Fluorescence and Its Applications

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Specific aim

Experiments designed in this laboratory are expected to help students learn basic concepts of electromagnetic radiation, absorption spectroscopy, and emission spectroscopy, use the absorption spectroscopy and emission spectroscopy to determine maximum absorbance and emission wavelength of three provided fluorescent molecules and green fluorescent protein, and use two provided fluorescent molecules to detect acidity of juice.

Concepts: Electromagnetic radiation, absorption spectroscopy, emission spectroscopy, fluorescence, green fluorescent protein, and acidity.

Materials provided

5 workstations, including
       SpectroVis Plus spectrophotometer + fluorometer
       Personal computer with molecular graphic software, data analysis software, spectrophotometer software

Cuvettes

Four solutions: 1 mM 7-hydroxycoumarin in phosphate buffer saline (PBS), 1 mM fluorescein in PBS, 1 mM rhodamine B in PBS, and 1 mg/mL green fluorescent protein in PBS

Juice: tomato juice and vinegar (provided by teachers)

Other materials required

Laboratory gloves
Closed eyeglasses
Laboratory coats
Distilled water in squirt bottle
Paper towel
Pippettes
Glue, scissors
Printer

Disposal Instructions
All solutions need to be diluted with 10 volume times of water and then poured into the sink drain.

Rhodamine B
Rhodamine B is a carcinogen. We recommend teachers instead of students to do the demonstration experiments.

Read this before you start

Hazards

The chemicals that are supplied with this experiment (for list, see above) may be harmful if swallowed, inhaled or absorbed through the skin. May cause skin, eye and respiratory tract irritation. In case of skin contact, wash off with soap and plenty of water. In case of eye contact, rinse thoroughly with plenty of water for at least 15 min. Contact a physician if irritation occurs. In case of ingestion, immediately contact a physician or poison control center.

Laboratory practices

The laboratory environment should always be treated as potentially hazardous. Therefore, eating, drinking and smoking are strictly prohibited in the laboratory. Wear closed shoes (no sandals). During experiments, always wear gloves and eye protection (closed goggles). It is a good practice to also wear a laboratory coat. After completing laboratory work, always wash hands with soap and water.

Setup of equipment

Prepare required materials (see list above)

Warm supplied aliquots to room temperature, no more than one hour before the experiment

Connect SpectroVis Plus via the supplied USB cable to computer

Disposal

The chemicals that are supplied with this experiment may be flushed down the drain with water.

Basic Concepts

Electromagnetic spectrum and visible light

The electromagnetic spectrum is the range of all possible electromagnetic radiation. It extends from long-wavelengths used for modern radio communication to gamma radiation at the short-wavelength end. It covers wavelengths from thousands of kilometers down to a fraction of the size of an atom. The visible spectrum is the portion of the electromagnetic spectrum that is visible to the human eyes. Electromagnetic radiation in this range of wavelengths is called visible light. A typical human eye responds to radiation wavelengths from about 390 to 750 nanometer (10^{-9} meter). Colors that can be produced by visible light of a narrow band of wavelengths (monochromatic lights) are called pure spectral colors. The various color ranges indicated in Figure 1 are an approximation. The spectrum is continuous with no clear boundaries between one color and the next.
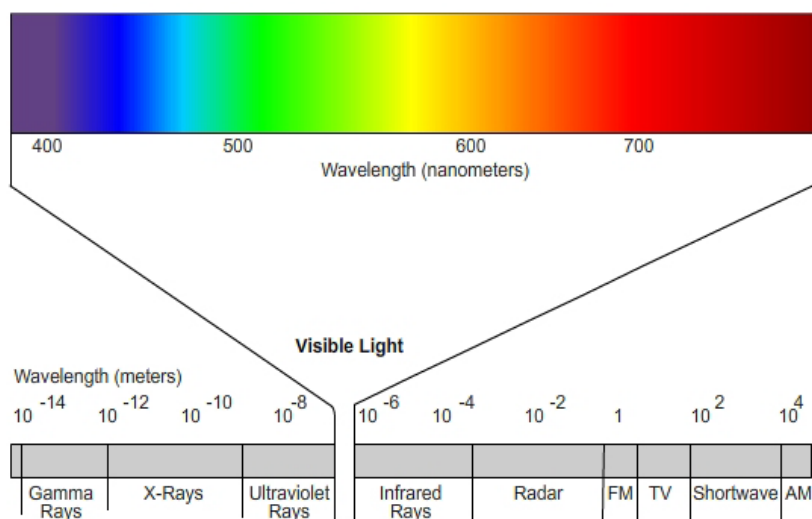

Figure 1: Wavelengths of the electromagnetic spectrum and visible light.

Absorption spectroscopy

Absorption spectroscopy is a technique that measures the absorption of radiation as a function of wavelength, due to its interaction with a sample. The sample absorbs energy from the radiating field. The intensity of the absorption varies as a function of wavelength and this variation is the absorption spectrum. A typical ultraviolet-visible (UV-vis) adsorption spectrum is shown in Figure 2 in which y-axis represents absorption intensity (abs stands for absorbance, a unit that represents absorption intensity) and x-axis represents wavelength of the light. A wavelength where absorption occurs, as well as its relative intensity, is related to a molecule itself. Usually chemicals with different structures have distinctive absorption spectra. By measuring absorption spectrum of a molecule, one can figure out at which wavelength the molecule shows maximum absorbance and the identity of the molecule. For a fluorescent molecule, its maximum absorbance wavelength is also a preferred wavelength for the light to excite the molecule for fluorescent emission because it can accept light energy more efficient at this wavelength than others.
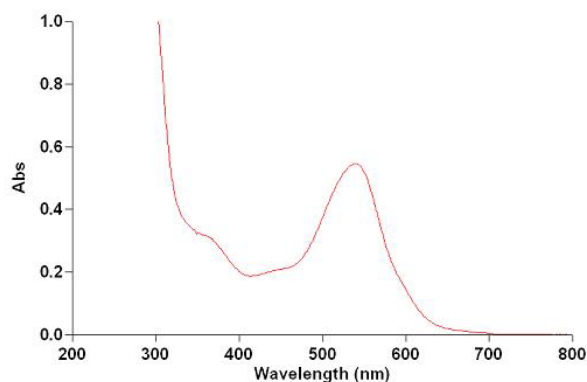
Figure 2: A typical UV-vis absorption spectrum.

