S5: Conducting a Relative Fluorescence Assay with the OPN Fluorometer

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To test the OPN Fluorometer we conducted a series of simple assays to determine the relative fluorescence of different concentrations of Rhodamine B and Acridine Orange in solution, and we describe these protocols below. We also discuss the major hazards associated with this activity; and, at the conclusion of this supplement, we provide some helpful hints for conducting the exercise as part of a teaching lab.

Relative Fluorescence Assay

The day before we conducted our experiments, we first wrapped two 1-L glass bottles in aluminum foil to prevent any ambient light from bleaching the fluorophores once they were placed in solution. Next, we prepared stock solutions of Rhodamine B (5 mg/L) and Acridine Orange (50 mg/L) in those respective bottles using deionized water (DI H₂O). From those stock solutions, we then prepared a series of 100-mL standard solutions, which we again placed in glass bottles that had been previously wrapped in aluminum foil. These standards equated with 5, 10, 20, 40, and 80 µL of each fluorophore solution diluted in 2 mL of DI H₂O. For the convenience of readers, we provide the mixing ratios for making these standard solutions, as well as their final concentration levels in parts per billion (ppb), in Table S5-1 below.

<table>
<thead>
<tr>
<th>Concentration (µL in 2 mL DI H₂O)</th>
<th>Fluorophore Soln. (µL)</th>
<th>DI H₂O (mL)</th>
<th>Rhodamine B (ppb)</th>
<th>Acridine Orange (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>250</td>
<td>100</td>
<td>12.5</td>
<td>125</td>
</tr>
<tr>
<td>10</td>
<td>500</td>
<td>100</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>20</td>
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<td>40</td>
<td>2,000</td>
<td>98</td>
<td>100</td>
<td>1,000</td>
</tr>
<tr>
<td>80</td>
<td>4,000</td>
<td>96</td>
<td>200</td>
<td>2,000</td>
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After preparing our standard solutions, we placed them in a drawer, closed it, and let the bottles sit overnight so that they would reach room temperature for the assays the next day. Then, on the day of the experiments, we set up the 3D-printed and wooden versions of the OPN Fluorometer as described in the Supporting Information (S1 and S4). Specifically, we used a 3D-printed OPN Fluorometer that was secured to a thin sheet of clear plastic with Scotch “Extreme” Fasteners ® (Fig. S1-12), and we used removable poster tack (i.e., mounting putty) to hold the components of a wooden OPN Fluorometer in place on our table top (S4). We further used either a standard glass cuvette or a small Pyrex test tube (VWR Disposable Culture Tube, No. 47729-572) to hold our solutions (S1, S4). Also, in our Rhodamine B assay, we used pieces of green and red cellophane as our excitation and emission filters, respectively. In our Acridine Orange assay, we used pieces of blue and green cellophane as our excitation and emission filters, respectively. For our light source, we used an Outlite WT03 tactical LED flashlight, which contained a “fresh” (i.e., recently charged) 3.7-V Lithium-ion battery as its power source. We further used a NSL-6910 photocell (i.e., light dependent resistor or LDR) connected to a Craftsman digital multimeter (No. 82170) as our detector (S1, S4).

Next, to warm up the electronics, we turned on the Outlite WT03 flashlight, placed it on the “high” setting, and fully extended its telescopic head to ensure that the brightest possible light would strike the solution. We then inserted the flashlight into the tube for the light source and waited 10 minutes for the flashlight, LDR, and multimeter to warm up. During this time, we further organized the other materials needed for the activity: a 1,000-μL commercial pipette, disposable pipette tips, plastic transfer pipettes, two large beakers (e.g., 500 mL) for waste, another beaker (e.g., 250 mL) filled with DI H₂O for rinsing out the cuvette or test tube, and the standard solutions (five bottles for each fluorophore, each wrapped in aluminum foil).
Also, at the start of the warm-up period, we recommend that students place their cuvette or test tube in the OPN Fluorometer and fill it with DI H$_2$O (or other applicable solvent). This is called a “blank” sample since it does not contain any fluorophores in it. Students should then record the resistance values displayed on the multimeter for this blank sample every minute at the 30-second mark (with the lid of the OPN Fluorometer on) to track the change in these numbers since this data should indicate whether the electronics have sufficiently warmed up to proceed with the assay. In addition, the final resistance reading for the blank can later be used for subsequent analysis (see the “Helpful Hints” below). Of course, at the conclusion of the warm-up period, students should use a plastic transfer pipette to empty out their cuvette or test tube, so that they can then begin their experiments.

