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Identification of Unknown Fluorescent Proteins: A Colorful Lab Module for Teaching Gene-to-Protein Information Flow and Protein Structure

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Fluorescent proteins impart specific colors to coelenterate marine organisms such as jellyfish and corals. Green fluorescent protein (GFP; from the jellyfish *Aequorea victoria*) and DsRed (from the coral *Discosoma sp*) have been adapted for use as fluorescent tags for the real-time imaging of proteins in living cells, leading to a revolution in cell biology research. These proteins also make fun and engaging tools for teaching biology and biochemistry. Here we describe a lab module in which students identify unknown fluorescent proteins by performing spectrophotometry and SDS-PAGE. Students are assigned an unknown gene from a panel of 12 cDNAs encoding various fluorescent proteins derived from GFP or DsRed. These fluorescent proteins differ in their fluorescence properties and the degree to which they aggregate to form higher order complexes. The students receive the genes in a bacterial expression vector and proceed to express the fluorescent proteins in *Escherichia coli* (*E. coli*), followed by rapid purification via a poly-histidine tag added by the vector. Three pieces of evidence allow for identification of the unknown. The color of the bacterial colonies allows students to determine if their protein is a derivative of GFP or DsRed. The absorption spectrum of the fluorescent protein is measured on a spectrophotometer, allowing unknowns from the same parent protein to be identified by their excitation max. Some of the unknowns, however, have excitation maximums that are identical or too close to determine. These can only be distinguished by determining their subunit composition through running heated (denatures complexes) and unheated (complexes remain intact) samples on SDS-PAGE and analyzing the banding pattern of the stained gel. Correctly interpreting the gel requires students to understand how different length polypeptides are encoded on the gene, how these come together to form a dimer or tetramer, and how these would run under native and denaturing conditions.

Keywords: protein structure, biochemistry, fluorescent proteins, SDS-PAGE

Introduction

This is a multi-day lab module for the expression, purification, and identification of unknown fluorescent proteins (FPs). Each lab in the module can easily be carried out in a lab classroom equipped to do molecular biology experiments. Student groups receive one or more genes encoding the unknown FPs. Following expression and purification of the proteins from *E. coli*, the FPs are analyzed by spectrophotometry and a modified version of SDS-PAGE to identify the unknowns. The experiments and analysis of the resulting data support the learning of gene to protein information flow, absorption and fluorescence, and the primary and quaternary levels of protein structure. The protein structure learning goal is tied into interpreting the electrophoretic banding patterns from protein complexes versus single-polypeptide proteins under denaturing and non-denaturing conditions.

What are Fluorescent Proteins?

Fluorescent proteins (FPs) are produced in a number of coelenterate marine organisms such as jellyfish and corals, giving these organisms vibrant colors. The fluorescence is sometimes coupled to bioluminescence, as is the case with green fluorescent protein (GFP) produced by the jellyfish *Aequorea victoria*. GFP, along with a chemiluminescent enzyme called aequorin, imparts a green glow to the rim of the hood of this jellyfish.

Fluorescence requires a source of light to excite the fluorophore (the light-emitting chemical groups) before the fluorescent light of a particular color can be emitted. Other organisms rely on sunlight to excite their FPs and produce their unique colors.

The gene for GFP was cloned in 1992, allowing the protein be genetically manipulated and expressed *in vitro* (Prasher *et al*., 1992). Point mutations to the GFP coding sequence have produced amino acid substitutions that have increased the brightness of its fluorescence and produced variations in color (Tsien, 1998). These GFP derivatives, along with FPs created from other fluorescent proteins such as DsRed, have been rapidly adopted as tools for imaging proteins in living cells. By adding an FP tag to a protein of interest, that protein's cellular localization, trafficking, and interaction with other proteins can visualized real-time, providing a research tool that has revolutionized the biological sciences (Lippincott-Schwartz and Patterson, 2003).

Fluorescent proteins have a characteristic beta-barrel protein structure. FP polypeptides are around 230 amino acids in length, and fold into a beta-pleated sheet secondary structure. The beta-strands then wrap around a central alpha-helical segment of the polypeptide to form a barrel-like conformation (Tsien, 1998). The alpha-helical segment in the center of the barrel contains the fluorophore, consisting of three amino acids which undergo a non-catalyzed rearrangement to form a unique double ring structure that absorbs and emits light. GFP from *Aequorea victoria* is a single beta-barrel. The red fluorescent protein DsRed, from the coral *Discosoma*, is a tetramer consisting of four identical beta-barrel subunits (Baird *et al*., 2000).

Summary of Laboratory Module

This module uses a panel of cDNAs in bacterial expression vector pRSET-B that encode various FPs derived from *Aequorea victoria* GFP or *Discosoma* DsRed (Table 1). The derivative proteins have amino acid substitutions that allow them to fold correctly at warmer temperatures, disaggregate multi-subunit complexes, and emit different colors of light. Three GFP derivatives include EGFP ("E" for enhanced, giving brighter fluorescence), ECFP (cyan) and EYFP (yellow) (Tsien, 1998). All of the GFP derivatives are monomers, consisting of a single beta-barrel polypeptide. Amino acid substitutions to DsRed have disrupted the subunit interfaces to produce dimer (dTomato) and monomer (mRFP1) derivatives (Campbell *et. al*, 2002). From mRFP1, a variety of different color derivatives of monomer red proteins have been produced (Shaner *et. al*, 2004).

