3 Standard Curves, Fluorescent Controls, Background Fluorescence & Sources of Error

Standard Curves

Fluorescence is linearly proportional to dye concentration in dilute samples (see Section 2). However, if the concentration is too great, quenching occurs and the relationship between fluorescence and concentration becomes curvilinear. Standard curves (shown in Figure 3-1) are constructed by analyzing serial dilutions of a single color of microsphere in solvent. They are used to evaluate a spectrophotometer's linearity as well as determine the number of microspheres per ml solvent added to each sample for a given microsphere lot. As shown in Figure 3-1 fluorescence is linear with respect to the number of microspheres in a sample at lower concentrations (generally 2,000 microspheres/ml of solvent) and becomes curvilinear at higher concentrations.

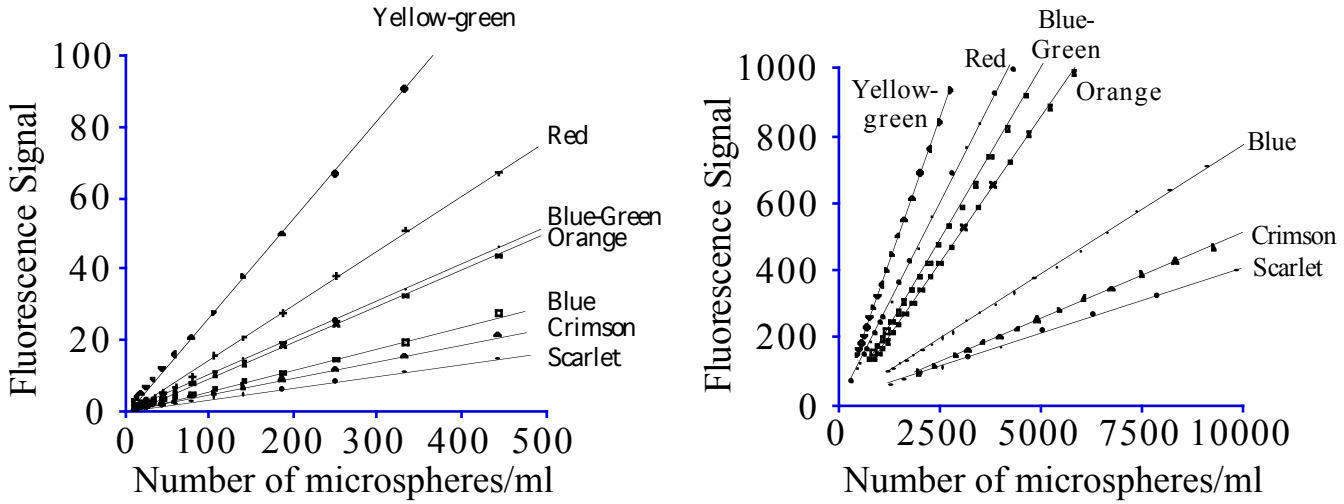

Figure 3-1 Relationship between the fluorescence intensity and the number of fluorescent microspheres per sample.

Each microsphere varies with respect to the quantum efficiency of the dye and the relative "dye load" for that lot of microspheres. These factors affect the fluorescence intensities per number of microspheres. A standard curve should be constructed for each new lot of microspheres to confirm the linearity and concentration range (see below).

Construction of Standard Curves

1) Take 5 µl of 0.2% FluoSpheres® (5,000 fluorescent microspheres) and add it to 10 ml of solvent (we use 2-ethoxyethyl acetate). This yields the fluorescence intensities for ~500 fluorescent microspheres/ml.

2) Depending on the range of your fluorimeter (based on the manufacturer's recommended operating range), calculate the number of µl required to add to 10 ml solvent to yield a fluorescent microsphere concentration that will be just above the upper intensity limit for your fluorimeter.

3) Using this high-stock solution, make multiple serial dilutions of microspheres with very accurate pipetting. Calculate the number of microspheres per ml for each dilution.

4) The fluorescence intensities per number of microspheres per ml are plotted to yield a standard curve for that lot of microspheres.

Fluorescence Controls

Samples of known fluorescence intensities should be measured in the fluorimeter prior to and during the running of a series of samples. Measuring these controls has three important purposes:

1) To make sure that all experimental parameters on the fluorimeter are appropriately set.
2) To check that the lamp and photomultiplier tube of the fluorimeter are functioning properly and the monochromators are appropriately set.
3) To monitor methodological noise.

A change in control intensities may be an early indication that the lamp is beginning to fail or your machine parameters are not properly set.

