



EDUCATION  
EXPERIMENT



[www.oceanoptics.com](http://www.oceanoptics.com)



[info@oceanoptics.com](mailto:info@oceanoptics.com)



**US** +1 727-733-2447

**EUROPE** +31 26-3190500

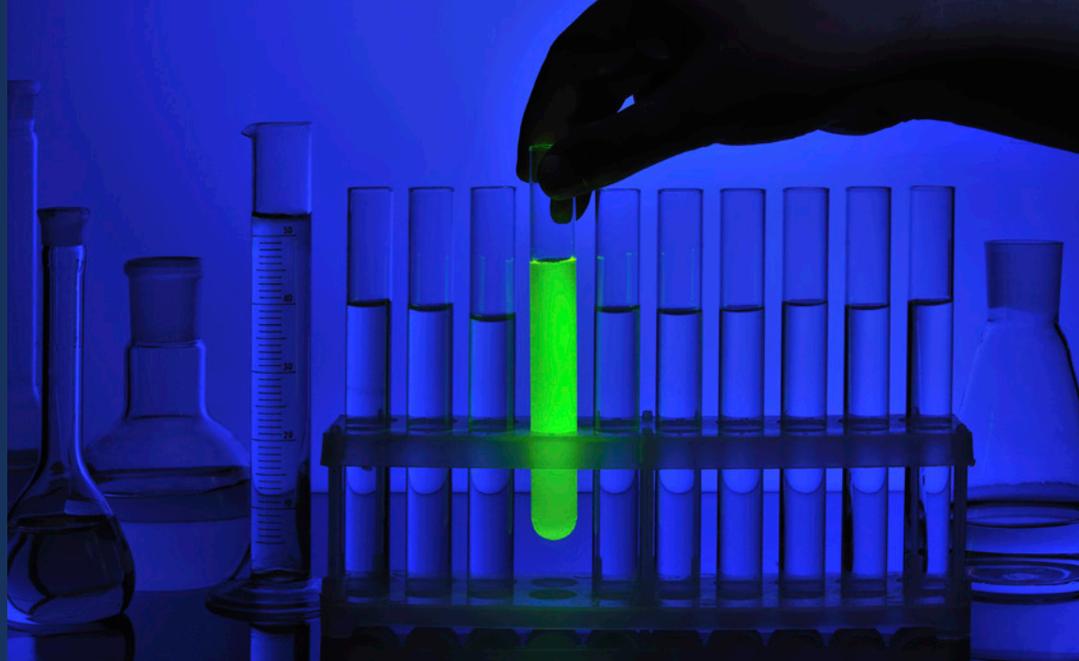
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| SPECTROMETERS |

| ACCESSORIES |

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| COMPLETE SOLUTIONS |



## Fluorescence of an Unknown Fluorophore

### Introduction

Fluorescent tags have become a powerful tool in research and diagnostics, capable of revealing cellular mechanics in real time, identifying abnormal cells, and sounding the alarm on the presence of pathogens. Still, they are only as powerful as the system used for detection, a key part of which is intelligent selection of excitation wavelength(s). Many fluorescence experiments are performed with a commercial fluorophore like TRITC, Fura-2, or one of the many variants of green fluorescent protein (GFP), in which case determining the best excitation wavelength (or band of wavelengths) is simply a matter of looking up the excitation spectrum. Occasionally, however, it may be necessary to determine the best excitation wavelength or band for an unknown fluorophore through experiment.

There are several approaches that can be taken when choosing an excitation light source for a fluorophore. If using an LED, it is best to choose one with a center wavelength close to the peak excitation spectrum wavelength. If using a laser, the excitation intensity will be so much higher that it is possible to even use a wavelength on the tail of the excitation curve. If using a broadband light source for excitation, it can be filtered using a single bandpass filter, taking care to ensure that the excitation light minimizes overlap with the emission spectrum.

Although the excitation spectrum for a given fluorophore is not exactly the same as its absorbance, the absorbance spectrum of a fluorophore can be used as a quick indicator of which wavelengths are likely to be good for excitation. When comparing an absorbance and excitation spectrum, most of the peaks will stay the same, though their relative heights may differ.

