Supporting Information to: Successful FCS experiment in non-standard conditions

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Additional figures (S1–S10) with calculated and experimental FCS correlation functions used to obtain the results presented in the paper and additional dependencies of the parameters of the CFs on the sample refractive index, penetration depth and window thickness for some objectives.

- Calculated correlation functions: Figure S1
- Experimental correlation functions
  - water immersion objective: Figure S2–S3
  - LD objective: Figure S4
  - silicon oil objective: Figure S10
- CFs parameters:
  - D/D_0 and N/N_0 as a function of n_s: Figure S5
  - τ and SP as a function of NA: Figure S6
  - D/D_0 and N/N_0 as a function of h for different objectives: Figure S7–S10
  - D/D_0 and N/N_0 as a function of window thickness: Figure S10

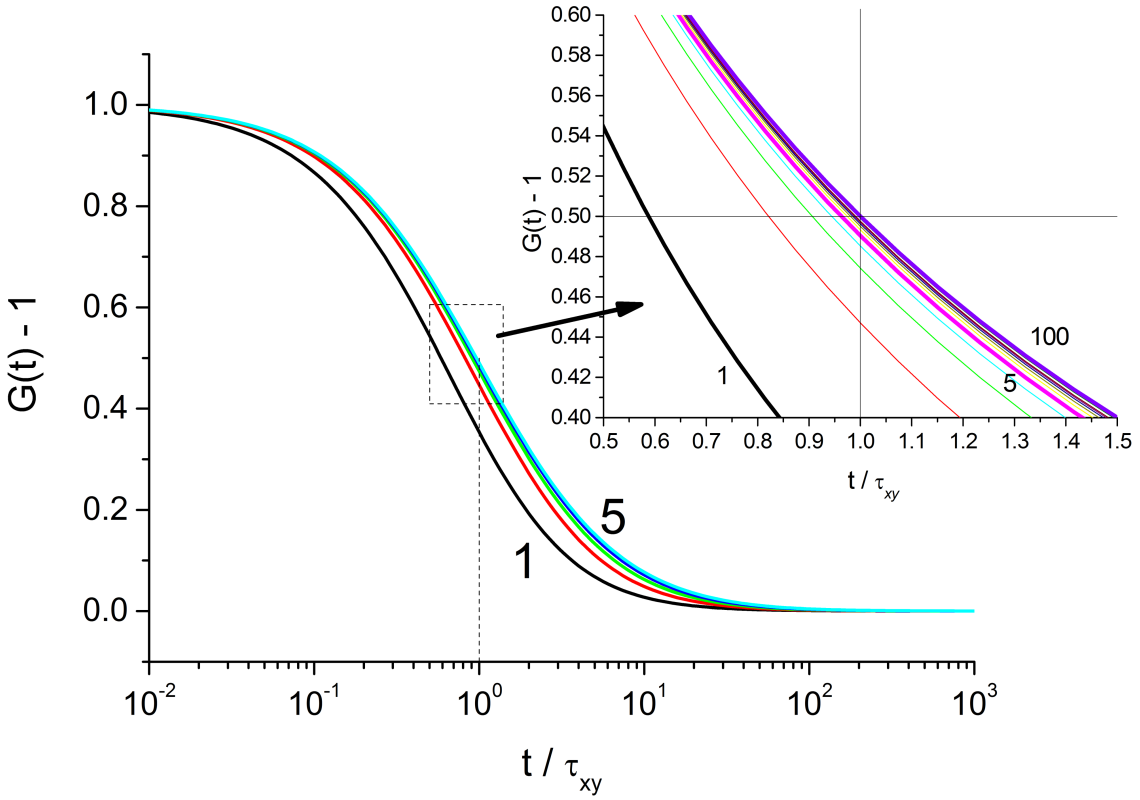

Figure S1: Total FCS correlation functions calculated using Eq. 4 for $T = 0, N = 1$ and SP equal to 1, 2, 3, 4, 5, from left to right. The inset shows magnified region marked as dashed rectangle with additional lines calculated for SP increasing from 10 to 100. Only such magnification allows distinguishing curves calculated for $SP > 10$. Here the correlation functions are very similar and practically overlap for $SP > 20$.

