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Quantitation Strategies in Optically Sectioning Fluorescence Microscopy

The development of microscopes that can record fluorescence intensity in five and more dimensions in one experiment (space, time and colour) makes parameters available for the scientific community that were previously beyond reach. A new realm of possibilities is opened by the observation of correlations between these dimensions. The goal of front-end microscopy is the extraction of quantitative information from certain correlations. In fluorescence recovery after photobleaching, for instance, diffusion coefficients are calculated from the correlation of space and time. In Förster resonance energy transfer imaging molecular interactions and concentrations are determined, and space and colour correlated.

To render the correlations that are determined in these experiments as valid, the recording system needs to be thoroughly calibrated. Otherwise artefacts arise that can lead to wrong conclusions, and the quantitation of the parameters is impossible. The first part of this thesis was determined to alleviate quantitation and optimization in optically sectioning microscopy. For this purpose the new tool for the assessment of imaging quality in optically sectioning microscopes, the sectioned image property chart (SIPchart), was fundamentally re-engineered obtaining the extended SIPchart (eSIPchart). SIPcharts are created by the analysis of an axial image stack of a thin uniform fluorescent layer. A pixelwise fit to the axial intensity distributions yields parameter maps that describe a certain imaging property in the field-of-view, e. g. field-dependent intensity variations.

The eSIPchart routine was developed in the course of this thesis to analyse axial image stacks of fluorescent solutions. It implements the following: a statistical analysis is performed with which arbitrary detector units are converted to real photon numbers. These allow the evaluation of the goodness-of-fit with a X^2_R- parameter map. They also enable the creation of concentration calibration curves. The implemented Voigt-fit gives a weight factor, on whose basis the quality of the illumination can be estimated. Great benefit can be taken from the transition from the recording of thin layers to fluorescent solution: i) the same dye can be used for calibration and for imaging. Hence calibration errors caused by deviating wavelengths and refractive indexes of the test sample, i. e. a thin layer, can be excluded. ii) fluorescent dyes can be found in places where fluorescence microscopy is performed. They are easily available, cheap, and versatile. iii) a fluorescent solution can contain several dyes and thus cover a big spectral range. iv) the refractive index of the solvent can be adjusted so that it matches the refractive index of the immersion medium. With the data obtained from eSIPchart analysis, a thorough calibration can be performed for chromatic and spherical aberrations and those which origin from the setting of the correction collar. Finally the eSIPchart gathers the characteristics of a particular setting in a microscope in a comprehensive manner. It thus becomes possible to judge upon the imaging properties and the suitability of an optically sectioning fluorescence microscope for a designated experiment at a glance.

The second part of the thesis deals with the application of a set of custom evaluation tools for the comparison of three microscopy systems. The ApoTome, a linear grid structured illumination microscope, is opposed to the confocal laser scanning microscope (cLSM), and conventional widefield epifluorescence microscopy. Different parameters of imaging are assessed with different techniques: the axial and lateral resolution is measured with subresolution fluorescent beads and thin uniform fluorescent layers. The field dependency is evaluated with eSIPcharts. The resolution anisotropy is measured by using linear dendrites from cell cultures which were prepared from olfactory bulb neurons of the larva of *Xenopus laevis*. Deep tissue imaging is evaluated eventually by using microinjected mitral cells in the olfactory bulb of the same animal. It was found out that the ApoTome achieves a better lateral resolution than a cLSM in standard imaging conditions, *viz.* with a confocal pinhole diameter of d_p = 1A:U: in the cLSM and the

recommended grid “VH” in the ApoTome. This is because the ApoTome’s backfocal plane (BFP) is illuminated homogeneously, whereas the cLSM’s BFP is illuminated with the clipped Gaussian intensity distribution of a laser. The axial resolution is better in the cLSM than in the ApoTome with one exception: the ApoTome exceeds the cLSM in the resolution of low spatial frequencies recorded at optimal resolution conditions (cLSM, d_p = 0:4A:U: and ApoTome, “VL”-grid). The axial resolution in the cLSM has a central-symmetric distribution over the field-of-view, mainly due to scanning-related aberrations, whereas the ApoTome’s axial resolution appears to be homogeneously distributed over the field-of-view. Grid-structured illumination microscopy features anisotropic resolution and selective enhancement of structures in the sample that have the same orientation and frequency as the grid.

Structured illumination microscopy is restricted to thin specimen because Poissonian noise contributions from out-of-focus fluorescence cannot be removed. In addition to that, the sample needs to remain stable for at least some seconds since the acquisition of the raw data for one optically sectioned image lasts ≈ 1 s.