

9025 Technology Dr. • Fishers, IN 46038-2886
 800.387.0672 • 317.570.7020 • Fax 317.570.7034
 info@bangslabs.com • www.bangslabs.com



BEADS ABOVE THE REST™

Contents:

- I. Introduction and Products
- II. Fluorescent Microsphere Standards in Confocal Microscopy
 - A. Spatial Resolution Determination
 - B. Misalignment Detection
 - C. Z-axis Registration
- III. Optimization of Imaging Parameters
 - A. Particle Dilution
 - B. Coverslip Thickness
 - C. Aperture Setting
 - D. Photobleaching
 - E. Scaling
 - F. Microsphere Immobilization
 - G. Microsphere Handling
- IV. Resources
 - A. Instrument Companies
 - B. Selected References

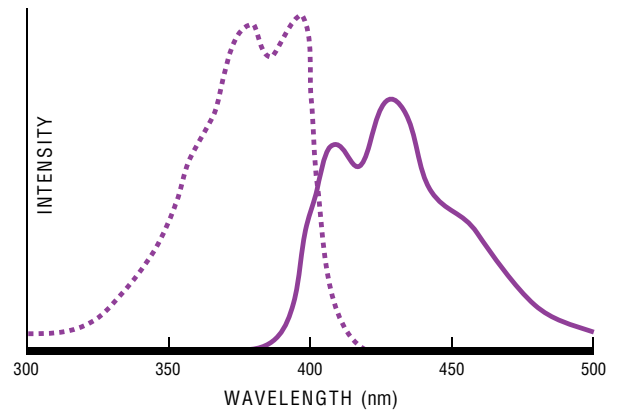
I. Introduction

Microspheres have long been utilized as standards for the validation of a broad range of scientific instruments, including flow cytometers, hematology analyzers, particle analyzers, and microscopes.

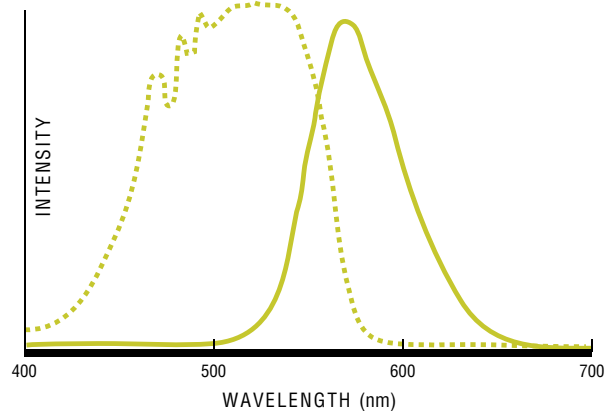
To aid in the performance assessment of confocal microscopes, we have developed a set of 60nm fluorescent microsphere standards:

Product Code	Description	Fl. Color
FS02F/2598	PS (360, 420) Plum Purple	Violet/Blue
FS02F/2599	PS (525, 565) Envy Green	Green
FS02F/2600	PS (660, 690) Flash Red	Red

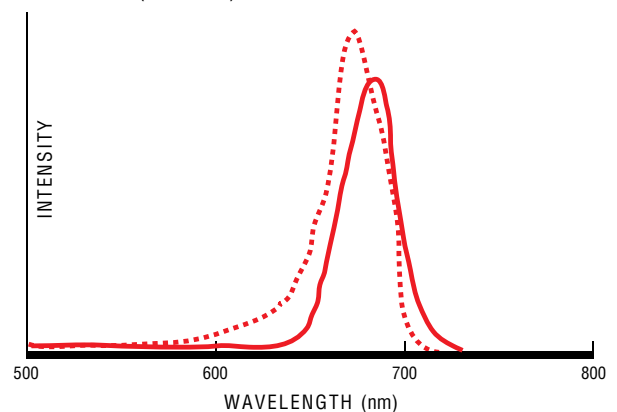
Plum Purple (360, 420)



Envy Green (525, 565)

