TechNote 103

Fluorescent / Dyed Microspheres

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Bangs Laboratories, Inc.

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Applications

Research
High Throughput Screening[5]
SNP Analysis[6]
DNA Hybridization Probes[7]
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- Neuronal Pathways[10]
- Phagocytosis[11]
- Environmental[12, 13]

Biosensors[14, 15]
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Standards
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I. INTRODUCTION

From their serendipitous development in 1947[1], microspheres have enjoyed respectable 'careers' in a variety of disciplines. Within the field of biomedicine alone, we have witnessed the evolution of microsphere applications from the earliest latex agglutination tests[2] to today's sophisticated multiplexed assays.[3, 35]

The proliferation of microsphere-based tests and assays is owed in no small part to the versatility of the beads themselves. Microspheres are available with a variety of functionalized surfaces, densities, and special properties (e.g. magnetic). The association of colored dyes or fluorophores adds a further level of flexibility. In fact, the incorporation of fluorophores has become a particularly important feature for assay development, conferring benefits such as multiplexing capabilities[35] and signal enhancement, and serving as a replacement for radioactive labels.[4]

II. DYEING TECHNIQUES

Microspheres are commonly dyed after synthesis, through dye entrapment or surface attachment.[29, 30]

A. Internal Labeling

Dye diffusion / entrapment involves the swelling of polymeric microspheres in an organic solvent / dye solution. The water-insoluble dye diffuses into the polymer matrix and is entrapped when the solvent is removed from the microspheres (through evaporation or transfer to an aqueous phase).

The great majority of Bangs' microspheres are internally labeled, which affords many benefits, including:

- availability of surface groups for coupling reactions;
- photostability, the protection of fluorophores from photobleaching;
- larger selection of dyes;
- wide range of sizes available, ~20nm - 90µm;
- greater dye loading / brighter microspheres.

Figure 1 illustrates the dye loading that is possible using the diffusion method. For visibly colored (non-fluorescent) dyes, an amount of dye equal

to approximately 10-40% of the bead weight may be entrapped within each, and up to approximately 1% of the bead weight for fluorophores (a lesser amount due to problems associated with intermolecular interaction of fluorophore molecules [fluorescence quenching]).

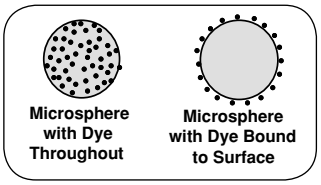


Figure 1: Dye Loading

B. External Labeling

Microspheres may also be externally labeled with dyes (generally fluorophores), as are some of the Bangs Flow Cytometry Products (see Table 1). Surface attachment of fluorophores offers other unique benefits, such as:

- environmental responsiveness of the dye;
- spectral retention (spectra are much like those of free dye);
- useful where particles will be used in solvent and will swell, as the dye will not escape.

III. FLUORESCENT MICROSPHERES

A. Fluorescence[31]

The phenomenon of fluorescence begins with the excitation of a molecule (fluorophore) to a higher energy level by absorption of light at a characteristic wavelength. As the fluorophore returns to the ground state, emission of photons (fluorescence) occurs. The emission spectrum is often a near mirror image of the excitation spectrum, although emission occurs at longer wavelengths and lower energy levels. The difference between excitation and emission maxima (Stokes shift) represents the energy that is lost during the excited state.

B. Reference Values

To assist clients in selecting a suitable product, useful reference values are provided below:

1. *Visible Spectrum*[32]

Wavelength (nm)	Region	Color Observed
< 380	Ultraviolet	Not visible
380-440	Visible	Violet
440-500	Visible	Blue
500-580	Visible	Green
580-600	Visible	Yellow
600-620	Visible	Orange
620-750	Visible	Red
> 800	Infrared	Not Visible

2. *Excitation Sources*[32]

Source	Wavelengths (nm)
Aluminum oxide doped with chromous oxide (ruby)	690
Argon	488-568
Carbon dioxide	337
Gallium-arsenic diode	325
Helium-cadmium	800-900
Helium-neon	633
Krypton	530-676 (647)
Mercury Arc	250-600
Organic dye	400-800
Tungsten Filament	350-1000
Xenon Arc	250-1000 (467)
Neodymium-yttrium aluminum garnet (YAG)	1060

C. Available Fluorophores

Listings of our most common fluorophores are presented in Table 1 (standard BLI) and Table 2 (Flow Cytometry).

