Fast sub-nanometer particle localization by Traveling Wave Tracking

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Abstract

Traveling Wave Tracking (TWT) is a new technique for the study of the motion of submicron-sized particles with a very high spatial and temporal resolution: the particle can be localized with sub-nanometer precision at the microsecond time scale. In this technique, a particle is moving through the traveling interference pattern, and measurements of the intensity and phase of the scattered light allow to precisely determine the particles position. In the present paper we describe the experimental setup and its performances. As an example of use of the TWT, we characterize the brownian motion of a submicron-sized bead in a confined environment.

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I. INTRODUCTION

An increasing research activity on single molecule techniques has been emerging over
the last ten years. This development is motivated by the necessity to better understand the
function and, particularly, the dynamical behavior of the enzymes [1–8].
In order to follow the details of the dynamics of one single protein a high spacial and
temporal resolutions are required. Recently, we have introduced an optical technique
allowing us to track sub-micron particles with nanometer space resolution and a few
microseconds time resolution. This technique was based on the simple idea that if the
light intensity used to observe the particle depends on space, then a measure of I(t), the
light emitted by the particle as a function of time, provides a direct measure of its position
[9–11]. In practice the spacial modulation was obtained by generating a standing wave
[12]. Although powerful, it had the drawback of loosing sensitivity at each antinode of the
standing wave and thus reduced continuous tracking to half a period.
In this manuscript we introduce a new technique by which a phase and intensity mea-

II. EXPERIMENTAL SETUP

Conventional tracking techniques are based on space resolved detectors, measuring the
light scattered from a relatively small probe, small bead or fluorescent dye, moving in a
uniformly illuminated field. These techniques use CCD cameras or quadrant photodiode
based detections.
The Traveling Wave Tracking (TWT) works in the opposite way: the scattered light is
recorded by a point detector (Avalanche Photodiode - APD), with a wide bandwidth (~10
MHz), while the probe moves through a pattern of fringes . The fringes are obtained by
interference of two laser beams with slightly different frequencies such that the pattern continuously shifts through the sample at constant speed. As a result, the sample is illuminated by a progressive sinusoidally modulated intensity:

\[ I(x, t) \propto 1 + \sin(qx + \Omega t) \]

where the fringe periodicity \( d \) is \( 2\pi/q \) and the pattern shifts with a speed \( v_f \equiv \Omega/q \) (In our setup \( d \approx 200 \text{ nm} \) and \( \Omega \approx 4 \text{ MHz} \)).

Let us imagine a small particle (a 100 nm polystyrene bead) immobile in the microscope: it will be alternately in the light and in the dark, with a frequency of \( \Omega \). As the bead scatters the light proportionally the local intensity, it appears to the APD as a blinking dot, and the APD records a signal \( I_{\text{APD}} \propto 1 + \sin(\Omega t + \varphi) \), where \( \varphi \) is a phase shift depending on the bead position in the fringes. If the bead moves over a small distance \( \delta x \) from the position \( x' \) to \( x' + \delta x \), the phase shift will change from \( \varphi' \) to \( \varphi' + (2\pi/d) \cdot \delta x \), where \( d \) is the fringe period. In such a way, any displacement of the bead can be precisely determined, on the condition that we measure the phase shift \( \varphi(t) \) as a function of time. In the following we will describe how the traveling fringes have been designed and how the phase shift has been measured.

The sinusoidal modulation is obtained by interference of two laser beams, with opposite wave vectors \( k_x \), undergoing total internal reflection at the glass/water interface. In this setup, the fringe periodicity is:

\[ d = \frac{\lambda_0}{2n_{\text{glass}} \cos \vartheta} \quad (1) \]

where \( \lambda_0 \) is the laser wavelength, \( \vartheta \) is the incidence angle at the glass/water interface, and \( n_{\text{glass}} \) is the glass refraction index (usually 1.52).

