

FROM MICRO TO NANORHEOLOGY: BASIC PRINCIPLES OF MICRORHEOLOGY MEASUREMENTS WITH FLUORESCENCE CORRELATION SPECTROSCOPY

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Abstract

Fluorescence correlation spectroscopy (FCS) represents one of the most sensitive fluorescence techniques due to single molecule detection. Microrheology serves as a tool to study viscoelastic properties of microenvironment of observed particles. We used 100 nm latex particles to study mean square displacement (MSD) of these particles in Newtonian fluids. The comparison of MSD determined by FCS compares well with those determined by dynamic light scattering method (DLS), classical microrheology (MR) or calculated theoretical MSD. FCS can be used to determine MSD in greater extent of time than both techniques and we can determine MSD in time range where MSD cannot be determined by MR or DLS. When pure diffusion model was used, MSD as a function of time exhibited curvature from theoretical curves. The fitting procedure of FCS autocorrelation function required triplet state model.

Key words:

Microrheology, fluorescence correlation spectroscopy, dynamic light scattering

1. INTRODUCTION

Fluorescence correlation spectroscopy uses fluctuations in fluorescence signal due to random motion of fluorescently labelled particles. Laser is focused to a diffraction-limited spot, where the diffusing molecules are observed. The observed volume is an ellipsoid (with volume typically around 1 fL and less) that is elongated along the optical axis. The concentration of molecules in the effective volume must be in range of molecules (or below 1 observed molecule) [1]. This is different when compared to classical microrheology or DLS microrheology, where much higher concentration of particles is needed. In microrheology measurements, local deformation of the sample by probe particles is used to calculate mean square displacement (MSD) of particles. Microrheology uses small amount of sample, typically in range of microliters which makes this method suitable in biological applications where small amount of sample is required [2], [3]. But classical microrheology has some limitations. For example, microrheology uses conventional microscope, so its limitation is the use of relatively large particles (typically micrometer sized particles), otherwise the results deviate from theoretical values. In fluorescence correlation spectroscopy and DLS is possible to use nanometer sized particles and to get results with relatively small deviations when compared to theoretical values. The use of fluorescence correlation spectroscopy in microrheological measurement was published by Rathgeber et al. [4], but the aim of this study is to present the basic principles of this method and the difference between microrheological measurements by three techniques in simple system such as latex particles and Newtonian fluids and to show the usefulness of FCS microrheology.

2. MATERIALS AND METHODS

100 nm fluorescently labelled latex particles from Sigma-Aldrich were used for all 3 methods. The MSD was determined for particles in water and water:glycerol mixtures. All solvents were purchased from Sigma-Aldrich and used as received.

FCS measurements were performed on MicroTime 200 with Olympus water immersion microscope. 30 μL of the sample were placed onto a cover glass. Sample was irradiated by pulsed laser head with wavelength of 510 nm. Fluorescence signal came through objective and 510/640 dichroic mirror and was directed through 50 μm pinhole and split into two τ -SPAD detectors to avoid afterpulsing and to perform cross-correlation.

DLS measurement was performed on ZetaSizer Nano ZS from Malvern Instruments. 500 μL of the sample were placed into the cuvette with appropriate amount of microrheological probes. Laser beam was single scattered on the particles and the particle movement was tracked by change in thermal energy in time. DLS microrheology software evaluates particle movement and generates mean square displacement as a function of time.

In microrheology measurement, amount of 20 μl of the sample were loaded into the glass chamber to prevent drying and convectional flowing of the sample. Glass chamber was made out of one glass slide and three cover slides pasted together using UV light cure Adhesive (CONLOC UV 651). Slide with the sample was placed on the top of the optical microscope (Nicon Ci-L). Trajectories of Brownian motion of inserted particles were captured by CCD (CMOS USB 3.0) camera with 1280 x 1024 resolution. 500 frames was captured with the frame rate 30 Hz in 2D plane. 100 nm particles were observed using 40X objective lens and the size of one pixel was 0.1846. Particles movements were analysed using particle tracking codes written in IDL language, first developed by Crocker [5].

All measurements were performed at 23°C.

3. RESULTS AND DISCUSSION

The correlation function was recovered by cross-correlation of two independent signal paths, because the fluorescence signal was divided by 50% beam splitter. The correlation function $G(t)$ is calculated as

$$G(t) = \frac{\langle I_1(t') I_2(t + t') \rangle}{\langle I_1(t') \rangle \langle I_2(t') \rangle} \quad (1)$$

where I_1 and I_2 represent fluorescence intensities of first and second detector, respectively. When we assume, that fluorescence intensity fluctuations can be converted into fluctuations in concentration, than the equation 1) can be written as

$$G(t) = \frac{\iint \text{PSF}(\vec{r}) \text{PSF}(\vec{r}') \langle \delta c_T(\vec{r}, t=0) \delta c_T(\vec{r}', t) \rangle d\vec{r} d\vec{r}'}{\left[\bar{c}_T \int \text{PSF}(\vec{r}) d\vec{r} \right]^2} \quad (2)$$