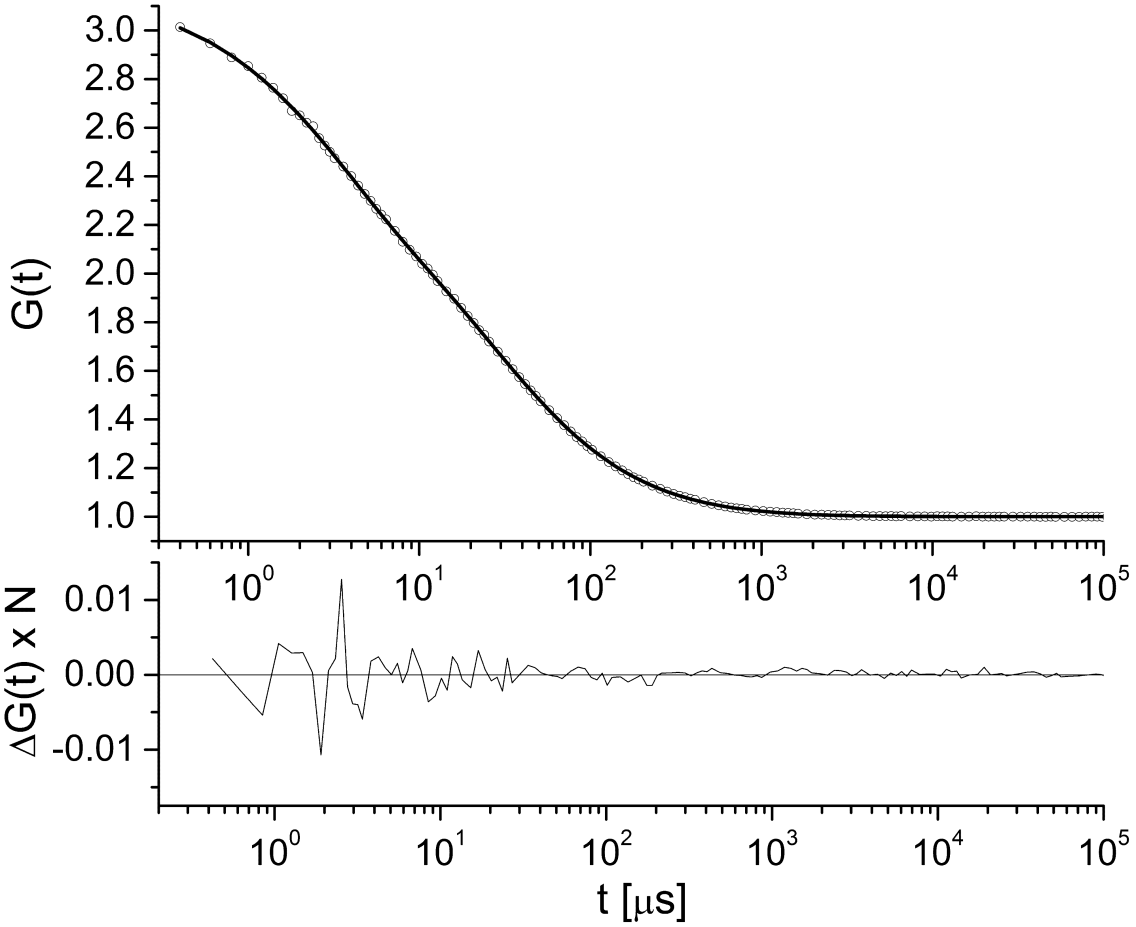

Figure S2: Typical FCS CF measured for Alexa488/water solution using the Zeiss water immersion objective (#1). The symbols represent the experimental data and the solid line represents a fit using Eq.4a. The lower part of the plot shows the deviation plot for this fit.