In particular, for each assay, we started by taking a sample from the first standard bottle (i.e., the one with the lowest fluorophore concentration), and we worked upwards from there (from lowest to highest concentration). Specifically, we first used a commercial micropipette to slowly and gently fill the glass cuvette or test tube with either a 2-mL (in the 3D-printed model) or a 4-mL (in the wooden version) sample from the standard bottle with the lowest fluorophore concentration. We slowly and gently empty the contents of the commercial pipette into the cuvette or test tube in order to prevent any air bubbles from forming in the solution, which could affect the readings on the multimeter. Also, we chose a larger volume of solution for the wooden version of the OPN Fluorometer to make sure that the light beam would pass through the sample. Then, we replaced the lid on the OPN Fluorometer, waited one minute, and recorded the corresponding resistance value displayed on the multimeter (since, by then, the readings had typically stabilized). Once finished, we used a plastic transfer pipette to remove the solution from the cuvette or test tube and then emptied the mixture into a waste beaker.
We next repeated these steps for the remaining standards (in order of increasing concentration levels). Once finished, we used a fresh transfer pipette to rinse out the cuvette or test tube with DI H₂O (3 times) before adding 2 or 4 mL from one of our standard solutions in order to later determine how well the data generated by the OPN Fluorometer could be used to predict the concentration of an “unknown” sample. After we had taken that reading (at the 1-minute mark), we then used a plastic transfer pipette to empty out the contents of the cuvette or test tube into a waste beaker.

At this point, students could again rinse out their cuvette or test tube with DI H₂O (3 times) and re-fill it with 2 or 4 mL of DI H₂O to record the resistance values associated with another “blank” sample (e.g., every 30 seconds for the next two minutes). Students could then use this information to later evaluate how the resistance of the LDR generally changed during the course of the experiment, which could lead to some additional interesting exercises (see the “Helpful Hints” section below).

Then, at the conclusion of our assay, we removed the cuvette or test tube and thoroughly rinsed it out (into the waste beaker) using DI H₂O. After finishing one round of assays, which took roughly 30 minutes per set-up, we repeated our tests to see if our results could generally be duplicated.

Once we had finished all of our tests, we entered our data into Microsoft Excel and graphed the results as scatter plots. We further used the “Add Trendline” command in Excel (by right-clicking on each set of data points) to fit a curve to each set of results. We also displayed the equation and R² value for each best-fit curve on each graph to see how well the trend line fit its respective curve.

Finally, we used the equation for each best-fit curve to estimate the concentration of the respective “unknown” solutions, and we further calculated the percentage error associated with each estimate. Then, we determined the mean and standard deviation
of these errors to see how well the data generated by the OPN Fluorometer could be
used to predict the concentration of an “unknown” sample.

Hazards
As explained in the Supporting Information for our OPN Scope paper,\(^1\) Rhodamine B
and Acridine Orange are hazardous chemicals that can cause severe irritation and even
damage on contact. In particular, Rhodamine B can cause serious skin and eye
damage upon exposure, and the chemical can be toxic if inhaled or ingested. Similarly,
Acridine Orange can cause skin and eye irritation on contact, and exposure to the
substance may also cause genetic defects. Therefore, readers should wear the proper
protective equipment (e.g., goggles, gloves, lab coats, masks or respirators, etc.) when
working with either one of these two chemicals, and readers should also make sure to
read the Material Safety Data Sheets for each one of these compounds before handling
them.

Furthermore, as recommended in our prior papers,\(^1-3\) given the risks described
above, we suggest that instructors prepare the stock and standard solutions of
Rhodamine B and Acridine Orange themselves in advance of any lab activities.