The light is totally reflected at the glass/water interface and the intensity exponentially decreases with a characteristic penetration depth \( \zeta \) in the direction perpendicular to the interface:

\[ \zeta = \frac{\lambda_0}{4\pi} \left( n_{\text{glass}}^2 \sin^2 \vartheta - n_{\text{H}_2\text{O}}^2 \right)^{-1/2} \quad (2) \]

The optical setup is described in Fig. 1. The laser beam is compressed to a diameter of 1 millimeter (\( L_1 \) and \( L_2 \) in Fig. 1) and split by using the beam splitter \( BS_1 \): two beams are produced, identical in intensity, phase and polarization. The lens \( L_3 \) focuses the beams on two, diametrically opposed, points of the objective back focus plane. In such a way a small
FIG. 1: Schematic of the experimental setup. Traveling Wave Tracking configuration through the TIRF objective. The two laser beams are focused on the diameter of the back focus plane, in order to light the sample with two parallel beams with opposite wave vector $k_x$. The light scattered by the probe is recorded using an avalanche photo-diode.

A region of the specimen ($15 \times 15 \ \mu m^2$) is illuminated by two antiparallel laser beams and an interference pattern is established at the glass/water interface.

In order to obtain a traveling wave, we chose to slightly shift the frequency $\omega$ of both the laser beams by $\Delta \omega_1$ and $\Delta \omega_2$ respectively. The frequency difference $\Omega = \Delta \omega_1 - \Delta \omega_2$ is typically set to $\Omega/2\pi = 4 \text{ MHz}$ in order to obtain a pattern moving much faster than any probe motion: the pattern speed is $v_f = \Omega d/2\pi \approx 1 \text{ m/s}$. The resulting electric field $\vec{E}(x, z; t)$ is the sum of the two beams:

$$
\begin{align*}
\vec{E}_1 &= E_0 e^{i (k \cdot x - (\omega + \Delta \omega_1)t)} e^{-z/\zeta} \\
\vec{E}_2 &= E_0 e^{i (-k \cdot x - (\omega + \Delta \omega_2)t)} e^{-z/\zeta}
\end{align*}
$$

and the total intensity on the specimen can be written as:

$$I(x, z; t) = |\vec{E}_1 + \vec{E}_2|^2 = 2E_0^2 [1 + \cos (qx - \Omega t)] e^{-z/\zeta}$$

The frequencies are modified by using two Acousto-Optic Modulators (AOM - IntraAction, AFM-402A1), whose frequency difference is electronically locked by a dual generator (In-
The light scattered from the probe is collected by the same objective, focused by the lenses $L_4$, $L_5$ and $L_6$ and measured by the avalanche photo-diode (Hamamatsu, bandwidth = 10 MHz). As explained, the intensity $I_{APD}$, measured by the photodiode, is proportional to Eq. (3). The resulting signal is a sine-like modulation at 4 MHz, whose amplitude and the phase depend respectively on the $z$ and the $x$ position of the probe. Both, phase and amplitude, can be extracted by comparing the measured intensity to the signal driving the Acousto-Optic modulators: $f(t) = \cos(\Omega t)$. The two signal $f(t)$ and $I_{APD}(t)$ are combined via a home-made lock-in amplifier, which measures the complex value:

$$V(t) = \langle I_{APD}(t')e^{-i\Omega t'} \rangle = V_C(t) + iV_S(t)$$

From $V_C(t)$ and $V_S(t)$, simultaneously recorded with a bandwidth of 300 kHz and a sample rate of 625 ksample/s, we can finally calculate the two coordinates $x(t)$ and $z(t)$:

$$\begin{align*}
x(t) &= \frac{d}{dz} \text{arg} V(t) \\
z(t) - z_0 &= -\zeta \ln |V(t)|
\end{align*}$$

Note that the absolute value of the intensity is unknown and we can only measure intensity variations. This implies that we do not know the absolute $z$ position of the probe, but we can only measure relative motions $z(t) - z_0$.