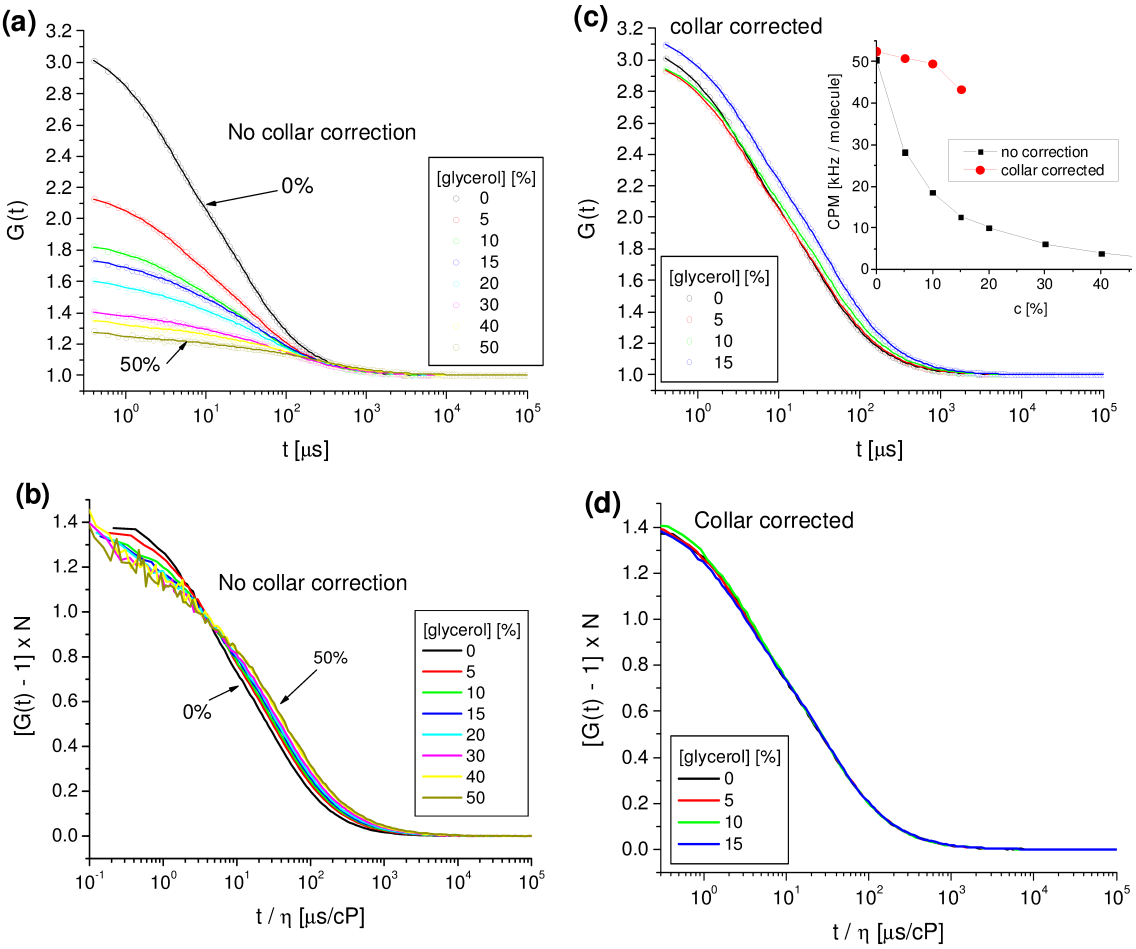

Figure S3: FCS CFs measured at a depth of 200 μm for Alexa488 in water/glycerol solutions of glycerol concentrations amounting to 0, 5, 10, 15, 20, 30, 40, and 50 % at a temperature of 22.5°C using the Zeiss water immersion objective (#1): a) CFs measured without collar correction the collar was optimized for the pure water solution and left unchanged for all other measurements. b) The same data with contrast normalized using N from the fit plotted vs. t/η. c) The same solutions measured with collar correction the position of the correcting collar was optimized for each solution separately. d) Data from (c) with contrast normalized using N from the fit plotted vs. t/η. Note that the scaled data points above one in (b) and (d) result from the triplet contribution. Inset in c) shows the concentration dependence of the molecular brightness (counts per molecule – CPM).

