P51™ Introduction to Fluorescence Lab

Glow Big or Glow Home

P51™ qPCR Learning Lab
Introduction to Fluorescence Lab
Instructor and Student's Guide

Version: 1.0
Release: August 2019

Student's Guide Contents

Background and significance

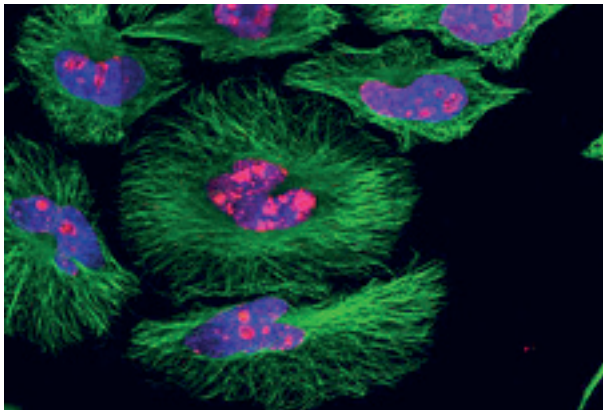
Fluorescence and biotechnology

When you bring to mind something that is fluorescent, you probably think of a color so bright that it practically glows. In fact, things that are truly fluorescent actually do emit light. And light being a form of energy, that energy must be supplied from somewhere. Fluorescence is a phenomenon where energy is absorbed from one part of the electromagnetic spectrum and is then released as a different part of the electromagnetic spectrum—visible light. If you have ever seen things glow under a blacklight, you are familiar with the phenomenon of fluorescence. The blacklight emits ultraviolet light, a form of light that your eye cannot see. The fluorescent colors absorb that ultraviolet light and release it back out as brilliant greens, oranges, and other colors.

Different fluorescent solutions illuminated using UV light. Photo: Maxim Bilovitskiy
Creative Commons license CC 4.0)

Many organisms have evolved to use fluorescent molecules, especially marine organisms living relatively close to the surface of the water. People also regularly use them in everyday life. Highlighters use fluorescent molecules to make important words stand out to you. Fluorescent lightbulbs save energy and light up our buildings. Fluorescent dyes help plumbers find leaks.

In the biology laboratory, fluorescence is an incredibly important tool because it has afforded scientists a way to visualize structures that were previously invisible or indistinguishable. Fluorescent proteins, such as the green fluorescent protein (GFP) and red fluorescent protein (RFP) are regularly used to track happenings inside the cell by making otherwise invisible structures light up. Fluorescent molecules can also be attached to antibodies which will then bind to specific structures in the cell showing us their location, shape and structure. And because different fluorescent molecules absorb and release unique wavelengths of light, often several of these chemicals can be observed in the cell at the same time, leading to some of the incredibly bright and colorful pictures of cells that you may have seen before.

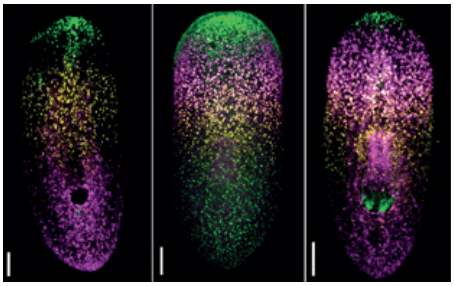


Cells grown in culture stained with three different fluorescent stains. Photo: Gerry Shaw
Creative Commons license CC 4.0)

Different fluorescent dyes have been developed that bind to DNA. The most common use of such dyes is to see DNA on an agarose gel, but fluorescent dyes can also be used to quantify DNA. For example, in a process called quantitative PCR (qPCR), DNA molecules are copied and as each new copy is made, a fluorescent dye binds to the DNA. A light beam and optical sensor then record the relative brightness of the dye and tell you how much DNA you have made.

Fluorescence has the added benefits that it makes images that are often quite striking to look at and that it is generally safe to use. Previous to using fluorescence, the most common way of tagging molecules was using radioactivity. While radioactive probes are still sometimes used today, they are generally much more dangerous to handle and not nearly as fun to look at.

What is fluorescence?



