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An Estimate of the Contribution of Spherical Aberration and Self-shadowing in Confocal and Multi-photon Fluorescent Microscopy

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Conventional histological sectioning and staining techniques produce tissue sections that are independent from each other (*i.e.* the staining intensity depends on the total stainable substance within the section, but is not influenced by the section above or below it). Although the hardness of the structure in a given section may influence the quality of the subsequent sections, nevertheless, each physical section can be generally regarded as an independent sampling event. However, due to spherical aberration and self-shadowing effects, the image intensity, relative contrast, and resolution in an optical section obtained from confocal or non-linear optical microscopy varies depending on the position of the section where it was obtained. Therefore, in sampling terms, optical sections are dependent sampling events. Artifacts caused by the geometry [1] and optical properties (*e.g.* absorption, scattering, refractive index) of objects within a specimen can interfere with the interpretation of the result and hinder the usefulness of 3D reconstruction and feature segmentation.

Objective lenses are optically corrected for spherical aberration under certain operating conditions, thus it is important to use the correct cover slip thickness, and to use the correct refractive index of the immersion medium used. The use of incorrect cover glass not only results in a significant shifting of the focal plane, but also re-introduces spherical aberration. A simple experiment using a fluorescent plastic slide was conducted to demonstrate the effect of using incorrect cover glass. Figure 1 shows the effect of a cover glass in an x-z view. A cover glass was placed on top of a fluorescent slide halfway into the field of view. The left half of the image was obtained without the cover glass while the right hand side of the image was taken with cover glass. Note the significant shifting of the focal plane. Optical sectioning obtained from various depths within a specimen may result in varying degrees of deterioration in spherical aberration correction. This will have a serious effect on both the intensity and integrity of the fluorescent signal obtained at various depths within a specimen when optical sectioning by confocal or multi-photon microscopy. The brightest plane in the fluorescent slide indicates the position of optimal spherical aberration correction (Fig. 2). Figure 3 shows a stack of photo-bleached planes written by two-photon excitation (λ=780nm) in a fluorescent plastic slide. The spacing between the photo-bleached planes was set at 10μm. Note the degree of photo-bleaching and the sharpness of the photo-bleached planes vary as a function of depth; this is due to the result of changing spherical aberration correction. Even the constituents of a cell can create enough optical inhomogeneity to cause significant shifting of the focal plane. Figure 4 shows the effect of a living CHO cell on the underlying fluorescent slide. Note the fluctuation of the slide surface and the intensity degradation under the cell. This effect can be dramatically demonstrated when a slice of maize stem was placed on a fluorescent plastic slide (Fig. 5). Even a simple object such as a latex bead (4μm) can generate a complex shadow (Fig. 7). A two photon fluorescent image of a clear Mylar flake (Fig. 6, arrow) suspended in FITC gel further demonstrates the effect of optical inhomogeneity, in this case resulting in long shadows and strong edge effect (Fig. 6). Intensity variation across the field is another important issue when attempting quantitative fluorescent imaging and feature segmentation. Figure 2a shows the changes in fluorescent intensity across the field and as a function of depth in the fluorescent plastic slide. The examples shown above demonstrate the complexity of

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interpreting confocal and multi-photon micrographs. Dynamic spherical aberration correction may be a possible way to minimize the problem.

[1] P. C. Cheng and W. Y. Cheng, Microsc. Microanal., 7 (Supp. 2)(2001) 1018-1019.