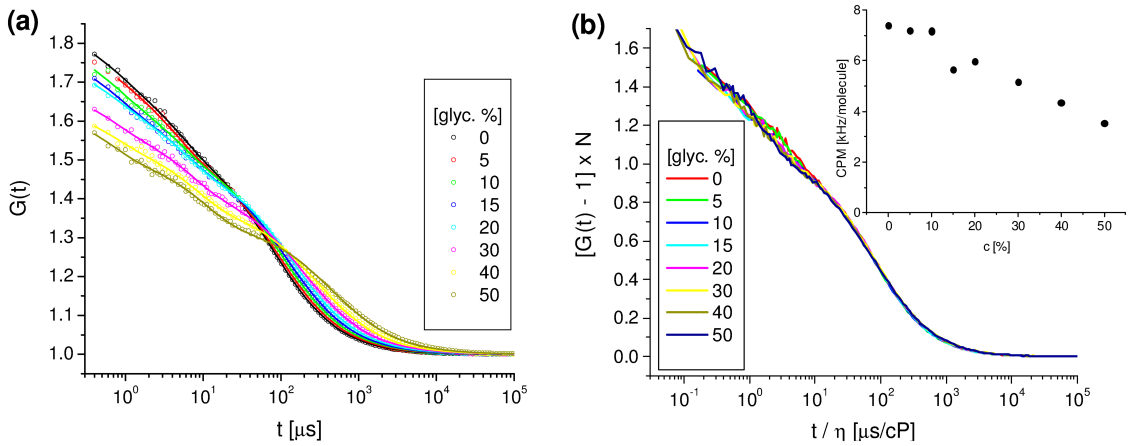

Figure S4: FCS CFs measured at the depth of 200 μm for Alexa488 in water/glycerol solutions of glycerol concentrations amounting to 0, 5, 10, 15, 20, 30, 40, and 50 % at a temperature of 22.5°C using the Olympus 40 × 0.6 LD objective (#3 in Table 1): a) experimental CFs, b) The same data with contrast normalized using N from the fit plotted vs. t/η. All measurements were performed with the correction collar in the optimum position for the pure water solution. Note that the scaled data points above one in (b) result from the triplet contribution. The inset in b) shows the molecular brightness CPM.