The best controls would be Lucite-embedded fluorescent dyes that could be used repeatedly. Unfortunately these are not currently available. We routinely make a set of control solutions of fluorescent microspheres dissolved in 2-ethoxyethyl acetate (see the following section on making a set of controls). We read these controls prior to every experimental run and after every 50 samples during a run. Using the internal control function found in the FAC or WINFAC programs (see FAC manual version 8 or higher), a control data file is generated to determine methodological noise. FAC has an automated control check that compares current control intensities to past control intensities stored in the control data file. If intensities differ by more than 5%, a message is sent to the operator that controls are out of range. Each technician should routinely evaluate controls to make sure they do not vary from day to day. Controls are also analyzed at the completion of an experiment to determine methodological noise (see components of methodological noise below). We plot the control intensities and calculate the coefficient of variation (standard deviation divided by the mean). Examples from two of our experiments, as well as "fluorimeter variability" are shown in Figures 3-2, 3-3 and 3-4. Our coefficient of variation (measurement of method noise) runs approximately 2-3% with good operator technique.

Methodological noise measured by the coefficient of variation consists of:

1) Fluorimeter (system) noise (i.e., the reproducibility of intensities without variables such as cuvette washing or cuvette orientation): The LS-50B used in our laboratory has a coefficient of variation of ~1% with the exception of scarlet, which varies up to 2% (Figure 3-2).

2) Other variability: cuvette cleaning, cuvette orientation and cuvette matching. Good technical methods are required to make sure the cuvettes remain clean. If a cuvette is not properly cleaned between samples, the method noise dramatically increases, directly decreasing the confidence in sample intensity. Other sources of operator variability include improper handling of the cuvettes (finger prints/ talcum powder) and particulate material in your sample. Poorly matched cuvettes will dramatically increase coefficient of variation. It is important to use the same set of cuvettes for all the samples from a single experiment.

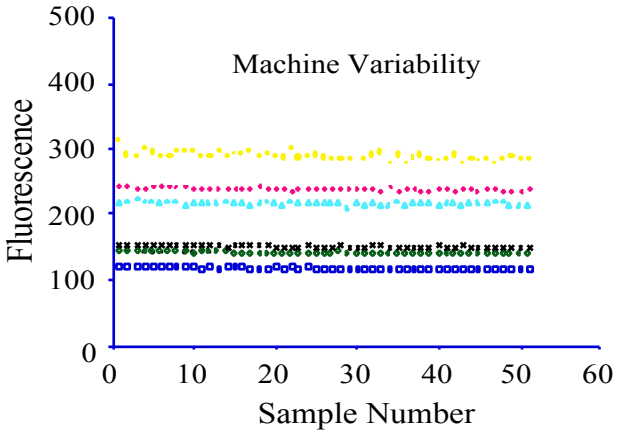

Figure 3-2
Example of machine variability from multiple reading of the control solution without moving the cuvette:

Coefficient of Variation
Blue 0.68%
Blue-Green 0.61%
Yellow-Green 0.63%
Orange 0.61%
Red 0.77%
Crimson 1.07%
Scarlet 2.11%

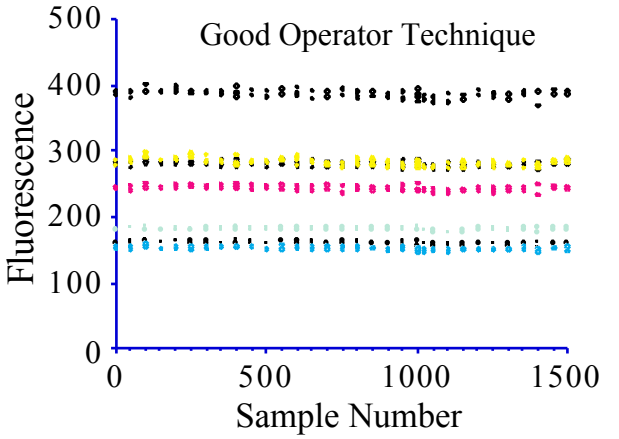

Figure 3-3
Example of a set of controls with good operator technique:

Coefficient of Variation
Blue 1.41%
Blue-Green 1.58%
Yellow-Green 1.57%
Orange 1.37%
Red 1.54%
Crimson 1.70%
Scarlet 1.93%

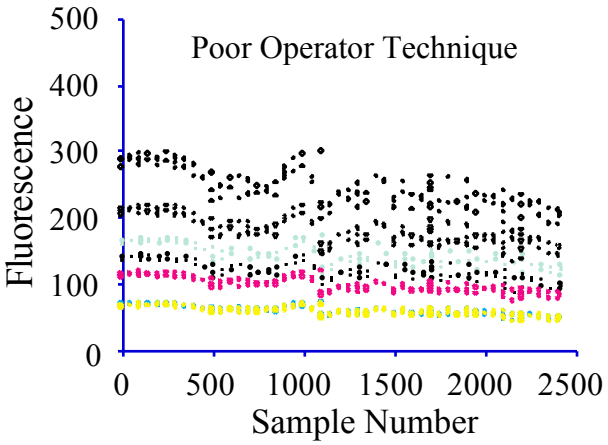

Figure 3-4
Example of a set of controls with poor operator technique

Coefficient of Variation
Blue 11.27%
Blue-Green 11.61%
Yellow-Green 11.99%
Orange 11.58%
Red 10.74%
Crimson 10.71%
Scarlet 10.39%

Figure 3-3 shows a set of controls from a "typical" data set with good operator technique. Figure 3-4 demonstrates a data set with a new technician who was not adequately cleaning the cuvettes between samples. Note the extremely high coefficient of variation.

