

# Fluorescence Techniques

by Tanya Schneider

In the Schepartz lab, fluorescence techniques are useful in evaluating protein•protein interactions, protein•DNA interactions, and protein folding. Both kinetic and thermodynamic measurements can be made using fluorescence. Three common fluorescence methods used to evaluate the above processes are fluorescence quenching, fluorescence resonance energy transfer (FRET), and fluorescence polarization (anisotropy). A brief discussion of each method follows.

## I. Covalent Modification of Peptides with Fluorophores

It is generally necessary to tag peptides with a fluorophore unless an intrinsic tryptophan is conveniently located for your experiment. The signal from tryptophan is typically too weak to use in polarization experiments where the polarizers reduce the intensity of signal. Fluorescein is an ideal fluorophore as it has a strong signal and is cooperative in labeling reactions. While other successful protocols exist for fluorescein labeling, the following protocol works well for me. I find that purifying a crude synthetic peptide before labeling works best – less mess on the HPLC trace. Crude labeled peptides made at Keck have been, in my experience, painful to purify.

The Molecular Probes website contains useful information about fluorophores, including a helpful (though general) protocol providing guidelines for labeling peptides with thiol-reactive probes (see <http://www.probes.com/media/pis/mp00003.pdf>).

### A. Modification of cysteine with fluorescein

Combine approximately 50 mM peptide with 500 mM 5-iodoacetamidofluorescein (Molecular Probes) in 20 mM sodium phosphate, pH 7.4. Fluorescein should be dissolved initially in DMF at a high concentration (1-10 mg/mL) to minimize the amount of DMF in the final reaction. Stir reactants at room temperature for one hour in the dark. Modified peptide can be separated crudely from free fluorescein through the use of a Nap-10 column (Pharmacia) in most cases with reasonably soluble peptides. Modified peptide can be separated from unmodified peptide or peptide dimer using HPLC. Usually the HPLC protocol used to purify the peptide originally can be used as a starting point. Modification with fluorescein often shifts the peptide to a later retention time, but be prepared for anything. Collect any HPLC peaks that have a signal at 280 nm (peptide) and 490 nm (fluorescein). Purified fractions can be run on a Phast gel to give an initial idea of whether the peptide is labeled – you can scan the unstained Phast gel on the STORM using the blue fluorescence option to detect fluorescein, and then stain the gel to detect peptides. Further confirmation through mass spectrometry is also necessary.

### B. Modification with other fluorophores

Rhodamine labeling is similar to fluorescein but more challenging. I have had success following the protocol above for fluorescein with a few modifications, mainly due to the low solubility of rhodamine in aqueous buffers (Tetramethylrhodamine-5-iodoacetamide is available from Molecular Probes.):

1. Use a lower concentration of both peptide and rhodamine in the reaction (closer to 25 mM) to avoid precipitating modified peptide, rhodamine, or both.
2. Do not use a Nap-10 column - material will get stuck in the column.

3. Use higher concentrations of DMF or other organic solvent (acetonitrile may also help) to keep rhodamine in solution if necessary.
4. If everything crashes out of solution, try separating precipitate from supernatant – the precipitate likely contains modified peptide which you may be able to redissolve in DMF and purify on HPLC.
5. All other fluorophores have proven more challenging in my experience than fluorescein, so, if possible, use that.
6. Words of caution regarding potential effects of fluorophores on peptide dimerization can be found in:  
Daugherty, D. L. and Gellman, S. H. A fluorescence assay for leucine zipper dimerization: avoiding unintended consequences of fluorophore attachment. *JACS*, (1999) **121**: 4325-4333.

### C. Other useful references

Waggoner, A. Covalent labeling of proteins and nucleic acids with fluorophores. *Methods in Enzymology*, (1995) **246**: 362-373.

Molecular Probes has some protocols supplied with their products.

