4 Fluorescent Microsphere Physical Characteristics

Fluorescent microspheres (FluoSpheres®) are available from Molecular Probes, Inc. and Triton Technologies (see Appendix A for addresses). Other Fluorescent microspheres are commercially available but have not been validated for measuring organ blood flow using the dye extraction method. The FluoSpheres® manufacturer provides the following information.

Physical Properties

Uniform polystyrene microspheres used in regional blood flow measurements are 10 or 15 µm in diameter, with 0.2% (w/w) suspension in 10 ml (Molecular Probes) or 20 ml (Triton Tech) of saline, with 0.02% Tween-80® and 0.02% thimerosal added. Each microsphere reagent contains a single fluorescent dye that is spectrally distinct from the others. The different fluorescent dyes have the following approximate maximal excitation and emission wavelengths (nm) (Table 4-1). The exact excitation and emission spectra depend on the solvent used to extract the fluorescent dyes.

Table 4-1
Optimal Excitation and Emission Wavelengths of FluoSpheres® in 2-ethoxyethyl acetate

Color	Excitation (nm)	Emission (nm)
Blue	360	423
Blue-Green	420	467
Green	450	488
Yellow-Green	495	506
Orange	534	552
Red	566	598
Crimson	610	635
Scarlet	646	680

Note: The excitation and emission wavelengths at which the dyes are measured can be altered to provide better separation between colors.

Storage

The microspheres are preserved from bacterial contamination by the addition of thimerosal. Microspheres can be stored at room temperature or refrigerated (DO NOT FREEZE). They should be protected from light when not in use. Sterile needles must be used to withdraw samples to avoid potential bacterial contamination. The microspheres are stable for at least one year when the recommended storage conditions are strictly observed. Do not use microspheres that show signs of deterioration.

Indications of Deterioration

Presence of large clumps of solid matter that do not break up completely after vigorous shaking or sonication, black residue, "fuzzy" objects, or evidence of leakage may indicate that the reagents no longer meet appropriate standards for use. If there are any questions concerning the reagents, contact the vendor.

Performance Characteristics

Purity

The purity of the fluorescent dyes in FluoSpheres® blood flow reagents is determined by HPLC and spectrophotometric analytical techniques. Each lot of reagents has a purity of greater than 98%.

Microsphere Uniformity

The size uniformity of FluoSpheres® is determined by flow cytometry analysis that identifies particle size distribution. Each lot has a coefficient of variation of ≤5%.

Stability

The stability of fluorescent microspheres in aqueous suspension is evaluated for the following adverse conditions: 1) leaching of dyes into the aqueous medium during storage, and 2) reproducibility of the signal obtained from the microspheres after prolonged storage. Each lot complies with the following specifications:

1) < 1% loss of dye from microspheres after 6 months storage in dark.
2) < 10% change in fluorescent signal (3,000 microspheres/ml in xylenes) after 6 months storage in dark.
3) ~50% loss of the yellow-green signal when the microspheres are exposed to 160°C for 15 min.
4) Once the microspheres are dissolved in the solvent, a significant loss of fluorescent signal results when the dyes are stored in light.

The polystyrene beads, along with the encapsulated dye, are inert to alkaline hydrolysis when temperature is maintained below 60°C. Higher temperatures may cause softening of the beads and degradation of the fluorescence.

Spillover of Fluorescence Into Adjacent Colors

Fluorescent dyes are designed to have narrow and well separated spectral emission bands. When excited at a specific wavelength, little spillover occurs from the emission of one color into the emission spectra of an adjacent color. For appropriately selected excitation and emission wavelength, up to seven color can be used without correction for spillover. When using more than 7 colors, the spillover of a fluorescent signal into the emission spectra of adjacent colors can be evaluated by measuring the fluorescence intensities of pure color solutions at each excitation/emission pair. These

values are used to construct a spillover matrix representing the quantity of the signal from a specific fluorescent color in each color band can be constructed (Table 4-2). Spillover can be minimized by appropriate selection of excitation and emission wavelengths, which can been seen by comparison of the following tables.