Experiments are carried out using an Olympus 60X TIRF objective (numerical aperture 1.45) and a CCD camera. The laser source is a second harmonic YAG (Coherent Verdi), with a wavelength $\lambda_0$=532 nm and a longitudinal coherence length of several meters. Its power is typically set to 500 mW.

III. RESULTS

A. Setup Performances

The resolution and the performances of this setup have been characterized using a piezoelectric stage, moving a bead stuck to the coverglass. The piezoelectric stage moves by 20 nanometers steps (the resolution of the stage is ±5 nm) every 200 ms. The motion is reflected by a phase shift: Fig. 3 shows how the phase $\varphi(t)$ evolves as a function of the
FIG. 2: Schematic of the demodulator (lock-in amplifier). The Photodiode signal $[PD]$ is amplified $[A1]$ and split into two channels $[Split]$. Channel one is multiplied $([\otimes])$ by the reference signal $\cos(\Omega t)$ and channel two by the reference signal shifted $[90]$ by $\pi/2$. The two low-pass filters $[300 \text{ kHz}]$ remove the higher harmonics and amplify the signals $V_C = \cos(qx) e^{-z/\zeta}$ and $V_S = \sin(qx) e^{-z/\zeta}$. $A1$ is a Minicircuits ZHL-6A, mixers $([\otimes])$ are Minicircuits ZAD-6, 90 shifter $[90]$ is a home-made $LC$ filter, lowpass filters are home-made 5th order $LC$ Butterworth filters.

We observe the well defined 20 nm steps, with sharp edges. The phase (left axis on the plot) changes linearly with the position (right axis). This allows us to calibrate the instrument: within the piezoelectric stage accuracy, the fringe period is $197 \pm 2nm$. By using Eq. (1) and (2) the penetration depth can be estimated: $\zeta = 230 \pm 90$ nm. The uncertainty associated to this estimation is large: we will see in section III B another way to calibrate this parameter.

Fig. 3 also reveals a constant positive drift. It gives an indication of the long-time stability of our setup, which is roughly one nanometer per second. This stability is not a limiting factor for extremely rapid events, like molecular motor motion at high ATP concentration, whose speed can reach $2 \mu m/s$. Conversely, it could become critical for observing slowly moving objects. We also notice in Fig. 3 that the phase is undetermined by a factor of $2\pi$: this is not a major problem for our experiments, because the observed object never covers more than few tens of nanometers within our sampling interval $\delta t$. This puts an upper limit of $v = d/\delta t$ on observable velocities, without any ambiguity. In practice this is not a severe
FIG. 3: A single colloid (diameter 200 nm) is fixed on the coverglass, while the sample holder is moved in 20 nm steps by means of a calibrated piezoelectric stage.

limitation; for instance, a globular protein (∼5 nm in diameter) in water, has a diffusion coefficient of 84 nm²/µs: it diffuses only ∼16 nm during the sampling interval of 16 µs. This value is small compared to the fringe period d = 200 nm.

Fig. 4a shows two 10-nanometers steps in a row, acquired with full bandwidth: the 10 nanometers displacement are easily observed and the internal structure of the step can be characterized with nanometer precision. The oscillatory motion after the step is due to the inertial behavior of the piezoelectric stage: without any feedback loop, it oscillates with exponentially damped amplitude after step-like impulses. The intrinsic noise of the experimental setup can be measured by analyzing the parts of the curve where the oscillations are completely damped (see Fig.4b). The plot shows that the intrinsic noise is 3 Ångström RMS; this noise, which is white and totally uncorrelated, is due the residual shot noise.
If we compare the TWT with a quadrant photodiode detection, we notice that the TWT improves the space resolution up to a factor of ten, with the same bandwidth. This improvement is due to the lock-in detection that filters out all the low frequency noises, including electronic (amplification and detection instabilities) and optical instabilities (laser intensity fluctuations and mechanical instabilities). This hypothesis is confirmed by the perpendicular resolution (along direction $z$), which measurement is based on the intensity amplitude instead of the phase shift: in the $z$ direction the bead can be localized with a precision of only 10 nanometers with full bandwidth. The bandwidth must be reduced down to a few of kHz in order to achieve a $1 − nm$ resolution, which is comparable with the resolution achieved by the other tracking techniques.

