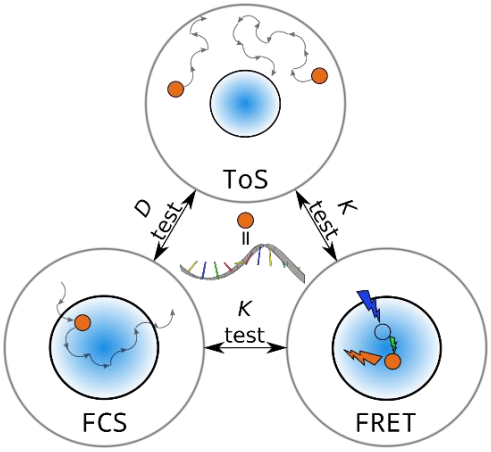
New method for determination of equilibrium constant for complex formation, concentrations and diffusion coefficients in solutions and living cells at nanomolar down to picomolar concentration of reagents. Robert Holyst

Research project objectives/ Research hypothesis: Complex-forming proteins in living cells often occur at nM down to pM concentrations. For example, there are 3—5 copies of lac repressor per an *E. coli* cell (~1 μm^3). The repressor forms a complex with a single operator on the DNA. Thus both reagents occur in *E. coli* at nM concentration. In this project we will develop a new method, which we called *Time of Search* (ToS), for studying motion and complex formation at extremely low concentrations (down to pM) in solutions and inside living cells. ToS will allow to determine diffusion coefficients of the reagents, their concentrations, and equilibrium constants for complex formation. A molecule of interest, **B**, slows down its motion upon forming a complex **AB**. The effective diffusion coefficient of **B** is then given by $D = (D_B + D_{AB}Kc_A)/(1 + Kc_A)$. Thus, D depends on effective concentration of reagent **A**, c_A, and the equilibrium constant, K. ToS, similarly to Fluorescence Correlation Spectroscopy (FCS), relies on detection of single photons emitted by fluorescent probes diffusing through the focus of a confocal microscope. However, different schemes of data analysis make the two techniques complementary. In short: in FCS we measure the time of flight of the probe across the focal volume. In ToS we measure the time needed for the probe to reach the focal volume, or in other words: the time of search of the focal volume by the probe. Both quantities depend on *D*. We will show that ToS will work down to pM concentrations, giving precise and accurate measurements (<0.5%) of diffusion coefficients for the studied molecules. We will demonstrate that equilibrium constants and concentrations of reagents can be retrieved from *D*. We will also show that the method works in living cells. ToS shall prove to be much easier than FCS in terms of data interpretation and give more precise and accurate value of *D*. The idea of ToS stems from more than 10 years of experience of my group (cf. e.g. *Nano Lett.* 2011, *Phys. Rev. Lett.* 2013, *Anal. Chem*. 2010, *Anal. Chem.* 2013) in the application of FCS and Taylor Dispersion Analysis (TDA) to the study of motion of reagents and complex formation.



Research methodology: FCS applied to formation of molecular complexes requires a full mathematical solution of the set of reaction-diffusion equations to interpret the data, especially when the time of flight across the focal volume is comparable to the inverse of reaction rate and thus when fast/slow reaction approximations known from the literature are not valid. FCS does not work below concentration of 0.5 nM. Due to different data analysis scheme, based on photon grouping and counting, rather than correlating them as in FCS, ToS allows to perform experiments at probe concentrations lower by orders of magnitude than in FCS (down to the pM scale). Instead of the autocorrelation function, ToS delivers a cumulative average number of molecules reaching the focal volume. Accumulation of data over time increases accuracy of measurements. We will use a relatively simple analytical equation of Smoluchowski for diffusion-limited reaction. We will additionally supplement the Smoluchowski model with excursion model, accounting for many returns of the same molecule to the focal volume. We will investigate hybridization of complementary DNA oligomers (**A**, **B**) as a model reaction leading to an **AB** complex. The thermodynamics of this reaction is well described in the literature and thus allows a thorough verification of the applicability of ToS to complex formation studies. Apart from literature data, we will also measure (for comparison of precision and accuracy) the equilibrium constants by FCS and FRET (Förster Resonance Energy Transfer). We will apply data filtering based on fluorescence lifetime to improve the signal-to-noise ratio and perform quantitative measurements even at extremely low oligonucleotide concentrations. In order to prove that ToS works *in vivo* we will perform experiments on *HeLa* cells (we have a mammalian cell lab in a group since 2014), studying motion of fluorescent probes in the cytoplasm. We will check the accuracy of ToS in *HeLa* cells for determination of probe concentration. **Expected impact**: We will introduce a new method beneficial for quantitative biology and biochemistry. The ToS will be used for determination of diffusion coefficients, in a simpler and more applicable than FCS way. We will also provide a useful tool for studies of formation of biomolecular complexes. **Pioneering nature of the project**: ToS reverses the paradigm of fluorescence-based molecular diffusion studies. We measure the motion of the probes around the focal volume rather than within it. This is not an obvious solution.