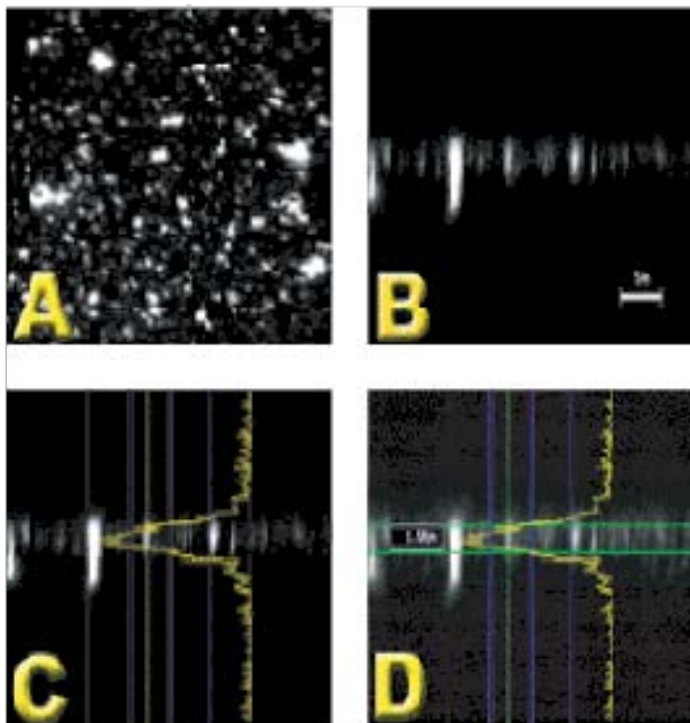


Flash Red (660, 690)



Many additional lots of fluorescent microspheres are available if larger sizes, different fluorophores, or other properties are required. Visit our online Products & Ordering Section for standard fluorescent or flow cytometry beads at www.bangslabs.com, or contact our Customer Service Department (info@bangslabs.com) for further information. See also the Technical Literature section of our website, which provides spectra for a number of available fluorophores.

II. Fluorescent Microsphere Standards in Confocal Microscopy



A. Spatial Resolution Determination

Determine the spatial resolution of a confocal microscope along the optical axis. Image microspheres along the optical axis to quantify the point spread function of the confocal microscope in this dimension. If one obtains an image of the microsphere along the optical axis (either from direct XZ imaging as in Figure B or from data compiled from stacks of XY images as in Figure A) and then plots the intensity of microsphere fluorescence as a function of distance along the optical axis (Figure C), the resolution may be simply quantified as the full-width of the intensity distribution at half-maximal values (Figure D).

B. Misalignment Detection

Image microspheres along the optical axis to determine if the point spread function is symmetrical above and below the central plane of focus. If the image of a single microsphere in the optical axis is not shaped like a cigar (e.g. if it is skewed to one side or 'slanted'), then the lasers or other optical elements may be out of alignment.

C. Z-axis Registration Determination

Mix microspheres in solution and image them after they contact and become affixed to a glass coverslip. Does excitation of individual microspheres in the mixture (by different laser lines) produce images that all coincide at the same z-axis position? If not, the laser lines are not focusing at the same point in space.

III. Optimizing Imaging Parameters

Note: These microspheres are extremely small (60nm), and for all applications it is important to optimize imaging conditions. Neglect of these parameters may strongly degrade results.

A. Particle Dilution

The particles are supplied at 1% solids (w/v). The goal when diluting is to get a sufficient number of particles in the field of view, but not so many that they pile up or bunch together. Try adding a couple of microliters into a milliliter of some desirable fluid, mix well, and look at a drop under a coverslip. More particles may be added if necessary.

B. Coverslip Thickness

Use the correct coverslip thickness for the specific microscope objective. If the lens has a coverslip correction collar, set it to match the measured coverslip thickness. If the lens does not have a coverslip correction collar, measure coverslips first, and then use a coverslip with the thickness that is appropriate for the lens (most objectives are optimized for 170µm coverslips).

Method: The most reliable measurement of coverslip thickness is taken with reflectance measurements in the confocal itself. For example, input 488nm light. Image the reflected light from the inner and outer surfaces of the coverslip, and use the confocal software to measure the distance between these two surfaces. Physical measurements with a micrometer may also be made, although this method is not suitable for determining thickness in a small region of the coverslip.

C. Aperture Setting

Set the size of the confocal pinhole aperture so that it is as small as possible. The objective is to increase confocality as much as possible, although this goal must be tempered by the need to obtain high quality images for quantification.

Method: Begin with bright imaging conditions (wide open pinhole, unattenuated laser lines, wide band pass emission filter). Determine whether photobleaching is occurring (see III-D), and reduce laser power until not bleaching is observed. Decrease pinhole and increase detector gain to compensate. The pinhole should be set such that it is below one Airy unit (in the Zeiss confocal, the pinhole macro can be helpful for determining these settings, given different excitation and emission wavelengths). Below this value, the relationship between signal strength and spatial resolution becomes less favorable. If it is necessary to image a defined set

of wavelengths to match a particular fluorophore, change the emission filter as needed (recognizing that the microspheres may not be sufficiently bright at some wavelengths).

D. Photobleaching

Although the internally-dyed microspheres are fairly resistant to photobleaching, it is possible to bleach any fluorescent beads with high input power and slow scan rate. It is likely that imaging will be performed while the confocal scanner is zoomed as high as possible. This increases the light flux at each individual point in the microsphere sample. If there is photobleaching during the measurement, the values recorded in the image will be internally skewed.