Students should further read the Material Safety Data Sheets for these chemicals in
advance of any lab exercises, follow the proper safety protocols during lab, and wear the
appropriate protective equipment when handling these diluted mixtures in lab. Finally,
because these compounds are aquatic contaminants, any solutions containing these
fluorophores should be disposed of as chemical waste at the end of any lab exercise or
in-class demonstration.

Helpful Hints
In addition to the helpful hints provided in the Supporting Information for the 3D-
printed (S1) and wooden (S4) versions of the OPN Fluorometer, we include some further
suggestions here, which might be useful in the context of a classroom demonstration,
teaching lab, or other educational exercise.
First, for optimal results, instructors should prepare fresh solutions of each fluorophore the day before each lab, so that the mixtures can reach room temperature before any experiments begin. Also, as discussed above, we recommend that instructors first wrap the containers for these solutions in aluminum foil to prevent any ambient light from bleaching the fluorophores prior to or during lab. As a result, if providing students with their own individual fluorescent samples (e.g., in small glass bottles), we recommend wrapping those containers in aluminum foil as well. To further reduce the effects of any photo bleaching or fading, instructors may want to consider dimming the overhead lights (or turning them off and lighting the room with a “soft” 25-W bulb or similar source) as long as this approach does not present any safety hazards for the lab exercise.³

Second, as explained above, we slowly and gently pipette each standard solution into the cuvette or test tube in order to prevent any air bubbles from forming, which could affect the readings on the multimeter. Also, because we proceed in order of increasing concentration, to conserve resources, we typically use the same disposable pipette tip on our commercial pipette to transfer the samples from each standard bottle and the same plastic transfer pipette to remove these solutions and empty them into the waste beaker. Of course, instructors could have their students use fresh pipette tips and transfer pipettes for each step, but this would increase the amount of these materials needed for the lab activity (by five times for each lab group). Nevertheless, after the final standard sample has been removed from the cuvette or test tube, in order to avoid any contamination, we do use a fresh transfer pipette to rinse out the container with DI H₂O. For the same reason, we then use a fresh pipette tip to transfer the “unknown” sample into the cuvette or test tube along with a fresh transfer pipette to rinse out the container again before starting a new assay.
Third, we wait ten minutes before taking a reading on our multimeters because, in our experience, the resistance value displayed on the readout has largely stabilized by that time. However, if using a different flashlight, a different LDR, a different fluorophore, or different concentration levels in solution, readers may need to adjust this time period. Relatedly, using a freshly charged battery may help the readings stabilize more quickly, so might using newer (as opposed to older) batteries in the flashlight.

Fourth, we have also found that there is some “drift” in the displayed resistance values over time, which may be due to several factors (e.g., the light beam becoming dimmer as the battery drains, the potential need for a more sensitive LDR, the possible bleaching or quenching of the fluorophore in solution, etc.). As a result, the resistance value displayed on the multimeter tends to increase over time (although this is not always the case – e.g., we have found that, sometimes, the resistance may decrease, depending upon the battery used). Based on our tests, we do not believe that this drift alters the general trends in the data, but we do discuss below some of the issues related to this drift along with some possible ways to address them.

Most importantly, the drift in resistance values primarily comes into play if students will be using the OPN Fluorometer to estimate the fluorophore concentration of an “unknown” sample after they have collected the data to generate their standard curves. In particular, because the resistance generated by the LDR will likely “drift” upwards over time, the resistance value associated with any unknown sample(s) will likely be higher than expected if they had been measured when the standard curve was actually created. As a result, students will most likely end up under-estimating the fluorophore concentration of their unknown sample(s) – possibly by a substantial amount, which may cause some confusion (or frustration) among the lab groups (particularly in an introductory class). Consequently, instructors may only want to use the OPN
Fluorometer to demonstrate some of the basic principles of fluorometry in a lab, such as how these instruments are put together, how they generate their results, and how fluorescence generally varies with fluorophore concentration.