Table 4-2: Spillover Matrix of Fluorescent Colors
(Excitation and Emission Wavelengths Used to Maximize Fluorescent Signal)

	Color Bands									
Color	Blue	Blue-Green	Green	Yellow-Green	Yellow	Orange	Orange-Red	Red	Crimson	Scarlet
Excitation	360	420	450	490	512	534	553	566	610	646
Emission	420	467	488	506	522	552	569	598	635	680
Blue	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Blue-Green	1.2	100.0	28.7	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Green	0.0	10.6	100.0	14.6	0.0	0.0	0.0	0.0	0.0	0.0
Yellow-Green	0.0	0.0	4.2	100.0	20.0	0.0	0.0	0.0	0.0	0.0
Yellow	0.0	0.0	0.0	3.2	100.0	1.4	0.0	0.0	0.0	0.0
Orange	0.0	0.0	0.0	0.1	6.1	100.0	12.6	0.3	0.0	0.0
Orange-Red	0.0	0.0	0.0	0.0	0.0	13.9	100.0	10.7	0.0	0.0
Red	0.0	0.0	0.0	0.0	0.0	0.2	9.6	100.0	0.7	0.0
Crimson	0.0	0.0	0.0	0.0	0.0	0.0	0.8	2.6	100.0	1.1
Scarlet	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	100.0

* Spillover matrix was constructed on a Perkin-Elmer LS-50B with excitation and emission slit widths of 4 nm and a red-sensitive photo multiplier tube.

Table 4-3: Spillover Matrix of Fluorescent Colors
(Excitation and Emission Wavelengths Used to Minimize Fluorescent Spillover)

	Color Bands									
Color	Blue	Blue-Green	Green	Yellow-Green	Yellow	Orange	Orange-Red	Red	Crimson	Scarlet
Excitation	360	430	460	493	512	530	557	578	613	655
Emission	423	467	490	506	524	545	568	598	635	680
Blue	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Blue-Green	1.2	100.0	7.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Green	0.0	7.2	100.0	8.5	0.0	0.0	0.0	0.0	0.0	0.0
Yellow-Green	0.0	0.0	3.2	100.0	12.0	0.0	0.0	0.0	0.0	0.0
Yellow	0.0	0.0	0.0	2.7	100.0	1.4	0.0	0.0	0.0	0.0
Orange	0.0	0.0	0.0	0.1	4.1	100.0	2.9	0.3	0.0	0.0
Orange-Red	0.0	0.0	0.0	0.0	0.0	5.0	100.0	1.6	0.0	0.0
Red	0.0	0.0	0.0	0.0	0.0	0.1	11.4	100.0	0.3	0.0
Crimson	0.0	0.0	0.0	0.0	0.0	0.0	0.8	2.6	100.0	0.5
Scarlet	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	100.0

* Spillover matrix was constructed on a Perkin-Elmer LS-50B with excitation and emission slit widths of 4 nm and a red-sensitive photo multiplier tube.

The values in table 4-2 represent an amount of a given color signal in each color band and are relative to an arbitrary value of 100 for the color signal within its own color band. This spillover matrix was constructed with excitation and emission slit widths of 4 nm. As the slit widths are increased, the relative spillover of each color changes very little because the relative strength of the fluorescence signals increases markedly. This spillover matrix was constructed using a Perkin Elmer LS-50B equipped with a red-sensitive photomultiplier tube.

Spillover Correction Methods

Currently, thirteen different colors of fluorescent microspheres are commercially available that have emission bands spaced such that spillover is low (generally less than ten percent of peak maxima) and only occurs for adjacent colors. Up to seven colors, can be used without correction for spillover. However when using more than 7 colors, spillover between some adjacent colors becomes significant and must be mathematically corrected to avoid over-estimation of some flows. Using spillover correction, up 13 colors can be used reliably to determine regional organ blood flow at 13 different points in time. Three different methods of spillover correction have been rigorously validated for blood flow measurement: 1) matrix inversion of fixed wavelength intensities 2) least squares fit of an over-determined system of linear equations for synchronous scan spectra and 3) least squares fit of combined Gaussian and Lorenzentian function for synchronous scan spectra. All three methods work well for spillover correction, and yield essentially identical results in most situations (Schimmel et al., 2001). A public domain program, FMSPILL, can be used to correct fluorescent spillover. FMSPILL enables correction for either fixed wavelength intensities, using a matrix inversion method, or for synchronous scans, using a least-squares over-determined method. Files produced from the WINFAC program can be directly read by FMSPILL. This program is available from the FMRC web site (http://fmrc.pulmcc.washington.edu/). The program currently requires a Windows® based operating system.

