An Inexpensive Fluorescent Graticule

Safraz Khan[1] and Joshua C. Brumberg[2,3]*

Departments of [1]Biology and [2]Psychology, Queens College, CUNY, 65-30 Kissena Boulevard, Flushing, NY 11367

[3]Biology and Psychology Departments, The Graduate Center, CUNY, 365 Fifth Avenue, New York, NY 10016

*joshua.brumberg@qc.cuny.edu

Introduction

Often investigators need to make accurate size measurements within microscope images. Images of an object of a known size are typically used to calibrate data collection and analysis software. Although this is an easy proposition using brightfield optics, it is more challenging when using an imaging system that does not have a brightfield path but rather relies on a fluorescent pathway (for example, epifluorescent, confocal, or two-photon microscopy). This article shows how to create a reliable, inexpensive means to calibrate images using purely fluorescent imaging.

Materials and Methods

Equipment. In order to make accurate measurements, we used a standard commercially available microscope slide (Figure 1A) that had two graticule grids on it (MBF Bioscience, Williston, VT). The slide was designed to allow for calibration of imaging systems to use a neural reconstruction and measurement software package. One was a 16×16 grid where each side was 250 µm for low-power objectives (<20×) and the other had a 20×20 grid with 25 µm divisions for higher-power objectives (>40×). Using a red Berol Laboratory Marker (Heat Resistant 365-T, Sanford, Inc., West Chester, PA), we evenly filled the grid squares with fluorescent red coloring on the side of the slide with the grid (see Figure 1B). These Berol markings could be removed later using acetone.

Microscopes. The slide works with both upright and inverted microscopes. For this article, it was observed using three different microscopes: (1) Olympus BX51 with epifluorescent illumination (Hg 100W bulb) equipped with a 10× air objective lens (Plan, Numerical Aperture (NA) = 0.25) and a 60× oil immersion lens (PlanApo, NA = 1.4) with an Optronics microfire camera; (2) Leica SP5 confocal microscope with a 10× objective lens (HC PL Fluotar, NA = 0.3) and a 63× oil immersion lens (PlanApo CS, NA = 1.4); and (3) custom-built two-photon microscope using an Olympus 10× (UPLAN FL N, NA = 0.30) and a 20× objective lens (XLumPlanFL, NA = 0.95).

Imaging. For the Olympus BX51 the standard epifluorescent pathway excitation was provided via appropriate filter cubes from Chroma Technology Corp. (Bellows Falls, VT). For the Leica confocal microscope, we used laser excitation wavelengths in the blue range (543 nm) and the red range (900 nm) along with appropriate excitation and emission filters. For the two-photon microscope, an excitation wavelength of 800 nm was used based on the finding that this wavelength led to optimal image acquisition. Images were digitally collected and stored on the dedicated Windows PC accompanying each microscope system. Collected images were enhanced for contrast using CorelPhoto for PC.

Results

The slide was placed in the focal plane with the etched side down (toward the illumination), and the original grid was evident because the colored areas fluoresced while the grid lines prevented any excitation/emission (see Figure 1B). Thus, it is possible to define a known distance in the appropriate data collection/analysis programs. This calibration can be done on virtually any microscope/imaging system. We made similar observations using conventional fluorescent illumination (Figure 1), two-photon excitation (Figure 2A), and confocal (Figures 2C and 2D). An advantage of the Berol marker is