Two of the FPs in the panel are tandem dimers (td prefix). These are not true dimers, but are created by joining the ends of two single beta-barrel polypeptides by splicing together the gene coding sequences. The resulting polypeptide is twice as long, with either end folding into a separate beta barrel. tdTomato is a single polypeptide of two identical red protein beta barrels. tdGO was created by fusing the polypeptides for EGFP and mOrange.

Table 1. The fluorescent protein panel.

Parent protein	Fluorescent protein	Subunit composition	Excitation max	Emission max
GFP	EGFP	monomer	488 nm	507 nm
	ECFP	monomer	434 nm (452 nm)	476 nm (505 nm)
	EYFP	monomer	514 nm	527 nm
DsRed	DsRed	tetramer	558 nm	584 nm
	dTomato	dimer	554 nm	581 nm
	tdTomato	tandem dimer	554 nm	581 nm
	mRFP1	monomer	584 nm	607 nm
	mOrange	monomer	548 nm	562 nm
	mGrape	monomer	595 nm	620 nm
	mStrawberry	monomer	574 nm	596 nm
	mCherry	monomer	587 nm	610 nm
Green-Red Hybrid	tdGO	tandem dimer	488 nm, 548 nm	507 nm, 562 nm

The individual lab sessions and activities that make up this module are described in Table 2. In the long version of the module, students receive *E. coli* cultures carrying plasmids with their unknown FP genes and extract the plasmid DNA. Following restriction analysis of the plasmids, they are transformed into *E. coli* strain JM109-DE3, which expresses the T7 RNA polymerase necessary to drive transcription of the FP genes from the pRSET vector. In the following lab session, the students are able to observe the colors of their FPs in the bacterial colonies on their transformation plates. The bacterial colonies are then scraped from the plates and the FPs are purified via a polyhistidine tag that is added by the vector. In the same lab session, the students perform spectrophotometry to measure the absorption spectra for their unknown FPs. In the next lab session, FPs are analyzed by carrying out SDS-PAGE with heated and unheated samples. The gels are Coomassie-stained and from the banding pattern, the students are able to determine the subunit composition (i.e., monomer, dimer, tandem dimer, or tetramer) of their unknown FPs. A shorter version of the module can be run in which students are provided with plasmid DNA for their unknowns, thus eliminating the plasmid prep and restriction analysis labs.

Three pieces of evidence allow the students to identify their unknown FPs. The first is the color of the bacterial colonies on their transformation plates. The DsRed derivatives will appear as a variety of shades of red, pink, and purple. The colonies with GFP derivatives will appear as a drab yellow as room lighting does not produce the shorter wavelengths required to excite the GFP fluorophores. However, by illuminating the colonies with a hand-held UV lamp, students are able to observe the green-hued fluorescence from EGFP, ECFP, and EYFP. Visual assessment of the colony colors is typically only precise enough allow students to categorize their unknowns as either GFP or DsRed derivatives. Determining the excitation max values from the absorption spectra allows for the precise identification of many of the unknowns (see Table 1). However, the excitation max values for some of the FPs are identical or too close to make an accurate determination. Determination of the subunit composition from the SDS-PAGE analysis is required to identify these FPs. Student lab groups are typically assigned one "easy" FP that can be identified by its excitation max, and one "difficult" FP that requires the SDS-PAGE to identify.

The SDS-PAGE analysis allows determination of the unknown subunit composition by comparing the band migrations for side-by-side samples of the same FP that were either heated (standard SDS-PAGE protocol) or were left unheated. Heating denatures the complexes and the individual subunits migrate to the molecular weight (MW) of the monomer polypeptides. When left unheated, the complexes remain as intact dimers and tetramers and migrate to that MW. Students are either told the MW position to which the monomer FPs migrate (e.g., mRFP1), or can deduce this from FPs already identified by their excitation max. The individual subunits from all the FPs (except tandem dimers) are roughly the same length polypeptide and will migrate to a consistent position in the gel (Figure 2). Monomer FPs show only a small difference in band position between heated and unheated samples due to denaturing of the beta-barrel. Any bands migrating to this range, approximately 30–40 kDa, can be concluded to represent single polypeptides. In order to identify the subunit composition of their FPs, students need to reason that when heated and denatured, the individual subunits of the dimer and tetramer proteins will migrate as free polypeptides to the monomer position. And that when left unheated, the FP complexes will remain intact and migrate more slowly to a position significantly further up in the gel. To distinguish dimers from tetramers, students must compare the unheated band position for their unknown FP (relative to MW markers) to the unheated band position that other student groups obtained when running their unknown dimers or tetramers. By sharing information and discussing their data between groups, they are able to figure out the relative band positions for the unheated dimers and tetramers. The tandem dimer polypeptides are just over twice the length of the beta-barrel subunits for the other FPs. They show little difference in migration between heated and unheated samples, but migrate to a position significantly above the monomer range, and are consistent with the migration of the unheated dimers.

Table 2. Outline of the laboratory module.

Activity	**Long version**	**Short version**	**Approximate time required**
Receive FP genes in *E. coli* DH5-α and plasmid preps	day 1		3 hours
Restriction digests and agarose gel electrophoresis	day 2		3 hours
Transform FP plasmids into *E. coli* JM109-DE3	day 3	day 1	3 hours
Purify fluorescent proteins and determine absorption spectrum	day 4	day 2	4 hours
Run samples on SDS-PAGE	day 5	day 3	3 hours
Examine stained SDS-PAGE gels	day 6	day 4	1 hour

The students enjoy this exercise because it allows them to apply their understanding of protein structure to interpret their SDS-PAGE results, and thus identify their unknowns. It requires them to do some critical thinking to interpret what the banding pattern on the gels means in terms of how the individual beta-barrel polypeptides and complexes will migrate under heated and unheated conditions.