## II. Fluorescence Quenching

It is possible to use fluorescence quenching to evaluate protein binding or folding if a unique fluorophore undergoes a change in environment in the binding or folding process that is reflected by a change in fluorescence signal intensity. For example, the fluorophore fluorescein quenches itself. If two protein monomers were covalently modified with fluorescein, one might be able to measure dimerization as a function of fluorescein self-quenching as the two fluorescein molecules were brought into close proximity. Similar changes in fluorescence can be seen during protein folding if a fluorophore moves from a hydrophobic to hydrophilic environment or vice versa. To execute these experiments, one would perform wavelength scans of the protein of interest over a range of desired conditions (ie, different concentrations for dimerization experiments, varying concentrations with DNA for DNA binding experiments) and measure the change in fluorescence of the fluorophore under investigation. These changes can then be plotted to give values for the equilibrium binding constant  $K_d$ .

Fluorescence quenching can be very convenient if your protein of interest has one intrinsic fluorophore (tryptophan is most useful) located in a useful position, thus removing the need to fluorescently label your protein. However, this technique has limitations. It may be difficult to ensure that fluorescence quenching is due only to the changes that you make in the system – and it may be hard to prove your case. Many other factors can lead to quenching, including photobleaching of the fluorophore, buffers, etc. A related, but perhaps more satisfying, technique is fluorescence resonance energy transfer.

For reference, measurements of dimerization and DNA binding using intrinsic tryptophan quenching were performed in:

DNA specificity enhanced by sequential binding of protein monomers. J.J. Kohler, S.J. Metallo, T.L. Schneider & A. Schepartz. *Proc. Natl. Acad. Sci. USA* (1999) **96**: 11735-11739.

## III. Fluorescence Resonance Energy Transfer (FRET)

FRET can also be used to measure protein•protein and protein•DNA binding. In contrast to a quenching experiment, FRET requires a pair of fluorophores. Here, a

donor fluorophore is excited and, when in close proximity with an appropriate acceptor fluorophore, transfers energy to the acceptor fluorophore. In this case, increased fluorescence is detected for the acceptor fluorophore and decreased fluorescence is detected for the donor fluorophore. One is able to detect binding by measuring the energy transfer that occurs when the molecules are proximal (usually donor quenching is quantified for  $K_d$  measurements). A successful donor-acceptor pair must have overlap between the emission wavelength of donor and the absorbance wavelength of the acceptor. The pair must also be able to transfer energy over the distance that you estimate to be relevant for your system. The characteristic transfer distance ( $R_0$ ) is known for common donor-acceptor pairs. Fluorescein and rhodamine are commonly used as a donor-acceptor pair due in part to the strong signal of fluorescein.

Unlike fluorescence quenching as described above, FRET gives an indication that the quenched signal of the donor fluorophore is related to an interaction with the acceptor molecule based on the acceptor signal. However, there are limitations here as well. If the donor and acceptor fluorophores are not positioned correctly, no transfer will be seen, so some thought and molecular modeling may be necessary prior to covalent modification. Also, it is important to make sure that donor quenching is not due to any factors except for the presence of the acceptor. One can perform control wavelength scans without the acceptor fluorophore to check this.

Useful references:

1. Molecular Probes catalog or website (<http://www.probes.com/>).
2. Fluorescence resonance energy transfer. P. R. Selvin. *Methods in Enzymology* (1995) **246**: 300-334.
3. Kinetic studies of Fos-Jun-DNA complex formation: DNA binding prior to dimerization. J.J. Kohler & A. Schepartz. *Biochemistry*, (2001) **40**, 130-142.
4. Hepatitis B Virus protein pX enhances monomer assembly pathway of bZIP-DNA complexes. T. L. Schneider & A. Schepartz. *Biochemistry*, (2001) **40**, 2835-2843.
5. Kinetic preference for oriented DNA binding by the yeast TATA-binding protein TBP. Y. Liu & A. Schepartz. *Biochemistry*, (2001) **40**, 6257-6266.

#### IV. Fluorescence Polarization

Fluorescence polarization measurements allow one to identify changes in the size of a complex based on the way the complex moves in solution. For example, when a fluorescently tagged DNA sequence is bound by a protein, the DNA likely will tumble more slowly in solution because the bound protein adds considerable size to the complex. This change is measured using a fluorimeter outfitted with polarizers. Polarized light is used to excite the sample, and emitted light is read in two dimensions, also through a polarizer. Polarization (P) is related to the difference between the parallel and perpendicular components of emitted light (I), when parallel excitation is used.

