

Make-up Lab: Investigating an Unexpected Fluorescence in Chlorophyll.

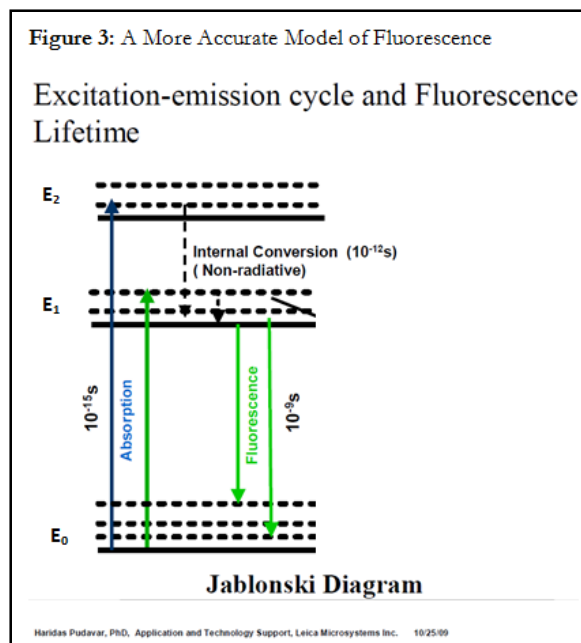
Introduction:

In this one-week lab, you will be further investigating the fluorescence of chlorophyll. Chlorophyll absorbs light in the UV, violet, and blue regions of the EM spectrum and fluoresces in the red region.¹ The intensity of the red color is a function both of how much chlorophyll is in the sample² and of what light source is used to excite the chlorophyll. Chemical compounds that exhibit fluorescence are commonly called *fluorophores* (or *fluorochromes*). By measuring the intensity and nature of this fluorescence, plant ecophysiology can be investigated.³

Your lab will consist of two parts: I) reproducing an 'unexpected' observed fluorescence of chlorophyll, and II) investigating the system to determine and explain the source of the fluorescence. The lab report you turn in at the end of today's lab should discuss answers to questions posed in the sections below as well as any insights you gain from your explorations and investigations, along with the data supporting those insights.

What we did in Lab 11:

In Lab 11 we explored the *in vitro* fluorescence of a chlorophyll system (a sample of spinach chlorophyll suspended in isopropyl alcohol and contained in a glass vial) when exposed to a variety of light sources (UV, Red, Green, and Blue LEDs & a white light source (incandescent bulb)). You determined that a perpendicular arrangement of light source, sample, and fiber optic detector cable produced the best spectroscopy results. The observed spectra were then used to compare and contrast models of fluorescence, such as the more complex Jablonski diagram shown in Figure 3 from Lab 11.



While we expected that the UV and Blue LEDs (and the white light source) would be capable of generating fluorescence in the chlorophyll, several lab groups noticed the chlorophyll system fluorescing when exposed to the Green LED. Samples of these groups' observations are shown on the next page. **Carefully compare** the light source spectra (first graph for each source) to the spectra for the light scattered and fluoresced by the chlorophyll system as sensed by the fiber optic (all other

¹Note: In fluorescence, there is a delay in time between when the light is absorbed and when it is emitted, typically a few nanoseconds for many fluorescent objects used in biology. Related physics is involved in phosphorescence where light is absorbed and lower energy light is emitted, but the time delay in phosphorescence is minutes to hours—this property is used in making glow in the dark objects that emit light for minutes to hours after being exposed to light.

²Taken from: http://www.vernier.com/experiments/bio-i/14/plant_pigments/.

³Murchie and Lawson (2013). "Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications." *Journal of Experimental Botany* 64 (13): 3983–3998. doi:10.1093/jxb/ert208

graphs). As you can see, the Green LED is producing a distinct (and unexpected!) fluorescence from our chlorophyll system.

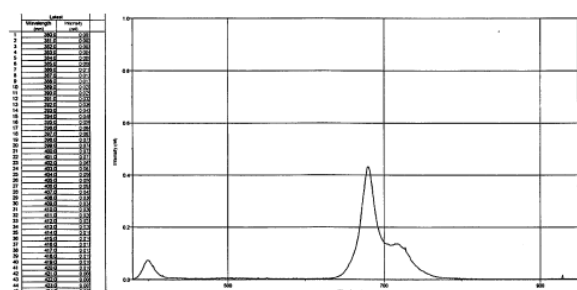
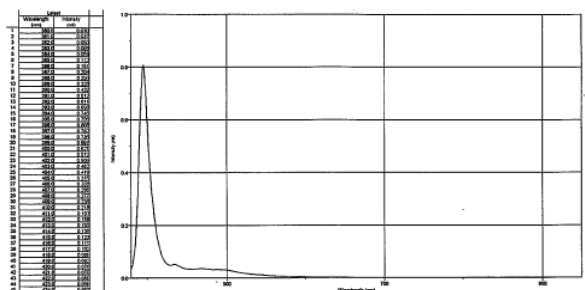
What was observed in Lab 11:

Spectra 2: "UV light"

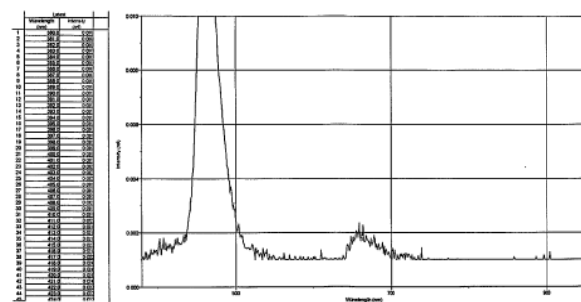
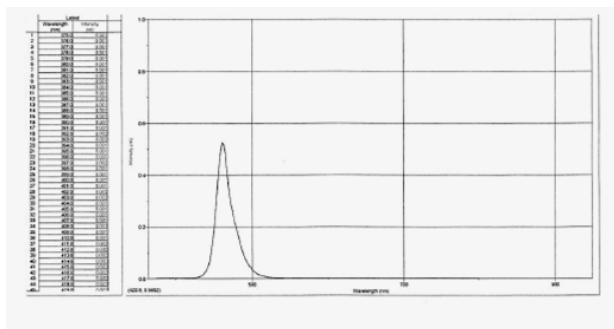
With UV LED:

A small peak of 400 nm is present along with a heightened, narrow peak at 680 nm.

UV through Chlorophyll



With Blue LED:



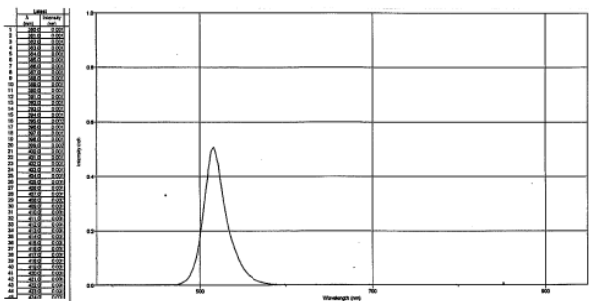
Blue Light (Note: Smaller Scale)

Spectra 5: "Green light"

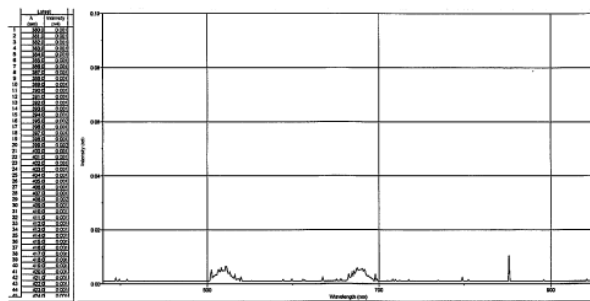
A sharp peak is seen at 550 nm, which represents the green light. Another peak is seen at 680 nm, which is an interesting result because this is the same wavelength observed for fluorescence.

With Green LED:

Green Baseline

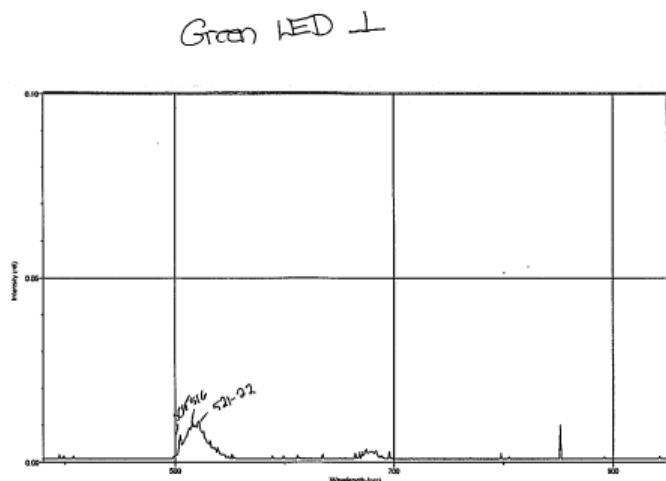
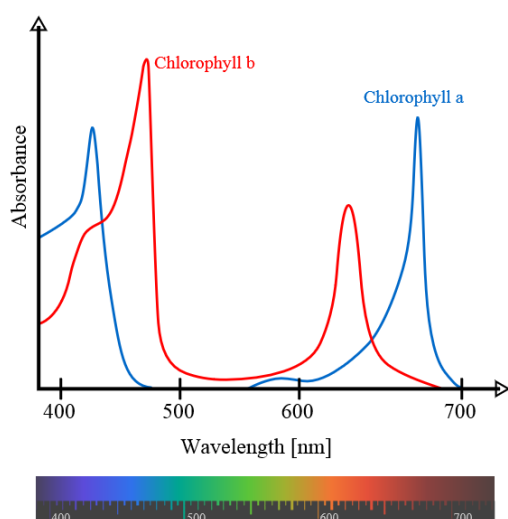


