Calibrating Differential Interference Contrast Microscopy with dual-focus Fluorescence Correlation Spectroscopy

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Abstract: We present a novel calibration technique for determining the shear distance of a Nomarski Differential Interference Contrast prism, which is used in Differential Interference Contrast microscopy as well as for the recently developed dual-focus fluorescence correlation spectroscopy. In both applications, an exact knowledge of the shear distance induced by the Nomarski prism is important for a quantitative data evaluation. In Differential Interference Contrast microscopy, the shear distance determines the spatial resolution of imaging, in dual-focus fluorescence correlation spectroscopy, it represents the extrinsic length scale for determining diffusion coefficients. The presented calibration technique is itself based on a combination of fluorescence correlation spectroscopy and dynamic light scattering. The method is easy to implement and allows for determining the shear distance with nanometer accuracy.

OCIS codes: (170.6280) Spectroscopy, fluorescence and luminescence; (180.1790) Confocal Microscopy; (300.2530) Fluorescence, laser-induced.

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1. Introduction

Differential interference contrast microscopy (DIC) is an optical microscopy illumination technique used to visualize refractive index variations across an unstained, transparent sample [1, 2]. DIC was invented in the early 1950's by Georges Nomarski [3-5]. DIC works by separating a polarised light source into two beams which take slightly different paths through the sample. Where the length of each optical path differs, the beams interfere after recombination. This produces an image that shows variations in optical density of the sample.

The core element of a DIC set-up is the Nomarski prism, which is a modification of a Wollaston prism. Like the Wollaston prism, the Nomarski prism consists of two optical quartz or calcite wedges cemented together at the hypotenuse. One of the wedges is identical to a conventional Wollaston quartz wedge and has the optical axis oriented parallel to the surface of the prism. The second wedge of the prism is modified by cutting the quartz crystal in such a manner that the optical axis is oriented obliquely with respect to the flat surface of the prism. The Nomarski modification causes the light rays to come to a focal point outside the body of the prism, and thus allows greater flexibility when setting up the microscope.

To evaluate DIC images several theoretical models were proposed in the last 25 years [6-10]. All theoretical approaches need to know the shear distance of the prism, i.e. the distance between the two light beams within the sample as generated by placing the DIC prism into the optical excitation path of a microscope.

There is a second, recent application of a DIC prism: dual-focus fluorescence correlation spectroscopy (2fFCS). Standard Fluorescence Correlation Spectroscopy (FCS) was originally invented by Magde, Elson and Webb in the early 1970's for measuring diffusion coefficients of fluorescent molecules [11]. The core idea of FCS is to detect fluorescence fluctuations within a very small detection volume of ca. one femtoliter and to calculate the second order correlation function (autocorrelation function, or ACF). On a millisecond time scale, fluctuations are usually dominated by the diffusion of fluorescent molecules in and out of the detection volume. The resulting decay of the ACF is directly related to diffusion coefficients of molecules. However, for a precise quantitative extraction of a diffusion coefficient form a measured ACF, one needs the exact shape of the so called Molecule Detection Function (MDF), which describes the position-dependent efficiency to excite and detect a fluorescence photon from a single molecule. Unfortunately, this knowledge is unavailable in FCS, which usually employs on a standard confocal epi-fluorescene microscope [12,13]. 2fFCS alleviates this problem by introducing an *external length scale* via the generation of two laterally shifted but overlapping detection volumes with the help of a DIC prism [14].

In 2fFCS, the obtained diffusion coefficients are highly sensitive to the shear distance of the Nomarski-prism. This sensitivity can be used to quantify the distance between the two propagating light beams within sample space as generated by the DIC prism. The core idea is to (i) measure with dynamic light scattering (DLS) the hydrodynamic size of commercially

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available fluorescently labelled and monodisperse spherical colloidal latex particles, and (ii) to measure with 2fFCS the diffusion coefficient of these particles. By comparing the hydrodynamic radius as obtained with both methods, one can directly determine the distance between the detection volumes in the 2fFCS measurement set-up, and thus the shear distance of the DIC prism.