Where $\text{PSF}(r)$ denotes the point spread function (impulse response of the focused optical system) and \bar{c}_T represents average concentration of fluorescent particle. Assuming Gaussian shape for point spread function, the illuminated volume can be determined from a measurement of sample with known diffusion coefficient according to equation 3):

$$G(t) = \frac{1}{V \bar{c}_T} \left(1 + \frac{4}{w_{xy}^2} Dt \right)^{-1} \left(1 + \frac{4}{w_z^2} Dt \right)^{-1/2} \quad (3)$$

The denominators in brackets in equation 3) describe the extension of the detection volume in the plane perpendicular (w_{xy}) and parallel (w_z) to the beam direction. The determination of illuminated or effective volume (V) is crucial part of each FCS measurement. Incorrect value of effective volume influences calculation of MSD and, when it is the case, the MSD as function of time exhibits deviation from theoretical values. As a first measurement, rhodamine 6G was used as a calibration sample. Müller et al. determined

diffusion coefficient of RH6G by dual focus FCS at 25°C to $414 \pm 5 \mu\text{m}^2/\text{s}$ ($392.5 \mu\text{m}^2/\text{s}$ at 23°C) [6]. By fitting the cross-correlation function of RH6G with equation 3), the values for the effective volume of $0.831 \pm 0.036 \text{ fL}$, $w_{xy} = 0.265 \mu\text{m}$ and $w_z = 2.125 \mu\text{m}$ were obtained. The calibration was double checked with 100 nm particles in water. Fitting of correlation function with triplet state model recovered diffusion coefficient of $4.01 \pm 0.04 \mu\text{m}^2/\text{s}$ which is in good agreement with diffusion coefficient determined by DLS ($4.03 \pm 0.03 \mu\text{m}^2/\text{s}$).

Because the diffusion coefficient term is related to MSD in a way that $D = \frac{\text{MSD}}{6t}$, equation 3) changes to

$$G(t) = \frac{1}{N} \left(1 + \frac{2}{3w_{xy}^2} \text{MSD} \right)^{-1} \left(1 + \frac{2}{3w_z^2} \text{MSD} \right)^{-1/2} \quad (4)$$

Equation 4) can be solved analytically to calculate MSD, but as described by Rathgeber et al., the right side of equation 4) can be neglected when $\text{MSD} \ll w_z^2/2$ and simple relationship for MSD can be obtained

$$\text{MSD} = \frac{3}{2} w_{xy}^2 \left(\frac{1}{NG(t)} - 1 \right) \quad (5)$$

Figure 1 shows that DLS and FCS methods agree well with theoretical value. Microrheology deviates from ideal values due to small particles used. Precise MSD value is not easy to determine with 100 nm particles, because of the microscopes inability to focus 100 nm particles with such high precision as well as in case of 1 μm particles. Nevertheless, the linear trend and the range, where particle microrheology can measure, is clear. It can be seen, that classical microrheology is useful in time range from 0.03 s up to 1 s and DLS method approximately from 3.5E-6 s to 1E-4 s. FCS covers the whole range of DLS and is also useful in time, where particle microrheology and DLS cannot be measured. Unfortunately, FCS is not able to cover whole measurement range potential of particle microrheology because of curvature of calculated FCS curve from theoretical values. This happens, because, in this case, we used approximated form of equation 4). In this case, equation 5) represents good approximation in times less than 0.1 s. When equation 5) is used, we can cover time range from 1E-6 s to 10 s.

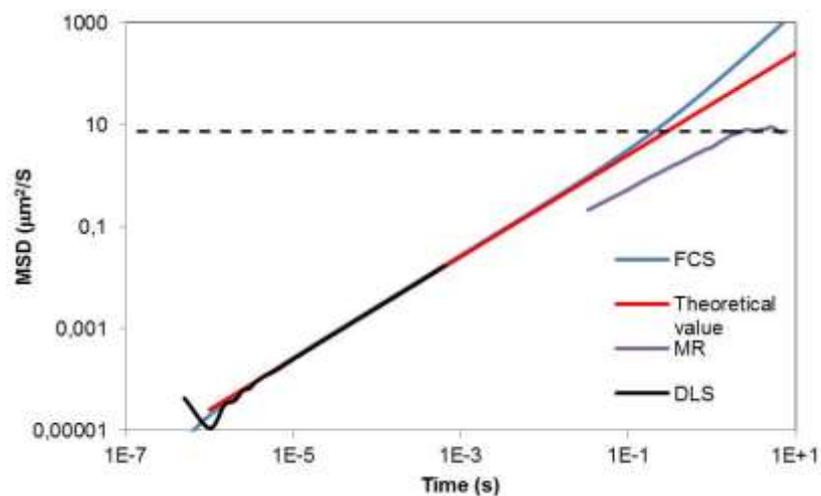


Figure 1 100 nm latex particles in water measured by three techniques. The dotted line represents value of MSD where MSD is equal or larger than $w_z^2/2$.