Green with Chlorophyll



Why this was 'unexpected':

"Chlorophyll absorbs light most strongly in the blue portion of the electromagnetic spectrum, followed by the red portion. Conversely, it is a poor absorber of green and near-green portions of the spectrum, hence the green color of chlorophyll-containing tissues."⁴ The types of chlorophyll most commonly found in plants are chlorophyll *a* and chlorophyll *b*. (It turns out that there are more than six different forms of chlorophyll!)



The **absorbance** spectra of free chlorophyll *a* (blue) and *b* (red) in a solvent are shown at left. The spectra of chlorophyll molecules are slightly modified *in vivo* depending on specific pigment-protein interactions. The measurement of the absorption of light is complicated *in vitro* by the solvent used to extract it from plant material, which affects the values obtained. **From this spectral information, which of the forms of chlorophyll *could* be responsible for absorption of green light leading to fluorescence?**

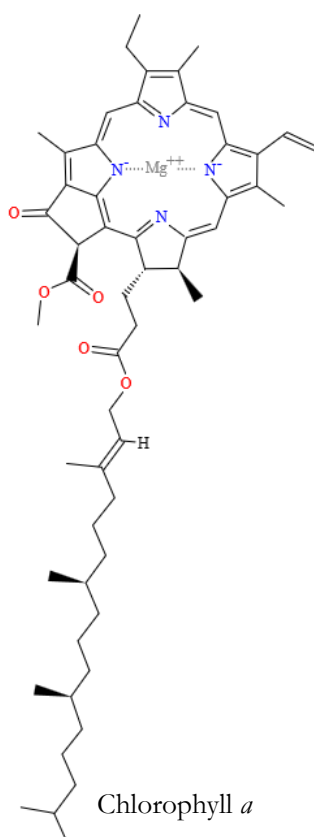
Here is a little more information about fluorescence, chlorophyll *a*, and chlorophyll *b*.

- "The capacity of a plant to carry out photochemistry is limited and will depend upon a range of factors including stresses caused by environmental conditions. Absorbed light energy in excess of that used for photochemistry must be effectively dissipated by non-photochemical processes. Such processes include the emission of heat and re-emission of small but diagnostically significant amounts of the absorbed radiation as longer wavelength red/far-red light energy. This re-emission of light is termed chlorophyll fluorescence. Although chlorophyll fluorescence emission from whole leaf systems is too weak to be viewed with the naked eye, it can be observed from illuminated extracts of a chlorophyll solution. Peak chlorophyll fluorescence occurs in the red region of the spectrum (685 nm) and extends into the infra-red region to around 800 nm. Each of these processes operate in direct competition for a finite pool of absorbed energy, any change in energy utilization by one process produces a complementary change in the others. This fact enables chlorophyll fluorescence to be used as a rapid and reliable non-invasive probe of photochemistry."⁵

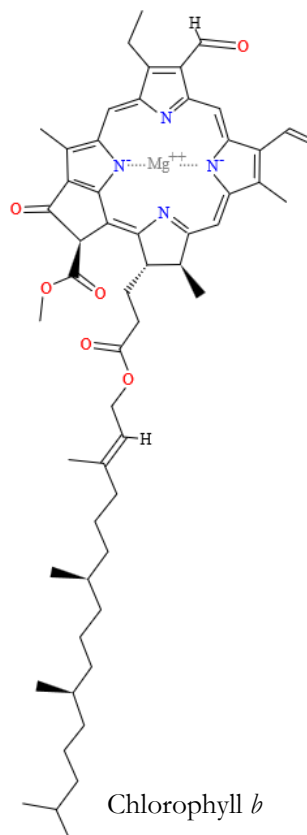
⁴ Taken from: <http://en.wikipedia.org/wiki/Chlorophyll>.

⁵ Taken from: <http://hansatech-instruments.com/products/introduction-to-chlorophyll-fluorescence/>.