Anisotropy is a similar measurement, and polarization and anisotropy are mathematically related.

$$P = \frac{I_{\text{para}} - \frac{I_{\text{perp}}}{I_{\text{para}}}}{I_{\text{para}} + I_{\text{perp}}}$$

$$\text{Anisotropy} = \frac{I_{\text{para}} - \frac{I_{\text{perp}}}{I_{\text{para}}}}{I_{\text{para}} + 2I_{\text{perp}}}$$

$$\text{Anisotropy} = \frac{2P}{3 - P}$$

The positioning of the fluorophore is also important for polarization measurements, but in a different way from FRET. Here, it is necessary that the fluorophore be linked to the peptide/DNA of interest through a tether which is not very flexible. It is important that the hydrodynamic properties of the fluorophore mirror those of the labeled peptide/DNA; otherwise, your measurements will not reflect the behavior of the peptide/DNA, only the floppy fluorophore. Ideally, the fluorophore is attached to the smaller peptide/DNA sequence in the complex as complexation will then show a greater difference in polarization. It is not considered possible to extract a precise measure of the size of a complex from a polarization measurement; instead, one merely judges relative changes that suggest complexation.

#### A. Useful references (in addition to Schepartz papers):

Jameson, D. M. and Sawyer, W. H. Fluorescence anisotropy applied to biomolecular interactions. *Methods in Enzymology*, (1995) **246**: 283-300.

Hill, J. J. and Royer, C. A. Fluorescence approaches to study of protein-nucleic acid complexation. *Methods in Enzymology*, (1997) **278**: 390-416.

#### B. Useful curve fits

1. Used for dimerization curve fit in several Schepartz papers, adopted from CD  $K_d$  fit in: Zitzwitz, J. A, Bilsel, O., Luo, J., Jones, B. E., and Matthews, C. R. *Biochemistry*, (1995) **34**: 12812-12819.

$$F_{\text{app}} = \frac{\sqrt{K_{\text{dim}}^2 + (8 * K_{\text{dim}} * [A_{\text{tot}}])} - K_{\text{dim}}}{4 * [A_{\text{tot}}]}$$

where  $F_{\text{app}}$  = apparent fraction of unfolded protein at any concentration and  $A_{\text{tot}}$  is the total protein concentration, expressed in terms of the monomer.

2. Used for binding curves in several papers, often for polarization data, taken from: Heyduk, T. and Lee, J. C. *PNAS*, (1990) **87**: 1744-1748.  
This is nice because it can be derived from first principles with no assumptions.

$$P = 0.5 \left( A_T + B_T + \frac{1}{K_a} \right) \pm \frac{\sqrt{(K_a * A_T + K_a * B_T + 1)^2 - 4K_a^2 * A_T * B_T}}{2K_a}$$

where  $P$  = polarization,  $A_T$  and  $B_T$  are total protein/DNA concentration, and  $K_a$  is equilibrium association constant. Typically, either A or B would be fluorescently labeled, and the concentration of the labeled molecule would be kept constant while the other species varied over a range of concentrations.

## V. Setting Up a Binding Experiment

Buffer: 1X PBS may be fine, or your peptide may demand detergents or other components to stay in solution. Check to make sure that additions to your buffer do not greatly change the polarization of your labeled molecule alone if you're doing polarization – glycerol has a large effect which masks many polarization changes due to binding.

Equilibration time and temperature: You can check to see when binding has reached equilibrium by watching polarization or FRET or quenching with time in the fluorimeter. This will give you an idea of how long you need to wait before taking measurements. The fluorimeter currently does not have temperature control. Some people have done 4 °C measurements by incubating their binding reaction on ice prior to measurement, then adding the sample to the cuvette and measuring immediately.

Concentration of fluorophore: Having at least 25 nM fluorescein-labeled peptide or DNA seems to be optimal for polarization experiments, though less may be possible. 5 nM fluorescein was sufficient in FRET assays performed in the PTI. Rhodamine has a somewhat weaker signal which photobleaches more easily, and tryptophan is weaker yet. Trial and error may be necessary to see how much peptide is necessary to give a good signal with limited noise. Some change in signal can be brought about by varying the slitwidth of the opening from the lamp to the sample and/or to the emission photomultiplier tube (PMT).

