Single Molecule Fluorescence Detection

Peck, K., L. Stryer, et al. (1989). "Single-molecule fluorescence detection: autocorrelation criterion and experimental realization with phycoerythrin." *Proc. Natl. Acad. Sci. U S A.* **86**(11): 4087-4091.

MODEL 1 Box diagram of a simple, single-wavelength spectrophotometer

1. Label each component in the diagram as sample, detector or source.

2. Explain in detail how you would measure the intensity of light *transmitted* by a red dye if you had a cuvette, some distilled water and a red dye dissolved in distilled water. What wavelength (range) would you use for the source in this experiment? Be specific about how you would measure the different intensities of light (I_o and I_{sample}) and how they would relate to the transmittance.

3. How might you convert the amount of light transmitted to the amount absorbed? What advantage is there to using absorbance rather than transmittance?

4. A monochromator is a wavelength selector that could be added to the instrument in Q1 to make it able to scan all wavelengths (one at a time) in the UV and visible region of the EM spectrum. Where would you place the monochromator in the box diagram?

5. The components of a monochromator include mirrors, grating and slits. How would these components be arranged to select for different wavelengths?

6. Draw a spectrum that explains the following statement about the performance of a monochromator. The narrower the slits the better resolution but the worse the sensitivity.

7. A possible problem with the instrument you constructed in Q4 is that its output is dependent on the source intensity, which may change over time. Given that transmittance is a ratio of intensities, is this a problem?

8. One method to minimize the problem of source variation is to measure a sample and a blank solution simultaneously in two cuvettes. What would such an instrument look like? We call such an instrument a double beam spectrophotometer. What are the advantages and disadvantages of such an instrument over the single beam?

9. Suppose you wanted to collect all wavelengths at rapidly as in an HPLC-DAD instrument. One way to do this is to use a detector that can simultaneously detect all wavelengths. Such a detector is called a diode array (DAD). Draw a box diagram for an instrument with a diode array detector?

10. What are some advantages and disadvantages of a DAD vs. a double beam spectrophotometer?

MODEL 2 Fluorescence Instrumentation

1. Draw a box diagram of a simple, single-wavelength fluorimeter and label each component in the diagram as sample, detector or source.

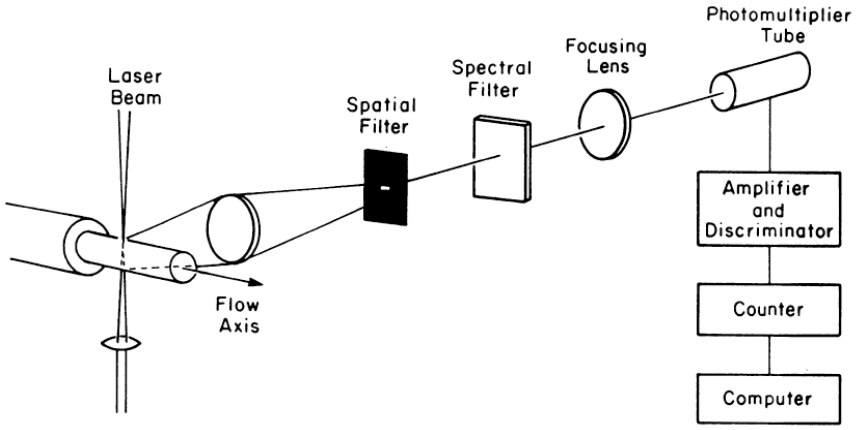
2. The fluorescence signal is given by the relation: $I = I_o \varepsilon lc$. In what important way does this differ from absorbance? What are the advantages and disadvantages due to this difference?

3. Explain how you would measure the concentration of fluorescein dissolved in a complex aqueous matrix with your instrument. How does this measurement differ from absorbance? Think of at least two advantages and disadvantages of the absorbance and emission measurements.

4. Monochromators can be added to the instrument in Q1 to make it able to scan excitation and emission wavelengths. Where would you place the emission and the excitation monochromators in the box diagram?

5. Draw an absorbance and corresponding emission spectrum. Label three points in the absorbance spectrum that "correspond" to the emission spectrum. Explain why they correspond.

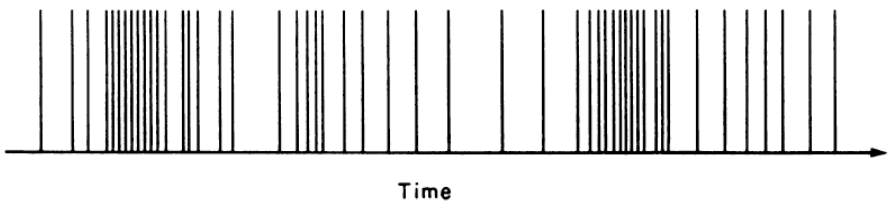
MODEL 3 Single molecule fluorescence detection instrumentation



1. The object of a high sensitivity fluorescence experiment is to maximize the amount of signal light collected while minimizing the amount of background light collected. How does each component maximize the amount of light collected or minimize the amount of background light.

2. How does a photomultiplier tube convert photons to current?

3. The diagram below shows any "count" that makes it past the amplifier and discriminator. Which counts are due to noise and which are due to signal? How do you know? What is the advantage of a counter/discriminator vs. detecting all photons?



4. The authors focus their laser to an 8μm *diameter* beam to get a probed volume of 120 femtoliter (fL). How do they arrive at this volume? What concentration solution would give a single molecule in such a volume? What is the advantage of small illumination volumes? What properties of a laser allow for this?

5. One significant source of noise is the Raman photons from water. What is the source of Raman photons and how do the authors deal with this?

MODEL 3 Abbreviated abstract

A theory for single-molecule fluorescence detection is developed and then used to analyze data from sub-picomolar solutions of β-phycoerythrin. The transit time of fluorescent molecules through the 120-femtoliter (fL) imaged volume was 800 μs. The optimal laser power (32 mW at 514.5 nm) gave an incident intensity of 1.8×10^{23} photons cm^{-2} s^{-1}, corresponding to a mean time of 1.1 ns between absorptions. The mean incremental count rate was 1.5 per 100 μs above a background count rate of 1.0. The distribution of counts for a 200 fM monomer demonstrate that single-molecule detection was achieved. At this concentration, the mean occupancy was 0.014 monomer molecules in the probed volume.

1. Given the laser power, how do the authors calculate the incident intensity of photons? How might they relate this to he time between absorption events?

2. Given the authors' stated transit time through the detector and the mean time between absorption events, how many photons are released by each molecule in the detector?

3. How many photons do the authors actually claim to see in 800 μs? Provide three reasons for the large difference between the observed and expected number of photons.

4. The authors claim the optimal laser power is 32 mW. What two things might happen to reduce S/N when the laser is above 32 mW? What might happen to reduce S/N when the laser is below 32 mW?

5. The authors claim that the mean incremental count rate was 1.5 per 100 μs above a background count rate of 1.0. Label the time increment on the figure from Q3 in Model 2. Verify the authors' statement.

6. Verify the authors last two sentences from the abstract.