Emission spectroscopy and fluorescence

Emission spectroscopy is a technique that examines the wavelengths and intensities of light emitted by molecules after they are excited by certain means such as photo exciting. Since a wavelength of light emitted by a molecule as well as its intensity is also related to a molecule itself, molecules with different chemical structures also have distinctive emission spectra. Fluorescence is a typical light emission. It occurs often from aromatic molecules. Some typical fluorescent substances are shown in Figure 3. One widely encountered fluorescent molecule is quinine, which is present in tonic water. If one observes a glass of tonic water that is exposed to sunlight, a faint blue glow is usually visible at the surface. The quinine in tonic water is excited by the UV light from the sun and the excited quinine emits blue light with a typical wavelength near 450 nm. Similarly to quinine, other fluorophores shown in Figure 3 can be excited and glow by emitting different colors of light.
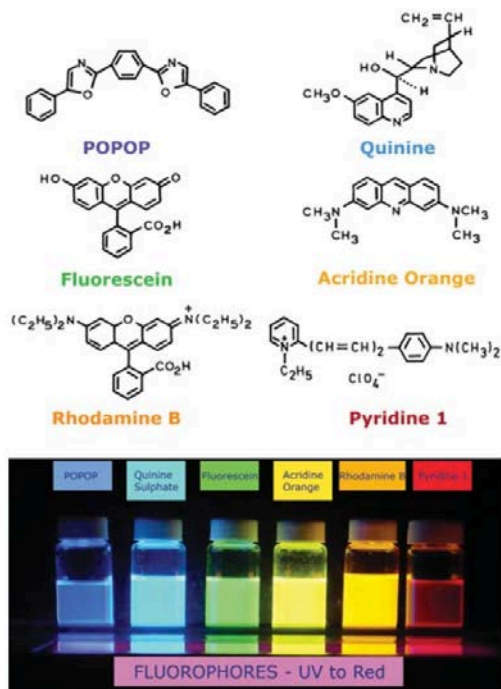

Figure 3: Structures of typical fluorescent molecules.

A typical fluorescent emission spectrum is shown in Figure 4 in which y-axis represents intensity and x-axis represents wavelength of emitted light. From the spectrum, one can determine the wavelength of maximum emission of a fluorophore and figure out the color the emitted light.
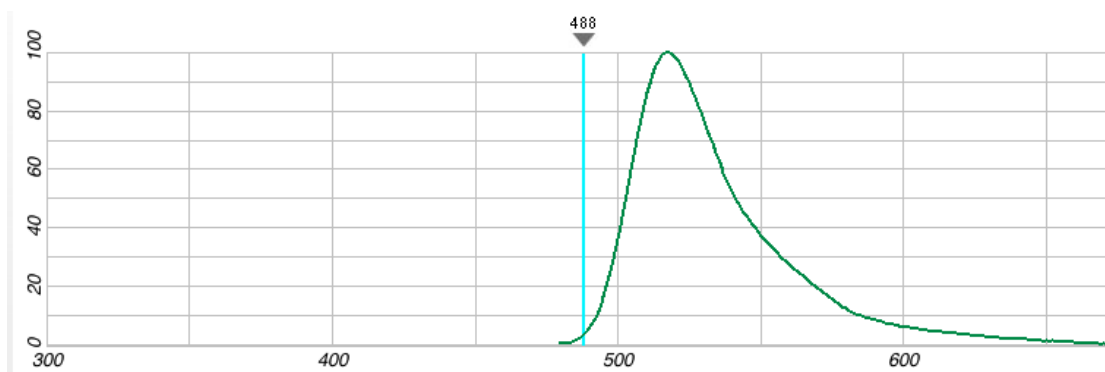

Figure 4: A fluorescent spectrum.

Activities

Part A

In the following experiments, we are going to use SpectroVis Plus spectrophotometer + fluorometer to measure absorption spectra of three small fluorescent molecules and green fluorescent protein and use absorption spectra to figure out maximum absorbance wavelengths of four fluorescent molecules. These maximum absorbance wavelengths are also their optimal excitation wavelengths for fluorescence emission.

The three fluorescent small molecules are 7-hydroxycoumarin, fluorescein, and rhodamine B. Their structures are shown in Figure 5. Green fluorescent protein shown in Figure 6 is a protein composed of 238 amino acid residues that exhibits bright green fluorescence when exposed to light. The discovery of development of green fluorescent protein led to the awarding of Nobel Prize in Chemistry to three scientists in 2008.
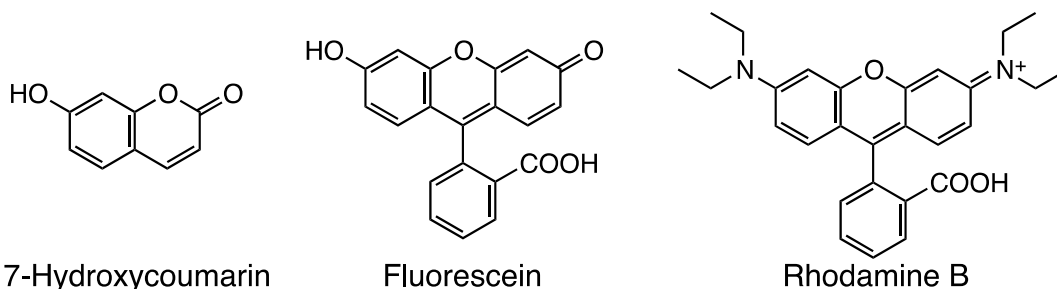