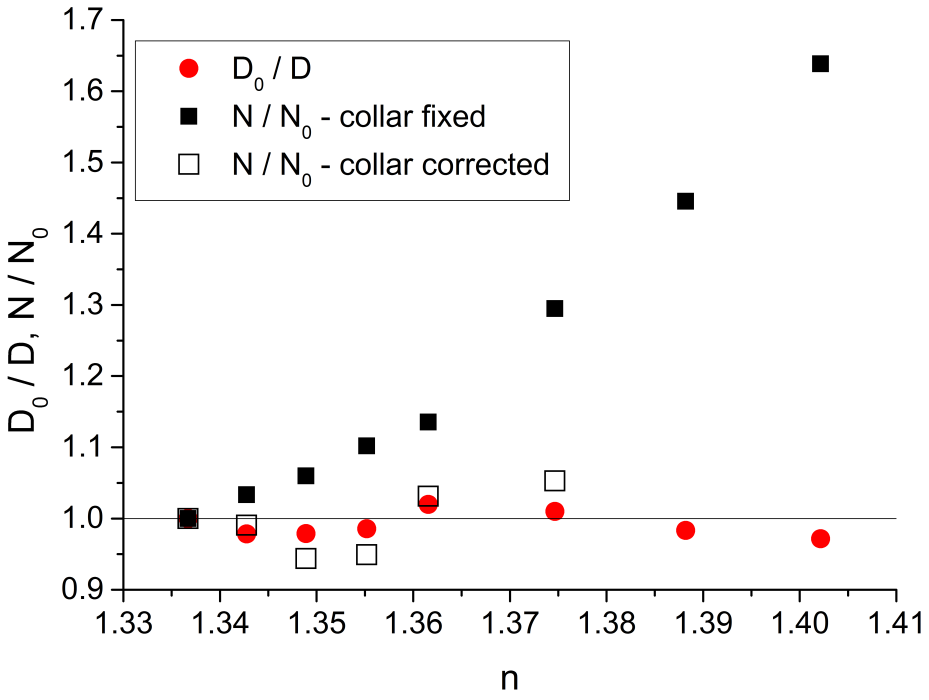

Figure S5: The normalized diffusion coefficient D/D_0 (●) and normalized number of particles N/N_0 (■) calculated from the CFs from Fig. S4 measured for Alexa488 in water/glycerol mixtures of different refractive index n_s with Olympus LD objective (#3 in Table 1) with the correction collar fixed to the position optimum for water (full symbols) and adjusted to the current conditions (open symbols). Note that each point represents a different sample. The measurements were performed for $h = 200$ μm (fixed collar) and $h = 100$ μm (collar corrected).

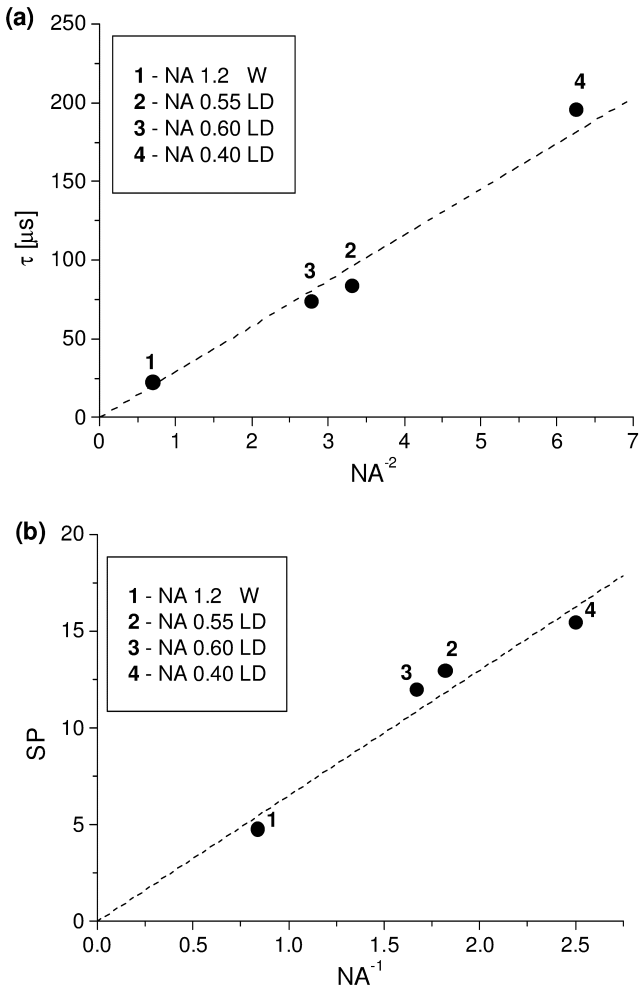

Figure S6: Correlation times (a) and the SP parameters (b) of the FCS CFs measured for Alexa488 in water solutions using objectives of different numerical aperture NA (numbers as in Table 1) plotted vs. NA^{-2} and NA^{-1}, respectively.

