

Note: A three-dimensional calibration device for the confocal microscope

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(Received 10 October 2012; accepted 29 December 2012; published online 16 January 2013)

Modern confocal microscopes enable high-precision measurement in three dimensions by collecting stacks of 2D (x - y) images that can be assembled digitally into a 3D image. It is difficult, however, to ensure position accuracy, particularly along the optical (z) axis where scanning is performed by a different physical mechanism than in x - y . We describe a simple device to calibrate simultaneously the x , y , and z pixel-to-micrometer conversion factors for a confocal microscope. By taking a known 2D pattern and positioning it at a precise angle with respect to the microscope axes, we created a 3D reference standard. The device is straightforward to construct and easy to use. © 2013 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4776672>]

In recent years, confocal microscopes combined with computers for image acquisition and analysis^{1,2} have become powerful tools for making high-precision 3D measurements in physics and biology.^{3,4} Accurate 3D measurements require that the pixel-to-micrometer conversion factors be known accurately in all three dimensions. This is particularly difficult along the optical axis (z -direction), because scanning in this direction is performed by a different physical mechanism than in the x - and y -directions. To create a 3D image, confocal microscopes acquire a series of 2D images by stepping either the sample or the objective lens along the z -axis, while whole images are taken in the other directions (x - y plane), either all at once by a CCD array or by a raster scan of the plane. It is essential to reconcile these two physically different scanning mechanisms to ensure that distance measurements in all directions are consistent. Although the microscope software uses nominal pixel-to-micrometer conversion factors, we find that these can be incorrect by as much as 40% in the z -direction.

We present a simple device for calibrating the x , y , and z pixel-to-micrometer conversions for a confocal microscope. The device is constructed to hold a 2D reference pattern of known length scale at a precise angle on the microscope. By taking a 3D image of the angled pattern and comparing it to the known dimensions of the reference, we can simultaneously determine the pixel-to-micrometer conversion factors in all three dimensions.

A detailed schematic of the calibrator is shown in Figure 1. The device consists of a 30° angled wedge mounted on a base plate such that the point of the wedge overhangs a large opening for viewing in the base. Both pieces were made of aluminum; the wedge was machined using sine bars to ensure accuracy of the angle. A large-area 0.17-mm-thick coverslip glued in place entirely covers and seals the bottom of the device, creating a viewing window and a reservoir that can be filled with a dyed fluid for fluorescence microscopy. Assembled and filled, the entire device weighs about 87 g.

We created a 2D reference pattern by etching a square array of dots into a 22 mm × 50 mm × 0.17 mm glass slide, using standard microlithographic techniques. Each dot is separated by 1.63 μm from its nearest neighbors. For imaging, the reference pattern is immersed in an index-matched fluid consisting of 37% water and 63% dimethyl sulfoxide (by volume) containing fluorescein-NaOH dye for fluorescence imaging. As a result, the pattern of dots appears dark against a bright background. The entire square pattern measures about 5 mm on each side.

To use the reference pattern with the calibrator, we cleaved the glass so that the reference pattern ran right up to the edge of the slide, and then mounted the patterned glass slide upside down on the calibration device with a flat bracket. The patterned slide is positioned so that the reference pattern is mechanically decoupled from the viewing window. This is easily achieved by cleaving the slide at a slight angle so that only a far corner makes contact with the window.

Once the patterned coverslip is in place, several milliliters of the dye solution are added so that the array of dots is entirely submerged. Wetting usually also occurs between the coverslip and the 30° wedge, which has the effect of making the coverslip adhere tightly to the wedge. At this point, the holding bracket is no longer required; capillary forces alone hold the reference pattern to the calibrator.

The entire device is set onto the sample stage of the confocal microscope, oriented so that the axes of the calibrator (shown in Figure 1) are aligned with the corresponding microscope image axes. The base plate of the calibrator fits easily onto the sample stage of the microscope, and is mechanically stable during imaging. The reference pattern is imaged in three dimensions and a stack of cross-sectional images is generated that shows the angled reference pattern in 3D. In this orientation, the separation in the x -direction is reduced to $\Delta x = 1.63 \mu\text{m} \times \cos 30^\circ$, while the separation $\Delta y = 1.63 \mu\text{m}$ between reference dots along the y -direction is unchanged. Adjacent columns of dots are separated in z by $\Delta z = 1.63 \mu\text{m} \times \sin 30^\circ$.

We show details of confocal images of the reference pattern in Figure 2, both (a) in a flat (0°) configuration to show

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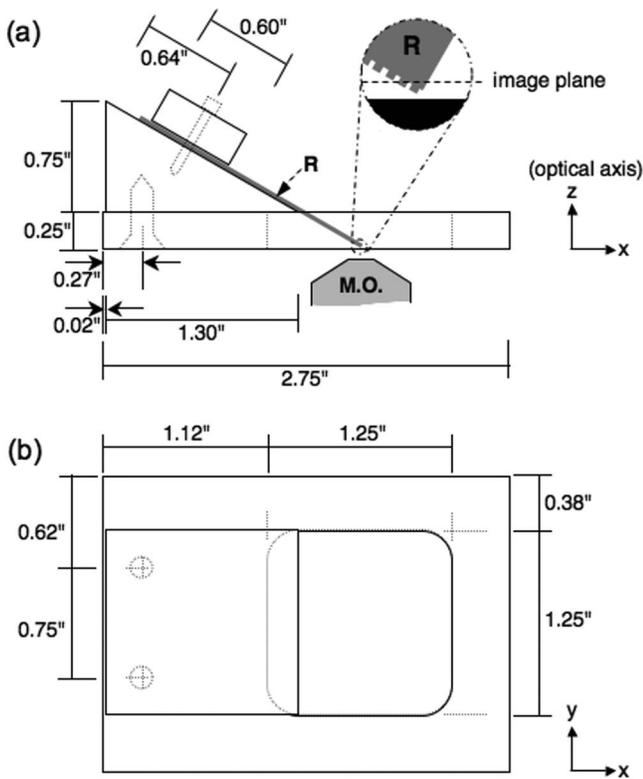