7-Hydroxycoumarin   Fluorescein   Rhodamine B
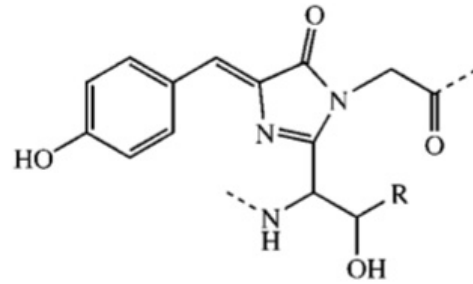
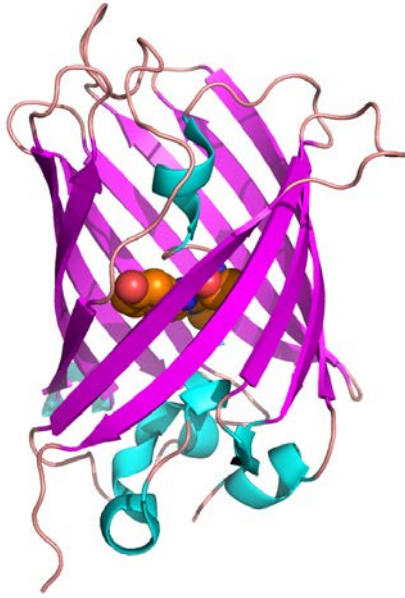Figure 5: Three fluorescent molecules.

Figure 6: Green fluorescent protein. Chromophore is shown on the right and also as spherical elements in the middle of the protein.

Please follow the procedures below to setup the SpectroVis Plus spectrophotometer + fluorometer for measuring UV-vis spectroscopy of three small fluorescent molecules and green fluorescent protein.

1) Connect SpectroVis Plus via the supplied USB cable to a computer that has been installed with Logger Plus 3 software