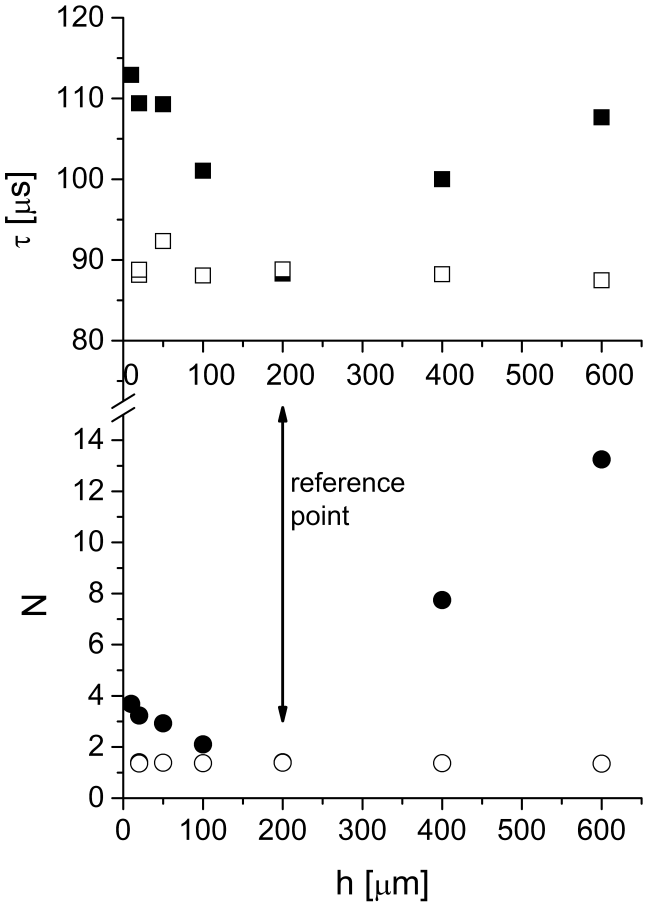

Figure S7: Correlation time τ and number of particles in the confocal volume N obtained from the fits to the FCS CFs of Alexa488 in water solutions at different penetration depth h of the incident beam into the sample: ■ - τ, ● - N obtained from measurements with the collar adjusted for $h = 200\mu$m chosen as a reference point; ❑ - τ, ❍ - N – collar adjusted for each h individually. Objective: LD Olympus 40×0.55 (#2 in Table 1).

