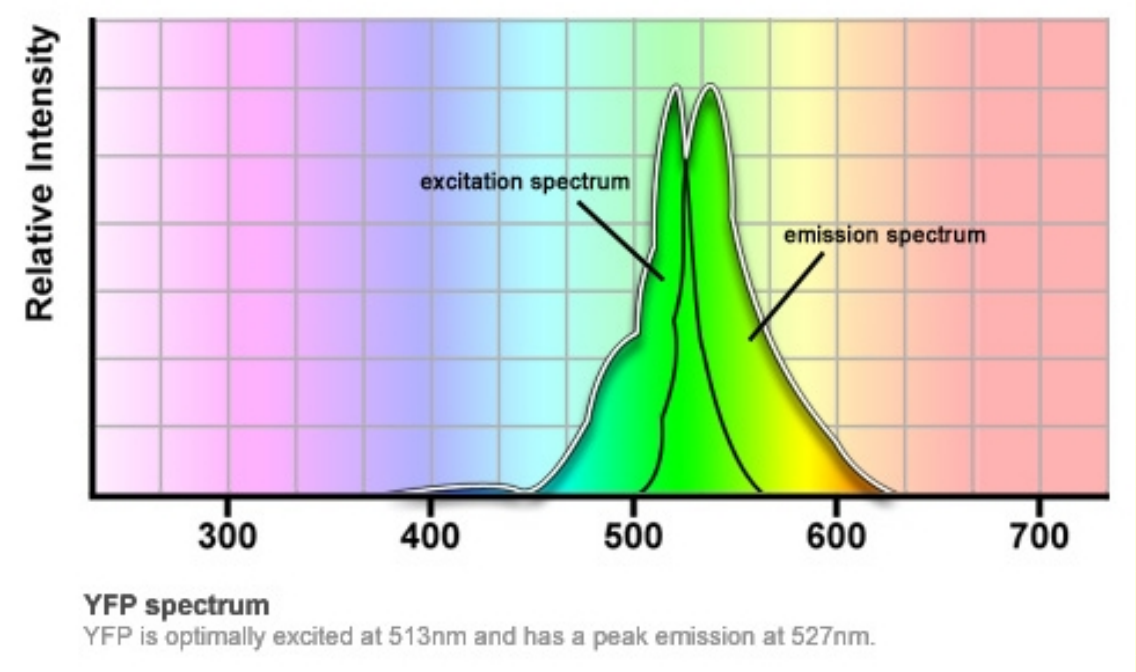
- The aim is to get a nice system for looking at protein targeting in chloroplasts
  - Looking in terms of Western blotting and
  - Looking using fluorescence microscopy
- Presently attempting thylakoid imports of James' n-strep-DmsA-YFP (bacterial Tat signal)

Progress

- Since the start of term in October (4 months)
- Setting up in the lab:
  - Collecting equipment and solutions
  - Collecting techniques (cloning and confocal independence left)
- Latest work has been on YFP and chlorophyll in relation to Lorenzo's question at an MCB seminar and Ale's concern about an *in vitro* import

Fluorescence Microscopy

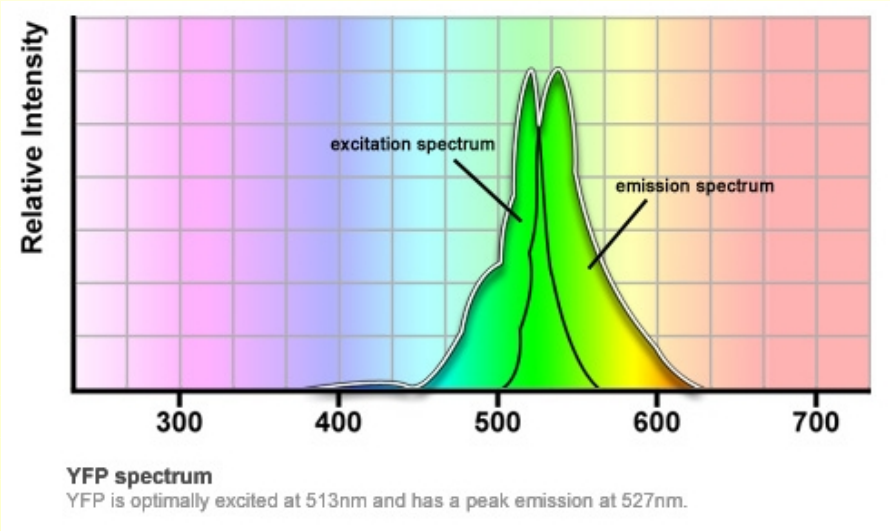
- Two related questions:
  - Will the background signal be a concern?
  - Will the signal from imported YFP be detectable?