2. Theoretical background

Here, we will briefly recall the theoretical basis of a 2fFCS measurement [14]. In 2fFCS, two overlapping detection volumes are generated y inserting a DIC prism into the excitation light path of an epi-fluoresescence confocal microscope. By measuring the ACF for each detection volume, and the cross-correlation function (CCF) between both volumes, and then analyzing the delay of the CCF decay in comparison to that of the ACFs, one can calculate an absolute value of the diffusion coefficients of fluorescent molecules or particles in solution, if one exactly knows the distance between the detection volume centres.
As was shown in detail in Ref. [14], an adequate model ACF/CCF is for a purely diffusion-generate correlation decay (neglecting any photophysics-related fluorescence fluctuations) is given by

$$\tilde{g}(t,\delta,\mathbf{v}) = \frac{c}{4}\sqrt{\frac{\pi}{Dt}}\int dz_1 \int dz_2 \frac{\kappa(z_1)\kappa(z_2)}{8Dt + w^2(z_1) + w^2(z_2)} \exp\left[-\frac{(z_2 - z_1 - v_z t)^2}{4Dt} - 2\frac{(\delta - v_x t)^2 + v_y^2 t^2}{8Dt + w^2(z_1) + w^2(z_2)} \right], \tag{1}$$

where δ is the lateral distance between detection volume centres, ε_1 and ε_2 are factors proportional to overall excitation intensity and detection efficiency in each laser, c is the concentration of fluorescent molecules or particles, and D their diffusion coefficient. Here, the functions $\kappa(z)$ and $w(z)$ are given by

$$w(z) = w_0 \left[1 + \left(\frac{\lambda_{ex} z}{\pi w_0^2 n} \right)^2 \right]^{1/2}, \tag{2}$$

and

$$\kappa(z) = 2\int_0^a \frac{d\rho\rho}{R^2(z)} \exp\left(-\frac{2\rho^2}{R^2(z)} \right) = 1 - \exp\left(-\frac{2a^2}{R^2(z)} \right), \tag{3}$$

with

$$R(z) = R_0 \left[1 + \left(\frac{\lambda_{em} z}{\pi R_0^2 n} \right)^2 \right]^{1/2}, \tag{4}$$

where λ_{ex} and λ_{em} are excitation and centre emission wavelengths, n is the sample refractive index, a is the confocal pinhole radius, and w_0 and R_0 are fit parameters. For calculating the ACF of each focus, one has to set, in Eq. (1), δ to zero, and to replace $\varepsilon_{1,2}$ by either ε_1^2 or ε_2^2, respectively. The integration in Eq. (1) has to be performed numerically. Fitting of experimental data is done globally for both the ACFs and the CCF, where one has the free fit parameters $\varepsilon_1 \cdot c^{1/2}$, $\varepsilon_2 \cdot c^{1/2}$, w_0, R_0, and D. Hereby, it is assumed that the distance δ between detection volumes is known *a priori*. Alternatively, if the value D of the diffusion coefficient is

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exactly known, one can use the above relations to fit the value δ of the distance between the detection volumes, which is exactly what will be done in the present paper.

3. Experiment

3.1 Materials

TetraSpeck 100 multi-fluorescent latex beads with a specified diameter of approx. 100 nm were purchased from Invitrogen (Karlsruhe, Germany) and used without any further purification in the DLS and 2fFCS experiments. TetraSpeck latex particles consist, by specification of manufacturer, of continuously fluorescent labelled spherical beads. The beads contain a mixture of four fluorescent dyes with well-separated excitation/emission peaks (365/430 nm, 505/515 nm, 560/580 nm, and 660/680 nm). The width of the absorption peaks allows for a proper excitation with laser sources at 470, 532 and 632 nm. For DLS, samples are diluted by a factor of 10 with LiChrosolv water (No. 115333), purchased from Merck KGaA (Darmstadt, Germany), to prevent multiple scattering. For single molecule experiments, carboxylic acid derivate of Atto655 (AD 655-2) was obtained from ATTO-TEC (Siegen, Germany).