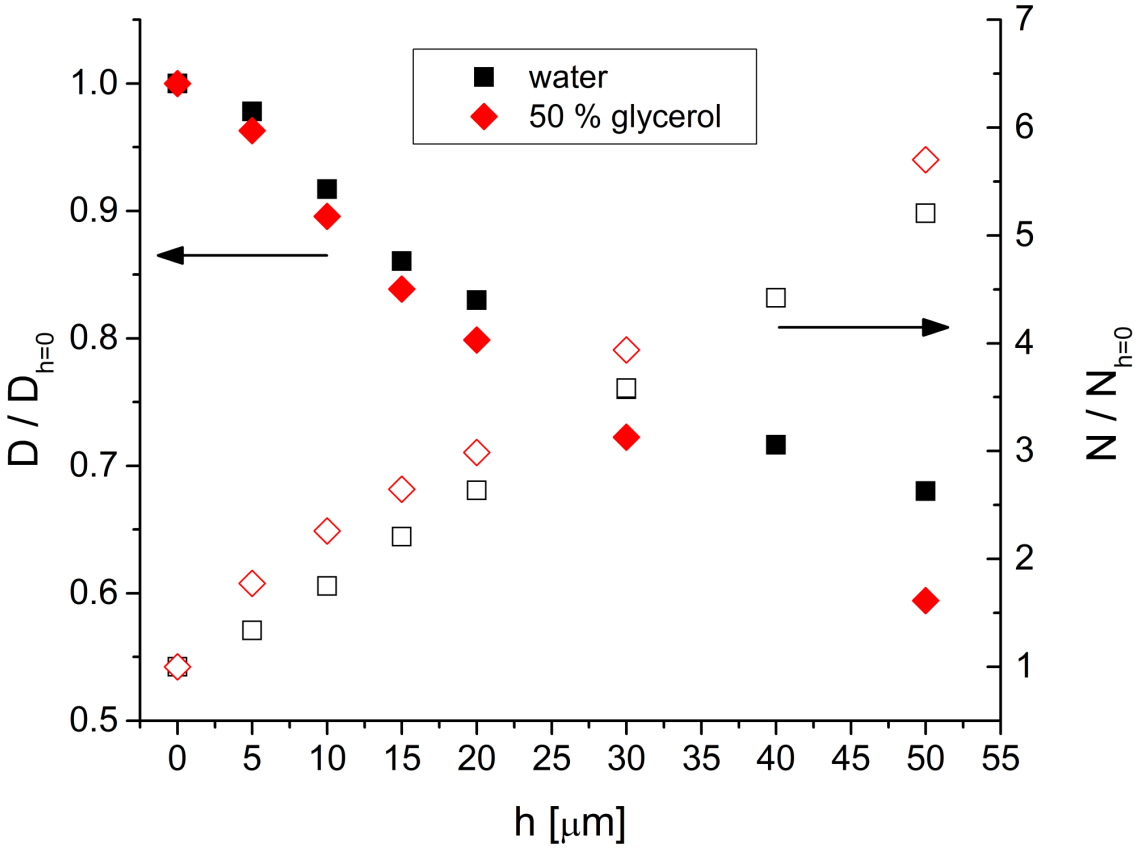

Figure S8: Relative changes of the diffusion coefficient $D/D_{h=0}$ (■, ◆) and the number of particles $N/N_{h=0}$ (❑, ✧) for different apparent penetration depth h of the incident beam into the sample for Alexa488 in water ($n_1 = 1.33$, ■, ❑) and in 50 % water/glycerol mixture ($n_2 = 1.402$, ◆, ✧), measured with the Zeiss oil immersion objective ($n_i = 1.51, NA = 1.4$, #5 in Table 1) without correction collar.

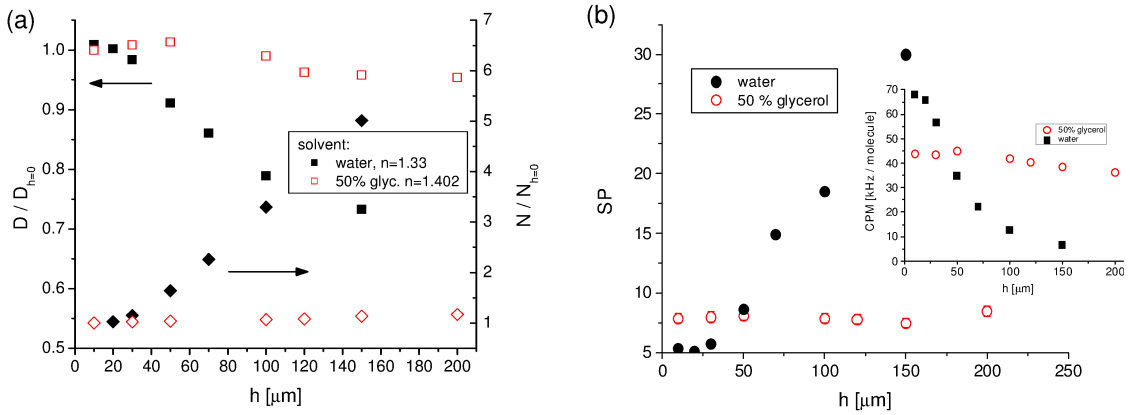

Figure S9: Relative changes of (a) - the diffusion coefficient $D/D_{h=0}$ (■,❑), number of particles $N/N_{h=0}$ (◆, ✧) and (b) - the structure parameter SP (●, ❍) for different apparent penetration depth h of the incident bean into the sample for Alexa488 in water (solid symbols) and in 50 % water/glycerol mixture (open symbols), measured with the Olympus silicon oil immersion ($n_i = 1.41$) objective, $NA = 1.3$ (#7 in Table 1) with the correction collar. The inset in b) shows the CPM depth dependence for both samples.

