Fluorescence Correlation Spectroscopy (FCS) is a powerful method to measure diffusional and rotational properties of molecules as well as intramolecular transitions (e.g. triplet transition rates and isomerization) and intermolecular interactions (e.g. protein-protein, DNA-protein, etc.). Since the introduction of the confocal measuring principle by Rigler et al. and single photon counting avalanche photodiodes, FCS techniques attained a tremendous revival due to an increased sensitivity enabling even the study of single fluorescent molecules traversing the open, optically confined volume element (so-called voxel). Despite these improvements it still remains desirable to further enhance both, the degree of confocality (i.e. to increase in practice the vertical resolution by minimizing spherical and chromatical aberrations) as well as the most important parameter in FCS measurements, the count rate per molecule (CRpM), in order to further improve the overall performance of FCS measurements (i.e. to enhance signal-to–noise and signal-to-background ratios of auto- and cross-correlation functions, and, simultaneously, to minimize acquisition times and the effective optical volume of measurement).

Here we present investigations showing an increase of the CRpM of up to 200 kHz (about factor 6 improvement) for Rhodamine Green (excit. wavelength 488nm, em. wavelength 520 nm +/- 20 nm (FWHM), excitation laser power ca. 1.3 mW) along with an effective confocal measuring volume in the subfemtoliter range. Fig.1 displays as an example the corresponding autocorrelation function of a 1 nM solution of Rhodamine Green. The improvements were achieved by utilizing achromatic lenses with special surface coatings (to minimize reflection) as well as via slight overfill of the back-aperture of the objective lens (Nikon 60*, water immersion with collar rim correction) enabling the reduction of spherical aberrations in aqueous media (provided the coverglass thickness is exactly determined by means of a µm screw and corrected by the collar rim) and, in addition, by using a Kr-Ar-Ionlaser with TEMoo mode. Finally, an optimised optical alignment of all components (e.g. laser, mirrors, lenses, fiber optics, etc.) resulted in the significant improvements described above.

![Figure 1: Autocorrelation function of an aqueous 1nM solution of Rhodamine Green (excit. 488nm, em. 520nm, laser power 0.13mW, acq. time 30s , CRpM=120 kHz, Veff = 0.76 femtoliter, r = 260 nm, z = 2.07 µm)](image-url)