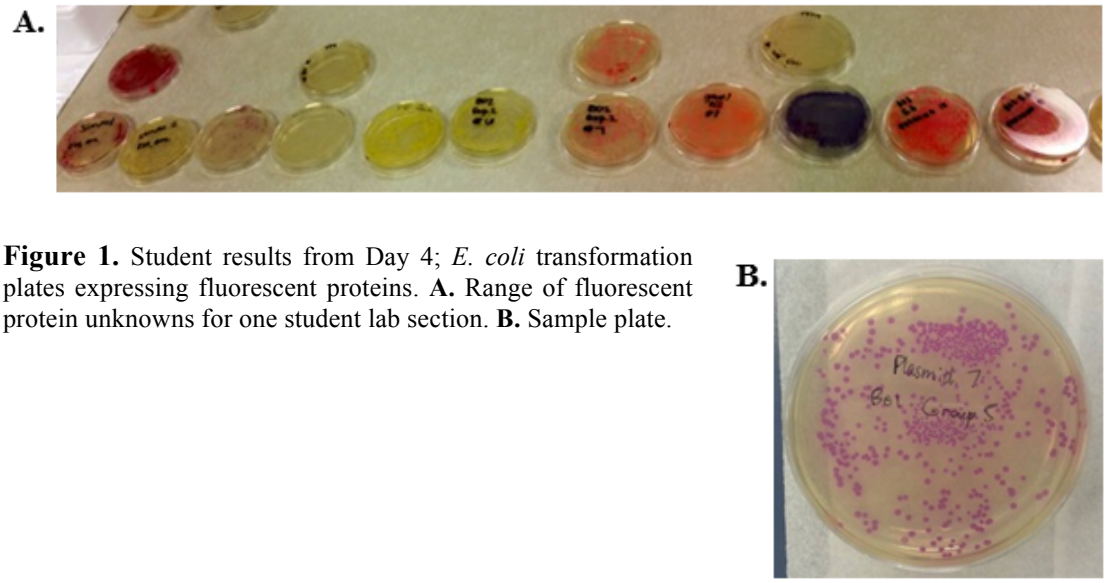

Figure 1. Student results from Day 4; *E. coli* transformation plates expressing fluorescent proteins. **A.** Range of fluorescent protein unknowns for one student lab section. **B.** Sample plate.

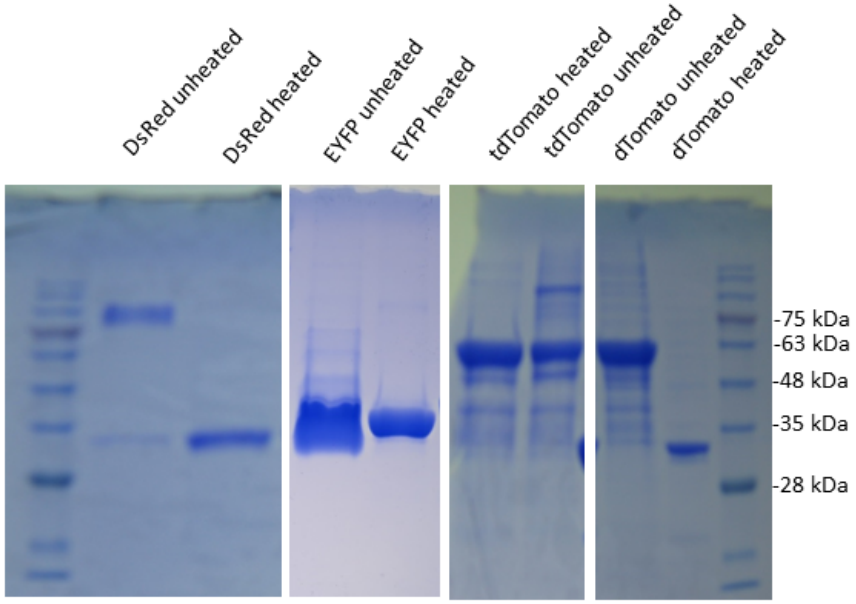

Figure 2. Student data from Day 6; SDS-PAGE of various fluorescent proteins under denaturing and non-denaturing conditions. Bands migrating between the 48 and 28 kDa size standards are consistent with the migration of monomers, although some variation inside this range may occur from denaturation of the beta-barrel.

Student Outline

Note: This outline contains the student introduction to the module, and the detailed background and procedure for the FP Purification and Spectrophotometry (day 4, long version) and SDS-PAGE (day 5) labs. The "Notes for the Instructor" section below contains a brief description of the activities for the other lab days, and the detailed protocol for preparing competent *E. coli* and transformation (day 3) is provided in Appendix A.

Introduction to the Module—Producing Recombinant Proteins in Bacterial Cells

In this project, you will express the genes for fluorescent proteins in bacterial cells, and then purify the proteins from the cells. By doing this, you will learn how foreign proteins can be expressed in a host organism (the *E. coli* bacterial cells) in order to more easily obtain large quantities of the protein. Proteins produced in this manner are referred to as recombinant proteins because the gene for the protein has been inserted into a plasmid vector that is taken up and carried by the host organism. Thus, the word recombinant refers to recombination of the gene encoding the protein, not the protein itself. It is typically easier to purify the proteins from bacterial cells than it is from the organism and tissue in which it is naturally produced. Large amounts of recombinant proteins can be expressed and purified from bacterial cells for research, medical, and commercial use. Insulin, used to treat patients with diabetes, is produced as a recombinant protein.

