Proceedings

High-precision Distance Measurements in Epifluorescent Microscopy — Simulation and Experiment

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The accurate localization of small target regions is of great importance in many applications. In quantitative fluorescence microscopy, efforts are made to localize small target regions such as genes with high precision. For example, for the diagnosis of chronic myelogenous leukemia (CML), a precise distance measurement of the two genetic regions abl (22q11) and bcr (9q34) in the interphase nucleus is important to support diagnosis. Furthermore the chromosomal organization in the nucleus can be investigated by means of distance measurements between labeled targets of known genomic separation [1,2].

The localization of small targets depends on different factors such as the conditions of the optical set-up, the method used for evaluating the data and the statistical noise of the image. A good knowledge of the influence of the noise is important for choosing the right image acquisition parameters, such as integration time and sampling rate. Their values should be chosen so, that the statistical localization error is well below other known systematic errors.

Simulations

Various Monte Carlo simulations allow to estimate several components of the localization precision $DX=F(I_{total}, I_{add\text{-}noise}, ?W_{Gauss}, I_{Clip}, exp_{COM})$• where I_{total} is the integrated intensity of the unclipped gaussian peak without additive noise, $I_{add\text{-}noise}$ is the mean intensity of the additive noise in each pixel, $?W_{Gauss}$ is the full width at half maximum of the gaussian peak to locate, I_{Clip} is the pixel intensity value to clip at and exp_{COM} is the exponent to apply to each intensity value before using the center of mass (COM) algorithm for localization.

If the influence of all these factors on the localization precision is known, the experimental set-up as well as the evaluation method can be optimized to minimize the statistical error in distance measurements.

Simulations were performed under the assumption that an object, small compared to the width of the point spread function (PSF) of the used system, was imaged. A gaussian peak was used as approximation of a true lateral PSF. For example a reduction of the statistical localization error by a factor of about 2 was achieved at a clipping-value of 15% ($?W_{Gauss}$ =4.0 pixel, I_{total} =5.5x10^6 quantum units) using an optimized method (see below) in contrast to a simple clipping method.

Improvement of the algorithm

The simulations showed that the localization precision can be optimized by choosing the right method of evaluation (Figure 1). The reason for the difference in localization precision is the fact that pixels near the threshold value are or are not clipped due to their statistical fluctuations. Pixels with an intensity value slightly higher than the clipping-value enter the COM algorithm with an already substantial weight, so that the statistical nature of the clipping-edge influences the localization to a great extent. Using the zero-clipping COM method suppresses these near-edge pixels sufficiently, so that the influence to the obtained localization is comparatively small.

To further suppress these near-edge pixels, a second method can be used. Applying a constant exponent to each pixel-value before application of the COM algorithm can suppress or enhance values of low intensity. This can also be seen as choosing a method somewhere between center of area (exponent 0, each pixels

gets the same weight 1), center of mass (exponent 1) and extraction of the maximal pixel (using a very high exponent). According to the simulations, the optimal exponent applied to the data depends on the clipping value and the statistical noise.

Experiments

Experimentally obtained pictures of fluorescent microspheres were evaluated with a Zeiss Axiophot microscope system [objective 40x] equipped with a sensitive CCD-camera, and the results obtained for the localization precision were compared with the simulations (Figure 2).

For this comparison it is important to know the exact amount of additive and multiplicative noise in the image. The multiplicative noise can be experimentally determined by recording successive images of a smooth intensity distribution and by histogramming the difference-image in dependence of the mean-image. Simulating the localization process then leads to an a-priori knowledge of the statistical localization error. This error was in good agreement with the experimentally obtained values.

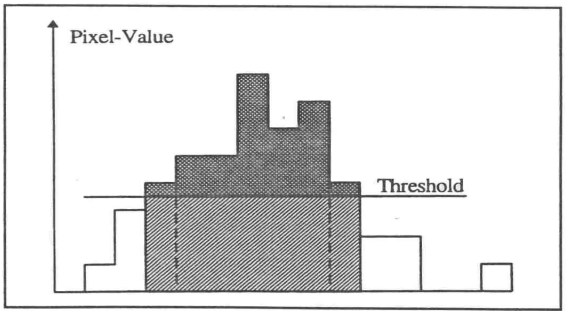

Figure1: The clipping of the peak in the obtained image can be done in different ways. In the first method, pixel-values lower than the threshold are set to zero. Both shaded regions remain to be further evaluated with the COM method. Simulations showed, that evaluating only the dark shaded region with the COM algorithm gives a much higher precision (improvement by a factor of 2). This is done by substraction of the threshold and zero-clipping.

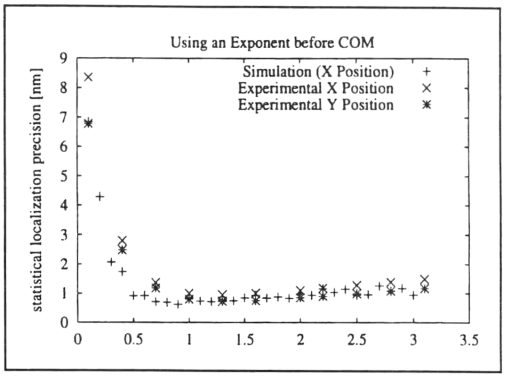

Figure2: Experimentally obtained values for the localization precision were compared with simulated localization. The width of the peaks to localize was 4 pixel (FWHM) (that corresponds to 670nm). The objects were tetraspectral microspheres (Ø=560nm). The maximal intensity in the CCD-image (Camera: Potometrics, Tucson, AZ) was 3200 analog to digital units (ADU). The threshold used for evaluation was 500 ADU.

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