Every Ocean Optics spectrometer has a unique instrument response function (IRF) that characterizes how the spectrometer responds to light across its wavelength range. The IRF arises from multiple wavelength-dependent effects including the attenuation of light in the fiber optic cable, absorbance of light by the mirrors in the spectrometer, the efficiency of the grating, and the responsiveness of the detector. Processed measurement modes like Absorbance, Transmission, Reflectance and Irradiance compensate for the IRF, thus providing accurate spectral shapes.

There are multiple measurement modes available in OceanView for measuring fluorescence. The Fluorescence wizard uses QuickView mode, displaying the raw number of counts for each wavelength without any processing or correction for the IRF. When this mode is used for fluorescence measurements, it is important to remember that the IRF can impact the spectral data, showing a peak that's generally in the correct location, but with a distorted shape and/or center wavelength.

In Relative Irradiance mode, the instrument response function is corrected by calibrating against a blackbody light source of known color temperature like a tungsten halogen lamp. Relative Irradiance measurements generate a spectrum of relative intensity as a function of wavelength, corrected for the instrument response function. The spectrum is scaled from 0 to 1 in arbitrary units.

While QuickView mode is an acceptable option for measuring fluorescence, it is very important to use Relative Irradiance mode when comparing measurements taken by different spectrometers, when determining the spectral shape, or when looking for peak location and shifts.

In this experiment, you will use Ocean Optics modular spectroscopy components to configure an absorbance system to measure the absorbance of an unknown fluorophore. You will then use the absorbance spectrum to select the best excitation LED for your fluorophore

and reconfigure your modular components to measure fluorescence from the sample. Fluorescence will be measured using two different measurement modes to show the impact of the instrument response function on the shape of the fluorescence spectrum.

## Materials

- QE Pro-ABS spectrometer with interchangeable slits
- INTSMA-200 interchangeable slit
- HL-2000 tungsten halogen light source
- LLS-365 LED light source
- LLS-470 LED light source
- Qty. of 2 QP600-2-VIS-NIR 600  $\mu$ m optical fibers
- CUV-ALL-UV cuvette holder
- Qty. of 2 74-MSP mirrored screw plugs
- Transfer/Beral pipettes
- Cuvettes
- Computer with OceanView software installed
- Unknown fluorophore and solvent used to suspend the fluorophore [e.g., tonic water, chlorophyll extract (see **Instructor Notes**), fluorescein, rhodamine]

## Safety

Wear safety goggles and other appropriate safety gear during the experiment. Take all proper safety precautions when handling solutions and wear proper eye protection when working with UV light sources.

## Procedure

### Determining the Excitation Wavelength for an Unknown Fluorophore

1. Connect the power cord to the HL-2000 light source. Turn the lamp on and allow the spectrometer to warm up at least 15 minutes.
2. Connect the spectrometer to your computer using the USB cable and allow the spectrometer to warm up for at least 15 minutes.
3. Use the fiber to connect the spectrometer to one of the collimating lenses on the cuvette holder.

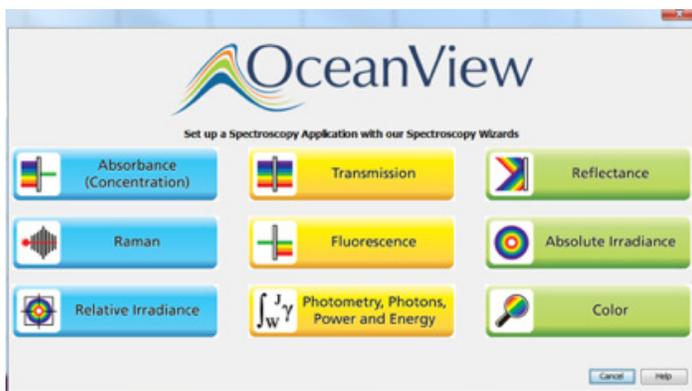
4. Use the other fiber to connect the light source to a collimating lens opposite the lens to which the spectrometer is connected – this is an Absorbance/Transmission measurement setup.