Method: The simplest way to detect photobleaching is to perform several scans of a small region under optimized and zoomed imaging conditions. Subsequently zoom out and take an image of the entire field of view. In the absence of photobleaching, the areas that were previously scanned should not be evident. To minimize photobleaching, decrease input laser power and compensate by increasing detector gain. If settings do not allow for a quality image, begin increasing the pinhole diameter.

E. Scaling

Ensure that all image data are on-scale, including the background. This is essential to ensure that the image of the microspheres is acceptable for quantitative measurements of intensity if (1) the image of the microsphere is so bright that it is off-scale or (2) the background is set to be so dark that it is off-scale.

F. Microsphere Immobilization

Ensure that imaged microspheres are immobilized. If the microspheres move, the measurement is polluted. When diluted into an aqueous medium, microspheres undergo significant Brownian motion. Unless a fast-scan confocal is being utilized, it is not possible to image the microspheres in the optical (z-) axis quickly enough to obtain good images. After some minutes, the microspheres will contact and lightly attach to glass coverslips. This is directly suitable for imaging with an inverted microscope. With an upright microscope, one must either image through solution to the lower surface (where the microspheres have become affixed) or embed the microspheres to immobilize them.

G. Microsphere Imaging

Image the microspheres in a medium of the appropriate refractive index. If imaging of glycerol-embedded tissues is to be performed, the microspheres should be imaged in the same embedding medium. Similarly, if cells are to be imaged in aqueous solutions, microspheres should be diluted into the same medium. Generally, microsphere suspensions are sufficiently concentrated that they may be diluted at least 1000-fold into the medium of choice (with this dilution, there will typically be sufficient microspheres to image in any given microscopy field with 40-100x objectives).

IV. Resources

A. Instrument Companies

There are a number of companies that have developed confocal microscopes, and offer technical support:

Carl Zeiss, Inc., www.zeiss.com/micro

Leica, www.leica-microsystems.com

Nikon, www.nikonusa.com

Olympus, www.olympusamerica.com

PerkinElmer Life Sciences, <http://las.perkinelmer.com>

B. Selected References: Confocal and Multiphoton Microscopy

1. **Carter, D.** 1999. Practical considerations for collecting confocal images. *Methods Mol Biol*, 122: 35-37.
2. **Emptage, N.J.** 2001. Fluorescent imaging in living systems. *Curr Opin Pharmacol*, 1(5): 521-525.
3. **Gratton, E., N.P. Barry, S. Beretta, A. Celli.** 2001. Multiphoton fluorescence microscopy. *Methods*, 25(1): 103-110.
4. **Konig, K.** 2000. Multiphoton microscopy in life sciences. *J Microsc*, 200(Pt. 2): 83-104.
5. **Lacowicz, J.R., I. Gryczynski, Z. Gryczynski.** 1999. High throughput screening with multiphoton excitation. *J Biomol Screen*, 4(6):355-362.
6. **Microscopy Online.** 2002. www.microscopy-online.com.
7. **Pawley, J., ed.** 1995. *Handbook of biological confocal microscopy, 2nd edition*. New York: Plenum Press.
8. **Pawley, J.** 2000. The 39 steps: a cautionary tale of quantitative 3-D fluorescence microscopy. *Biotechniques*, 28: 884-886.
9. **Rost, F.W.D.** 1991. Tutorial on practical confocal microscopy and the use of the confocal test specimen. In: Pawley, J., ed. *Quantitative fluorescence microscopy*. Cambridge: Cambridge University Press, p. 1-55.
10. **Sheppard, C.J.R., D.M. Shotton.** 1997. *Confocal laser scanning microscopy*. New York: Bios Scientific Publishing, p. 15-44.
11. **Williams, R.M., W.R. Zipfel, W.W. Webb.** 2001. Multiphoton microscopy in biological research. *Curr Opin Chem Biol*, 5(5): 603-608.
12. **Xu, C., W. Zipfel, J.B. Shear, R.M. Williams, W.W. Webb.** 1996. Multiphoton fluorescence excitation: new spectral windows for biological nonlinear microscopy. *PNAS*, 93: 10763-10768.
13. **Zucker, R.M., O. Price.** 2001. Evaluation of confocal microscopy system performance. *Cytometry*, 44: 273-294.



Acknowledgements:

Our heartfelt thanks to Marshall (Chip) Montrose, Ph.D. (Indiana University School of Medicine) and the confocal microscopy newsgroup <confocal@ubvm.cc.buffalo.edu> for invaluable assistance in the development of these products. Dr.Montrose kindly evaluated the 60nm confocal bead standards and provided information on their use.

**Copyright 2007, Bangs Laboratories, Inc.
All Rights Reserved**