2) Start Logger Plus 3
3) Choose Change Units -> Spectrometer:1 -> absorbance from the experiment menu.
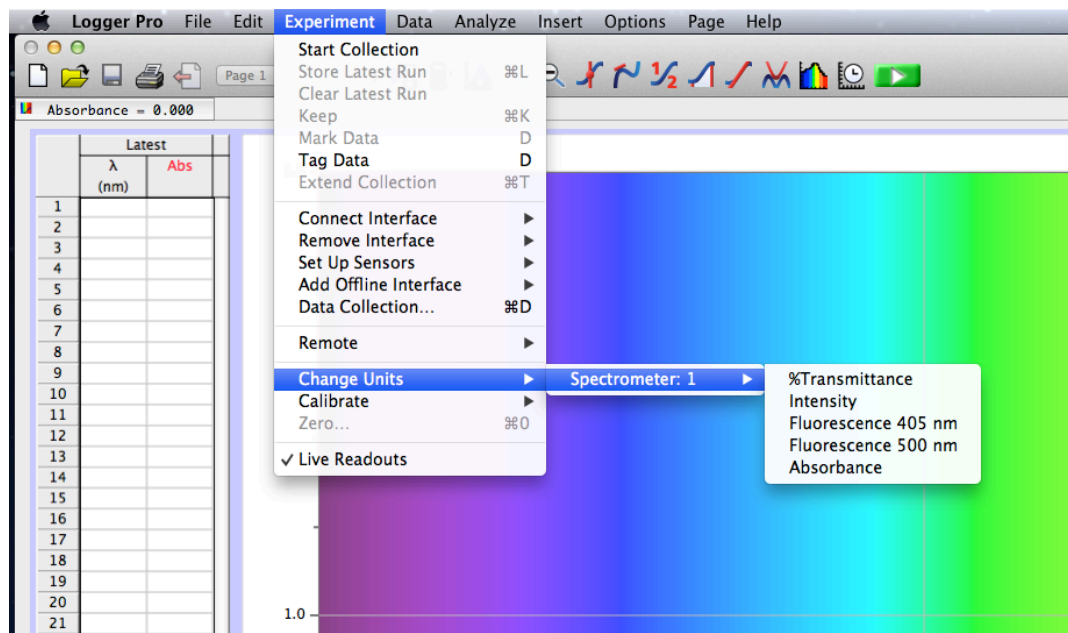
4) Choose Calibrate -> Spectrometer:1 from the experiment menu. Wait for about 90s for the lamp to warm up.
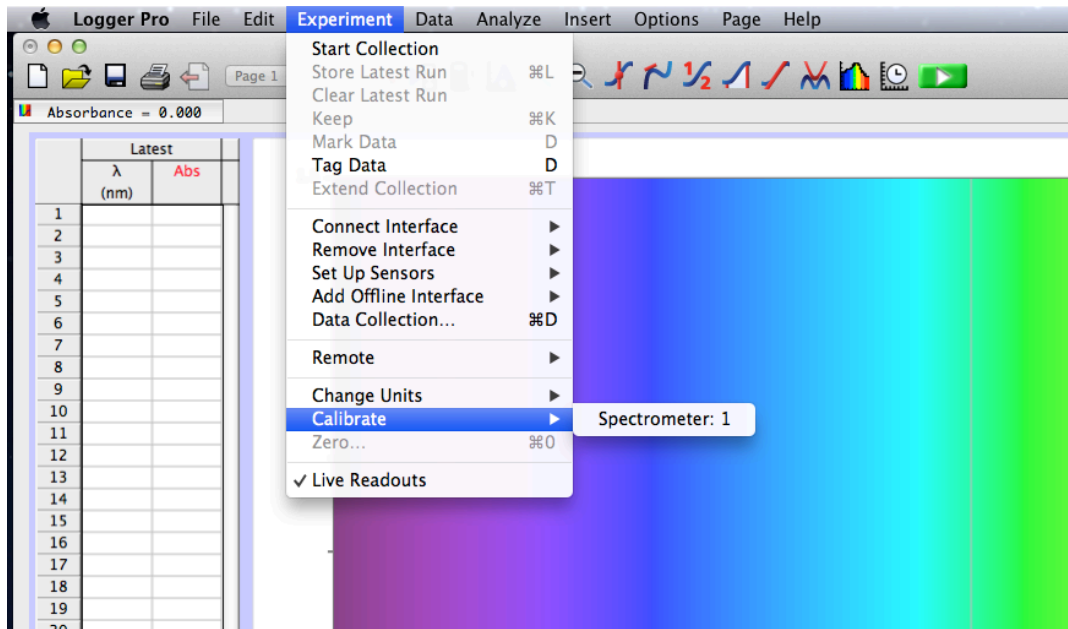
5) After the lamp has been warmed up, fill a cuvette about ¾ full with distilled water and place it in the cuvette holder. Press the "Finish Calibration" key in in the "Calibrate Spectrometer" dialog box. When the scan finishes, press the "OK" button in the "Calibrate Spectrometer" dialog box.
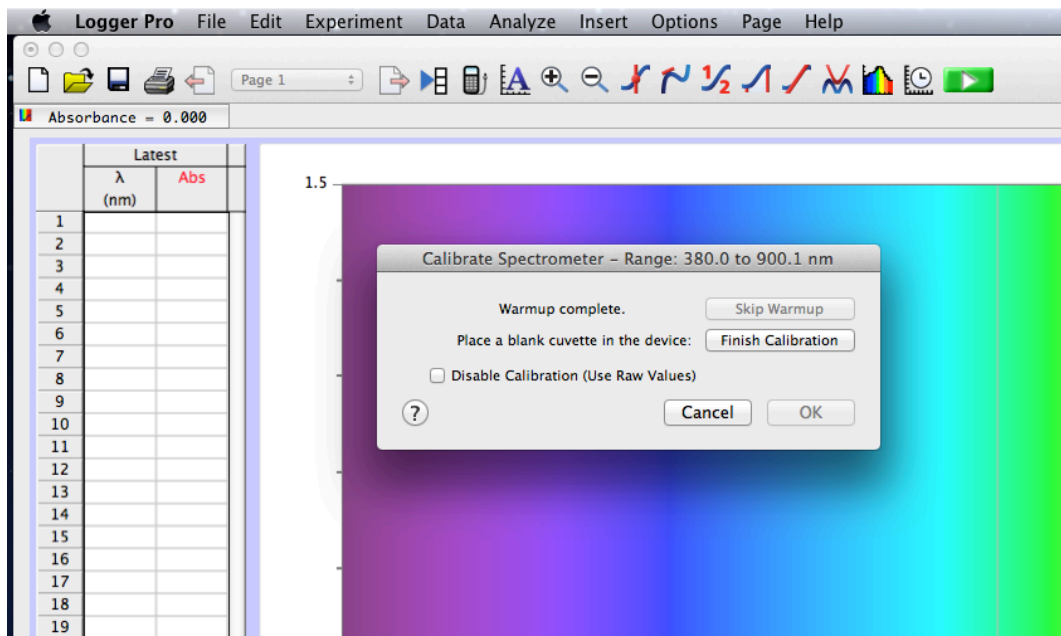

