Experimental Variation of Magnification Calibration for Localization Microscopy

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Abstract: We study the variation of a localization microscope from temporal and thermal factors, enabling elucidation of the discrepancy between transillumination brightfield and epi-illumination fluorescence of an aperture array for magnification calibration.

Magnification is the fundamental function of optical microscopy, and the scale factor between the size of image pixels and camera pixels is its primary quantity. A common but potentially inaccurate assumption in localization microscopy is that the scale factor equals its nominal value. A less common but potentially insufficient calibration of the scale factor involves imaging a coarse reference material. Distortion is an additional complication [1], rendering even methods that accurately determine mean values of magnification [2] subject to position errors across a wide field. In a previous study [3], we showed that assumption of a nominal magnification can result in position errors extending into the micrometer scale, while even an accurate calibration of mean magnification can result in position errors of hundreds of nanometers across a wide field. To solve this problem, we developed aperture arrays as reference materials for localization microscopy, achieving a widefield calibration of image pixel size that is accurate to one part in 5×10^3, which is our current limit of fabrication accuracy. However, we also found a comparable discrepancy in magnification between the imaging modes of transillumination brightfield and epi-illumination fluorescence, with unclear origins and important implications for the use of brightfield imaging in the production of reference materials and for the use of fluorescence imaging in the calibration of localization microscopes.

In this study, we quantify several aspects of experimental variation to elucidate the effects of illumination optics on magnification calibration. Our microscope and methods are similar to those of our previous study [3], but we better match the imaging wavelengths to minimize any discrepancy from chromatic aberrations (Fig. 1a). For widefield imaging of aperture arrays with high throughput (Fig. 1b), and for calibration of localization data from particle tracking of microelectromechanical systems [4-6], we select an objective lens with a nominal magnification of 50×, a numerical aperture of 0.55, and a working distance of 9 mm with air immersion. The platinum surface of the aperture array faces the objective lens. We localize each aperture by light-weighting and apply a similarity transformation to register the positions to an ideal array with a pitch of 5000 nm, determining the scale factor [3].

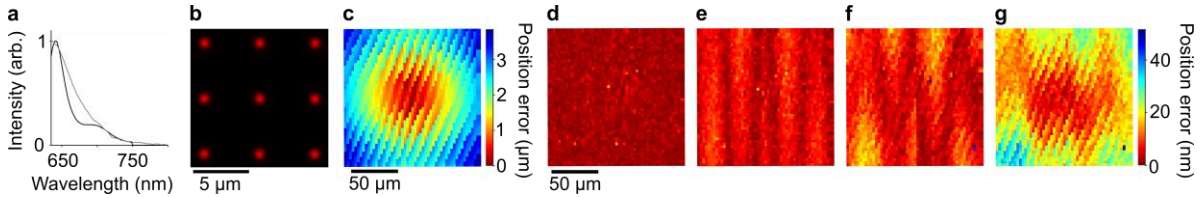

Figure 1. (**a**) Plot showing imaging wavelengths for (gray line) transillumination brightfield and (black line) epi-illumination fluorescence. **(b)** Optical micrograph (false color) showing a region of an aperture array under transillumination brightfield. (**c-g**) Scatter plots showing the total magnitude of position errors at each aperture location resulting from (c) inadvisable assumption of the nominal value of magnification, (d) *in situ* calibration for transillumination brightfield, (e) application of the calibration from (d) to a replicate a few hours later, (f) application of the calibration from (d) to a replicate a few days later, and (g) application the calibration from (d) to data from epi-illumination fluorescence.

In the worst case of assumption of the nominal magnification, gross errors of position result (Fig. 1c). These position errors increase with radius from the field center, extending to several micrometers at the field corners, while striations indicate inadvertent motion of the microscope system during readout of the imaging sensor with a rolling shutter. In the best case of an *in situ* calibration, in which the calibration both derives from and applies to a single image of an aperture array, the resulting position errors are due mostly to fabrication precision (Fig. 1d) [3]. Over several hours, the mean value of image pixel size [2] varies with a standard deviation of 0.002 nm in transillumination brightfield, or a relative standard deviation of one part in 6×10^4 (Table 1). Over several days, this variation increases to 0.006 nm for transillumination brightfield (Table 2) and 0.009 nm for epi-illumination fluorescence (Table 3), possibly due to variation of ambient temperature from 22 °C to 25 °C. Position error striations during tests of temporal variation of

magnification further indicate microscope motion during sensor readout (Figs. 1e-f). We test the thermal dependence of magnification by using an enclosure to control the air temperature around the aperture array, objective lens, filter set, and tube lens of the microscope system. The image pixel size decreases significantly by 0.04 nm as temperature increases from 22 °C to 40 °C (Table 4).

