Using Fluorescence in Biotechnology Instruction to Visualize Antibiotic Resistance & DNA

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ABSTRACT

Fluorescence technology has many useful applications for both research and teaching, among them the detection of fluorescence in live transgenic organisms and of DNA in agarose gels. However, dedicated fluorescence imaging systems can be expensive and complex. We describe a simple apparatus for non-microscopic fluorescence imaging using affordable and readily available parts. We describe three activities of increasing complexity that utilize fluorescence illumination and teach principles of fluorescence. At the high school level, our more advanced activities can be used for lessons addressing NGSS performance expectations in HS-LS3 and HS-LS4.

Key Words: antibiotics; electrophoresis; fluorescence; illuminator; imaging; selection.

Introduction

Research in microbiology, molecular genetics, cell biology, and developmental biology often uses fluorescence for microscopy and DNA visualization. While powerful and useful, fluorescence microscopes are expensive and complex, and traditional DNA visualization with ultraviolet (UV) transillumination requires skin and eye protection. Here, we present a simple and inexpensive method for non-microscopic fluorescence illumination with many potential applications and advantages for education and/or research at the secondary and college levels. Biotechnology instruction at the high school level can be facilitated by our inexpensive fluorescence illuminator, which is constructed from readily available parts and takes advantage of smartphone cameras to increase student engagement with fluorescence technology. Our “fluorescence macroscope” portably and easily permits fluorescence illumination for a variety of applications. We describe three activities, of progressively increasing complexity, to teach students about fluorescence and some of its potential uses in biotechnology. The first is a simple demonstration of fluorescence that is appropriate to any grade level. The second is a theoretical experiment illustrating the use of fluorescently labeled strains to track the survival of antibiotic-resistant cells under antibiotic selection (we also provide a link to a detailed experimental protocol for high school or undergraduate instructors who are interested in conducting the actual experiment). The third uses fluorescence to visualize DNA fragments and thus is appropriate for high school students. This authentic research technique can generate excitement about science among students at the high school and undergraduate levels.

Background

Fluorescence is the absorption of light at a particular wavelength and reemission of light at one or more longer wavelengths. Electric lights are a common application of fluorescence. Fluorescent tubes use mercury vapor to produce UV light that is absorbed by a phosphor coating and reemitted as white light. Modern light-emitting diode (LED) lamps also typically use blue or UV-emitting diodes in concert with phosphors to create white light. In nature, the isolation of green fluorescent protein (GFP) from jellyfish by Osamu Shimomura in 1962 (for which he and two colleagues were awarded the 2008 Nobel Prize in Chemistry) led to the use of GFP and other fluorescent proteins in research. Fluorescently labeled proteins have been used with microscopy to watch previously undetectable processes in cell and developmental biology (Lukyanov et al., 2005; Chalfie, 2009; Renz, 2013). Fluorescent dyes can be used to specifically stain biomolecules such as DNA, making them visible with appropriate illumination. Finally, just as fluorescence can be used with a microscope...
to visualize proteins or subcellular structures, whole cells can be engineered to produce a soluble fluorescent protein within their cytoplasm, making them glow. Fluorescent cells can then be distinguished from non-fluorescent cells via fluorescence imaging.

The key to fluorescence imaging is excitation — illuminating a specimen at a wavelength that will cause it to emit at its characteristic emission wavelength. All fluorescent molecules have one or more excitation and emission wavelengths, and the excitation wavelength is invariably shorter (that is, higher-energy) than the emission wavelength. For example, many green-fluorescent molecules are maximally excited at 450–490 nm (which appears blue) and emit in the green spectrum at 510–550 nm (Figure 1A). Typical fluorescence illumination utilizes a wide-spectrum light source with an excitation filter that blocks all but a narrow spectrum of wavelengths centered on the excitation wavelength (a bandpass filter). For imaging, an emission filter blocks the excitation wavelength but permits the emission wavelength to pass, allowing specific detection of fluorescence (Figure 1A, B).