- "Chlorophyll is a chlorin pigment, which is structurally similar to and produced through the same metabolic pathway as other porphyrin pigments such as heme. At the center of the chlorin ring is a magnesium ion. This was discovered in 1906, and was the first time that magnesium had been detected in living tissue. For the structures depicted [below], some of the ligands attached to the Mg^{2+} center are omitted for clarity. The chlorin ring can have several different side chains, usually including a long phytol chain. There are a few different forms that occur naturally, but the most widely distributed form in terrestrial plants is chlorophyll *a*."⁴
- "The trapping of light energy is the key to photosynthesis. The first event is the absorption of light by a photoreceptor molecule. The principal photoreceptor in the chloroplasts of most green plants is chlorophyll *a*, a substituted tetrapyrrole. The four nitrogen atoms of the pyrroles are coordinated to a magnesium ion. Unlike a porphyrin such as heme, chlorophyll has a reduced pyrrole ring. Another distinctive feature of chlorophyll is the presence of *phytol*, a highly hydrophobic 20-carbon alcohol, esterified to an acid side chain. Chlorophylls are very effective photoreceptors because they contain networks of alternating single and double bonds. Such compounds are called *polyenes*. They have very strong absorption bands in the visible region of the spectrum, where the solar output reaching Earth also is maximal. The peak molar absorption coefficient of chlorophyll *a* is higher than $10^5 \text{ M}^{-1} \text{ cm}^{-1}$, among the highest observed for organic compounds."⁶



	Chlorophyll a	Chlorophyll b
Molecular formula	$C_{55}H_{72}O_5N_4Mg$	$C_{55}H_{70}O_6N_4Mg$
C2 group	$-CH_3$	$-CH_3$
C3 group	$-CH=CH_2$	$-CH=CH_2$
C7 group	$-CH_3$	$-CHO$
C8 group	$-CH_2CH_3$	$-CH_2CH_3$
C17 group	$-CH_2CH_2COO-$ Phytyl	$-CH_2CH_2COO-$ Phytyl
C17-C18 bond	Single (chlorin)	Single (chlorin)
Occurrence	Universal	Mostly plants



⁶ Berg JM, Tymoczko JL, Stryer L. *Biochemistry*. 5th edition. New York: W H Freeman; 2002. Section 19.2, *Light Absorption by Chlorophyll Induces Electron Transfer*. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK22535/>.

About the Equipment:

Each group has access to:

- a spectrometer and **fiber optic cable (BE GENTLE WITH THE CABLE—no bending, no crushing)** that can interface with the computer via a USB port,
- Logger Pro software for the computer,
- a variety of vials, cuvettes, and solutions, (further detailed below)
- 1 mL eyedropper pipettes,
- a UV light source, and
- small LED light boxes emitting different colors of light (Green and Blue).

Part I: Reproducing the Observed 'Unexpected' Fluorescence of Chlorophyll

Using the chlorophyll solution from Lab 11, reproduce the observed 'unexpected' fluorescence when exposed to the Green LED light source. It may be easiest to accomplish this if the room is dark and/or the sample, source, and detector are shielded from ambient light (try overturning a lightweight cardboard box to cover the set-up). You may also find it necessary to mess with some of the LoggerPro detector data display settings. Ask your TA to show you how to change the sample time from the ~30 ms default to 100ms via the "Intensity" button above the graphical display. Consider the following questions:

- Are you able to detect red fluorescence due to the exposure to the Green LED? How does the emitted light (fluorescence) compare to the absorbed light (light source)?
- Is this a high intensity fluorescence or a low intensity fluorescence? How can you tell?
- What factors affect the intensity of the observed fluorescence?

Part II: Investigating the System to Determine and Explain the 'Unexpected' Fluorescence

Now that you also have observed this 'unexpected' fluorescence, it is your task to figure out what is going on here. Consider the following questions:

- Could something else in the system be responsible for the observed fluorescence? If so, what?
- If nothing else in the system is responsible, is the chlorophyll truly fluorescing due to the green light? What other options are there?

To fully explore these questions, you will need to **design an experiment** from the available equipment (see lists above and on the next page) and **construct an argument to explain the 'unexpected' fluorescence** you have observed. **Be careful** transferring fluids between containers and be sure to replace the chlorophyll solutions in the original glass vial and **seal the vial tightly** when you are finished (***do not** pour out the chlorophyll solution when you are finished*). Do not leave the isopropyl alcohol or chlorophyll solutions in the plastic cuvettes for extended time periods, as the

isopropyl alcohol in both solutions will eat away at the plastic. Do not dilute the chlorophyll solution with additional isopropyl alcohol.

- Empty glass vials
- Empty plastic cuvettes
- Glass vials with the chlorophyll solution (from Lab 11)
- Isopropyl alcohol

For your report:

The **lab write-up** should include your discussion of your reproduction of the 'unexpected' fluorescence of chlorophyll in Part I and your investigation of the system in Part II, along with your determination and explanation of the source of the 'unexpected' fluorescence. Be sure to include any necessary data/graphs to support your arguments.

Approximate timing:

- Introduction 15 min.
- Part I: Reproduce Observation 15 min.
- Part II: Investigate the System 50 min.
- Finalize Report 30 min.