3.2 Instruments

Dynamic light scattering (DLS) measurements were performed on a standard ALV 5000 system, equipped with a laser of 633 nm wavelength. Scattering intensity was detected at angles of 60°, 90°, and 120°, respectively, and the hydrodynamic radius was calculated with a second order cumulant fit using the Stokes-Einstein relation. The measurement system was equipped with a temperature controlled water bath giving a precision in sample temperature stabilization of $\pm$ 0.2 K. During measurements, the sample chamber was sealed to prevent solvent evaporation and convection.

The 2fFCS measurement system was based on a MicroTime200 inverse time-resolved Fluorescence Microscope (MT200, PicoQuant, Berlin, Germany) as described in [16], see also Fig. 1. The dual-foci modifications is described in Ref. [14]. The setup is equipped with two identical 470 nm lasers (LDH-P-C-470B), two identical 635 nm lasers (LDH-P-635), as well as one 532 nm laser (PicoTA 530N). All lasers are linearly polarized pulsed laser-sources (PicoQuant, Berlin, Germany). The light of each of the two pairs of identical wavelength lasers was combined by two polarizing beam splitters (broadband polarizing cube by Ealing Catalogue, St. Asaph, UK) into single beams. The light of the 532 nm laser was split into two equal-intensity beams using a combination of a zero-order half-wave plate (WPQ05M-532, Thorlabs, Munich, Germany) and a polarizing beam splitter. To create a time delay between both beams, one of the beams is coupled into a short (1.56 m) and the other into a long optical fibre (6.4 m), resulting in a relative time delay of both pulse trains by 25 ns (single mode fibres PMC 400, Schäfter und Kirchhoff, Hamburg, Germany). Afterwards, both beams are reunited by a polarizing beam splitter as is done with the light of both pulsed diode-laser pairs. The 632 nm and 532 nm beams are combined by a dichroic (560 dcxr), and the resulting beam is combined with the 470 nm light by another dichroic (490 dcxr), resulting in a virtually single light beam containing three wavelengths with pulse trains of alternating polarization in each wavelength.

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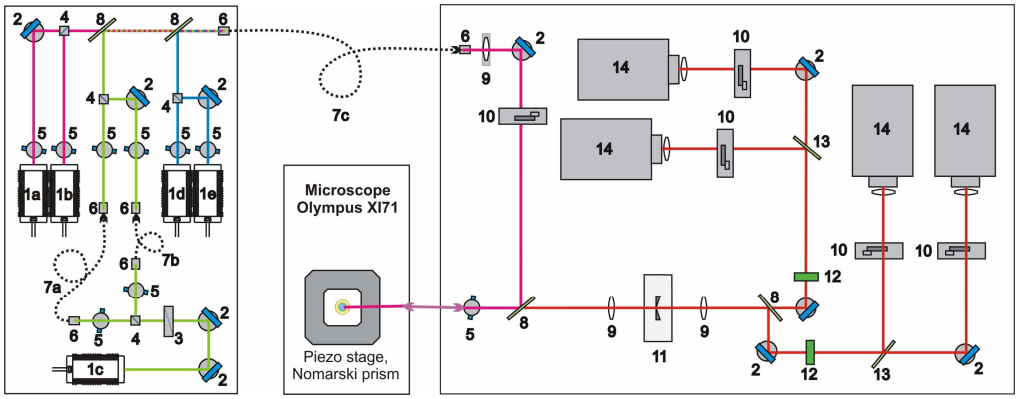

Fig. 1. Confocal setup with PicoQuant MicroTime 200, equipped with: 1a+b.) Laserhead 637 nm, 1c.) Laserhead 532 nm, 1d & e.) Laserhead 470 nm, 2.) Mirror, 3.) Adjustable zero order half-waveplate, 4.) Polarizing cube, 5.) Beamdisplacer, 6.) Fiber coupler, 7a-c.) Single mode fiber, 8.) Dichroic, 9.) Lens, 10.) Shutter, 11.) Confocal aperture, 12.) Fluorescence filter, 13.) 50/50 Mirror, 14.) Single photon avalanche diode