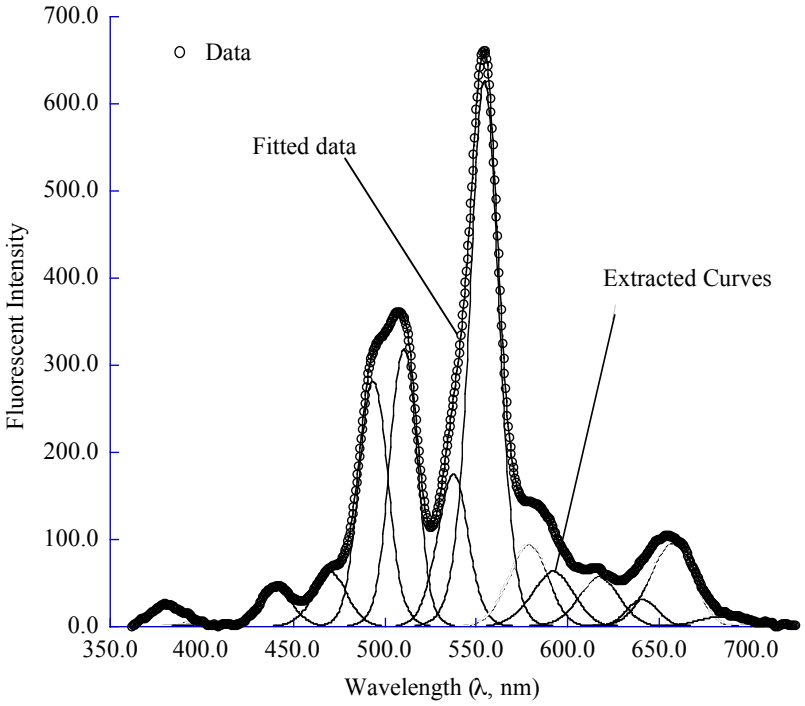

Figure 4-1 Curve extraction of 13 colors from a synchronous scan of a tissue sample with 13 different colored fluorescent microspheres.

Choosing Appropriate Colors for an Experiment

A judicial choice of colors prior to performing an experiment will help insure optimal results. The first decision is to determine how many different measurements will be performed (separated by time and/or physiological interventions). Each measurement requires a different color. To minimize spillover and take advantage of some inherent characteristics of specific dyes, we suggest the following color combinations for a given number of colors (Table 4-4):

Any color can be used for an experiment using only one color. However, blue is the least favorable because of the high background due to tissue auto-fluorescence and solvent background in that region. Crimson and scarlet are less favorable because their quantum efficiency is relatively poor. Injecting more microspheres can compensate for this reduced efficiency. In our fluorimeter, scarlet signals are comparable to crimson even though the quantum efficiency of scarlet is less. We have found very low crimson and scarlet signals to be very linear and reliable. Spillover becomes a significant issue if colors other than those listed below are used. Hence spillover will occur if more than seven colors are used in a single experiment or if colors with excitation and emission wavelengths that fall between those listed below are used together.

Experiments using aerosols and intravenous fluorescent markers will produce fluorescent signals with significantly different intensities. Ventilation signals are usually an order of magnitude less than blood flow signals. If enough colors are used in an experiment to cause spillover problems, the colors used to mark ventilation and perfusion must be carefully selected. Colors that will have low fluorescent signals should be grouped together in the color spectrum and similarly, colors that will have high fluorescent signals should also be grouped together in the color spectrum. This will minimize the error introduced when spillover correction is used.

Table 4-4
Color Selection Guide in order of Preference. All colors except Scarlet, Blue and Blue-Green are equivalent when using a single color (see comments). The first four colors can be used without correction for spillover. Additional colors having increasing spillover in some adjacent colors.

	Color Band	Ex WL	Em WL	Comments:
1	Yellow-Green	493	506	
2	Orange	530	545	
3	Red	578	598	when using Carmine FWL = 578/595
4	Crimson	610	635	when using Carmine FWL = 618/636
5	Green	460	490	spillover correction required, <5%
6	Yellow	512	524	spillover correction required, 2-8%
7	Orange-Red	557	568	spillover correction required, 2-11%
8	Scarlet	654	680	Correlation w/ RM are a few % lower than other FM
9	Blue-Green	430	461	2-ethoxy may contain impurities which interact with BG in tissue
10	Blue	360	418	Tissue fluorescence may be significant
11	Dark Red	639	650	spillover correction required, 30%
12	Carmine	585	617	spillover correction required, 30-45%

References

Schimmel, C., D. Frazer and R.W. Glenny. 2001. Extending Flourescent Microsphere Methods for Regional Organ Blood Flow to 13 Simultaneous Colors. *Am. J. Physiol. Heart. Circ. Physiol.* 280: H2496-506.