Of course, instructors could still have their students to put their standard curves to good use. For example, instead of giving students an actual physical sample to analyze, instructors could simply explain how a standard curve could be used to estimate the concentration of an unknown sample. Then, the instructor could give each lab group a “hypothetical” unknown to analyze. Specifically, by looking at the curves generated by each lab group, an instructor could pick one or more resistance values located on each curve as if those were the values for any unknown samples assigned to the group. The students could then use the equation for their best-fit curve to estimate the concentration their hypothetical sample (e.g., if $y = a\ln(x) + b$, then $x = e^{(y-b)/a}$), which would demonstrate the general concept of using a standard curve to estimate the concentration level of an unknown while avoiding any complications caused by the drift in resistance values over time.

Relatedly, because of this issue of “drift,” we only use fresh (i.e., recently charged) batteries for one assay, which should last about 30 minutes. After that, for each additional test, we replace the rechargeable battery with a new one that has been freshly charged. Instructors may further want to keep track of how long (and how many times) each battery has been used in order to determine whether any need to be replaced.

Fifth, instructors should also know that we did not see a linear fit to our data given the concentrations of the standard solutions set forth above. However, at lower concentrations of Rhodamine B (under 20 ppb) or Acridine Orange (under 40 ppb), we often did obtain near linear fits to our data, although a quadratic equation frequently provided a perfect fit ($R^2 = 1$) in these instances (Fig. S5-1A). However, it could take
longer than one minute for the readings to stabilize at these lower levels. Also, when these lower concentration levels were included with the data for higher levels, it occasionally became difficult to fit a natural logarithmic, quadratic, or power function through all of the data points (unlike the fits that we obtained for samples equating with 5 to 80 µL of stock fluorophore solution in 2 mL of DI H₂O). Of course, in these instances, instructors could simply use the “smooth line” scatter plot function in Excel to show the general trend of the data (Fig. S5-1B) and, then, have their students fit a line to the data in the linear range (Fig. S5-1A).

Figure S5-1. Sample data showing (A) a near linear fit for low concentrations of Rhodamine B (under 20 ppb) using a glass cuvette in the 3D-printed OPN Fluorometer and (B) a “smooth line” scatter plot of the overall data set, which shows the general trend. Readers should also know that, despite the near linear fit to the data points in Panel (A), a quadratic equation \( y = 0.0414x^2 - 1.1804x + 31.493 \) provides a perfect fit \( (R^2 = 1) \) for these results.
Sixth, on a related note, while we used the “Add Trendline” function in Microsoft Excel to fit curves to our data, instructors could instead simply have students plot their results using “smooth line” scatter plots to see the general trend in the data (Fig. S5-1B). Although this approach would not enable students to see the explicit mathematical relationship in their data, the resulting graphs might be well suited for students who have not yet reached this stage in their studies. Also, in instances like those described above, where a simple mathematical function does not fit the data nicely, this approach might avoid any confusion or frustration among the students.

Seventh, in addition to having students record the resistance values associated with different concentrations of Rhodamine B or Acridine Orange, instructors may want to have their students actually look at the solutions as their concentrations increase to see firsthand the corresponding effect on fluorescence (Fig. S5-2). Plus, as a practical matter, curious and inquisitive students may take it upon themselves to look at these solutions anyway before replacing the cuvette or test tube lid. As a result, instructors may simply want to build this into their lab exercises, so that every student has the chance to observe this phenomenon. We simply suggest that instructors have their students look at their different fluorophore solutions after recording the resistance value for each one in order to reduce the amount of bleaching that might occur from any overhead light.
Figure S5-2. The effects of increasing concentrations of Acridine Orange in deionized water (DI H$_2$O): (A) 125 ppb; (B) 500 ppb; and (C) 2,000 ppb

Eighth, readers should also be aware that, like the LDRs that we use in other OPN instruments, the resistance of the NSL-6910 photocell is inversely proportional to the intensity of the light striking it. As a result, the corresponding graphs that students generate will appear to be “flipped” versions of a typical calibration curve since they decrease (instead of increase) with concentration levels (Fig S5-3A). We suggest that instructors explain this phenomenon at the start of lab to avoid any confusion among