http://www.mekentosj.com/science/fret/spectrum2.html

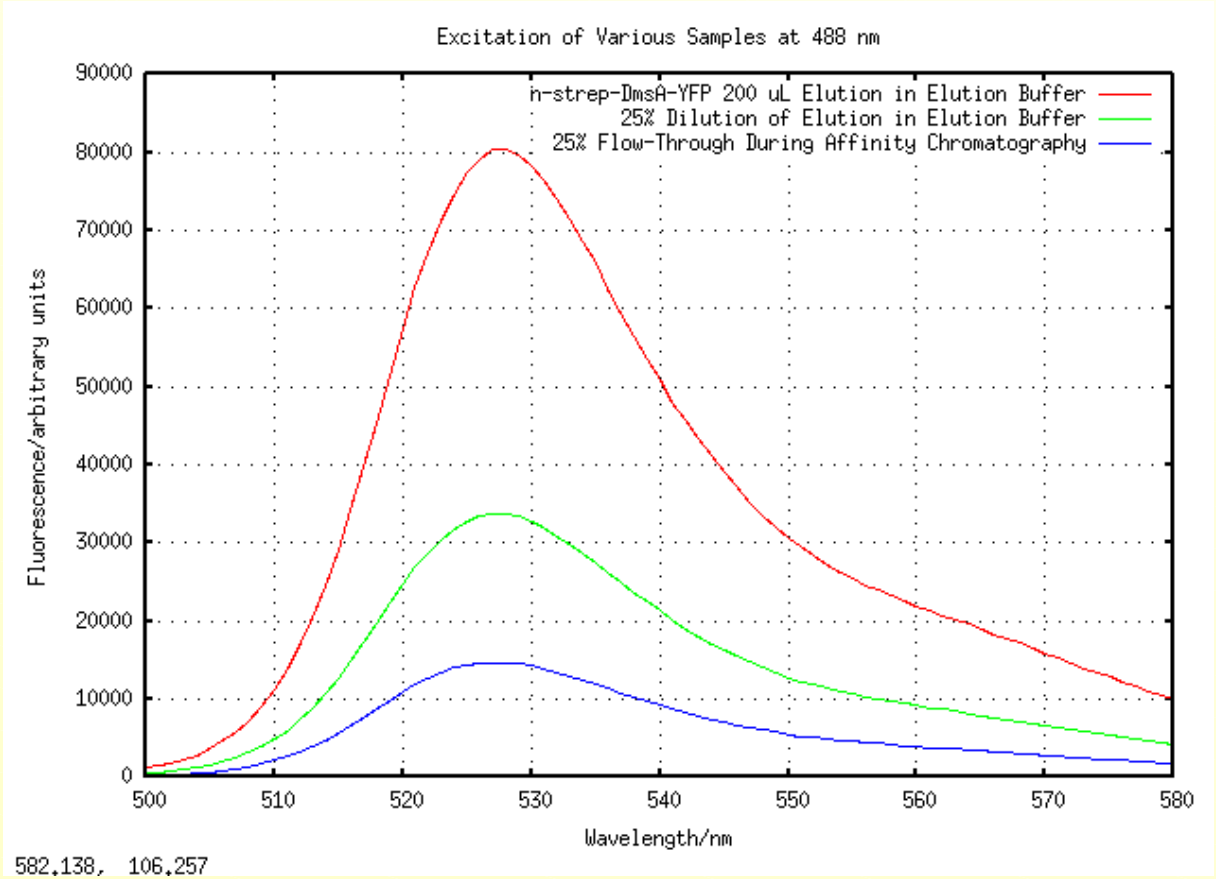
Excitation of YFP

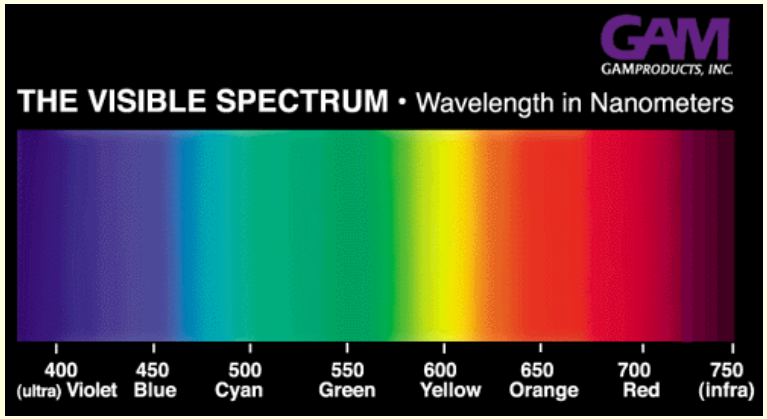
- The 514 nm Argon laser line is the best choice for excitation near the 513 nm excitation max.
  - Could also use the 488 nm Argon laser line as used for GFP
- Measurements are shown that use 488 nm to keep away from the max. emission of 527 nm



Yellow Fluorescent Protein

- The fluorometer (detection at 90 degrees to excitation) suggests the YFP isn't very yellow (is yellow, judging by eye)