Fluorescence Illuminator Construction

We constructed our illuminator from a Fenix UC35 V 2.0 LED rechargeable 1000-lumen flashlight (from Amazon.com; Figure 2A) fitted with a one-inch-diameter bandpass filter (from

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**Figure 1.** Schematic of fluorescence illuminator and principle of operation. (A) Visible-light spectrum showing excitation and emission regions using the green- and red-fluorescence filter sets described here. (B) Illustration of fluorescence illumination principles and the use of bandpass and longpass filters to obtain specific wavelengths of light, thereby allowing stimulation of fluorescence emission and subsequent imaging. (C) Schematic of an LED flashlight equipped with a bandpass filter, allowing excitation of green fluorescent protein-expressing bacterial colonies. The emitted fluorescence is detected by placing a longpass filter (>550 nm) over a digital camera, allowing the specific detection of fluorescent colonies.
Figure 2. Fluorescence imaging setup. (A) Fenix UC-35 V2 LED flashlight. (B) Bandpass (excitation) filter (580 ± 5 nm, for red fluorescence, pictured). (C) Longpass (emission) filter (610 nm cutoff, for red fluorescence, pictured). (D) Setup for green-fluorescent illumination, with the illuminator mounted to a stand clamp and imaging performed with an iPhone 8 Plus. The emission filter is secured with a rubber band.

Discussion of the Science

Ask students what they observed. How did the drawings look under fluorescence illumination compared to standard white illumination? Did having the emission filter in place make a difference? Some colors will not fluoresce under the blue excitation light used for green fluorescence, because the excitation wavelengths of those colors do not match the blue illumination. An example of this is blue highlighter (see Figure 3C). Meanwhile, yellow and green highlighters do not glow under red fluorescence illumination, whereas pink and orange fluoresce strongly (Figure 3B). It is also clear from this experiment why the emission filter is important to block the excitation light. Without an emission filter in place, green fluorescence illumination appears mostly blue due to the excitation light, drowning out the emission light from the green, orange, and pink markers (Figure 3D). This exercise is suitable not only as a means to understand the use of fluorescence in biology and biotechnology, but also to teach the principles of fluorescence from a physical-science perspective.

Critical-Thinking Exercises & Questions

• Ask students to predict which colors will fluoresce with each setup before they do the experiment. Were their predictions correct? If a color fluoresced with a particular illumination setup, what does that mean about its excitation and emission wavelengths? Why does a yellow marker fluoresce with a green setup but not with a red setup?

• Ask students: Why do you think highlighters appear brighter than other colored markers? Why did some colors not show up at all under fluorescence illumination?
Activity 2: Selecting for Antibiotic Resistance

Introduction
An antibiotic is a compound that specifically kills or inhibits the growth of bacteria while doing no harm to eukaryotic cells (e.g., human cells). Antibiotic selection is simply defined as the preferential survival of the most antibiotic-resistant bacteria in any environment containing antibiotics, even when the antibiotic concentration is insufficient to kill most bacterial cells. It’s the survival of the fittest — the bacterial cells that can best resist the antibiotic are least likely to die. Thus, antibiotic selection favors growth in the relative population of antibiotic-resistant cells. Because the prevalence of human infections with antibiotic-resistant bacteria is increasing, there is growing concern about the emergence of antibiotic resistance worldwide (World Health Organization, 2015). The fact that antibiotic selection is constantly occurring wherever antibiotics and bacteria coexist has led many governments to implement restrictions on antibiotic use. For instance, in the United States, antibiotics for human use are available by prescription only, and veterinary use is likewise subject to regulation.