Both dichroic mirrors were purchased from AHF-Anaysentechnik (Tübingen, Germany). Pulse width of all laser pulses was ca. 50 ps, with an overall repetition rate of 10 or 20 MHz, adjustable to the specific fluorescence lifetime of the measured fluorophore. Alternate laser pulsing is accomplished by a dedicated laser driver electronics (PDL 828, Sepia-II, PicoQuant, Berlin, Germany). The final three-wavelength beam is coupled into a polarization maintaining single mode fibre for optical cleaning, and, after re-collimation, reflected towards the microscope's objective (UPLAPO 60x W, 1.2 N.A., Olympus Europe, Hamburg, Germany) by a triple-band dichroic (z470/532/638rpc, AHF-Analysentechnik, Tübingen, Germany). Before entering the objective, the laser beam is passed through a Nomarski prism (U-DICTHC, Olympus Europe, Hamburg, Germany) to deflect both polarization contributions in two parallel but laterally shifted beams. After focusing through the objective, two overlapping foci are generated. The fluorescent light is collected by the same objective and focused onto a single circular aperture (diameter 200 µm). After passing several emission filters (HQ505/30m for λ_{ex} = 470 nm, HQ580/70m for λ_{ex} = 532 nm, and HQ687/70m for λ_{ex} = 635 nm, all purchased from AHF-Analysentechnik, Tübingen, Germany), the light is split by a non-polarizing beam splitter cube and focused onto two single photon avalanche diodes (SPAD, PDM series, Micro Photon Devices, Bolzano, Italy). A dedicated single photon counting electronics (PicoHarp 300, PicoQuant, Berlin, Germany) is used to record single-photon detection events with 4 ps temporal resolution (time tagged time resolved or TTTR detection mode, see. [16]).

In the final measurements, laser excitation was done in pulsed interleaved mode (PIE) [17] with a repeating pulse sequence of four excitation pulses (two pulses at one wavelength and differing polarization followed by two pulses at the second wavelength and again differing polarization). The overall repetition rate of this four pulse sequence was, as already stated above, either 10 or 20 MHz. Thus, when histograming photon arrival times with respect to the first laser pulse of the sequence (time-correlated single photon counting or TCSPC, [18]), one will observe four subsequent decay curves, corresponding to the four excitation pulses in one sequence. Because the fluorescence decay time of the TetraSpeck dyes is much shorter than the time between pulses in the sequence (~25 ns), one can use the arrival time of the photons with respect to the laser pulse sequence to distinguish which laser did excite which photon. Because the two different polarizations of the excitation light are focused into two different positions, this allows one to distinguish which photon was excited in which focus [14]. Using

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this information, ACFs and the CCF between foci were computed with a custom made Matlab routine [19] by cross-correlating only photons from different SPAD detectors to eliminate afterpulsing and dead time effects of the detectors.

3. Results and discussion

In a first step, we measured with DLS the hydrodynamic size of fluorescently labelled TetraSpeck 100 latex beads at 298.15 ± 0.1 K using a detection angle of 90°. A typical set of measured ACFs and CCF is presented in the inset of Fig. 2. For each wavelength, measurements were repeated over fifty times to obtain a sufficiently small standard deviation.

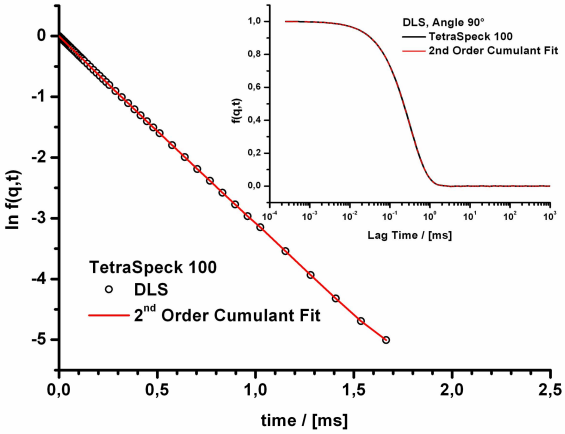

Fig. 2. Main panel: DLS at 90° of mono disperse TetraSpeck 100 latex particles, fitted with a 2nd-order cumulant fit. Inset: standard plot of ACF.