You will be assigned two unknown fluorescent proteins that you will have to identify. In Lab 1, each group will be given two different cultures of the bacterium *E. coli* strain DH5α that contain a plasmid with the gene for a fluorescent protein. Strain DH5α is used to produce large amounts of the plasmid but is unable to express the fluorescent protein because it lacks the gene for T7 RNA polymerase. In Lab 1, you will lyse the DH5α bacteria and purify the plasmid DNA. In Lab 2, you will learn how to digest a plasmid with restriction enzymes and analyze the DNA fragments by agarose gel electrophoresis. In Lab 3, you will transfer the plasmid DNA into a different *E. coli* strain (JM109-DE3) that has the gene for T7 polymerase. The bacteria with the plasmid will be plated out to grow and synthesize the proteins. In Lab 4, the proteins will be purified, and their absorption spectra measured. In Labs 5 and 6, you will further analyze the proteins by polyacrylamide gel electrophoresis.

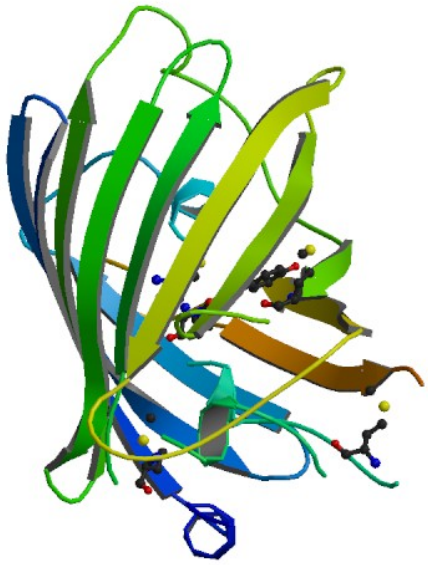

Figure 3. Beta-barrel protein conformation of GFP. The fluorophore is shown as the ball and stick structure in the center of the barrel. Image from the RCSB PDB (www.rcsb.org) of PDB ID 1EMA (Ormo *et al*., 1996).

Background on Fluorescent Proteins

Fluorescent proteins (FPs) are found in light and color-producing cells of many coelenterates (e.g., jellyfish and corals). Fluorescence is the process by which light is absorbed by a molecule and then reemitted at a longer wavelength, producing a particular color. The function of FPs in these marine organisms remains an intriguing mystery. When excited by light, the proteins fluoresce without requiring ATP or any other cofactor. A fluorophore is defined as the chemical structure in a fluorescent molecule that absorbs and emits the photons of light (the term chromophore is sometimes also used). In FPs, the fluorophore is typically a double ring structure formed by three amino acids. It is enclosed in a barrel formed by 11 strands of beta-pleated sheet, with additional amino acid sequence closing the top and bottom. For this reason, the structure is described as a beta barrel.

FPs are of great interest because they can be used as "molecular tools" to carry out experiments. They can often be attached to other proteins without interfering with their functions. This makes it possible to label and detect molecules, cells, and organisms while they go about their normal activities. Currently, there is an explosion of knowledge in areas such as cell and developmental biology, neurosciences, and ecology where FPs are making important contributions. FPs are also being used for screening drugs, evaluating viral vectors for human gene therapy, biological pest control, and monitoring genetically-altered microbes in the environment, among other applied efforts.

You will receive two unknowns from a panel of 12 different fluorescent proteins (Table 3). All of these were derived from one of two naturally-occurring "parent" proteins, either green fluorescent protein or DsRed. The derivative FPs were created by introducing point mutations into the genes on which they are encoded. These point mutations produce specific changes in the amino acid sequence, referred to as **amino acid substitutions**. By changing the amino acid sequence of a protein, you can change its biochemical properties and alter its function. Green fluorescent protein (GFP) is produced by the jellyfish *Aequorea Victoria*. The GFP protein is composed of a single beta-barrel polypeptide. EGFP, EYFP, and ECFP were derived from the wild-type GFP. The amino acid substitutions in EGFP increased ("E" is for enhanced) the fluorescent light output. The color of the emitted light was changed in ECFP (cyan) and EYFP (yellow).

We also have nine different red fluorescent proteins. The wild-type red fluorescent protein, DsRed, is from the coral, *Discosoma*. DsRed is a bulky tetramer formed from four identical beta-barrel subunits. dTomato is a dimer (two subunits), and mRFP1 is a monomer (single subunit form with mutations that eliminate aggregation). There were 33 amino acid substitutions that were needed to derive mRFP1, the first functional monomer red protein from DsRed. mRFP1 was further modified to produce mOrange, mStrawberry, mGrape, and the other red FPs. The article by Shaner *et al*. provides a flowchart detailing the lineage for how the different red fluorescent proteins were derived from DsRed (2004). The "td" prefix indicates those proteins are tandem dimers. These are not true dimers, but are created by joining the ends of two single beta-barrel polypeptides by splicing together the gene coding sequences. The resulting polypeptide is twice as long, with either end folding into a separate beta barrel. tdTomato is a single polypeptide of two identical red-protein beta barrels. tdGO was created by fusing the polypeptides for one green and one red protein.

Table 3. Panel of 12 different fluorescent proteins.