Fluorescent *in situ* hybridization (FISH) image of planaria

Fluorescence is emitted electromagnetic radiation that results from the release of energy after a substance is struck with electromagnetic radiation of a different wavelength. When a photon (particle of light) strikes an electron, it transfers energy to the electron, causing the electron to jump to an excited (higher energy) state. This excited state is unstable, so the electron eventually falls back to its lower energy state, known as the ground state. But the energy that the electron absorbed from the light must go somewhere. Some of this energy is distributed as heat, and then some may be emitted as electromagnetic radiation in the visible range-light. Because some electromagnetic radiation was lost as heat, the emitted radiation has less energy than when it was initially absorbed, which means the photon emitted will have a longer wavelength than the photon that was absorbed; this is called *Stokes shift.* Therefore, fluorescent substances give off light of a different color than they absorb.

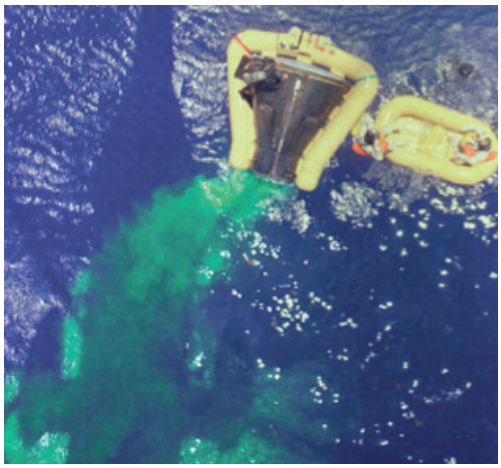
Fluorescent molecules only absorb specific wavelengths of light, and then only let off (emit) specific wavelengths of light. This is what allows scientists to use multiple fluorescent molecules at the same time. For example, most green fluorescent proteins absorb blue light (480 nanometers, nm), and emit green light (509 nm), while a red fluorescent protein may absorb yellow light (580 nm) and emit reddish/orange light (610 nm). To make GFP fluoresce, the scientist must use a blue light and the appropriate filter. But under blue light the scientist will see a diminished response from the RFP. To see RFP fluoresce, the scientist must use a yellow light and appropriate filter, which will get a dimmer response from the GFP.

Today's lab

In this lab, you will explore the basic uses of fluorescence in biotechnology. You will use the fluorescent dye fluorescein, a molecule commonly used in biological applications, to explore how fluorescence can be utliized as an indicator. First, you will establish the concentration of an unknown sample, much like how fluorescence is used in applications such as qPCR and DNA quantification. Second, you will measure the amount of fluorescein used in a common task, highlighting a word with a highlighter.

Fluorescein – the basics

A bright, fluorescent molecule, fluorescein has been used in biological research, medicine, air rescue, and even plumbing. Having been first synthesized in 1871, we've been using fluorescein for a while. Fluorescein has been used by NASA to more easily spot spacecraft that splash down in the ocean upon return to Earth. For decades, plumbers have used fluorescein to find leaks in water systems. The city of Chicago even used fluorescein to dye the Chicago River green for their annual St. Patrick's Day celebration until they switched to more environmentally friendly dyes in the 1960s.



The Gemini IV spacecraft releases fluorescein into the surrounding ocean so it can be more easily spotted by the recovery helicopter.

Fluorescein is also widely used in biotechnology applications. In some cases, fluorescein is attached to molecules so that we can visualize their location in a tissue or inside a cell. Sometimes it is attached to other molecules in a way that it requires a chemical reaction to release the fluorescein and allow it to fluoresce; thus specific enzymatic reactions can be tracked, seeing where and when the fluorescence occurs. Fluorescein can also be attached to probes used to detect specific macromolecules, such as nucleic acid probes used for in situ hybridization and antibodies used to detect proteins.

Fluorescein has a strong maximum absorption at 494 nm, which is seen as blue light, and maximum emission at 512 nm, which is seen as green light. This means that blue wavelengths of light will cause the most fluorescence and we will see that fluorescence as green. When using strong blue light, it helps to view your sample through an amber filter. Because amber absorbs blue light it will subtract out the background and allow the non-blue fluorescence to stand out.

Laboratory guide

Gloves and protective eyewear should be worn for the entirety of this lab

Observation: Behold fluorescein

Observe a tube of diluted fluorescein compared to water.