FIG. 1. Schematic drawings of the calibrator. (a) Side view, with reference pattern slide (R) shown held in place on the 30° wedge by the metal bracket, and the microscope objective (M.O.) below the reference pattern. The inset shows a not-to-scale magnified view of the edge of the patterned slide close to – but not touching – the glass viewing window at the bottom of the calibrator. An example of a cross-sectional image plane is drawn as a dashed horizontal line. (b) Top view of the calibrator, without the reference pattern slide and holding bracket.

the square pattern, and (b) and (c) two different cross sections of the pattern mounted at 30° on the calibrator. In this small example, the cross sections are separated by $n = 36 \pm 1$ pixels in z , corresponding to $m = 5$ columns of dots. Hence, in this example, each pixel in z corresponds to $m \Delta z/n = 0.113 \pm 0.003 \mu\text{m}/\text{pixel}$, which is about 10% smaller than the conversion factor used by the microscope software, $0.1259 \mu\text{m}/\text{pixel}$.

Usually, larger image stacks are used to achieve higher precision. Figure 3 shows z -direction calibration data ob-

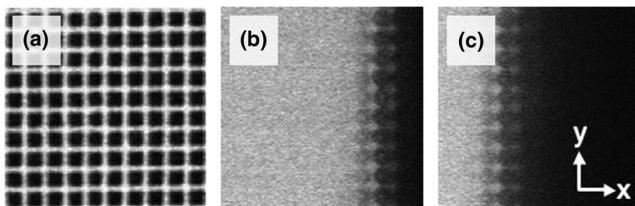


FIG. 2. Details of the reference pattern imaged on the confocal microscope. (a) The square reference pattern at 0° (flat), surrounded by fluorescently dyed fluid. (b) A cross section through the reference pattern held at 30° by the calibrator in a pool of the same fluid. (c) The same field of view as (b), 5 rows of dots and 36 scan steps higher in z . Bright regions in the images indicate the presence of the dyed fluid; dark sections indicate the glass of the patterned slide.

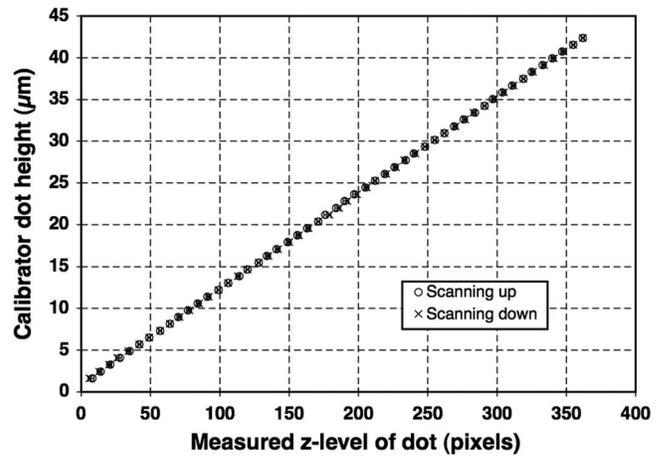


FIG. 3. Measured calibrator dot positions over a larger range than the example of Figure 2. The calibrated pixel-to-micrometer conversion factor is the slope of a straight-line fit to these data. Horizontal error bars indicate a z -level measurement uncertainty of ± 1 pixel, which translates into a $\pm 0.0002 \mu\text{m}/\text{pixel}$ uncertainty in computing the calibrated conversion factor.

tained over a larger stack with scope and resolution chosen to match those of an upcoming experiment. In this example, we performed calibration measurements scanning both up and down the angled reference pattern, as inconsistency between the scanning directions could indicate mechanical instability. Within the measurement uncertainty, we find no difference between the two scanning directions, nor do we see any deviation from a straight line along the length of the scans.

By fitting a straight line to the data, we obtain the calibrated pixel-to-micrometer conversion factor. For the data obtained while scanning up, we find a conversion of $0.1149 \pm 0.0002 \mu\text{m}/\text{pixel}$, and for scanning down we obtain $0.1146 \pm 0.0002 \mu\text{m}/\text{pixel}$.

We have used this calibration device to determine the pixel-to-micrometer conversion for several confocal microscopes in our laboratories, varying the scanning method (sample motion versus objective lens motion), scan speed, and resolution (over a range of nominal pixel-to-micrometer conversion values). We find that the x - y conversion is usually accurate, but that the z conversion can vary significantly between different microscopes and between different scanning methods on the same microscope. We find that the z conversion can differ by as much as 40% from the nominal value used by the confocal microscope software. For a given microscope and scanning method, however, neither the scanning rate nor the resolution affect the result, and the calibrated conversion factors are usually consistent over weeks to months. The source of the problem remains unclear; as it is consistent over time, it seems likely to be a software problem rather than a hardware malfunction. However, since the microscope software often uses an incorrect pixel-to-micrometer conversion, an independent calibration is essential to ensure accuracy of results.

This work was supported by National Science Foundation (NSF) through the Materials Research Science and Engineering Center at Harvard (MRSEC) (Contract No. DMR-0820484). The 2D reference pattern was fabricated at

the Center for Nanoscale Systems (CNS) at Harvard University, a member of the National Nanotechnology Infrastructure Network (NNIN), which is supported by the NSF (Award No. ECS-0335765). We thank Stan Cotreau for help with the design and fabrication of the device and Tom Kodger for useful discussion.

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