5. Start the OceanView software. Click the **Start** button, then select **All Programs | Ocean Optics | OceanView | OceanView** or use the **Desktop shortcut**  created when you installed the software.

6. Select the **Spectroscopy Application Wizards** option on the Welcome Screen.



7. Select the **Absorbance (Concentration)** option in the **Spectroscopy Wizards** window to start the Absorbance Wizard.



8. Select the **Absorbance only** option.

9. Use a transfer pipette to fill a cuvette  $\sim\frac{2}{3}$  full with solvent for your reference measurement. Wipe the cuvette with a KimWipe to remove any fingerprints, place it in the cuvette holder and set your acquisition parameters in the **Set Acquisition Parameters** window.

a. Click the Automatic button  to automatically adjust the Integration Time to the

optimum value.

b. Set **Scans to Average** to 10 and **Boxcar Width** to 5 to reduce measurement noise and improve the measurement. You can set **Scans to Average** to a higher value but this will slow down your acquisitions (total measurement time equals the number of averages multiplied by the integration time).

c. Place a checkmark in the **Nonlinearity Correction** box if this feature is available for your spectrometer.

d. Click **Next**.

10. Click the **Store Reference**  button to store a reference spectrum. Click **Next**.

11. Close the shutter on the light source or block the light from entering the spectrometer for your background measurement. Do not turn your light source off. Click the **Store Background**  button to store a background spectrum.

12. Open the shutter or unblock the light source and click **Finish**. You are now ready to measure the absorbance spectrum for your unknown fluorophore.

13. The Absorbance spectrum will be displayed in the **Absorbance View** window. You can adjust the graph appearance using the graph tools  above the graph. For example, click the **Scale Graph Height to Fill Window** button  to zoom in on the spectrum.

14. Pour the solvent out of the cuvette and use a transfer pipette to fill the cuvette  $\sim\frac{2}{3}$  full with the unknown fluorophore. Wipe the cuvette with a KimWipe to remove any fingerprints. Place it in the cuvette holder in the same orientation you used for the reference measurement. The absorbance spectrum for the fluorophore is displayed.

15. Record the wavelength where the maximum absorbance occurs in the table in the **Data and Analysis** section.

16. Save your absorbance spectrum by clicking the **Configure Graph Saving** button  to configure the

## File Writer.

- Select the directory where you want to save the file.
- Enter a filename for your spectrum.
- Click **Apply** and then **Exit** to close the dialog box.

17. Click the **Save Graph to Files** button  to save your absorbance spectrum.

18. Print a copy of the graph showing the unknown fluorophore absorbance spectrum by clicking the **Print** button . Close the OceanView software.

## Measuring Fluorescence for an Unknown Fluorophore

1. Reconfigure the spectrometer system for fluorescence measurements.

- Change the slit on the spectrometer to a 200  $\mu\text{m}$  slit.
- Disconnect the fiber connecting the light source to the cuvette holder and reconnect it to a collimating lens that is adjacent to the collimating lens connected to the spectrometer.
- Place the 74-MSP mirrored screw plugs into the other two ports of the cuvette holder. The mirrored screw plugs should be adjacent (next to one another) in the cuvette holder.
- Select the best LED for fluorescence excitation based on your absorbance measurement. Record the LED wavelength you selected for fluorescence excitation in the table in the **Data and Analysis** section.

## Measuring Fluorescence with the QuickView Fluorescence Wizard

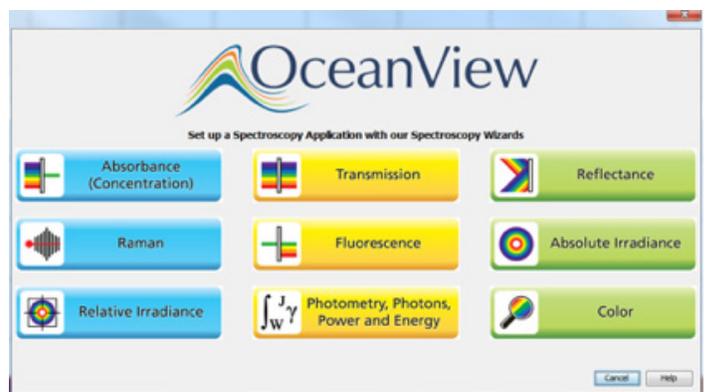
1. Start OceanView. Click the Start button, then select **All Programs | Ocean Optics | OceanView | OceanView** or use the **Desktop shortcut**  created when you

installed the software.