A semi-logarithmic plot of the data is shown in the main panel of Fig. 2, together with a 2nd-order cumulant fit. The good fit quality proves the good monodispersity of the bead sample. The hydrodynamic radius R_h of the beads was determined to be 55.6 ± 0.6 nm.

In a second step, 2fFCS measurements were performed at the three excitation wavelengths of λ_{ex} = 470 nm, λ_{ex} = 532 nm, and λ_{ex} = 637 nm, respectively. Due to the high label density of the beads, total excitation power was reduced to less than 0.1 μW within each detection volume. During each measurement, fluorescence was collected for 5 minutes, and each measurement was repeated more than eighty times per excitation wavelength. A few correlation functions had to be discarded due to distortions generated by the transit of large bead clusters through the detection volume. A typical measurement result is shown in the inset of Fig. 3.

To obtain the distance between the overlapping detection volumes each set of ACFs and CCF was globally fitted by the model function of Eq. (1) to obtain a value of the diffusion coefficient D and thus hydrodynamic radius R_h, assuming that the distance δ between the detection volumes had a certain value between 360 and 416 nm. The obtained hydrodynamic radius R_h as a function of assumed distance δ is shown in Fig. 3. The intersection of this curve with a horizontal line at the actual value of the hydrodynamic radius as obtained from the DLS measurements gives the actual distance between the detection volumes, and thus the shear distance of the DIC prism.

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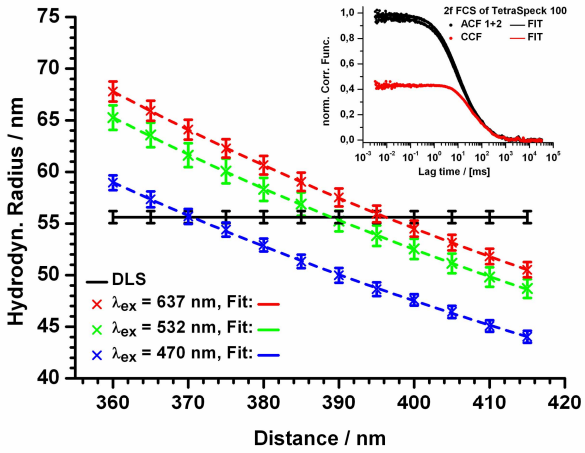

Fig. 3. Main picture: Wavelength dependent determination of Nomarski-DIC-prism shear distance, by comparison of DLS and 2f-FCS measurements, obtained from enhanced model for multi labelled particles. Inset: 2fFCS measurement of TetraSpec 100 latex particles. Autocorrelation (ACF) and cross-correlation (CCF) functions, fitted with 2fFCS model.

Table 1 lists the obtained values of the shear distance for the three different excitation wavelengths. Standard deviation of the 2fFCS measurements is better than 3 %, or ± 1.5 nm, as indicated by the error bars in the main panel of Fig. 3.

Table 1. Wavelength dependent shear distances obtained from comparison of DLS and 2fFCS experiments.

Excitation Wavelength (λ_{ex}) / nm	Shear distance / nm
470	370
532	389
637	395

The observed wavelength dependence of the shear distance is remarkably large and mostly due to chromatic aberration effects of the objective. When considering the wavelength dispersion of ordinary and extraordinary refraction of quartz (or calcite, which may be also used in DIC prisms), one does not expect such a strong wavelength dependence of the shear distance. Our result emphasizes how important it is to measure the shear distance of DIC prisms as function of wavelength, if one is interested in precise evaluations of experiments involving such an optical element.

4. Conclusion

We have presented a precise method for measuring the shear distance of a Nomarski DIC prism using a combination of DLS and 2fFCS. The achieved precession is less than ± 1.5 nm for shear distance values around 400 nm. This method will be useful for calibrating DIC microscopes as well as 2fFCS measurement systems. Especially for 2fFCS, a relative error of the shear distance value leads to a doubled relative error in the determined value of an diffusion coefficient. Thus, knowing a DIC's shear distance as precisely as possible is paramount for obtaining precise absolute values of diffusion coefficients with 2fFCS.

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