The usefulness of equation 5) rises with increased viscosity of samples. MSD in time decreases with increased viscosity. Above mentioned means, that the limitation of equation 5) disappears when determined MSD in the time-limit of FCS measurement is less than 10 (of course the value depends on determined extension of the detection volume in the plane parallel to the beam direction – this value changes with different lasers used or different laser intensity). We can see in Figure 2 that FCS can again cover the range of DLS measurement, but also can cover part of the MR measurement. From Figure 2 is obvious that there are some deviations between all methods and theoretical value. The mistake was probably caused in sample preparation.

We can observe the same pattern in Figure 3 as in previous case. All methods vary in a small way from each other or from the theoretical value, which is clearly caused by pipetting glycerol during sample preparation. But important is that all methods copy the same pattern as theoretical value. Most importantly, when viscous sample is measured, FCS can cover the whole range of time as well as DLS and MR and of course the empty space between both methods.

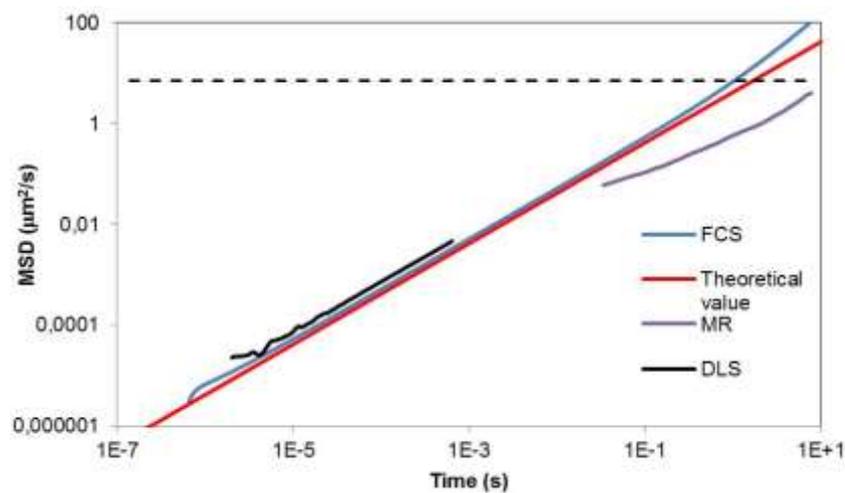


Figure 2 100 nm latex particles in 40% water-glycerol mixture measured by three techniques. The dotted line represents value of MSD where MSD is equal or larger than $w_z^2/2$.

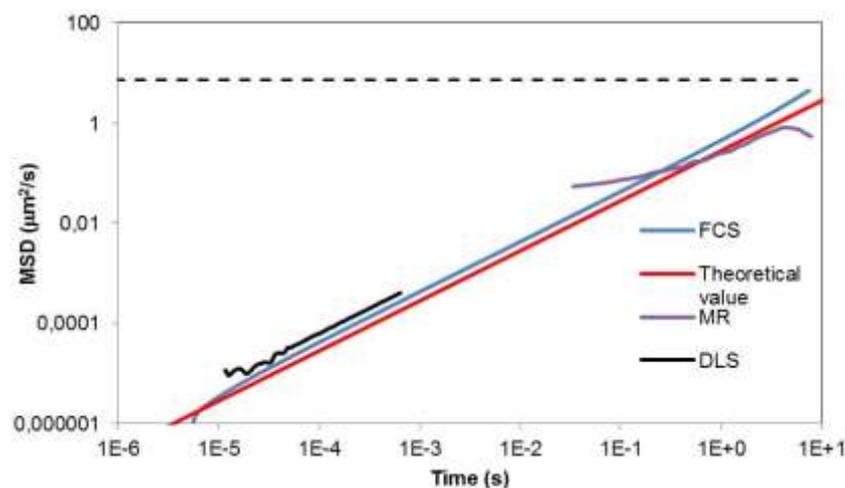


Figure 3 100 nm latex particles in 80% water-glycerol mixture measured by three techniques. The dotted line represents value of MSD where MSD is equal or larger than $w_z^2/2$.

4. CONCLUSION

This paper is interested in study of microrheology properties of 100 nm latex particles in Newtonian fluids by three techniques. We compared commonly used particle tracking microrheology, dynamic light scattering and rarely used fluorescence correlation spectroscopy. FCS showed great potential in study viscoelastic properties in microrheological conditions. It is possible to use exact or approximated equation for determination of MSD (equation 4) and 5)). The great advantage of FCS microrheology is the sensitivity and amount of sample needed for measurement. We don't need as many particles as in case of DLS or microrheology. The amount of sample needed is around 30 μL for FCS, 100 μL for microrheology and 500 μL for DLS. Above mentioned reasons make FCS cheaper method for MR measurement in sample material point of view. Of course the sensitivity of the machine has its price, meaning that FCS equipment is incomparably more expensive than classical equipment for particle tracking microrheology. Nevertheless, when small particles (let's say the 100 nm particles or smaller) have to be measured, particle tracking MR can be used only with large deviations caused due to impossibility of precisely focus such small particles by conventional microscopes. In this case FCS or DLS have to be used.

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