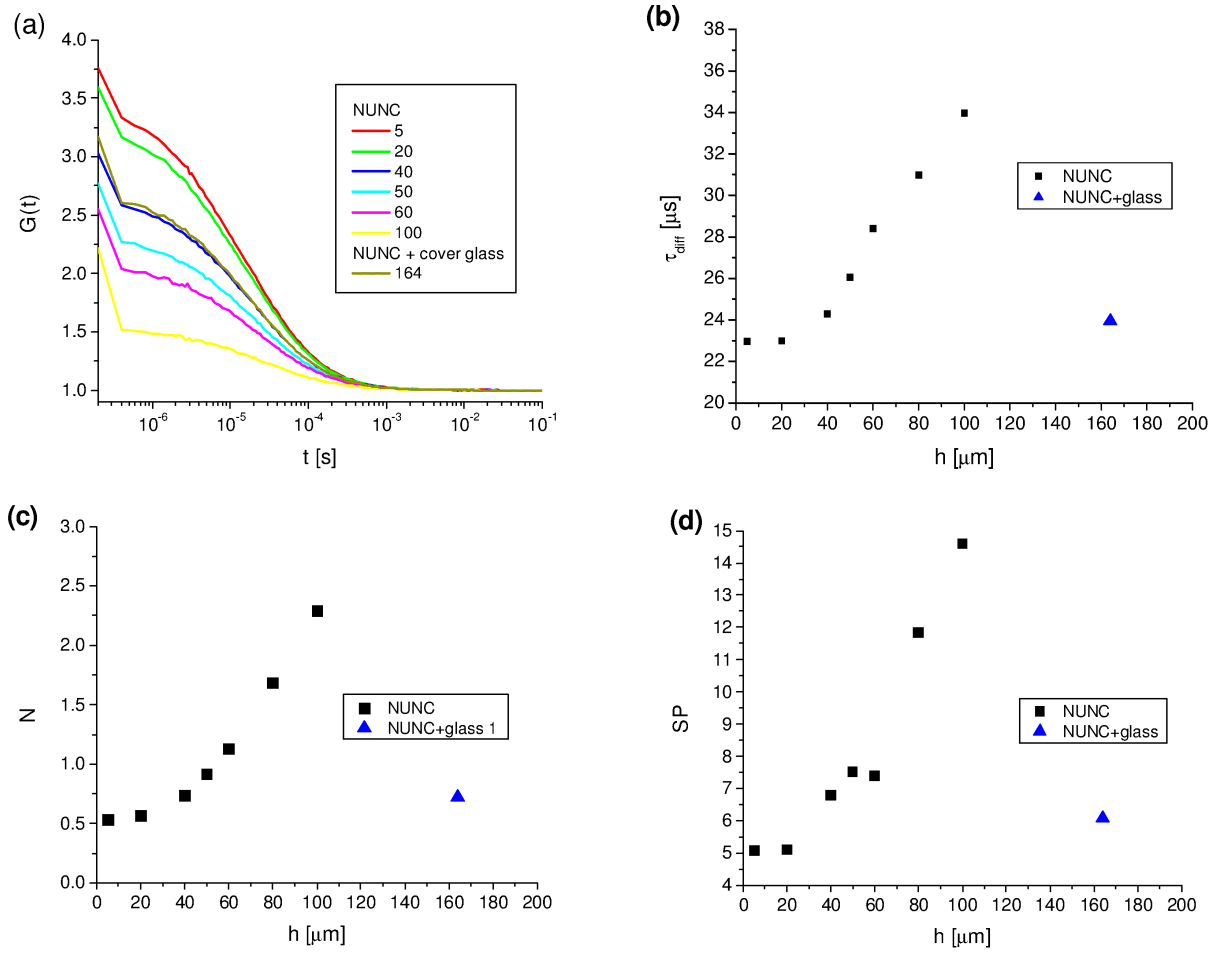

Figure S10: Correlation functions (a) and their fitted parameters (b-d) obtained for measurements of Alexa488 in water performed using the silicon oil immersion objective (#7) at different depth h indicated in (a) in a regular NUNC cell (rectangles in b-d) and in a NUNC with 1 cover glass attached to the bottom (triangle in b-d).