Table 1. Hourly variation (transillumination brightfield)

Image pixel size (nm)
127.3276
127.3298
127.3322
127.3313

Standard deviation = 0.0020 nm

Table 2. Daily variation (transillumination brightfield)

Image pixel size (nm)
127.3190
127.3298
127.3257

Standard deviation = 0.0055 nm

Table 3. Daily variation (epi-illumination fluorescence)

Image pixel size (nm)
127.3333
127.3477
127.3488

Standard deviation = 0.0086 nm

Table 4. Temperature variation (transillumination brightfield)

Temperature (°C)	Image pixel size (nm)
22.0 ± 0.5	127.3109
30.5 ± 0.5	127.3313
40.0 ± 0.5	127.3575

Uncertainties are limits of error

Table 5. Illumination variation (from transillumination brightfield to epi-illumination fluorescence)

Imaging mode	Image pixel size (nm)
Transillumination brightfield	127.3257
Transillumination brightfield with ground-glass diffuser and DMF	127.3242
Transillumination brightfield with scattering nanoparticles	127.3304
Epi-illumination fluorescence	127.3488

Having estimates of the effects of temporal and temperature variation, we vary the illumination optics between transillumination brightfield and epi-illumination fluorescence (Table 5). Image pixel size varies by one part in 5×10^3, resulting in significant errors upon application of a brightfield calibration to a fluorescence image (Fig. 1g). This discrepancy remains after the addition of a ground-glass diffuser in the illumination path, which reduces partial coherence of the light-emitting diode, and a droplet of dimethyl-formamide (DMF) in water with a volume fraction of 80 % on the silica substrate of the aperture array, which is more representative of fluorescence measurements. Addition of a suspension of silica nanoparticles that scatter light reduces the discrepancy slightly to one part in 7×10^3. This indicates that the solid angle of light incident on the aperture array is a probable cause of the discrepancy between transillumination brightfield and epi-illumination fluorescence. Confirming this, calibration for transillumination fluorescence produces nearly identical results to epi-illumination fluorescence (not shown).

In conclusion, temperature variation from ambient to physiological conditions can significantly affect magnification calibration for localization microscopy. Periodic striations of position errors during magnification calibration indicate microscope motion during sensor readout and warrant further study, ranging from potential elimination of the effect by use of a global shutter, to potential application of the effect for motion measurement at the temporal resolution of a rolling shutter. Transillumination brightfield is appropriate for critical-dimension localization microscopy, but this imaging mode results in a magnification that differs significantly from that of epi-illumination fluorescence. The importance of matching the conditions between calibration and experiment motivates our ongoing development of built-in reference materials for *in situ* calibration of localization microscopy measurements.

References

1. C. R. Copeland, C. D. McGray, J. Geist, J. A. Liddle, B. R. Ilic, and S. M. Stavis, "Aperture arrays for subnanometer calibration of optical microscopes," in *2017 International Conference on Optical MEMS and Nanophotonics (OMN)*, (IEEE, 2017), 1-2.
2. X. Dai, H. Xie, C. Li, Z. Wu, and H. Geng, "High-accuracy magnification calibration for a microscope based on an improved discrete Fourier transform," Optical Engineering **52**, 114102 (2013).
3. C. R. Copeland, J. Geist, C. D. McGray, V. A. Aksyuk, J. A. Liddle, B. R. Ilic, and S. M. Stavis, "Subnanometer localization accuracy in widefield optical microscopy," Light: Science & Applications **7**, 31 (2018).
4. C. McGray, C. R. Copeland, S. M. Stavis, and J. Geist, "Centroid precision and orientation precision of planar localization microscopy," Journal of Microscopy **263**, 238-249 (2016).
5. C. R. Copeland, C. D. McGray, J. Geist, V. A. Aksyuk, and S. M. Stavis, "Transfer of motion through a microelectromechanical linkage at nanometer and microradian scales," Microsystems & Nanoengineering **2**, 16055 (2016).
6. C. R. Copeland, C. D. McGray, J. Geist, and S. M. Stavis, "Particle tracking of microelectromechanical system performance and reliability," Journal of Microelectromechanical Systems **27**, 948-950 (2018).