6) Choose Data Collection from the Experiment menu and then choose "Full Spectrum" in the "Data Collection" dialog box.
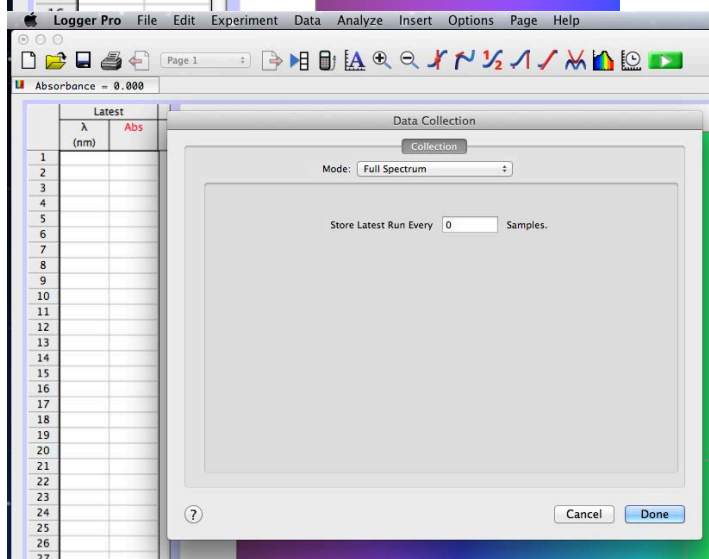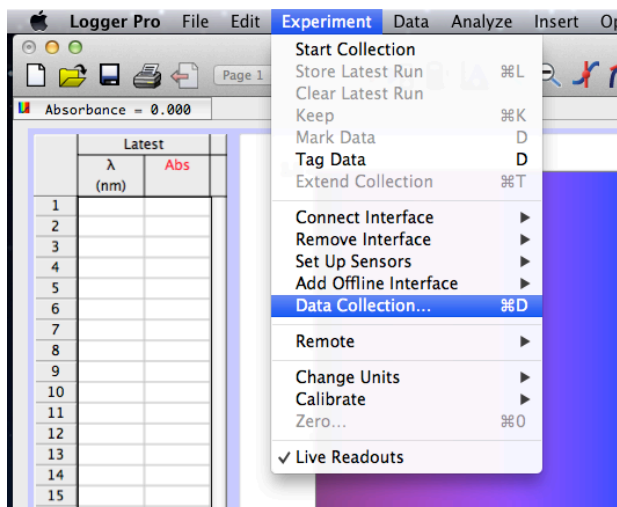
7) Fill a cuvette with 3 mL of distilled water and then add 30 μL of the provided 7-hydroxycoumarin solution. Put the cuvette in the cuvette holder.
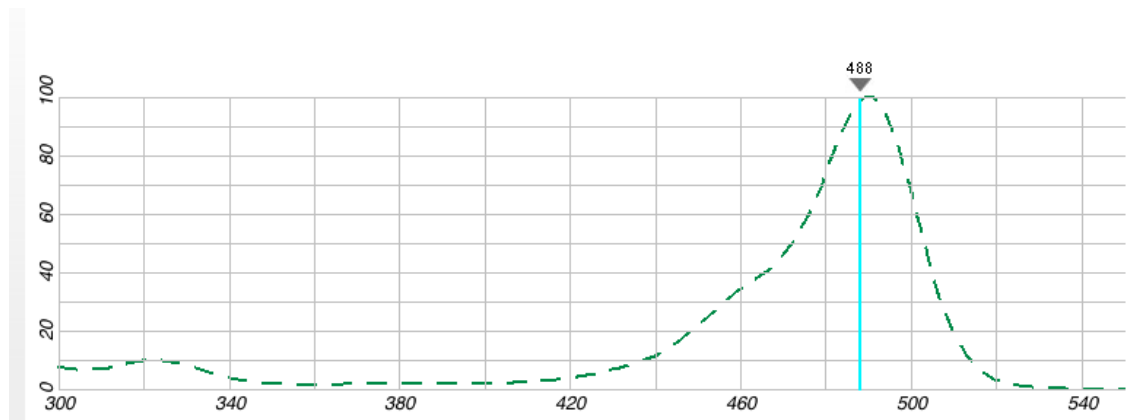
8) Then start "Start Collection" from the Experiment menu.
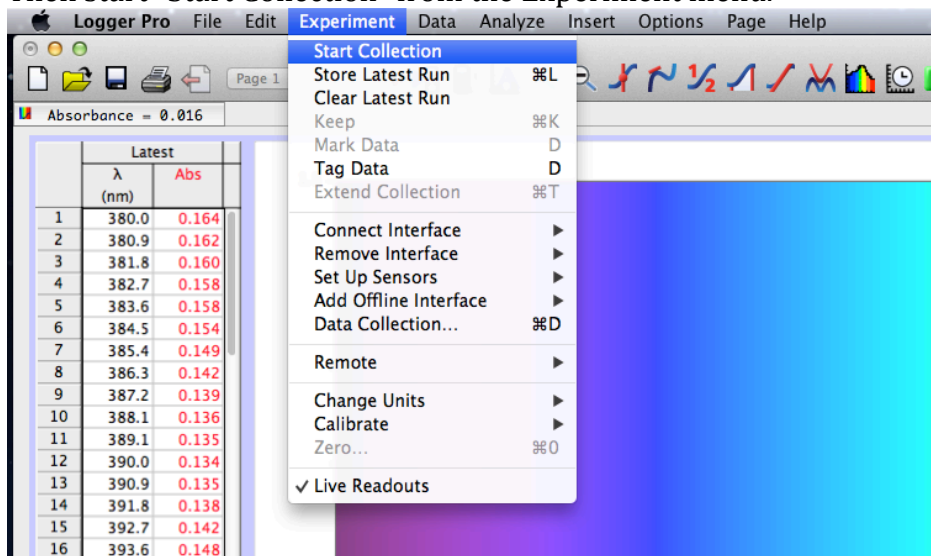
9) After a stable spectrum shows up, press "Stop Collection" from the Experiment menu.
10) Choose "Print Graph" from the "File" menu to print the spectrum
11) Paste the spectrum below and compare the spectrum with the one provided by Invitrogen Inc. Since SpectroVis does not provide absorbance below 390 nm, you will not see the maximum absorbance wavelength of 7-hydroxycoumarin. But you are expected to see a small absorbance peak around 400 nm.
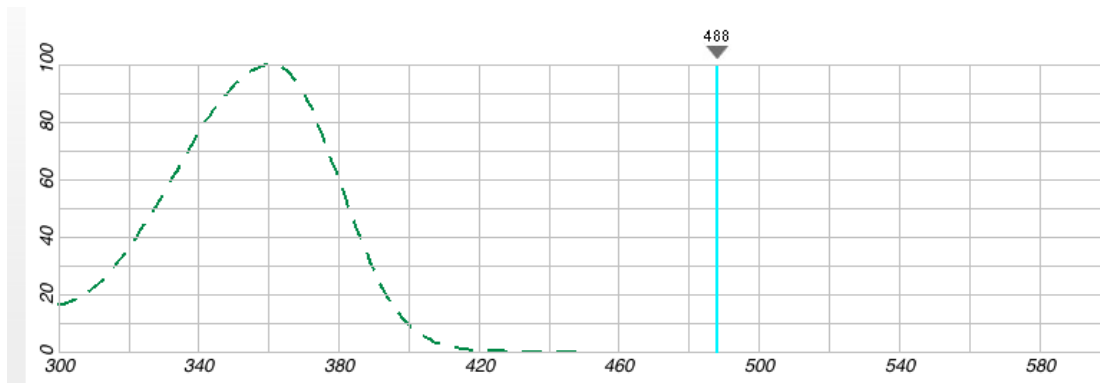
12) Dispose the solution in the cuvette and wash it three times with distilled water.
13) Add 3 mL of distilled water into the cuvette and then transfer 30 µL of the provided fluorescein solution to the cuvette.
14) Place the cuvette into the cuvette holder.
15) Start "Start Collection" from the Experiment menu.
16) After a stable spectrum shows up, press "Stop Collection" from the