Table 1: Standard Fluorophores*

Fluorophore	Excitation/Emission Maxima	Labeling	Fluorescence
Plum Purple	360, 420	Internal	Violet
Ultra Violet	360, 390	Internal	Violet
Glacial Blue	360, 450	Internal	Blue
Surf Green	470, 525	Internal	Green
Dragon Green	480, 520	Internal	Green
Envy Green	525, 565	Internal	Green
Yellow Green	441, 486	Internal	Yellow/Green
Suncoast Yellow	540, 600	Internal	Yellow
Europium Chelate	365, 610	Internal	Orange
Flash Red	660, 690	Internal	Red
Far-Out Red	475, 663	Internal	Red

** Notes:*

- Values for each fluorophore were determined by using a single lot of fluorescent polymeric microspheres.
- Supporting excitation and emission spectra are presented on page 3.
- It is important to note that the visually apparent color of the microsphere suspension will likely differ from the color of fluorescence.

*Table 2**: Flow Cytometry Division*

Fluorophore	$Excitation_{max}$	$Emission_{max}$	Labeling	Fluorescence
Acridine Orange	500	526	Internal	Green
Alexa Fluor® 488	499	519	Surface	Green
Alexa Fluor® 647	652	668	Surface	Red
APC	650	660	Surface	Red
APC-Cy™7	650, 755	767	Surface	Far-Red
Chlorophyll	430, 453	642, 662	Surface	Red
Cy™5	649	666	Surface	Red
Dansyl Chloride √	334	465	Internal	Blue
DAPI	350	470	Internal	Blue
Far-Out Red	475, 590	663	Internal	Red
FITC	490	525	Surface	Green
Fura-2	340, 380	512	Internal	Blue
Glacial Blue	360	450	Internal	Blue
Hoechst ≤	346	375, 390	Internal	Blue
Indo-1	350	405, 482	Internal	Blue
Oxazine 1	250, 640	660	Internal	Red
Phycoerythrin	480, 565	578	Surface	Green
PE-Cy™5	480, 565, 650	670	Surface	Red
PE-Cy™7	480	767	Surface	Far-Red
PE-TR	480, 565	613	Surface	Red
Propidium Iodide	536	617	Internal	Orange
Rhodamine 123	511	534	Internal	Green
Starfire Red™	488	685	Internal	Red
Rhodamine B	540	625	Internal	Red
Rhodamine WT	550	590	Surface	Yellow
Tetramethyl Rhodamine	557	576	Internal	Green/ Yellow
Texas Red®	589	615	Surface	Red

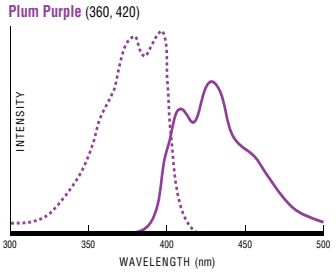
*Table 2**: Flow Cytometry Division, cont.*

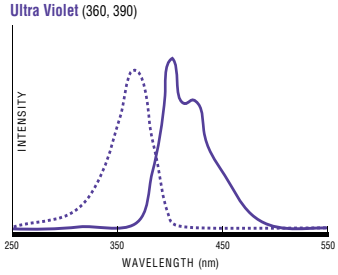
***Notes:*

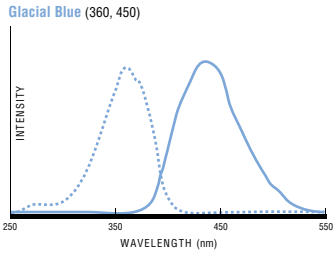
- Most BLI Flow Cytometry products are in the range of 7-8µm nominal mean diameter. These products have been validated for use in flow cytometers, and may not be suitable for less sensitive instruments.
- Values for each fluorophore were determined by BLI using a single lot of fluorescent polymeric microspheres (if designated with √), or were obtained through references 33 or 34 (no designation).
- It is important to note that the color of fluorescence and visually apparent color (e.g. of the microsphere suspension) may differ.