Materials
- Colony illustration worksheet (provided as a supplemental material with the online article)
- Fluorescent yellow highlighters and non-fluorescent yellow markers (these colors are difficult to distinguish under white light but are clearly distinguishable under green light illumination)

The principle of this exercise is that, given a population of bacterial cells with some antibiotic-resistant cells and some antibiotic-sensitive cells, the resistant cells will outcompete the sensitive cells in the presence of an antibiotic. We present this activity as a theoretical exercise using actual experimental data that we generated in our laboratory. For instructors who seek to conduct a microbiology experiment (or who are curious about how the data were generated), we provide a detailed experimental protocol on our laboratory website (https://cabeenlab.okstate.edu/resources). Notably, this experiment is a clear, microbiology-based demonstration in support of NGSS PE HS-LS4-3, helping to explain how organisms with an advantageous heritable trait tend to increase in proportion (National Research Council, 2012; NGSS Lead States, 2013). The experiment also supports AP Biology topics 7.2–3 (Natural and Artificial Selection), as we are selecting (artificially in this case) for antibiotic-resistant bacteria.

Figure 3. Fluorescence using highlighters (Activity 1). (A) A drawing using colored highlighters and photographed under white light. “CABEEN LAB” written in yellow highlighter, “follow” and “twitter” written in blue highlighter, coccoid cells drawn in pink highlighter, top left and bottom right bacilli drawn in green highlighter, top right and bottom left bacilli drawn in orange highlighter. (B–D) The same drawing photographed under three types of fluorescence illumination as noted.
fluorescence imaging; in our testing, Sharpie Accent Liquid Highlighter and Crayola Classic Markers worked very well)
- Fluorescence illuminator with green filter set

**Procedure**

1. Hand out worksheets and markers to each student. Explain that the worksheets represent bacterial colonies on an agar plate. Explain that each colony is composed of millions of cells that grew from a single cell, so the number of colonies reflects the number of cells originally spread on a plate.

2. We have two different types of bacterial cells. The **first type** is antibiotic resistant and marked with fluorescence, meaning that it will grow normally in the presence of the antibiotic and will glow green. We will represent colonies grown from these cells using the fluorescent highlighter. The **second type** is non-fluorescent and sensitive to the antibiotic, meaning that its growth will be impaired in the presence of the antibiotic. We will represent colonies grown from these cells using the yellow (non-fluorescent) marker.

3. We first mix the two types of cells in a test tube, in the absence of antibiotic, at a 1:1 ratio – an equal number of each cell type. If we spread that mixture on a plate, what is students’ prediction of what the plate will look like with respect to the relative numbers of fluorescent and non-fluorescent colonies? Ask them to draw their prediction on the top plate of their worksheet. Then darken the room or a spot in the room, and have each student hand their

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**Figure 4.** Representative results of an experiment selecting for antibiotic resistance (Activity 2). (A) Representative images of plates showing green-fluorescent (chloramphenicol-resistant) and non-fluorescent (nonresistant) colonies of *B. subtilis* (green-fluorescent colonies are brighter than non-fluorescent colonies when depicted in black and white). (B) Tabulated results of actual experiments conducted in the presence of 0.5 or 1.0 µg/mL chloramphenicol for different time periods.
worksheet to another student, take turns photographing the worksheet using the fluorescence illuminator and green filter set, and count the numbers of each type of colony. If the cells were mixed at an equal ratio, the prediction is that there should be a roughly equal proportion: about 15 colonies of each color.

4. Now imagine that we take the 1:1 mixture, add the antibiotic, and let cells grow for an hour. Remind students that the antibiotic will slow the growth of the sensitive cells but not the resistant (fluorescent) cells. On the bottom plate of the worksheet, ask them to draw their prediction of what the relative proportions of each cell type will be, and have them switch papers and photograph and count them as before. Here, the prediction is that there will be more fluorescent than non-fluorescent colonies (but there is no “right answer” as to how many more).

Discussing the Science
Ask students to describe how their worksheets looked under white light and under fluorescence illumination. Did fluorescence help them distinguish the types of colonies? Explain that fluorescent proteins are used in research labs to distinguish between two types of cells in experiments like the one they just performed. Did their predictions match the expectation that the fluorescent cells would grow in their relative proportion but only in the presence of the antibiotic? Here you can show students the actual experimental data obtained from an experiment just like this (Figure 4). Explain that this is an example of selection – the survival of the fittest. You can also explain the rising concern about antibiotic resistance because of antibiotic selection as described in the introduction of this activity.