- Take two individual 200 µl tubes.
- Add 20 µl Phosphate Buffer (labeled "B") to each tube.
- Add 5 µl Concentrated Fluorescein (labeled as "F") to ONE of the tubes.
- Pipette up and down to mix the two liquids.
- Place tubes in P51™ or other blue light illuminator.
- Record your observations.

Observations

With Fluorescein	Without Fluorescein

The "Without Fluorescein" tube can be considered a negative control. Why is it important to have a negative control? Why might just looking at one tube not give enough information?

Investigation A: Estimating concentrations

Making a dilution series

When investigating or establishing concentrations, scientists often make what is called a dilution series. A dilution series is a sequential dilution of a solution. This results in dilution factors increasing on an exponential scale instead of a linear scale. For example, if making a 10-fold dilution series, you might put 100 microliters (µl) of your original solution in your first tube. You might then set up 7 more tubes, each with 90 µl water. You would take 10 µl of your original solution and add it to the 90 µl in the second tube. This second tube is now 1/10th the concentration of the original solution. You would then take 10 µl from tube 2 and dilute it in the next tube of 90 µl of water. This third tube would have 1/10th the concentration of tube 2, and 1/100th the concentration of the original tube. To continue, you would take 10 µl of this and add it to the 90 µl of water in the next tube and so on. To make a 5-fold dilution series, you might take 20 µl of your solution and add it to 80 µl of water, then take 20 µl of that solution and dilute it in your next tube of 80 µl of water and so on and so on.

Fill in the remaining boxes in the table to establish how different dilution factors affect concentration. The fluorescein we will be using today is provided to you at a concentration of 50 nanograms (ng) per microliter.

	Tube	1	2	3	4	5	6	7	8
10-fold series	Dilution factor	1	10	100	1,000	10,000			
	Concentration	1	1/10	1/100	1/1,000				
	Amount of fluorescein	50 ng/µl	5 ng/µl	.5 ng/µl					
5-fold series	Dilution Factor	1	5	25					
	Concentration	1	1/5						
	Amount of fluorescein	50 ng/µl	10 ng/µl						
2-fold series	Dilution factor	1	2	4					
	Concentration	1	1/2						
	Amount of fluorescein	50 ng/µl							

What might be the advantages of using a larger dilution factor, like in a 10-fold dilution series?

Disadvantages?

What might be the advantages of using a smaller dilution factor, like in a 2-fold dilution series?

Disadvantages?

What is the effect of always taking from the previous tube when creating a dilution series? For example, why not just add a smaller and smaller amount from your original stock solution to each tube?

Estimating concentration

You have been given an unknown concentration of fluorescein, labeled "U". Your job is to figure out how concentrated your unknown is.

1. Get a new strip of eight tubes.
    - Label the tubes 1-8.
2. Add 20 µl of your Unknown Concentration (labeled "U") to tube 8 of your 8-tube strip.
3. Add 20 µl of your Concentrated Fluorescein (labeled "F") to tube 1 of your 8-tube strip.
4. To view fluorescence, place the strip in the P51™ Viewer or other blue light illuminator and record your observations.

Your group must now decide on whether you would like to make a 10-fold, 5-fold or 2-fold dilution series.

What fold dilution series will your group make?

Why did you decide this?

Make a dilution series

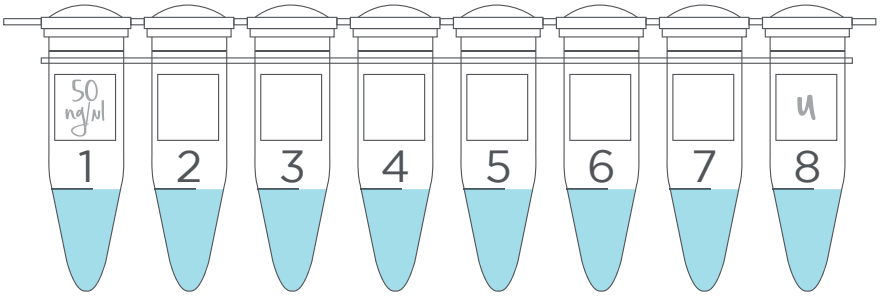
1. Follow the table below to create your dilutions series.
2. First, add the appropriate amount of Phosphate Buffer (labeled "B") to tubes 2-7.
3. For tube 2, add the fluorescein solution from the original stock solution (tube "F").
4. For tubes 3-7 add the solution from the previous tube.
5. Mix thoroughly before removing solution to add to the next tube in the dilution series.
    - To mix, pipette up and down repeatedly.