Parent protein	Fluorescent protein	Subunit composition	Excitation max	Emission max
GFP	EGFP	monomer	488 nm	507 nm
	ECFP	monomer	434 nm (452 nm)	476 nm (505 nm)
	EYFP	monomer	514 nm	527 nm
DsRed	DsRed	tetramer	558 nm	584 nm
	dTomato	dimer	554 nm	581 nm
	tdTomato	tandem dimer	554 nm	581 nm
	mRFP1	monomer	584 nm	607 nm
	mOrange	monomer	548 nm	562 nm
	mGrape	monomer	595 nm	620 nm
	mStrawberry	monomer	574 nm	596 nm
	mCherry	monomer	587 nm	610 nm
Green-Red Hybrid	tdGO	tandem dimer	?	?

Procedures

A. Visualization of Fluorescent Proteins in Bacterial Colonies Following Transformation—Lab 4

Now that the JM109-DE3 cells have expressed the FP genes carried in the pRSET-B vector, you will be able to see the proteins from the colors they produce in the bacterial cells. A visual assessment of the color of the bacterial colonies is the first piece of data you have to determine the identity of your unknowns. The absorption spectrum of the purified FP will be your second piece of data. In the next lab, you will run your purified FPs on an SDS-PAGE gel to observe their MW under heated and unheated conditions, giving you your last piece of evidence. From these data, you should be able to determine the identity of your two unknown FPs.

Take a moment to look at all the plates in your section to see the range of colors that are produced. You should be able to distinguish the GFP derivatives from the DsRed derivatives by eye. To see the true colors of the GFP proteins, you will need to shine a handheld UV-lamp on the colonies. Place all of the GFP protein plates in a dark corner of the lab, remove the lids, and shine the UV lamp directly onto the colonies. Can you tell which is EGFP, EYFP, and ECFP? In your notebook, record the color of the colonies on your two plates and give a rough estimate of the number of colonies.

B. Purification of Polyhistidine-Tagged Fluorescent Proteins Using NTA-Nickel Resin

The pRSET vector adds a polyhistidine tag (six histidine amino acids in a row) to the N-terminus of the fluorescent proteins. This doesn't interfere with the folding of the protein and it provides a method for rapidly purifying it on a nickel affinity column. Nickel is chelated to nitrilotriacetic acid (NTA) spacer molecules attached to agarose beads. Each nickel atom has six spatially-defined coordination positions that can participate in charge interactions (similar to hydrogen bonding). Binding to the NTA fills four of the coordination positions. The side chains of two adjacent histidines (more precisely the imidazole rings of the side chains) have just the right spatial orientation needed to fill the remaining two coordination positions on the nickel. A single histidine in a protein could fill one of the coordination positions on its own, but this binding would be very weak. A single pair of histidines forms a more stable interaction, but the binding affinity would still be relatively low. However, three tandem pairs of histidines (a His-tag) can form bonds with three nickel ions that are next to each other on the same bead, and the binding affinity for this interaction is very high. Six histidine amino acids in a row are never found on naturally-occurring proteins.

The lysis and column wash buffers contain a low concentration of free imidazole (10 mM), which competes out low-affinity binding to the resin of a single or two adjacent histidines in a protein. In the elution buffer, the imidazole concentration is raised to 250 mM, high enough to compete successfully for the nickel and displace the His-tagged protein.

C. Purification Procedure—Lab 4

To purify the protein from our bacterial cells, we need to remove the cells from the plate by gently scraping off the colonies in luria broth (LB; this is a liquid bacterial growth medium), and then transferring them to a microfuge tube. You will use a bacterial cell spreader to scrape off the colonies. Dip the cell spreader in ethanol and pass it through a Bunsen burner flame to kill the bacteria when you are done using it.

1. For each plate, pipet 700 µL of LB medium onto the surface and gently scrape off bacteria with a spreader. Push the suspension together on one side of the plate and transfer it to a microfuge tube using a disposable, wide-mouth transfer pipette. Repeat with a second 700 µL-aliquot of LB and add this suspension to the first tube (~1.5-mL cell suspension). Avoid digging up and transferring pieces of agar. The LB may soak into the agar; you can add more if necessary. Remove all of the cells from one plate before beginning the second.

2. Centrifuge one minute at high speed (13,000–14,000 rpm) to pellet the bacteria.

3. Discard the supernatant into a beaker with some bleach. Estimate the size of the pellet and resuspend it in five volumes of lysis buffer. Stir with the pipet tip and pipet up and down to break up the cell pellet. Make sure the pellet is completely resuspended before proceeding.

4. Add 10 µL of 10% SDS per 500 µL of lysis buffer. **Do not exceed** this amount of SDS—if there is too much SDS, the bacteria will release their DNA and a gel will form making further separations impossible. Gently stir in the SDS with

your pipet tip. Avoid any vigorous mixing, as this will cause the SDS (a detergent) to foam, which will make your sample difficult to work with.

5. Incubate the tubes on the platform rocker for 30 minutes at room temperature. Lay them on their side so that the suspension rolls back and forth down the length of the tube.

6. Centrifuge the lysate at high speed for one minute to pellet the debris. Transfer the supernatant to a fresh tube. Save the pellet. Is the supernatant fluorescent (colored)? If not, add 50% more SDS and gently stir with the pipet tip to mix and resuspend the pellet, then repeat from step 5.

7. Save 10 µL of each of the lysate supernatants to run on the gel in the next lab. Label it "crude lysate." Put each aliquot in a well-labeled microfuge tube and store it in the freezer.

8. Cut the end off a pipet tip (remove about 0.25 cm) so it is wide enough to take up the beads of the Ni-NTA resin. Mix the resin slurry well before pipetting, and add 50 µL to each tube of supernatant.