Making Control Solutions

When making an appropriate set of control solutions, it is important to consider the number of fluorescent microspheres per volume of solvent being used and their "typical" intensities.

1) Control solutions should have a fluorescent signal that is approximately the mean signal for each color in your sample, and be on the linear part of the standard curve, as described earlier.

2) Take 5 µl of 0.2% FluoSpheres® (5,000 fluorescent microspheres) and add it to 10 ml of solvent (we use 2-ethoxyethyl acetate). This yields fluorescence intensities of ~500 fluorescent microspheres/ml and gives a reference intensity for making your control solution.

3) Depending on the number of different colored microspheres being used ("n"), a stock solution is made by dissolving one color of microspheres in 2-ethoxyethyl acetate to yield an intensity that is the "n" times more concentrated than average intensities from an experimental sample.

4) Stock solutions are then combined to yield a control solution in the range of our "typical" sample intensities.

For example, if we use three fluorescent microsphere colors (blue-green, orange and crimson), and our "typical intensities" are 100 for blue-green, 200 for orange and 50 for crimson, we should make a stock solution 3 x 100, or 300 for blue-green, 3 x 200, or 600 for orange, and 3 x 50, or 150 for crimson. The three stock solutions are then combined to yield a 1:3 dilution to make a control solution that reads 100, 200 and 50, respectively, for blue-green, orange and crimson.

Solvent Blanks

Solvent "blanks" are routinely read at the beginning, during, and at the end of each fluorimetry session. This allows for correction of "background" fluorescence to be subtracted from the fluorescence intensity. In our system, 2-ethoxyethyl acetate produces significant background intensities in the blue region only.

Reading solvent blanks also helps assure clean cuvettes. A high blank fluorescence intensity should flag the operator to a dirty cuvette or other problems.

Organ Fluorescence

Tissue can fluoresce causing an increase in fluorescence intensities. With direct extraction of microspheres from air dried lung tissue soaked in 2-ethoxyethyl acetate, there is a significant contribution in fluorescence intensity in the blue region. This tissue fluorescence has an optimum peak excitation and emission pair of 320 and 405- nm, respectively. There is significant background tissue fluorescence only in the blue region. This background tissue intensity can be subtracted from the measured fluorescence intensities.

We have encountered no significant background signal caused by tissue fluorescence in microsphere extractions filtered to remove tissue particulate. Prior to beginning an experiment with new tissue types or recovery methods, we recommend the organ of interest be harvested from an animal that received no fluorescent microspheres, tissue digested, and fluorescence determined for each excitation and emission wavelength pair of interest.

Particulate: It is important that the sample solution be free of particulate, by careful pipetting or filtering prior to analysis. The presence of particulate in solution causes the exciting light to scatter, potentially increasing and/or decreasing sample fluorescence intensities.

Potential Sources of Error

An insufficient number of microspheres per tissue sample will introduce error into the flow estimate. The generally accepted number of microspheres needed for accurate flow measurement is 400/piece (Buckberg, 1971). This problem is solved by increasing the number of injected microspheres.

Under low-flow situations (i.e., small pieces of tissue with very low perfusion having less than 400 microspheres in a given sample), one can determine the relative error of a measurement by the number of microspheres in the reference blood sample and the number of microspheres in the tissue sample (Nose *et al*., 1985).

Low fluorescence intensities will introduce error into the flow estimates. The lowest acceptable intensities will depend on the sensitivity of the fluorimeter being used. Using a Perkin Elmer LS-50B, the mean of the intensities should be greater than 10 (preferably greater then 50) above background tissue or background solvent intensities.

Microspheres can be lost during the filtering process and/or when transferring the filtered material into the vials in which solvent is added. Microsphere loss will be reduced if the sample can remain in a single vessel during the entire process. Correction for microsphere loss is now presented in Section 5.

If solvent volumes are not precisely reproducible, the measured concentration of fluorescence will not accurately reflect the amount of fluorescent dye per sample. An accurate repeating pipette must be used to add solvent to each sample.

Dilution errors will lead to incorrect estimates of sample fluorescence. Dilutions should be performed with an accurate pipetting technique or by weight.