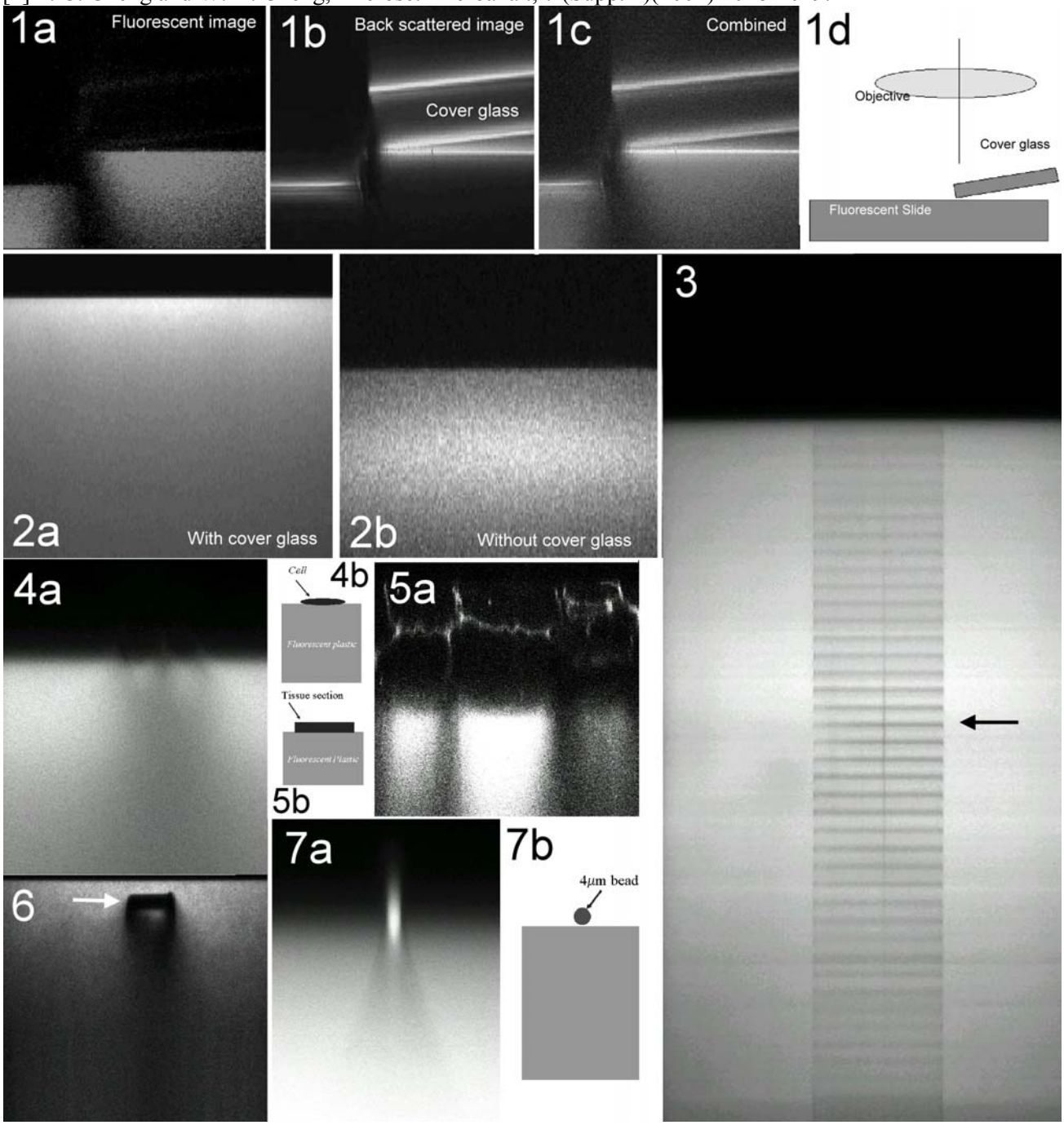

FIG 1a-1d: Fluorescent (1a), back scattered (1b) and combined image of 1a and 1b (1c) of the setup shown in1d. Note the shifting of the surface of the fluorescent slide. (2P, λ=780nm)

FIG 2a and 2b: Intensity variation in images obtained with and without cover glass (2P, λ=780nm)

FIG 3: Photo-bleached planes written in a fluorescent slide. Arrow: best beam profile position.

FIG 4a, b; 5a, b: Effect of biological samples on the image obtained of an underlying fluorescent slide (Fig 4:1P, λ=488nm; Fig 5: 2P, λ=780nm)

FIG 6: Mylar flake suspended in FITC gel (2P, excitation 780nm).

FIG 7: Effect of a 4μm latex bead on the image of an underlying fluorescent slide (1P, 488nm)

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