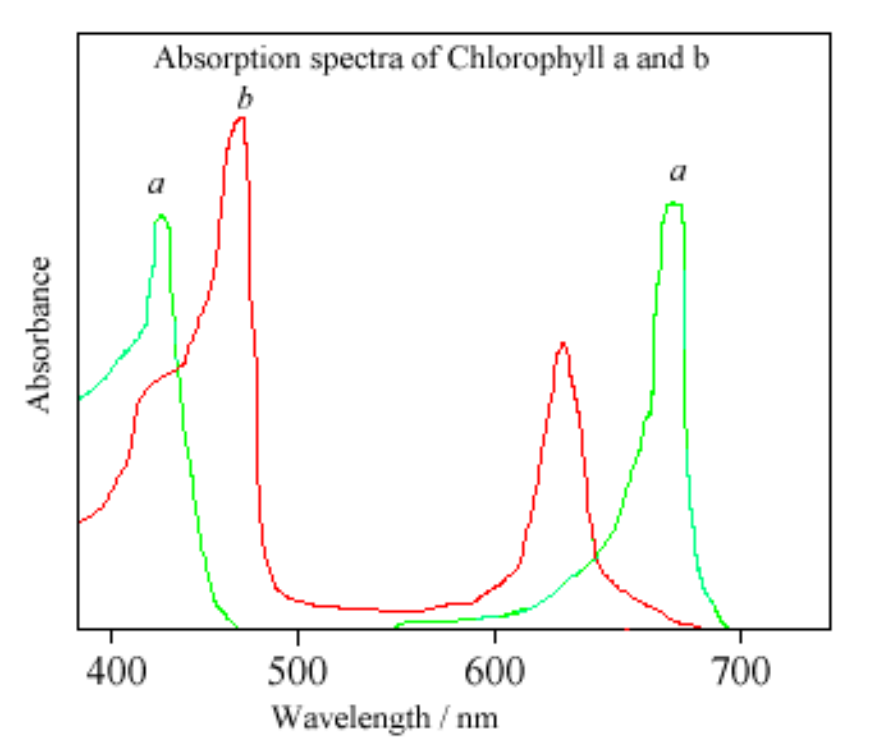


Still working on estimation of YFP concentration

- Complicating factors:
  - Buffer is changed from elution buffer to import buffer by dialysis and found to reduce volume from 200 uL to around 75 uL
  - Need to check exact YFP mutant being used (lots of molar extinction coefficients available for various versions – of the order 10^5 M cm^{-1})
- Other options include standard protein assays (keeping in mind elution is not pure) or comparison of ECL Western blotting bands with purchased GFP/YFP (expensive)