Critical-Thinking Exercises & Questions
- What do you think would happen to the relative numbers of the two strains if you grew them for some time in the absence of antibiotics? (They would stay approximately equal.)
- What if you grew them for longer in the presence of the same antibiotic concentration? What about if a higher concentration of antibiotics was used? Either scenario would more strongly favor the antibiotic-resistant strain, so it would dominate even more (see Figure 4).
- Where do you think antibiotics come from? Most antibiotics are made by microorganisms! Bacteria have been making antibiotics to compete with each other for millions of years, long before humans appeared on Earth. Only relatively recently did humankind discover and take advantage of antibiotics for medicinal use.

Activity 3: Making DNA Visible

Introduction
One of the commonest uses of fluorescence imaging in biotechnology is to visualize DNA in agarose gels following gel electrophoresis. Agarose gels are used within an electric field to separate DNA molecules by size, thus allowing different samples to be compared. DNA is negatively charged thanks to the many phosphate groups in its backbone, so it is propelled through the gel matrix toward the positive electrode, with smaller DNA fragments moving faster (they are less impeded by the matrix). Some applications of this technology include forensic DNA fingerprinting, gene editing, and numerous research applications.

Resources for DNA Samples & Gel Electrophoresis
Our primary aim in this activity is to make DNA gel imaging accessible to instructors who do not have ready access to a commercial gel-documentation system. The principles and procedures for isolating and/or obtaining DNA samples and separating them via agarose gel electrophoresis are well known and are outside the scope of this procedure. However, we refer science instructors who are not already set up to do such experiments to such excellent resources as a gel electrophoresis apparatus made from household materials (Ens et al., 2012), the MiniOne electrophoresis system (https://theminione.com), and DNA extraction from green peas (https://www.ssrc.org.uk/wp-content/uploads/2013/07/NCBE_PeaDNA.pdf or https://learn.genetics.utah.edu/content/labs/extraction/howto/).

Materials
- Green nontoxic DNA gel stain (such as SYBR Safe, dsGreen, or a comparable product)
- 1–2% agarose gel with electrophoretically separated DNA molecules (see Resources above)
- Fluorescence illuminator with green filter set

Procedure
The agarose gel must be stained with the DNA gel stain (either before or after electrophoretic separation; follow manufacturer’s instructions). Figure 5. DNA gel imaging with SYBR Safe (Activity 3). Gel image of DNA ladders (L; New England Biolabs 1 kb plus) and PCR products (1.2 kbp, lanes 1–9, and 500 bp, lanes 10–12) loaded on a 1.5% TAE-agarose gel made with 1X SYBR Safe stain. Imaging was performed with an iPhone 8 Plus using green-fluorescence filters (excitation 470 ± 5 nm, emission >550 nm).
instructions or electrophoresis protocols) before imaging. Darken the room or a part of the room and produce an image using the fluorescence illuminator and a smartphone. An example of a SYBR Safe-stained gel image is shown in Figure 5.

**Discussing the Science**

To visualize DNA, it must be specifically stained, because DNA in a gel is invisible to the naked eye. A specific fluorescent DNA stain can be very sensitive, because when fluorescently illuminated, the stained DNA will emit light. Typically, DNA stains specifically bind to DNA by intercalation – they have ring structures that insert themselves between adjacent nucleotide bases. Molecules that can intercalate into DNA can also cause mutations in living cells, which is why some DNA stains, like ethidium bromide, are also considered mutagenic agents and require special handling (e.g., gloves). New “safe” formulations, such as SYBR Safe, are considered to be safer on the basis of toxicity testing in animals, but they likely stain DNA via a similar mechanism. Another advantage of dyes that are excited by blue rather than UV light is that eye protection is not necessary when imaging agarose gels.

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