9. Incubate on the rocker table 30 minutes at room temperature.

10. During this incubation, begin re-extracting your left over bacterial cell pellet from step 6. Add 100 µL lysis buffer and 2 µL of SDS, and stir to mix and resuspend the pellet. Place this on the rocker. This will give you some additional extracted FPs to help in obtaining a strong absorption spectrum if you do not get enough from the nickel-resin purification.

11. After the 30-minute incubation of the lysate supernatant with the resin, centrifuge at 5,000 rpm for 20 seconds to pellet the resin. Remove the supernatant. Save 20 µL of the supernatant (label it "unbound lysate") to run on gel in the next lab, and then discard the rest.

12. Wash the pellet (resin + bound protein) by adding 100 µL of lysis buffer (without SDS). Stir with the pipet tip to mix well. Centrifuge at 5,000 rpm to pellet the resin. Discard the supernatant.

13. Elute the protein from the resin by adding 30 µL of elution buffer (lysis buffer plus 250 mM imidazole). Stir with the pipette tip and mix on the rocker for 5 minutes. Centrifuge to pellet the resin. Remove the supernatant and save it in a clean tube labeled "elution 1." You will use this in part D.

14. Elute each FP again with another 30 µL of elution buffer. Mix on the rocker for 5 minutes, centrifuge to pellet the resin, and remove each supernatant to separate, clean tubes labeled "elution 2." Store these at room temperature for the SDS-PAGE gel in Lab 5.

D. Collect Excitation and Emission Spectra—Lab 4

Fluorescent proteins absorb and emit light at characteristic wavelengths, based on the chemical structure of their fluorophore. You will now determine the excitation (absorption) spectra for your unknown proteins. Compare the excitation max values for the two spectra to the known values for each fluorescent protein given in Table 3. This is your best evidence yet as to the identity of your unknown proteins!

1. For each of your unknown proteins, place 1 mL of deionized water in a cuvette and add all 30 µL of elution 1. Cover with Parafilm and invert to mix.

2. Measure the absorbance of your fluorescent proteins from 400 to 700 nm using a scanning spectrophotometer. Use a scan interval of not greater than 5 nm (a 1–2 nm is preferable to get better resolution of the absorption peaks). Collect a baseline (blank the instrument) with water plus 30 µL elution buffer, then collect the excitation spectra for your two unknowns.

3. **Optional** If you do not get a strong absorption peak for one or both of your proteins, you may need to add more protein from your re-extracted cell pellet (step 10 in part C). To do this, centrifuge for 1 minute at high speed, remove the supernatant, and add all of it into the cuvette with your elution 1. Do not dump out elution 1, the idea is to add more protein to what you already have.

Examine your data to determine the identity of your unknowns. The λ_{max} values for some of the fluorescent proteins are very close together and it may be difficult to discriminate between them. The SDS-PAGE gel you will run in the next lab will help you to determine some of these. For mRFP1 and mCherry, the excitation max values are only 3 nm apart, and these will be difficult to discriminate unless you have very strong peaks. An overlay of the excitation spectra for both proteins is shown in the Shaner *et al*. article (2004). Examining this overlay of the excitation spectra will provide a useful clue as to which of the two proteins you may have.

E. SDS-PAGE of Fluorescent Proteins—Labs 5 and 6

In today's lab, you will run your elution 2 samples from Lab 4 on an SDS-PAGE gel to separate all the proteins present in the sample by MW. In this experiment, you will deviate from the standard SDS-PAGE procedure in that you will heat half of your elution 2 sample and leave the remaining half unheated. Remember that heating the sample is necessary to denature the proteins before running them on the gel, and this breaks apart any protein complexes allowing everything to run as a single polypeptide. The heating will separate potential dTomato dimers and DsRed tetramers into their constituent monomer subunits. In the unheated samples, the subunits of these proteins will remain together, and these proteins will run on the SDS-PAGE at the higher MW of the dimer and tetramer.

Running the Gel

We will be using pre-cast 12% polyacrylamide gels for this electrophoresis. Each group will run their own SDS-PAGE gel. For each fluorescent protein, you will run the following samples on the gel (therefore, including the size standards, you will run nine lanes on the gel):

a. FP-heated;
b. FP-not heated;
c. the crude lysate from the purification in part C; and
d. the unbound lysate from the purification in part C.

1. Prepare 600 mL of 1X electrophoresis buffer.

2. Secure the gel in the electrophoresis tank and add the buffer. Fill the inner chamber almost to the top.

3. Remove the comb and use a Pasteur pipette to rinse out the wells.

4. Obtain your elution 2 samples from the previous day. Measure the volume of each sample (you should have about 30 µL) and divide it evenly in two, placing each half in a clean tube. Prepare each tube as a separate sample by adding 4 µL of 5X sample buffer. Add water, if necessary, to a final loading volume of 20 µL.

5. Take one tube for each FP, and heat at 95°C for 10 minutes. Keep the other tube at room temperature; it will not be heated.

6. For both the crude lysate and unbound lysate, take 16 µL of each (or as much as you have and add H_2O to 16 µL) and place in separate tubes with 4 µL of 5X sample buffer. Mix gently. Heat these tubes for 10 minutes at 95°C.

7. After heating, cool the tubes briefly on ice (not too long or the SDS will precipitate), and then spin the tubes for 30 seconds at full speed in a microfuge to bring down any condensation.

8. Load 5 µL of the molecular weight standards into one well. Don't forget to load your gel near a power supply so that you will not have to move it once it is loaded.