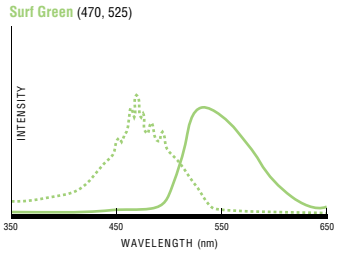
D. Spectra

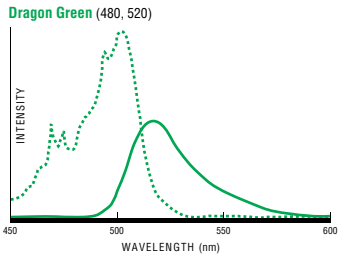
Spectra for the fluorophores listed in Table 1 follow. It is important to note that spectra are provided to assist clients with the selection of appropriate fluorescent microspheres, and are not offered as a guarantee of performance, i.e. spectra may shift slightly based upon characteristics of the base microsphere, method of fluorophore incorporation / attachment, and other factors. For a given fluorophore, excitation and emission intensities are relative to each other. Comparison of fluorescence intensities of different fluorophores has not been conducted.

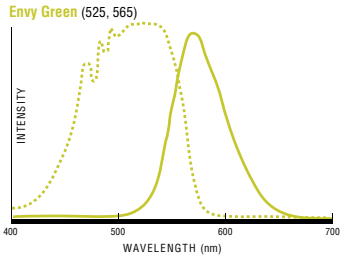


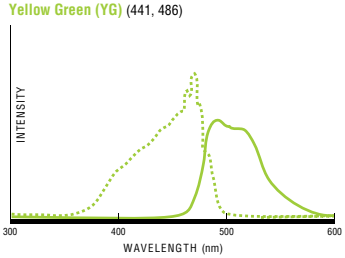


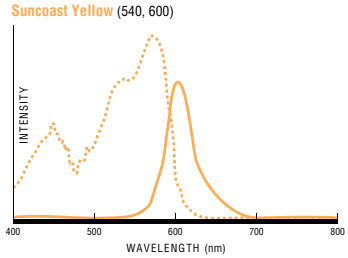


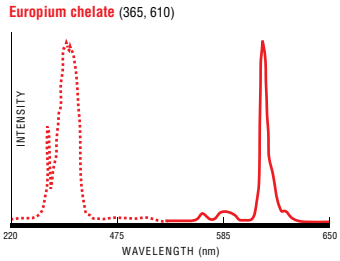


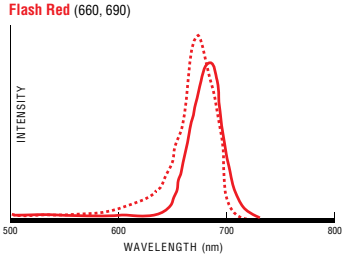


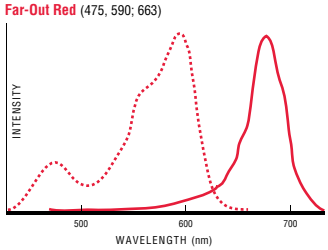












IV. VISIBLY DYED MICROSPHERES

A. Common Colors

Visibly dyed microspheres are used for all sorts of things. They are most commonly utilized in rapid diagnostics, including lateral flow and agglutination tests (see TechNotes 301 and 303). They have also been used to visualize fluid flows, and as a biological surrogate.

Our dyed microspheres are available in colors that span the spectrum, from brilliant blues to vibrant reds. Standard colors follow, as well as our visible dye palette. Please note that the following color palette is provided to serve as a general reference only. Actual product hue may vary due to differences in microsphere composition and size, as well as the concentration of the suspension.

Raspberry Purple
Crimson Red
Tangerine Orange
Basic Black
Slate Blue
Sapphire Blue
Cabo Blue
Shamrock Green

Basic Black (K)	Shamrock Green (G)
Cabo Blue (B)	Crimson Red (R)
Sapphire Blue (B)	Raspberry Purple (V)
Slate Blue (B)	Tangerine Orange (O)

Product availability varies; please refer to our website (**www.bangslabs.com**) or contact our Customer Service Department (**info@bangslabs.com**) for details. If we do not have a suitable product available 'off-the-shelf,' colors and intensities can be created/matched to meet your specific requirements.