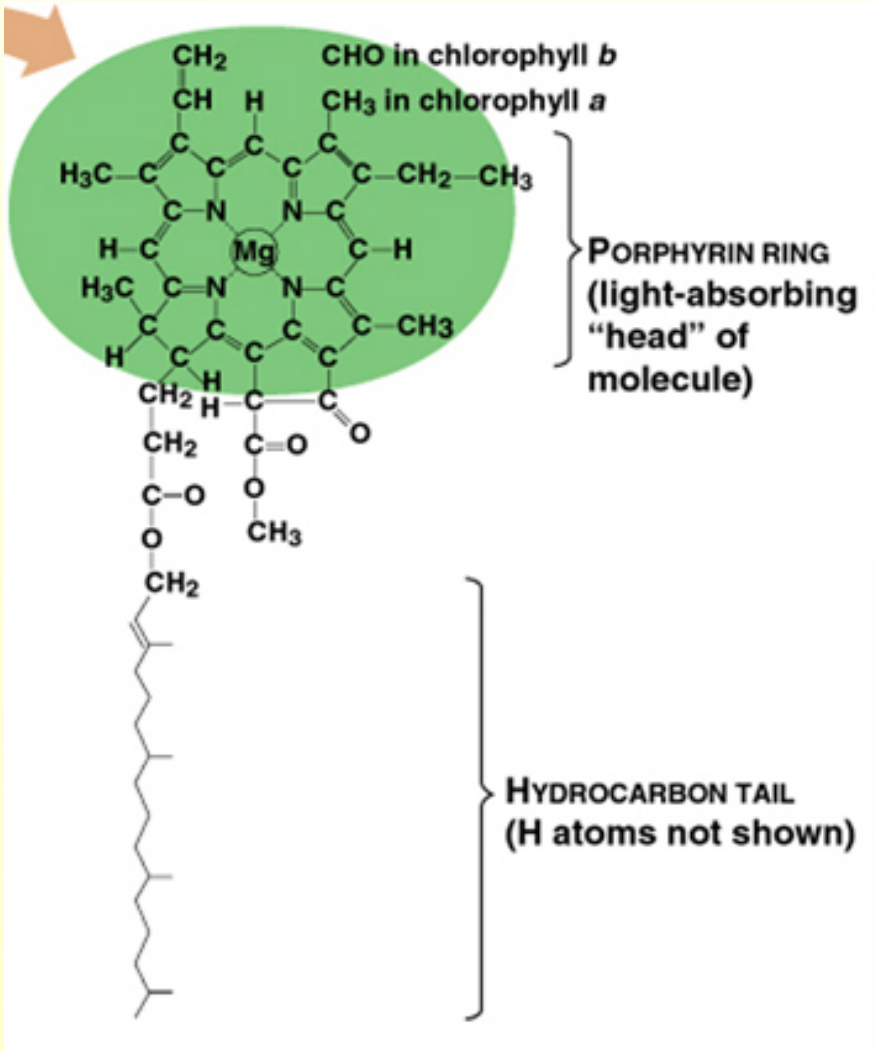
Chlorophyll in Thylakoids

- Chlorophyll is held by chlorophyll binding proteins in the thylakoid membrane
- Chlorophyll b has an excitation maximum near 488 nm and this could potentially be a problem



Spectra from the net (Paul May)