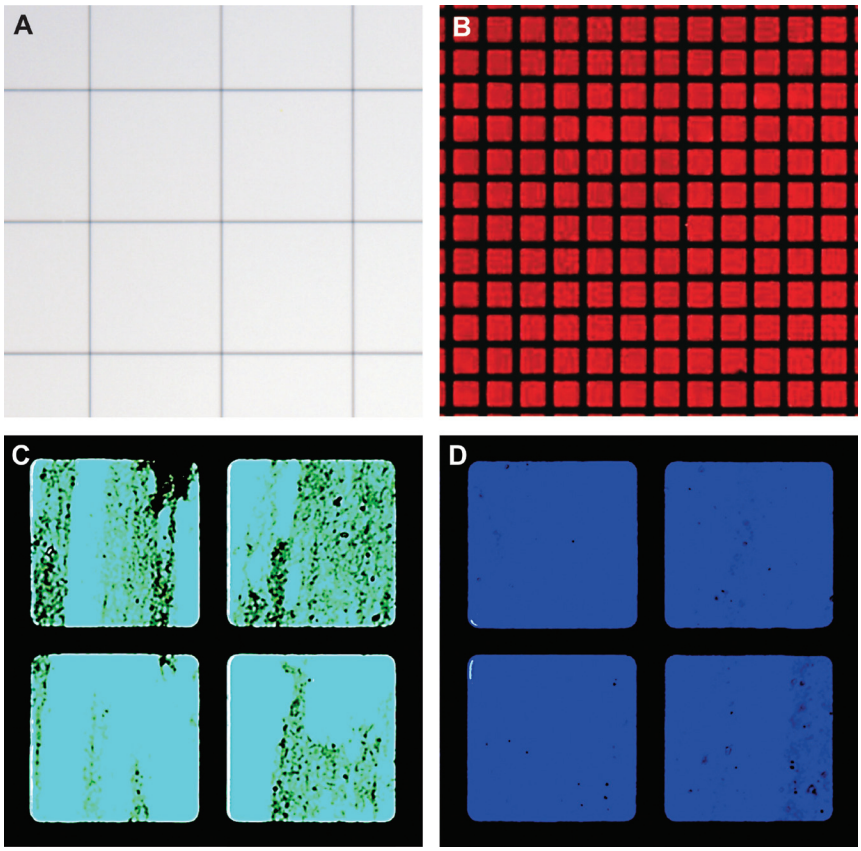

Figure 1: Fluorescence calibration grid. (a) 250 µm grid in its native state imaged using brightfield optics prior to the application of the Berol marker. (b)(c)(d) After application of the marker material, the grid may be observed using various filter sets (excitor/dichoric/emitter) and standard epifluorescence. The Berol marker coated grid is revealed using commercially available filter sets (Chroma Technology Corp. Bellows Falls, VT) for three standard fluorophores: (b) Texas red (560 nm/595 nm/630 nm), (c) fluorescein (480 nm/505 nm/510 nm), and (d) DAPI (350 nm/400 nm/460 nm).

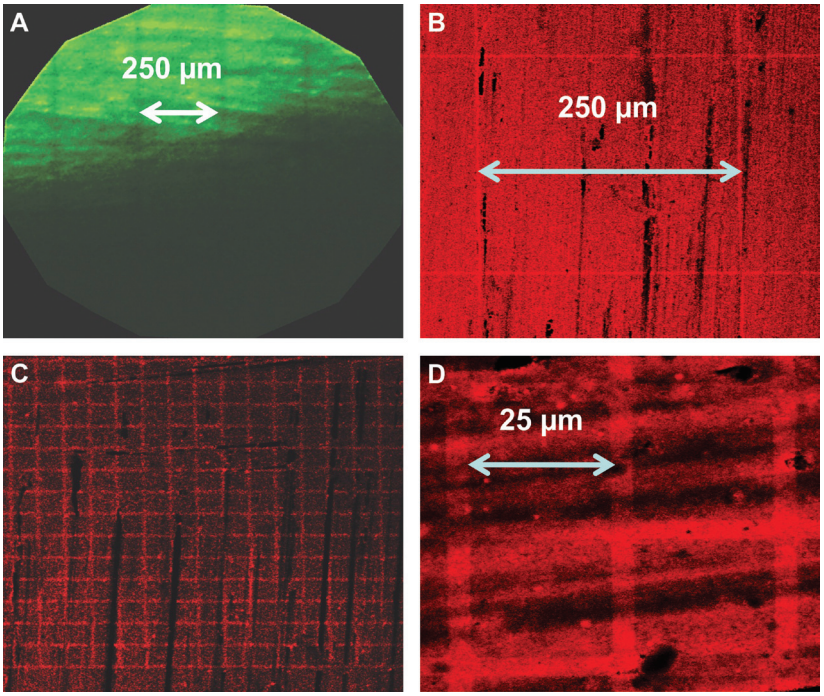

Figure 2: Calibration grids in use. (a) Two-photon image of the 250 µm Berol marker coated grid taken with a 10× objective lens (UPLAN FL N, NA = 0.30). The grid squares can be seen in the background and used to calibrate software programs that quantify images. (b) Image of the 250 µm Berol marker coated grid taken on a Leica SP5 confocal microscope using a 10× objective lens (HC PL Fluotar, NA = 0.3). (c) Image of the 25 µm Berol marker coated grid taken on the confocal microscope with 10× (HC PL Fluotar, NA = 0.3). (d) Same as (c) but with a 63× (PlanApo CS, NA = 1.4) objective lens.

that it fluoresces in response to a wide spectrum of excitation wavelengths and thus can be used equally well in response to illumination from 350 nm to 560 nm (see Figure 1). As a control, we imaged the same grid slide using bright-field illumination, and the same objectives as our epiflourescent microscope. The resultant measurements were comparable to those made with the fluorescent grid.

Conclusion

Many modern fluorescence microscopy studies rely on accurate quantitative measurements, and there is often a need to calibrate a microscope system that does not have a bright-field pathway. Using items readably available in most laboratory settings, it is possible to create a fluorescent graticule at little to no additional cost. The advantage of the described method is that the same reticle used to calibrate bright-field systems can be used to calibrate fluorescent systems emitting from 350 nm to 560 nm.

Acknowledgements

Thanks to Geoff Greene and Casey Johnson of MBF Biosciences Inc. for making the initial suggestion of this methodology. Thanks to Ethan Skowronski-Lutz and Chris Moore for help with the two-photon imaging and Dr. Areti Tsiola for helpful comments on the manuscript.

MT