	Tube	2-7
10-fold dilution series	Volume of buffer added to each tube	18 µl
	Volume of fluorescein or liquid added from the previous tube	2 µl
5-fold dilution series	Volume of buffer added to each tube	20 µl
	Volume of fluorescein or liquid added from the previous tube	5 µl
2-fold dilution series	Volume of buffer added to each tube	20 µl
	Volume of fluorescein or liquid added from the previous tube	20 µl

Through comparison, estimate the concentration of fluorescein in your unknown

1. It may help to separate tube 8 from the other tubes.
2. Separate the other tubes where you think tube 8 will best fit in the sequence.
3. Put all 8 tubes in the illuminator in order.

In the graphic below, write the concentration of fluorescein that is in each tube.



Circle the tube that your unknown seems closest to. If it appears to be between two tubes, circle both tubes.

Approximate concentration of **unknown solution:** _____

Were you able to estimate an approximate concentration using the dilution factor you chose?

If you were to try this again, what dilution factor would you use? (It may help to compare your results to those from another group)

Justify why you think the dilution factor you chose in the previous question is a good choice

Save this dilution series for Investigation B.

Investigation B: Calculating molecules

How much fluorescein makes the word fluorescent fluoresce?

Highlighters can use a few different molecules to make them look very bright. One molecule that some highlighters use is fluorescein. For this investigation we will assume that the highlighter you are using contains fluorescein.

Make a solution of fluorescein from a highlighted word

1. Use a fluorescent yellow/green highlighter.

2. On the right side of this page use a highlighter to highlight the word "Fluorescent".

3. Use scissors to cut the highlighted word out of the paper.

4. Label a 200 µl tube "H" for highlighter.

5. Put your cut out piece of paper into the tube. You will need to fold or roll the paper some to fit it in.

6. Add 100 µl of Buffer to the tube. If you are using a 20 µl micropipette you will need to add 20 µl five times.

7. Close the lid and shake the tube well. Holding the tube with one hand and using a finger to flick the tube repeatedly works well. Or, just vigorously shake the tube repeatedly. Continue shaking for at least 30 seconds.

8. Shake all the liquid to the bottom of the tube by flicking your wrist.

9. Open the tube and remove the paper. You will likely need to use a pipette tip or something similar to fish the paper out, while making sure that the liquid stays in the tube.

10. Close the lid to your tube.

Fluorescent

Calculate the concentration of the highlighter fluid in solution

1. Compare this tube to your dilution series from Investigation A.

    • Remove your old unknown from the dilution series. Twist and pull to break off the tube if needed.

2. Place your dilution series from Investigation A and your highlighter fluid extract in the P51™. Turn on the P51™ and view.

Can you tell how concentrated you new solution is by comparing it to your original dilution series?

What can you do to make a solution that you can measure? Make a plan with your group and describe it here. You have buffer and an 8-tube strip to use how you see fit.

Your teacher may want to approve your plan before proceeding.

CER Table

Question:
What is the concentration of your original tube of highlighter solution?

Claim

Evidence

Provide data from the lab that supports your claim

Reasoning

Explain clealry why the data you presented supports your claim.

Practicing calculations with scientific notation

How many molecules of fluorescein made the word fluorescent fluoresce?

Important values for calculating:

- 1 nanogram (ng) is equal to 10^{-9} grams (g).
- 1 microliter (µl) is equal to 10^{-6} liters (l).
- The mass of a single molecule of fluorescein is 5.5166 **x** 10^{-22} g.

Metric prefixes for small numbers		
mili (m)	10^{-3}	0.001
micro (µ)	10^{-6}	0.000001
nano (n)	10^{-9}	0.000000001
pico (p)	10^{-12}	0.000000000001

Using the concentration you calculated for your highlighter solution, can you calculate approximately how many molecules of fluorescein you used when you highlighted the word fluorescent?

The concentration of my highlighter solution is _____ **(from previous page)**

Knowing the concentration of your solution and the mass of one molecule of fluorescein, how many molecules are in one microliter of your solution? Use the space provided to show your work.

How many total molecules were in your original 100 µl of solution? Use the space provided to show your work.

I used _____ **molecules of fluorescein to make fluorescent fluoresce!**