9. Load all 20 µL of each of the other samples.

10. Put the cover on the tank, connect the leads to the power supply and run the gel at 120 V for about an hour, or as much time as is necessary for the blue dye front to move all the way to the bottom of the gel. Always check that there is current (look for bubbles coming off of the electrodes). If not, something is wrong; most likely the level of buffer in the upper reservoir has fallen below the top of the short inner plate. If this happens, disconnect your gel from the power supply, add more buffer.

Staining the Gel

1. When the run is finished, turn off the power supply, pour off the buffer, and remove the gel cassette.
2. Carefully pry off one of the plates (TA will demonstrate).
3. Look for fluorescence in your gel (it is easier to see with one of the plates removed).
4. Obtain a staining dish and label it with your group number. Pour 50 mL of Coomassie staining solution into the dish.
5. Carefully lift the gel off of the other plate and place it into the staining dish. Cover with plastic wrap and leave it on the rotator to stain overnight.

Materials

This module requires a teaching laboratory with facilities for carrying out basic molecular biology protocols including culturing *E. coli.* It also requires equipment for SDS-PAGE as described below. The specific materials required for Days 4 and 5 are described here. To obtain the plasmid panel, contact the author at abcoleman@ucsd.edu.

Day 4 – Purify Fluorescent Proteins and Determine Absorption Spectrum

Equipment

1. Rocking platform (preferred) or orbital shaker, one per class of 24 students
2. Hand-held UV lamps, two per class of 24 students
3. Microcentrifuges, one per student group
4. Spectrophotometer with scanning capability between 400–700 nm, one or two per class of 24 students. It is possible to use a spectrophotometer without scanning ability and to have the students make individual absorbance measurements over the desired range of wave lengths, but additional time should be allowed for this.
5. Bacterial culture cell spreaders, one per student group
6. Bunsen burners, one per group
7. Micropipettes and tips.

Consumable Materials and Reagents

Volumes provided are more than sufficient for a class of 24 students.

1. Lysis buffer, 100 mL: 50 mM monobasic sodium phosphate, 300 mM NaCl, 10 mM imidazole. Adjust pH to 8.0 with NaOH.
2. 10% SDS (sodium dodecyl sulfate), 10 mL
3. NTA-Ni agarose resin, 1 mL: Pre-charged slurry from Qiagen, catalog number 30210
4. Elution buffer, 25 mL: Lysis buffer (no SDS) plus 250 mM imidazole.
5. Luria broth (LB), 200 mL: Per liter, 10 g casein peptone, 5 g yeast extract, 10 g NaCl, 1.5 g Tris/Tris-HCl, pH 7.5. Phosphate buffered saline (PBS) may be substituted for LB if it is more readily available.
6. Ethanol, 300 mL
7. Wide-mouth disposable plastic transfer pipettes, three per student group

Day 5 – Run Samples on SDS-PAGE

Equipment

1. Mini-vertical electrophoresis gel apparatus, one per student group
2. Electrophoresis power supplies, enough to run the number vertical gel units used
3. Rocking platform (preferred) or orbital shaker, one per class of 24 students
4. Heating block, capable of 95°C, one per class of 24 students
5. Micropipettes and tips

Consumable Materials and Reagents

1. Precast tris-glycine 12% polyacrylamide gels for SDS-PAGE, one per student group. Precast gels are recommended. If students cast their own gels an additional lab day should be provided for this.
2. 10X electrophoresis buffer, 60 mL per student group (students dilute to 1X before using): 0.25 M Trizma base (30.275 g/L), pH 8.3; 1.92 M glycine (144.13 g/L); 1% SDS (10 g/L); pH should be approximately 8.3, do not adjust with NaOH or HCl.
3. 5X sample buffer, 2 mL per class of 24 students: 0.375 M Tris, pH 6.8; 45% glycerol; 25% 2-mercaptoethanol (hazardous, requires fume hood); 11.25% SDS; 0.023% bromophenol blue.
4. Gel Staining Solution, 50 mL per student group: 40% methanol; 0.2% Coomassie Brilliant Blue; 10% acetic acid; 50% dH_2O; dissolve Coomassie Blue in methanol first, then add acetic acid and H_2O.
5. Destain Solution, 100 mL per student group: 40% methanol; 10% acetic acid; 50% dH_2O.
6. Molecular weight markers, 5 µL per student group, AccuRuler Prestained Protein Ladder, LambdaBio catalog number G02101. Any markers with bands that span 100–10 kDa can be used.

Notes for the Instructor

To establish permanent stocks of the plasmids, they should be transformed into an *E. coli* strain the does not produce T7 RNA polymerase such as DH5-α. Glycerol stocks of cultures carrying each plasmid should be prepared and stored at -70°C. Stocks of the plasmid DNA should also be prepared for each, and there are several commercially available plasmid DNA extraction kits that are the most convenient method for doing this (e.g., Qiagen Plasmid Plus Midi Kit, catalog number 12943). Explanation and protocols for all of the required plasmid manipulation procedures are beyond the scope of this manuscript, however there are many good molecular

biology protocol manuals that provide this information (Ausubel, 2007).

Both the long and short versions of the module have certain advantages and disadvantages that should be considered. The long version provides students with the opportunity to purify their own plasmid DNA for transformation. This allows additional molecular biology techniques to be taught, and provides a better context for understanding gene to protein information flow. However, while transformations with student prepared plasmid have a high success rate, some student groups may not have a sufficient number of colonies on their transformation plates to purify enough fluorescent protein to do the analysis. Some of this can be remedied by providing instructor-prepared plasmid to student groups who appear to have poor plasmid quality or concentration based on their restriction digest gels in day 2. Somewhat better results though, in terms of the number of colonies on their transformation plates and the amount of fluorescent protein that can be purified, are obtained from the short version of the project where students are provided with the plasmid.