B. Determination of Absorbance / Optical Density

For investigators who wish to determine maximum absorbance wavelength and optical density of dyes within the microspheres, a sample protocol follows.

Procedure:

1. To a 0.1mL volume of dyed microsphere suspension (10% solids), add 4.9mL acetone and mix by shaking or vortexing for 2 minutes.

2. Allow the mixture to sit for 2 hours, with periodic shaking or vortexing, while the acetone swells the microspheres and extracts the dye.
3. Pellet the microspheres using a high speed (Eppendorf) centrifuge at ~20,000 rpm for 5 minutes.
4. Decant the supernatant for analysis. Dilute aliquots of the supernatant with acetone to the following concentrations: 250x, 500x, and 1000x.
5. To find the wavelength of maximum absorbance (λ_{max}), place the 250x sample in a cuvette and program your UV-visible spectrophotometer to scan from 400nm to 800nm. Record the maximum absorbance wavelength.
6. To obtain optical density (OD) or absorbance measurements, manually set the resultant wavelength (λ_{max} from Step 5) and measure the OD for all three diluted samples, using acetone as a blank.
7. Multiply each OD by its respective dilution factor (250, 500 or 1000) and average these numbers to obtain the average optical density of the extracted dye solution.

   Note: If no λ_{max} is observed, it is likely that light scattering is occurring due to the presence of microspheres in the test samples. To remedy, centrifuge (Step 3) at a higher rpm or for a longer period of time.

V. SPECIALTY PRODUCTS

A. QuantumPlex™ Microspheres for Multiplexing

QuantumPlex™ is an innovative bead kit for multiple analyte-detection research applications in flow cytometry. They have also been utilized to visualize fluid flows, and as a biological surrogate.

The QuantumPlex™ beads come in 5-bead sets of two sizes. The two kits are also sold together as a set of 10. Each set consists of 5 populations internally dyed with varying intensities of Bangs' proprietary dye, Starfire Red™. Bead kits are available with anti-Mouse IgG, streptavidin, or COOH surfaces and, when conjugated with ligand, will allow for the detection of up to 10 different analytes per sample. See our website, **www.bangslabs.com**, and reference 35 for further information.

B. Flow Cytometry Standards

The Flow Cytometry division of Bangs Laboratories provides controls and standards for flow cytometers and related equipment. These products span all of the major aspects of instrument quality control from daily QC to optical alignment. Specialty products include compensation standards, reference beads, certified blank beads, and beads for use in MESF and antibody binding capacity (ABC) determinations. Some of the fluorophores that are available are included in Table 2. See the flow cytometry portion of our website, **www.bangslabs.com**, for further product information.

C. Confocal Standards

Single-label fluorescent microspheres are available for use as confocal microscopy standards. Fluorophores correspond to standard filter sets in blue, yellow and red wavelengths. Primary applications include spatial resolution determination, misalignment detection and Z-axis registration. Their nominal mean diameter of 0.06µm and narrow size distribution allow for optimum imaging conditions. See TechNote 106, *Confocal Standards*, for further information.

Custom Products

If we don't offer a product that meets your specific requirements, ask about our capabilities for customization. We offer custom dyeing and protein coating of polymeric and superparamagnetic microspheres.

VI. HANDLING

Dyed and fluorescent microspheres should be handled in accordance with standard practices, i.e. storage of aqueous suspensions at 2-8˚C (no freezing) and use of aseptic technique where practicable. Additional recommendations may apply to ProActive® (protein-coated) and magnetic microspheres; refer to appropriate product-specific literature for details.

The exposure of fluorescent microspheres to light should be limited to minimize the potential for irreversible photobleaching. Internally dyed microspheres (fluorescent and non-fluorescent) should not be exposed to organic solvents, as this will cause swelling of the polymer matrix and leaching of the dye. If microsphere aggregation occurs, surfactant (e.g. 0.01-0.1% Tween® 20) may be added to the suspension, followed by careful sonication, i.e. ensuring that the temperature of the suspension is not significantly elevated. Further information is provided in TechNote 202, *Microsphere Aggregation*.

See our series of handling-specific TechNotes (200's) for additional information. TechNotes may be downloaded from the Technical Support section of our website, **www.bangslabs.com**.

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