Chlorophyll in Thylakoids

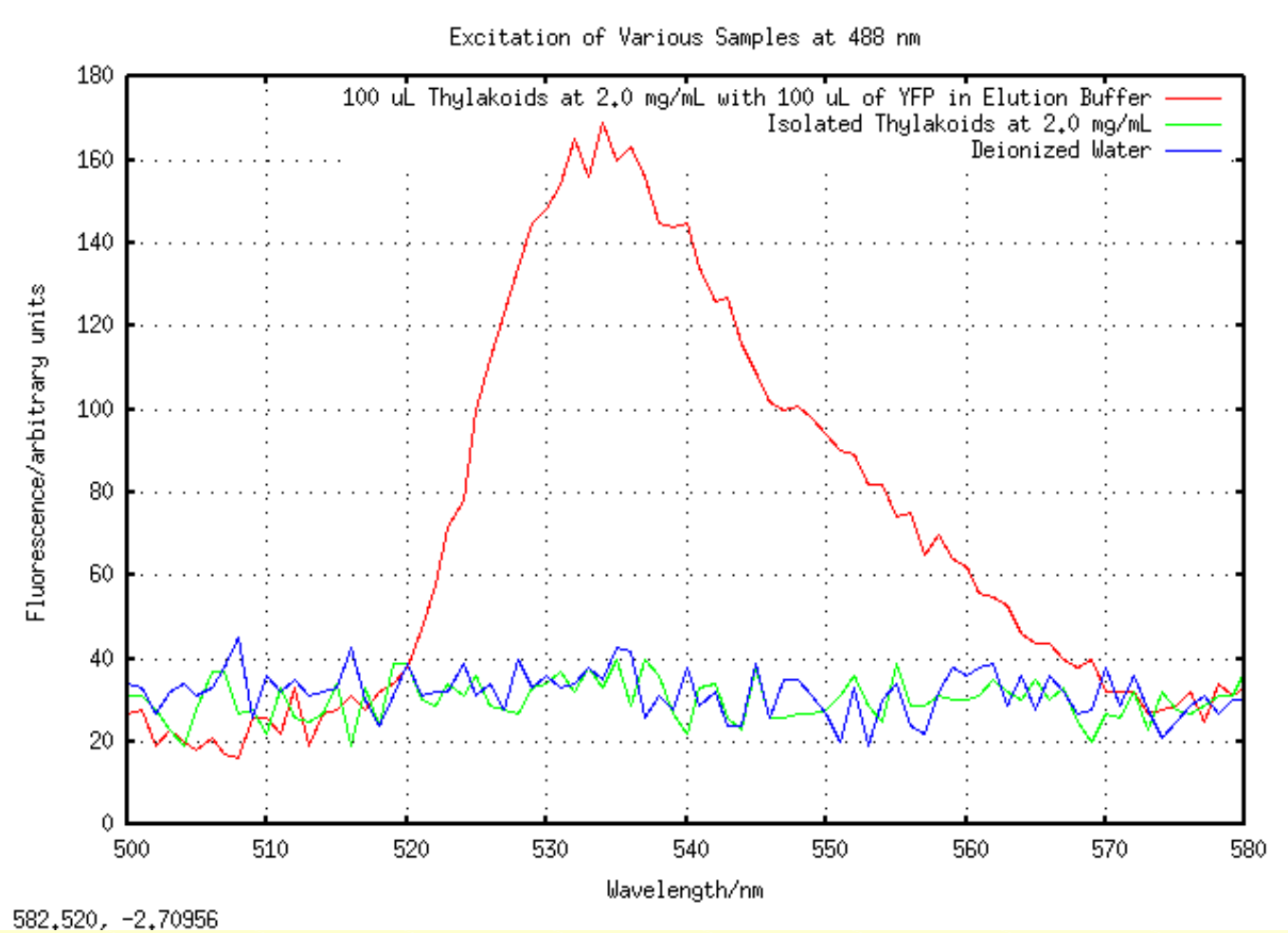


- Luckily, chlorophyll is known to emit in the orange-red region; the 650-700 nm region
- So we don't expect a problem (YFP max. emission 527 nm; EYFP even better at red-shifted values – closer to yellow)

http://fig.cox.miami.edu/~cmallery/150/chemistry/chlorophyll.jpg

Seeing YFP with a Chlorophyll Background

- With the correct filters, chlorophyll is no more trouble than water using 488 nm for excitation



Things to Note

- First tests only (further work will only be useful if YFP localisation is visible under the confocal)
- Measurements made on day after isolation of thylakoids
  - This should actually make the background worse because of damage to the photosynthesis system so chlorophyll is only able to absorb and emit (need to check on a fresh preparation)
- Signal not as smooth due to non-specific optical density of the thylakoid sample (also to check)

Possible Continuations

- Dilutions of fluorescent protein to see behaviour of excitation versus emission
- 3D measurements where the excitation is allowed to vary
- Various combinations pre- and post-import with thylakoids
- All very interesting if successful imports are visible under confocal: academic if not visible

Short Term Plan

- Need to optimize immuno-blotting for YFP after thylakoid import
  - Dot blot needed to optimize antibody concentrations
  - Import volumes may have to be scaled up (12.5 uL thylakoids at 2.0 mg/mL chlorophyll in 100 uL reaction volume)
- Not a problem if it doesn't work straight away as system could be used to try to set up and maintain a delta-pH gradient (useful for reconstituting the Tat system in lipsomes)

Take Home Message

- Going back to the two questions:
  - The chlorophyll background shouldn't mask the YFP signal and
  - The YFP should be detectable (~4 times brighter than EGFP)
  - (but it all depends on the confocal)
- The Sim Aminco 8100 Spectrofluorometer complements use of the confocal for detection of active chromophores (fluorescent proteins and chlorophyll)

Thank you, and good morning!