Experiment menu.
17) Choose "Print Graph" from the "File" menu to print the spectrum
18) Paste the spectrum below and compare the spectrum with the one provided by Invitrogen Inc.

19) Base on the spectrum, please determine the maximum absorbance wavelength of fluorescein.
_____ nm.
This wavelength is also the optimal wavelength used for the excitation of fluorescein for fluorescent emission.
20) Dispose the solution in the cuvette and wash it three times with distilled water.
21) Add 3 mL of distilled water into the cuvette and then transfer 30 µL of the provided rhodamine B solution to the cuvette.
22) Place the cuvette into the cuvette holder.
23) Start "Start Collection" from the Experiment menu.
24) After a stable spectrum shows up, press "Stop Collection" from the

Experiment menu.

25) Choose "Print Graph" from the "File" menu to print the spectrum

26) Paste the spectrum below and compare the spectrum with the one provided by Invitrogen Inc.

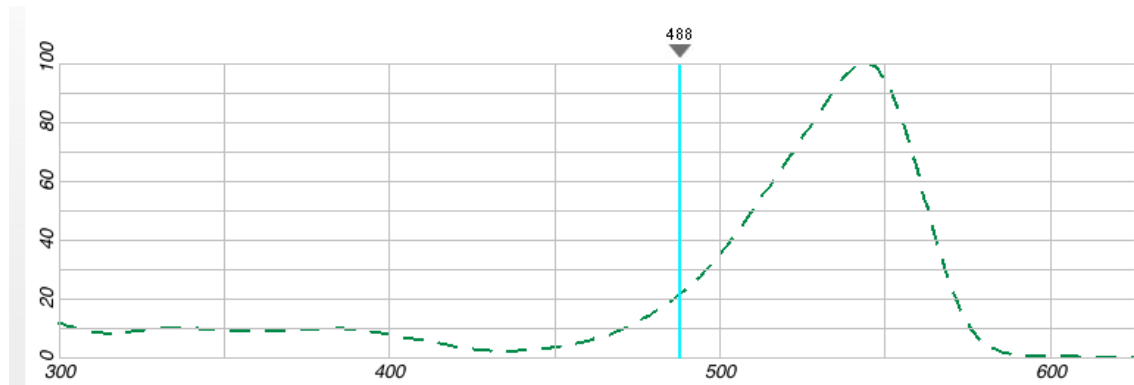27) Base on the spectrum, please determine the maximum absorbance wavelength of rhodamine B.

_____ nm.

This wavelength is also the optimal wavelength used for the excitation of rhodamine B for fluorescent emission.

28) Dispose the solution in the cuvette and wash it three times with distilled water.

29) Add 3 mL of distilled water into the cuvette and then transfer 30 μL of the provided green fluorescent protein solution to the cuvette.

30) Place the cuvette into the cuvette holder.

31) Start "Start Collection" from the Experiment menu.

32) After a stable spectrum shows up, press "Stop Collection" from the Experiment menu.
33) Choose "Print Graph" from the "File" menu to print the spectrum
34) Paste the spectrum below.

35) Base on the spectrum, please determine the maximum absorbance wavelength of green fluorescent protein.
_____ nm.
This wavelength is also the optimal wavelength used for the excitation of rhodamine B for fluorescent emission.
36) Dispose the solution in the cuvette and wash it three times with distilled water.

Part B

Now, we know the optimal wavelength of light to excite three small molecules and green fluorescent protein for their fluorescent emission tests. In this part, we are going to use the obtained information in part A to setup experiments to measure fluorescent emission of three small fluorescent molecules and green fluorescent protein.

Based on the information in part A, we know the optimal excitation lights for 7-hydroxycoumarin, fluorescein, rhodamine B, and green fluorescent protein should be 360 nm, 490 nm, 480nm, and 550 nm, respectively. However, our SpectroVis Plus only provides two possible excitation wavelengths, 405 and 500 nm. So we are going to choose a wavelength that is more close to the optimal wavelength of a specific fluorescent molecule when we do the fluorescent emission test. That means we are going to use 405 nm as the excitation wavelength for 7-hydrocoumarin and

500 nm as the excitation wavelength for fluorescein, rhodamine B, and green fluorescent protein.

Now we are going to setup our SpectroVis for fluorescent emission tests of three small molecules and green fluorescent protein.

1) Choose Change Units -> Spectrometer:1 -> Fluorescence 405 nm from the Experiment menu.
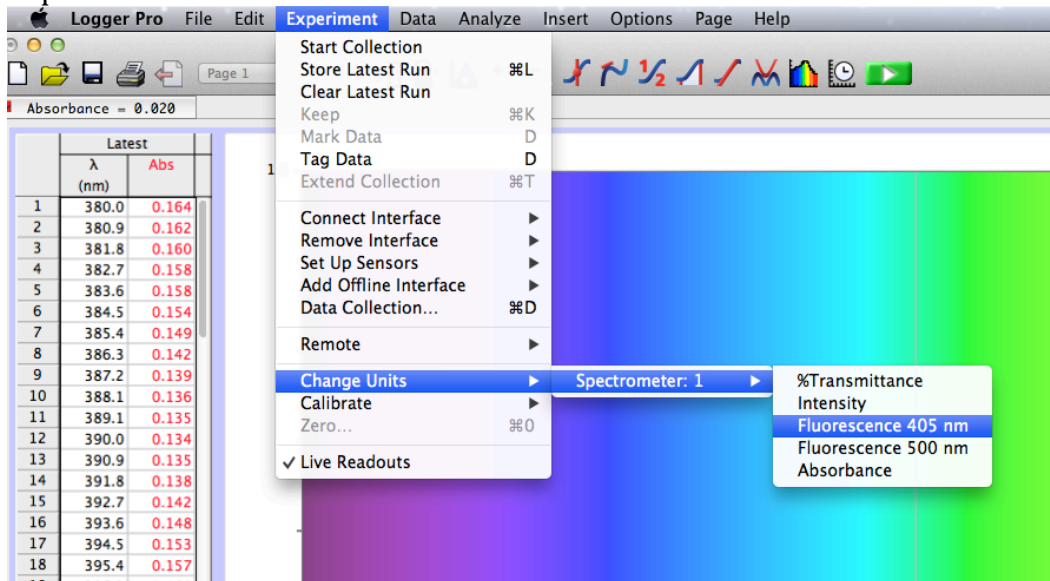
2) Choose "Clear Latest Run" from the Experiment menu.
3) Add 3 mL of distilled water into a clean cuvette and then transfer 30 μL of the provided 7-hydroxycoumarin solution into the cuvette.
4) Put the cuvette into the cuvette holder of SpectroVis.
5) Start "Start Collection" from the Experiment menu.
6) When a stable emission spectrum shows up, press "Stop Collection" from the Experiment menu.
7) Print out the spectrum and paste it low.