Samples must be properly stored and handled once the 2-ethoxyethyl acetate has been added. All samples should be stored in the dark until ready to read as light can decay the fluorescence intensities with time. **Note:** Free dye is less stable when not bound in the microspheres; prompt processing of the samples once solvent is added is important.

Quenching of the fluorescence intensities at high concentrations will cause an underestimate of the true amount of microspheres within a sample. Samples with high fluorescence should be accurately diluted until the fluorescence intensities are within linear range of the fluorimeter. The true fluorescence intensities are then calculated from the diluted sample intensities.

Light scattering caused by particles either on the surface of the cuvette or in the solution will lead to incorrect fluorescence intensities. Cuvettes must be cleaned regularly and talc-free gloves worn when handling cuvettes. **Note:** Particulate becomes a greater problem when read at larger slit widths.

Although spillover of one color into an adjacent color is small, error can occur if adjacent color intensities are of disparate magnitudes. This can be avoided by choosing colors that do not overlap or can be corrected mathematically for spillover.

Dye degradation over time. A single dye solution should consist of solvent and the fluorescent color to be used. This solution should be read daily to determine if degradation occurs in specific color/solvent combinations. The solution intensity should be stable for as long as samples are stored in solvent prior to reading.

Fluorimeter variability will introduce error into an experiment if the characteristics of the fluorimeter change over the course of sample measurement from a given experiment. Sources of variability can be the excitation lamp, the photomultiplier tube, or the parameters set by the operator. Measuring the intensity of the known control solution (described earlier) on a routine basis provides the first indication of a fluorimeter change. It is good technique to read all samples from a given experiment within a short period of time (1-2 weeks).

Improper reference blood withdrawal methods will lead to a systematic error (multiplication factor) in calculated piece flows.

Poorly matched cuvettes will dramatically increase your coefficient of variation and decrease accuracy of your measurements. Approximately 40% of the cuvettes we receive are returned because they do not meet our variation requirement of less than 5%.

Cuvette breakage and replacement: If a different cuvette must be used while reading a series of samples from the same experiment, the differences in the cuvette intensities can be corrected by using the percent change in control intensities from initial cuvettes to new cuvettes. Figure 3-5 shows a data set corrected for a new cuvette. Figure 3-5a shows the uncorrected values and Figure 3-5b shows the corrected values.

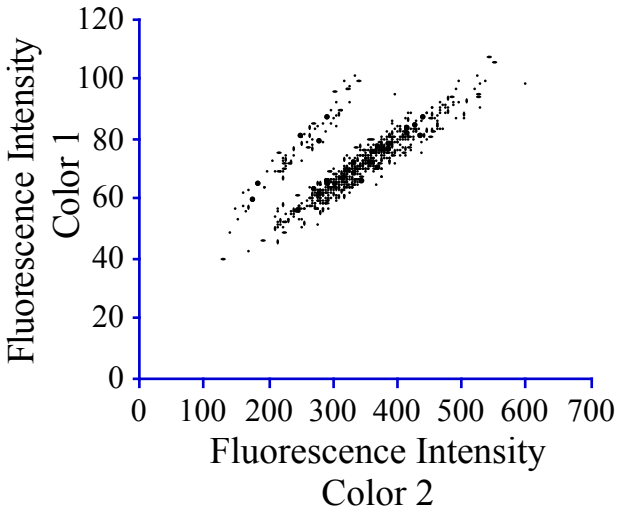

Figure 3-5a: Simultaneous injection of fluorescent microspheres. A cuvette broke during experimental readings and a new set was used to finish the experiment. The points that appear as outliers can be corrected by the percent change in control values.

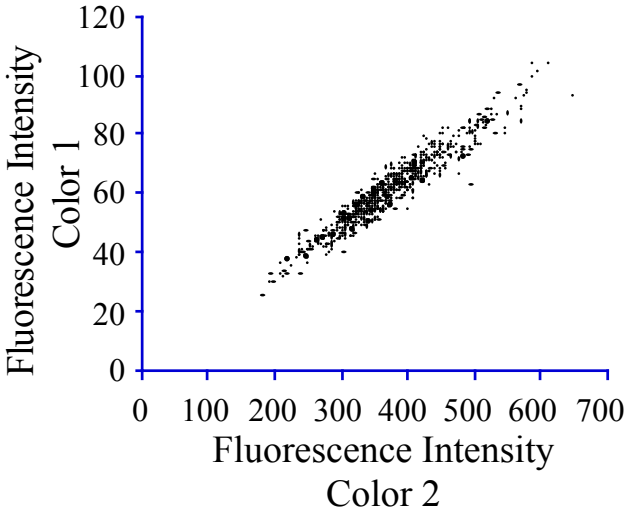

Figure 3-5b: These points have been corrected by the percent change in control values.