## VI. Using the PTI Fluorimeter

The following is a guide to aid you in use of the instrument. This does **not** replace personal instruction from a lab member well acquainted with the instrument.

### A. Start-up

1. Sign in and record start time in order to keep track of lamp hours.
2. Make sure that all other components (computer, motor) are turned off, and turn on the lamp power. The lamp will ignite automatically after a few seconds. Allow the lamp to warm up for at least 15 minutes at ~60 watts. Set lamp to 70-75 watts after warm up. **\*\*Igniting the lamp with the computer on can cause damage to the computer. Also, it is better to leave the lamp on if you're only leaving the system for an hour or so - ignition is what really wears on the lamp.\*\***
3. Turn on Motor Drive Box, which powers most of the system. Check to see that the PMT digital readout is set at 1000V (max = 1100V).
4. Turn on computer. Operating software for the PTI system is Felix. In the Felix program, first choose **Configure**. Under **Hardware**, choose **Initialize** to set the monochromators. Check to see that the monochromators are actually set to the values that the computer gives after initialization.

### B. Taking measurements

Wavelength scans: useful for FRET or quenching experiments. Choose **Emission scan** under **Acquire** to bring up a relevant window. Input the desired excitation wavelength for your sample and the emission wavelengths you wish to scan. I find that the default settings for step size (1 nm) and integration time (1 sec) are generally reasonable, but can be changed as needed. Adjust slitwidth as necessary - each turn of the screw = 2 nm. I find that 8 - 10 nm is fine for fluorescein-labeled samples. Data can be saved as .txt files and imported onto a Mac using Excel.

**Polarization:** Choose **time-based scan** under **Acquire** to allow a measure of polarization over time - I generally average polarization over 30 - 60 seconds. Before each polarization experiment, it is necessary to set the G factor for the PTI. The G factor is a ratio of the relative transmission efficiencies of the emission channel for horizontal and vertically polarized light. The G factor will vary for each fluorimeter (as the lamp and polarizer set up does) and is also wavelength dependent. Setting the G factor cancels out these differences.

$$\text{G factor} = \frac{I_{hv}}{I_{hh}}$$

To measure the G factor, set both polarizers to the horizontal position (90°) ( $I_{hh}$ ). Start a time based scan which is the same as your experimental time will be. I generally collect 1 point/second for 30 seconds. Then, switch the emission polarizer to the vertical position (0°) and repeat the measurement ( $I_{hv}$ ). The G factor is simply the ratio of the two measurements as described above. To set the G factor, select the G factor curve where it is listed on the left of the screen and also highlight the scan on the screen. Choose **Polarization** under **Configure**, and click “capture” to set the G factor. For this instrument, it is usually roughly 0.7. If you are using a low concentration of labeled sample, it may also be advisable to subtract out background from your buffer by doing the same measurements with just the buffer in the cuvette. Subtract these hv and hh measurements from your sample hv and hh measurements before calculating the G factor.

Polarization measurements, as described above, are based on measuring the sample with the polarizers in two different positions; the excitation polarizer is always in the vertical position, but measurements are taken with the emission polarizer in the vertical and horizontal positions. Thus, you will collect two sets of data for each sample, and then use these values to solve for polarization. Felix will do this calculation from your two sets of data - simply choose **Polarization** under **Transform** and select the correct data sets as listed in the left hand column. Choosing **Average** under **Math** and highlighting the polarization curve allows one to measure the average polarization over the time period of the experiment. As with most experiments, at least three independent sets of data are needed.

### C. Shut down

1. Close Felix and shut down computer after saving data to disk if needed.
2. Turn off Motor Drive Box.
3. Turn lamp down below 60 watts, and then turn off.
4. Sign out and log total lamp hours.

### D. Final notes

The cuvette should be stored with distilled water in it after cleaning. Occasional careful cleaning with nitric or hydrochloric acid can be handy and often cleans up your measurements!

The shutter to the PMT on the PTI closes to protect it when the cover to the instrument is open. However, it is easy to lean on the shutter that will cause it to open and expose the PMT, leading to very noisy signal and potential damage to the PMT from overexposure to light.