8) Based on the spectrum, please determine the maximum emission wavelength.

_____ nm. Then check with the visible light spectrum and tell why the 7-hydroxycoumarin-emitted light has a blue color.

9) Dispose the solution in the cuvette and wash it three times with distilled water.
10) Choose Change Units -> Spectrometer:1 -> Fluorescence 500 nm from the Experiment menu.
11) Add 3 mL of distilled water into a clean cuvette and then transfer 2 μL of the provided fluorescein solution into the cuvette.
12) Put the cuvette into the cuvette holder of SpectroVis.
13) Start "Start Collection" from the Experiment menu.
14) When a stable emission spectrum shows up, press "Stop Collection" from the Experiment menu.
15) Print out the spectrum and paste it low.

16) Based on the spectrum, please determine the maximum emission wavelength.

_____ nm. Then check with the visible light spectrum and tell why the fluorescein-emitted light has a green color.

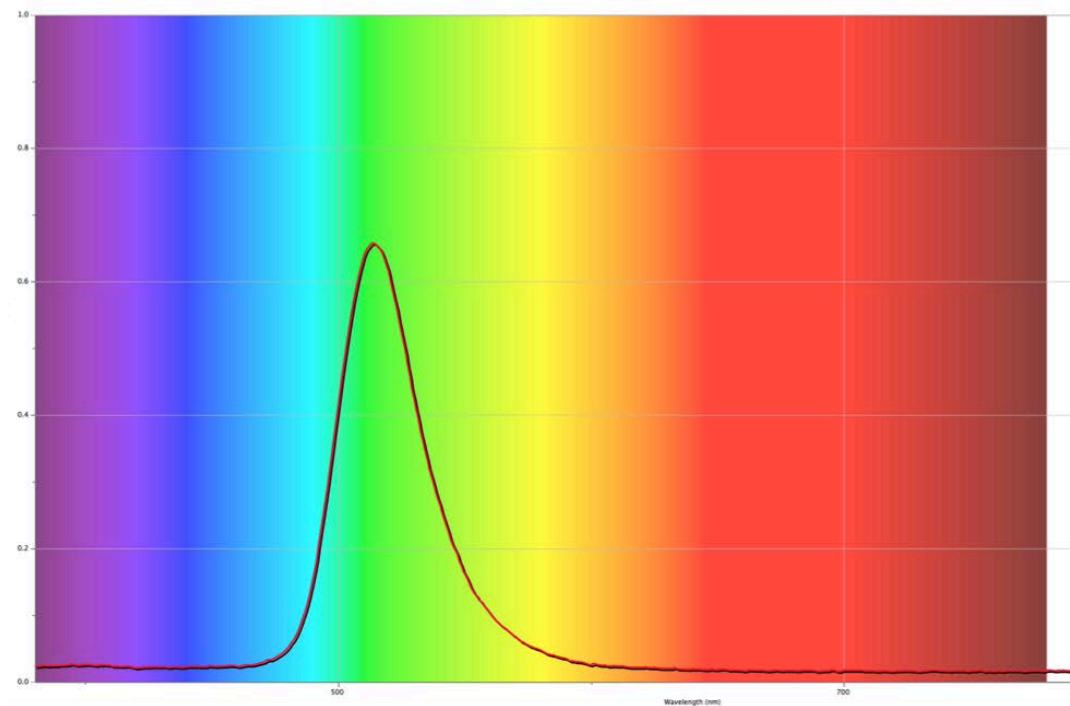17) Dispose the solution in the cuvette and wash it three times with distilled water.
18) Add 3 mL of distilled water into a clean cuvette and then transfer 10 μL of the provided rhodamine B solution into the cuvette.

19) Put the cuvette into the cuvette holder of SpectroVis.
20) Start "Start Collection" from the Experiment menu.
21) When a stable emission spectrum shows up, press "Stop Collection" from the Experiment menu.
22) Print out the spectrum and paste it low.

23) Based on the spectrum, please determine the maximum emission wavelength.

_____ nm. Then check with the visible light spectrum and tell why the rhodamine B-emitted light has a red color.

24) Dispose the solution in the cuvette and wash it three times with distilled water.
25) Add 3 mL of distilled water into a clean cuvette and then transfer 2 μL of the provided green fluorescent protein solution into the cuvette.
26) Put the cuvette into the cuvette holder of SpectroVis.
27) Start "Start Collection" from the Experiment menu.
28) When a stable emission spectrum shows up, press "Stop Collection" from the Experiment menu.
29) Print out the spectrum and paste it low.

A standard green fluorescent protein emission spectrum is shown below.

30) Based on the spectrum, please determine the maximum emission wavelength.

_____ nm. Then check with the visible light spectrum and tell why the green fluorescent protein-emitted light has a green color.

31) Dispose the solution in the cuvette and wash it three times with distilled water.

Part C

Now we have used both absorption spectroscopy and emission spectroscopy to study three small fluorescent molecules and green fluorescent protein. In the part, we are going to use one interesting feature of 7-hydroxycoumarin and fluorescein to test the acidity of juice and vinegar. Both 7-hydroxycoumarin and fluorescein exist in two forms, one neutral form and one anion form. The anion form for both molecules is more fluorescent than the neutral form. As shown in Figure 7, the exchange between the two forms is driven by the hydrogen cation

(or proton) concentration in water. The hydrogen cation concentration of a solution is related to its acidity. When the solution is acidic, its hydrogen cation concentration is high so that both 7-hydroxycoumarin and fluorescein will stay as a neutral form and emit low fluorescence.