A brief description of the activities for the lab days that were not covered in the student outline is provided here, along with some additional technical information.

Day 1: Cultures of *E. coli* DH5-α carrying the different FP plamids are prepared in LB + 50 µg/mL ampicillin. Student groups receive 1.5 mL of culture and the plasmid DNA is extracted with a standard mini-prep procedure. The DNA concentration is quantified by absorbance at 260 nm. Spin column-based mini-preps will allow for isolation of better quality plasmid DNA, however a simple alkaline lysis followed by precipitation with isopropanol can also be used.

Day 2: The students perform restriction digests on the plasmid DNA with *Bam*HI and *Hind*III. Most of the cDNAs are cloned into the *Bam*HI and *Eco*RI sites of pRSET-B. This yields an approximately 0.75 kb-insert fragment (1.5 kb for the tandem dimer constructs) and a 2.9 kb-vector fragment. The DNA fragments are separated on 1% agarose gels with 1 kb ladder (New England Biolabs –N3232) size standards. The DNA fragments are visualized with a 1:10,000 dilution of SYBR safe added to the gel only.

Day 3: The students prepare competent *E. coli* JM109-DE3, and then transform these cells with the plasmids. If the students are using plasmid DNA that they have prepared it is recommended that they use a relatively large amount of DNA for the transformation (500 ng or more) to compensate for poor DNA quality and errors in quantifying the concentration. If instructor-prepared plasmid DNA is used, then 100 ng per plasmid will provide ample colonies. Transformed cells are streaked onto LB + amp plates and incubated at 30°C for two days. The lower temperature incubation is essential for folding of some of the FPs. Following the two-day incubation, the transformation plates may be stored at room temperature for up to a week. We have found that additional incubation time at room temperature actually enhances protein expression. Full procedures for the preparation of competent cells and transformations are provided in the appendix A.

Day 4: Full procedure in Student Outline. Imidazole is an irritant and can cause burns. Students should wear gloves when handling the lysis buffer and elution buffer.

Day 5: The full procedure for setting up and running the SDS-PAGE is provided in the student outline. The student should wear gloves and lab coats when handling the polyacrylamide gels. The students place the gels into the Coomassie stain following the electrophoresis. The gels should be allowed to stain overnight. The gels should be destained by placing them through two washes with the destain solution, 50 mL each per gel. The first wash should go for a few hours, followed by overnight in the second wash. The gels are stable in the destain for a week or more.

The crude lysate and unbound lysate samples that were taken during the FP purification in Day 4 provide controls for the effectiveness of the His-tag/nickel resin purification. Because of the high expression levels achieved by the vector, the FP band will be the most prominent band in the crude lysate sample, although bands for bacterial proteins will also be present. The FP band intensity should be much reduced or not visible in the unbound lysate sample if the FPs successfully bound to the nickel resin, and bacterial bands will be present.

Day 6: The students examine the stained gels. It is recommended that the gels be transferred from the destain solution into deionized water before being viewed by the students.

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About the Author

Aaron Coleman obtained his Ph.D. from the University of California, Riverside for his dissertation work on the role of fibroblast growth factor (FGF) in the early development of melanoma. He continued studying FGF in his post-doctoral research at the Beckman Research Institute at City of Hope National Medical Center, where he examined how FGF-initiated signal transduction pathways may modulate the sensitivity of tumor cells to chemotherapy. Aaron is now an Associate Teaching Professor at the University of California, San Diego, where he teaches biochemistry lecture and laboratory courses. His current research interests are centered on biology laboratory education. Current studies include assessing how varying degrees of inquiry in biochemistry lab modules affect student outcomes, and measuring the impact of undergraduate biology curricula on the development of science identity.

Appendix A
Fast Protocols for Competent *E. coli* and Transformation

Prepare Log-Phase Cells (done by instructor before class)

The day before the students will do the transformations, inoculate 5 mL of LB (no ampicillin) with *E. coli* JM109-DE3. Grow overnight at 37°C with agitation. The morning of the transformations, add the entire 5 mL overnight culture to 100 mL LB (no ampicillin) in a 500 mL-flask. Incubate at 37°C with agitation. Grow until the culture reaches a density where the absorbance at 590 nm is equal to 0.4. This usually takes about three hours, but begin checking the absorbance periodically at 1.5 hours.

Making Competent Cells (done by the students)

1. Transfer 1.2 mL of the log-phase culture to a sterile microfuge tube and centrifuge for one minute at high speed.
2. Discard supernatant and re-suspend pellet in 0.6 mL ice-cold 0.1 M $CaCl_2$. Do not vortex. Re-suspend the pellet of bacterial cells by gently stirring with pipette tip. Incubate 30 minutes on ice.
3. Centrifuge 45 seconds at high speed (minimize exposure to room temperature), discard supernatant, and gently re-suspend in 120 µL ice-cold 0.1 M $CaCl_2$. Keep on ice until use.

Transformation

1. Transfer 25 µL competent cells to a fresh sterile thin-walled 0.5 mL-tube and add 100–500 ng plasmid DNA. Mix gently.
2. Incubate on ice 30 minutes.
3. Heat shock the cells in a 42°C heat block for one minute. Rapidly transfer to ice to cool.
4. Remove from ice and add 150 µL LB medium without ampicillin.
5. Incubate 30 minutes at 37°C with gentle agitation.
6. Spread onto an LB/agar plate with ampicillin (50 µg/ml). Incubate at 30°C for two days.

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