Localization microscopy (e.g. PALM/STORM) is a powerful tool for imaging biological structures on the nanoscale. Determining the molecular composition of a sample requires that localization microscopy images are quantifiable in terms of the density of fluorescently labeled molecules or of binding sites. However, for techniques relying on reversibly switching fluorophores, quantification is severely hampered by overcounting due to the unknown number of switching cycles a fluorophore undergoes. Additionally, the stoichiometry of the number of fluorophores on a marker (e.g. an antibody) is often unknown.

Here, we present how these effects can be accounted for. We introduce a novel three-state model for the on-off switching and bleaching of single fluorophores and measure the transition rates in this model by analyzing the time dependence of spatial correlations in the acquisition [1]. In addition, we show how the number of fluorophores per marker (e.g. antibody) that contribute localizations during an acquisition can easily be calibrated using Fluorescence Correlation Spectroscopy. Together, this allows us to estimate the average number of localizations per marker with a precision on the order of 10 - 20%.

We show how our method is tested on experimental images of DNA oligomers labeled with single Alexa Fluor 647 fluorophores and neutravidin tetramers labeled with multiple Alexa Fluro 647 fluorophores, and applied to experimental images of a constituent protein of the nuclear pore complex labeled with nanobodies (see figure) and of Immunoglobulin E receptors.