Alternatively, to generate graphs that look more like a typical concentration curve (i.e., one that initially increases, but then levels off at higher concentration levels), instructors could have their students subtract the resistance value associated with each fluorescent sample from that of a “blank” (i.e., a cuvette or test tube filled only with DI H$_2$O or other applicable solvent), which was taken before the assay began. To do so, students would only need to follow these steps. First, at the start of the warm-up period for the electronics, students should fill their cuvettes or test tubes with 2 or 4 mL of their solvent (e.g., DI H$_2$O in the assays described above). Then, at the end of the warm-up period, students should record the resistance value associated with this “blank” solution. This measurement will indicate the amount of fluoresced or reflected
generated by the cuvette or test tube and the solvent itself. Next, students should use a plastic transfer pipette to empty out the cuvette or test tube, and they can then begin their assay as described above. Finally, once they have collected all of their data, students should subtract the resistance value associated with each fluorescent sample from that of their “blank” solution to control for any light not associated with the fluorescent solutions (i.e., \( R_{\text{final}} = R_{\text{blank}} - R_{\text{sample}} \)). The resulting curve should appear much more like a traditional calibration curve, which increases significantly at low concentration levels, but then begins to level off at higher ones (Fig. S5-3B).

![Figure S5-3](http://pages.stolaf.edu/opn-lab/equipment/12/27/16/Page12of14)

**Figure S5-3.** The effect of subtracting the resistance associated with each fluorescent sample from that of a “blank” solution taken at the end of the warm-up period. (A) The raw resistance values. (B) The difference in resistance values between the blank and various samples (\( R_{\text{final}} = R_{\text{blank}} - R_{\text{sample}} \)).
Of course, depending upon the exact resistance value associated with the “blank” sample, it is possible that students may obtain some negative differences (i.e., ones that are below zero) when they subtract the resistance of their sample from the resistance of their blank. Nevertheless, in our experience, the resulting curves themselves typically follow a general trend that is similar to the ones shown in Figure S5-3B above.

Also, in more advanced classes, at the end of their assays, students could take another set of “blank” readings (e.g., every 30 seconds for two minutes) after they have thoroughly rinsed out their cuvette or test tube in order to see how the resistance value associated with a blank sample has changed over the course of their experiment. Then, based on prior tests conducted by the instructor, students could use a linear, quadratic, or other type of mathematical model to estimate the rate of change in the resistance values of the blank solution over time. This information could then be used to refine the \( R_{\text{final}} = R_{\text{blank}} - R_{\text{sample}} \) equation described above since the value of \( R_{\text{blank}} \) could now be estimated based on the time that the sample was tested. Of course, this approach would also require as part of the lab that students to note the time when they measured the resistance of each blank and each fluorescent sample, but this type of attention to detail is one of the skills that students should be learning in their lab activities.

Otherwise, many of the helpful hints set forth in the Supporting Information for our OPN Colorimeter\(^2\) and OPN Spec\(^3\) papers also apply to the use of the OPN Fluorometer, and we briefly summarize the most relevant suggestions here for the convenience of readers.

Most notably, while we used a 10-minutes warm-up period before beginning our fluorescence assays, students could instead begin their experiments as soon as the readings on their multimeters begin to level off (e.g., by showing less than a 1% drop in the resistance of their “blank” solution per minute).\(^2\,^3\) Depending upon the LED
flashlight used and the age (or level or charge) in the batteries, we have found that readings can often level off within 5 minutes. However, instructors should conduct a sufficient number of pilot tests with their equipment in advance to make sure that students are using an appropriate warm-up period.

In addition, in each relative fluorescence assay that we conducted, we left the LED flashlight on throughout the experiment. In fact, turning off the flashlight or even moving it around in the tube may render subsequent results incomparable to earlier ones given how the NSL-6910 photocell responds to changes in light intensity. Instructors may therefore want to have their students tape their flashlights into place before starting an assay, so that the position of the light does not change during the experiment.

Indeed, in our experience, we have found that keeping the position and intensity of the beam relatively constant during an assay is extremely important due to the sensitivity of the NSL-6910 photocell that we use. As a result, inadvertently bumping or moving a flashlight, removing a cuvette or test tube from an OPN Fluorometer during an experiment, or pulling the plug or cork containing the LDR out of the OPN Fluorometer while an assay is in progress may render subsequent results incomparable to earlier ones (and, thus, require students to start the experiment over again).

References