More fluorescent
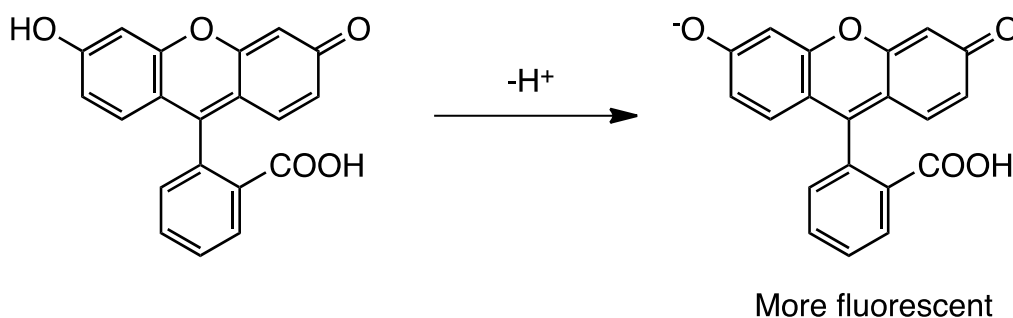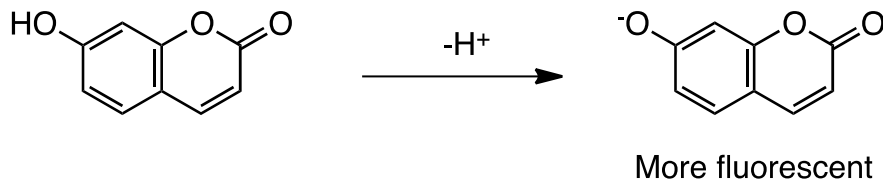
More fluorescent

In part B, we tested fluorescent emissions of 7-hydroxycoumarin and fluorescein in PBS, a solution which is weakly basic instead of acidic. Now, we are going to see whether adding an acidic solution to these two fluorescent molecules will drive their fluorescence decrease and test the acidity of tomato juice.

We will start with 7-hydroxycoumarin.

1) Setup SpectroVis as discussed in part B for the fluorescent emission test of 7-hydroxycoumarin (choose fluorescence 405 nm).
2) Transfer 3 mL of distilled water into a clean cuvette and then add 30 μL of the provided 7-hydrocoumarin solution.
3) Put the cuvette into the cuvette holder.
4) Start "Start Collection" from the Experiment menu.
5) When a stable spectrum shows up, press "Stop Collection" from the Experiment menu.
6) Print out the emission spectrum and paste it below.

7) Take the cuvette out. Add 10 μL of commercial vinegar into the cuvette and mix it well with the rest solution through pipetting the solution up and down several times.
8) Put the cuvette into the cuvette holder and then start "Start Collection" from the Experiment menu.
9) When a stable spectrum shows up, press "Stop Collection" form the Experiment menu.
10) Print out the spectrum below and compare it to the spectrum obtained before adding vinegar.

The intensity of the fluorescence emission after adding vinegar is low than that before adding vinegar.

11) Dispose the solution in the cuvette and wash it three times with distilled water.
12) Transfer 3 mL of distilled water into a clean cuvette and then add 30 μL of the provided 7-hydrocoumarin solution.
13) Put the cuvette into the cuvette holder.
14) Start "Start Collection" from the Experiment menu.
15) When a stable spectrum shows up, press "Stop Collection" from the Experiment menu.
16) Print out the emission spectrum and paste it below.

17) Take the cuvette out. Add 10 μL of tomato juice into the cuvette and mix it well with the rest solution through pipetting the solution up and down several times.
18) Put the cuvette into the cuvette holder and then start "Start Collection" from the Experiment menu.
19) When a stable spectrum shows up, press "Stop Collection" form the Experiment menu.
20) Print out the spectrum below and compare it to the spectrum obtained before adding vinegar.

Please determine whether tomato juice is acidic.

Now we change to use fluorescein.

1) Setup SpectroVis as discussed in part B for the fluorescent emission test of fluorescein (choose fluorescence 500 nm).
2) Transfer 3 mL of distilled water into a clean cuvette and then add 2 μL of the provided fluorescein solution.
3) Put the cuvette into the cuvette holder.
4) Start "Start Collection" from the Experiment menu.
5) When a stable spectrum shows up, press "Stop Collection" from the Experiment menu.

6) Print out the emission spectrum and paste it below.

7) Take the cuvette out. Add 10 μL of commercial vinegar into the cuvette and mix it well with the rest solution through pipetting the solution up and down several times.
8) Put the cuvette into the cuvette holder and then start "Start Collection" from the Experiment menu.
9) When a stable spectrum shows up, press "Stop Collection" form the Experiment menu.
10) Print out the spectrum below and compare it to the spectrum obtained before adding vinegar.

The intensity of the fluorescence emission after adding vinegar is low than that before adding vinegar.

11) Dispose the solution in the cuvette and wash it three times with distilled water.

12) Transfer 3 mL of distilled water into a clean cuvette and then add 2 μL of the provided fluorescein solution.
13) Put the cuvette into the cuvette holder.
14) Start "Start Collection" from the Experiment menu.
15) When a stable spectrum shows up, press "Stop Collection" from the Experiment menu.
16) Print out the emission spectrum and paste it below.

17) Take the cuvette out. Add 10 μL of tomato juice into the cuvette and mix it well with the rest solution through pipetting the solution up and down several times.
18) Put the cuvette into the cuvette holder and then start "Start Collection" from the Experiment menu.
19) When a stable spectrum shows up, press "Stop Collection" form the Experiment menu.
20) Print out the spectrum below and compare it to the spectrum obtained before adding vinegar.

Please determine whether tomato juice is acidic.