2. Select the **Spectroscopy Application Wizards** option on the Welcome Screen.



3. Select the **Fluorescence** option in the Spectroscopy Wizards window to start the QuickView Fluorescence wizard.



4. Select the **Active Acquisition (Recommended)** option.

5. Wipe the cuvette with a KimWipe to remove any fingerprints, place the cuvette containing your unknown fluorophore in the cuvette holder and set your acquisition parameters in the **Set Acquisition Parameters** window.

a. Click the **Automatic** button  Integration Time to automatically adjust the Integration Time to the optimum value.

b. Set **Scans to Average** to 10 and **Boxcar Width** to 5 to reduce measurement noise and improve the measurement. Set **Scans to Average** to a higher or lower value depending on your integration time. Use fewer averages with longer integration times to shorten the total measurement time, if necessary (total measurement time equals the

number of averages multiplied by the integration time).

c. Place a checkmark in the **Nonlinearity Correction** box if this feature is available for your spectrometer.

d. Click **Next**.

6. Block the light from entering the spectrometer. Do not turn your light source off. Click the **Store Background**  button to store a background spectrum.

7. Unblock the light source and click **Finish**. You are now ready to measure the fluorescence spectrum for your unknown fluorophore in QuickView Fluorescence mode.

8. The Fluorescence spectrum will be displayed in the **View Minus Bkgnd** window. You can adjust the graph appearance using the graph tools  above the graph. For example, click the **Scale Graph Height to Fill Window** button  to zoom in on the spectrum.

9. Save your QuickView Fluorescence spectrum by clicking the **Configure Graph Saving** button  to configure the **File Writer**.

- Select the directory where you want to save the file.
- Enter a filename for your spectrum.
- Click **Apply** and then **Exit** to close the dialog box.

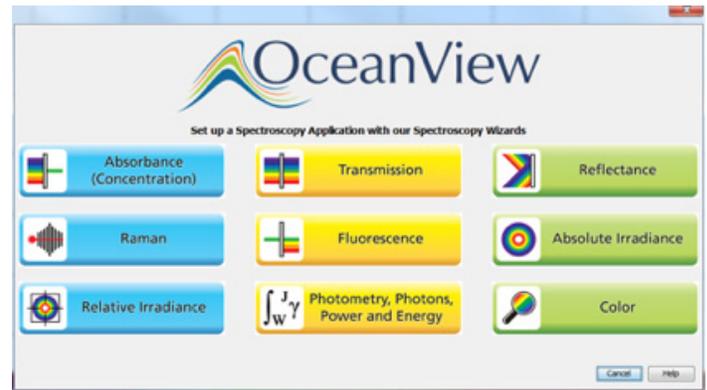
10. Click the **Save Graph to Files** button  to save your QuickView Fluorescence spectrum.

11. Print a copy of the graph showing the QuickView Fluorescence spectrum for your fluorophore by clicking the **Print** button .

## Measuring Fluorescence with the Relative Irradiance Wizard

1. Click the **Create a New Spectroscopy Application**  button in the top menu to open the **Spectroscopy Application Wizards** window.

2. Select the **Relative Irradiance** option in the Spectroscopy Wizards window to start the **Relative Irradiance** wizard.



3. Select the **Active Acquisition (Recommended)** option.

4. Direct the light from the HL-2000 (or any other blackbody source with a known color temperature) into your spectrometer using an angled sampling accessory like the CVD-DIFFUSE or by rearranging the fibers so they are opposite one another in the cuvette holder.

