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Possible Effects of Fluorescence Bleaching/Saturation on the Definition of the Paper Surface in CLSM Measurements

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We have observed that scanning a paper surface with a low f-number objective (10×0.3) in the "reflection mode" at 488 nm on our Leica TCS4D, an ambiguous definition of the paper surface may occur, when using a modified centre of mass algorithm for detection of the surface. This ambiguity depends among other things on the laser power, and it manifests itself both in the texture of the SFP images, and in the calculation of surface roughness parameters. But as this "reflection" mode also includes the fluorescent emission of the object we tend to interpret the result as an effect of fluorescence bleaching and saturation (Visscher et al. 1994).

We have several indications for this.

1) a given laser power seems to give a repeatable result for the surface roughness. This means that a measurement of laser power P3 gives approximatly, the same result as a measurement of laser power P1 if P3=P1, even if a measurement of power P2 at a higher power level than P3 has been made in between. All measurements are made over exactly the same area.

2) the short time fluctuations in laser power seems to be insignificant, when tested using a flat mirror.

3) the effect depends on the averaging mode. Line averaging gives a stronger effect than frame averaging for the same energy exposure. The repetition frequency experienced by each voxel is in order 0.001 s for line average and 0.1 s for frame average.

POSSIBLE MECHANISMS

This inconsistency may come from one or more of several sources.

-the topography of the paper surface may change mechanically due to the exposure of the laser.

-the optical properties of the upper surface layer may change due to the laser light.

-the laser radiation may be erratic itself creating high frequency laser power fluctuations.

-electronic or digital noise from the recording and an imperfect optical impulse response function of the objective may give random errors in detection of the surface.

-the surface detection algorithm may be instable or there are some error in the software.

Having analyzed these factors, a change in the optical properties of the surface layer may be the most likely, as the constitutents of paper may have optically unstable properties in the 480-500 nm region. These instabilities show up as photo yellowing, changes in the luminescent absorption and emission spectra and fluorescent bleaching (Olmstead and Gray 1993). Athough the paper may be very inhomoge-neous with fines and fibre parts in the sub resolution regime which could change form during exposure, the scattering cross section of these are so small that topological change in these would be less likely to cause the observed changes.

MAGNITUDE OF THE EFFECT

For the laser power high (3-5 mW), medium (1-2 mW) and low (<1.0 mW) with line averaging (8 x), the calculated roughness parameter Ra. (average magnitude of height deviation from mean height) was respectively 1.3μm, 1.6μm and 2.3μm. Whereas for 8x frame and average medium laser power the calculation gave Ra=1.3μm. Apart from the change in laser power and averaging method and proper adjustment of the dynamic range, all other sampling parameters were equal. Sampling distance in z-direction was 1.0 μm. The variation in the 1 μm

range are to be compared with the FWHH of 4. μm in the axial resolution curve. Even without centre of mass calculation of the surface position, the numeric noise contribution to Ra should be approximately: 1μm/512*512=0.002 μm.

IMPACT

We feel that a qualified answer to the cause of the observed deficiency is not yet found. Alterations in the fluorescence absorption, and thus the reflection properties of the fluorphores may influence their measured spatial distribution. The surface roughness measurements of papers by use of confocal microscopes as well as by laser profilometers must be closely examined, if a deficiency relating to fluorophore properties can be confirmed.

REFERENCES

Visscher K, GJ Brakenhoff, TD Visser. 1994. Fluorescence saturation in confocal microscopy. J. of Microscopy **175:** 162-165.

Olmstead JA, DG Gray. 1993. Fluorescence emission from mechanical pulp sheets. J. Photochem. Photobiol. A: Chem. **73:** 59-65.