5. Set your acquisition parameters in the **Set Acquisition Parameters** window.

a. Click the **Automatic** button  **Automatic** to automatically adjust the Integration Time to the optimum value.

b. Set **Scans to Average** to 10 and **Boxcar Width** to 5 to reduce measurement noise and improve the measurement. Set **Scans to Average** to a higher or lower value depending on your integration time. Use fewer averages with longer integration times to shorten the total measurement time, if necessary (total measurement time equals the number of averages multiplied by the integration time).

c. Place a checkmark in the **Nonlinearity Correction** box if this feature is available for your spectrometer.

d. Click **Next**.

6. Click the **Store Reference**  button to store a reference spectrum. Click **Next**.

7. Close the shutter on the light source or block the light from entering the spectrometer for your background measurement. Do not turn your light source off. Click the

**Store Background**  button to store a background spectrum.

8. Open the shutter or unblock the light source and click **Next**.

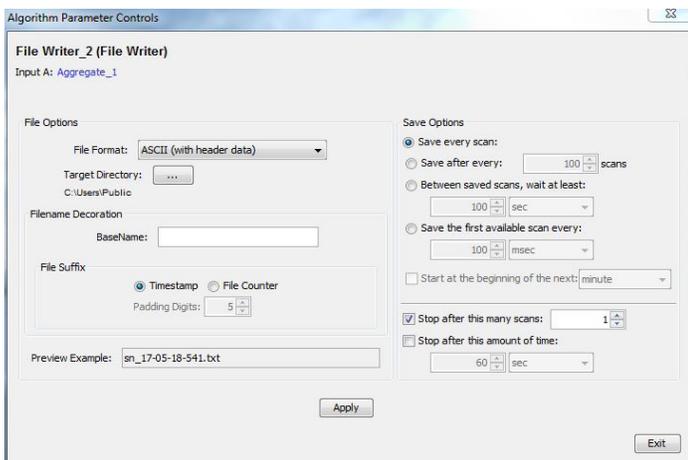
9. Enter the color temperature for your blackbody source or select it from the Color Temperature table. Click **Finish**.

10. Wipe the cuvette with a KimWipe to remove any fingerprints and place the cuvette containing your unknown fluorophore in the cuvette holder. You are now ready to measure the fluorescence spectrum for your unknown fluorophore in Relative Irradiance mode.

11. The Fluorescence spectrum will be displayed in the **RelativeIrradianceView** window. You can adjust the graph appearance using the graph tools  above the graph. For example, click the **Scale Graph Height to Fill Window** button  to zoom in on the spectrum.

12. Save your relative irradiance spectrum by clicking the **Configure Graph Saving** button  to configure the File Writer.

- Select the directory where you want to save the file.
- Enter a filename for your spectrum.
- Click **Apply** and then **Exit** to close the dialog box.



13. Click the **Save Graph to Files** button  to save your relative irradiance spectrum.

14. Print a copy of the graph showing the Relative Irradiance Fluorescence spectrum for your fluorophore by clicking the **Print** button .

## Data and Analysis

Unknown #	Wavelength of Maximum Absorbance ( $\lambda_{max}$ )	LED Chosen for Excitation ( $\lambda_{EX}$ )

Compare the spectral data measured in QuickView Fluorescence and Relative Irradiance mode.

Do the fluorescence spectra look the same?

Does the maximum emission occur at the same peak wavelength? Are the peak shapes the same? Why not?

## Additional Tasks

1. Try placing 74-MSP mirrored screw plugs at different locations in the cuvette holder to see how the fluorescence intensity changes.

	Fluorescence Peak Intensity
With no 74-MSPs	
74-MSP across from light source	
74-MSP across from spectrometer	
74-MSP in both locations	

2. Excite fluorescence with each LED wavelength and an HL-2000 to see how the fluorescence intensity changes. Record the peak intensity in each case.

	Fluorescence Peak Intensity
LLS-365	
LLS-470	
HL-2000	

## Instructor Notes

Chlorophyll has a strong fluorescence spectrum and is easily extracted from dark, green leaves like spinach using rubbing alcohol. Prepare the extract by tearing spinach or other dark, green leaves into small pieces and placing them in rubbing alcohol. The solution will turn green over time as the chlorophyll is extracted from the leaves.

Contact us today for more information on setting up your spectroscopy system from Ocean Optics.



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