B. Wall limited Brownian motion

In order to illustrate this novel technique, the TWT has been used to characterize the brownian motion of a sub-micrometer particle diffusing close to a wall. The two dimensional motion (parallel and perpendicular to the surface) has been recorded for many seconds with full bandwidth, as shown in Fig.5 (to improve the visibility only every hundredth point have been plotted). We have measured the motion of spherical polystyrene beads of diameter 0.2, 0.3 and 0.5 $\mu$m. The diffusion coefficient can be measured from the fluctuations of the bead as a function of time:

$$\Delta x(t)^2 = \langle [x(t) - x(0)]^2 \rangle = 2D_0 t$$

$$\Delta z(t)^2 = \langle [z(t) - z(0)]^2 \rangle = 2D_0 t$$

For a spherical particle of radius $R$, in a Newtonian fluid far from any boundary, the diffusion coefficient $D_0$ is given by the Stokes-Einstein equation [13]:

$$D_0 = \frac{k_BT}{6\pi\eta R}$$

where $\eta$ is the viscosity of the fluid. Brenner [14] demonstrated that the diffusion coefficient is strongly affected by a confined environment, and decreases in the vicinity of a wall. Therefore, the diffusion coefficient is a function of the distance between the bead and the surface [15, 16]. In addition, it also depends on the direction of motion and two diffusion
FIG. 4: A) A single colloid (diameter 200 nm) is fixed on the coverglass, while the sample holder is moved in 10 nm steps by means of a calibrated piezoelectric stage. B) the residual shot noise limits the resolution to 3 Å $RMS$. 
coefficient should be defined: $D_x(z)$ and $D_z(z)$ for the motion parallel and perpendicular to the surface respectively. Those diffusion coefficients are different ($D_x \neq D_z$) and they should be calculated for each position $\bar{z}$ above the surface. In order to take into account this effect, the data has been analyzed with a time window of 1.6 ms; within each window we compute $\Delta x(t)^2$, $\Delta z(t)^2$ and the mean position above the surface $\bar{z}$. Fig. 6 shows the mean square of the fluctuations $\bar{\Delta x(t)^2}$ for a 200 nanometers bead diffusing at $\sim 170$ nm from the coverslip. The average has been done on about 1000 realizations, and the time over which Eq. 6 holds, is restricted to windows such $\sqrt{\overline{\Delta x(t)^2}} \ll \bar{z}$. The straight line is the best fit of $D_x^{200}(50nm)$ according to Eq.(6).

The diffusion coefficients $D_x$ and $D_z$ have been measured for different altitudes $\bar{z}$ and for
FIG. 6: Mean square of the bead fluctuation as a function of time: $\langle \Delta x^2 \rangle$, for a 200 nm bead. The initial distance between the bead and the surface is 170 nm, which corresponds to $z/r = 1.7$ in Fig. 7.

beads of 200, 300 and 500 nanometers of diameter. The values of $D_x$ are in good agreement with the analytical equations proposed by Brenner for the lateral and perpendicular correction:

$$D_x = D_0 \left[ 1 - \frac{9}{16} \frac{r}{z} + \frac{1}{8} \left( \frac{r}{z} \right)^3 - \frac{45}{256} \left( \frac{r}{z} \right)^4 - \frac{1}{16} \left( \frac{r}{z} \right)^5 \right]$$

\( (8) \)
The experimental data are fitted to Eq.(8) with two adjustable parameters: $D_0$ and $z_0$. $D_0$ is the diffusion coefficient $D_x(\infty)$ far from the wall and $z_0$ is an arbitrary offset due to the relative measurement of $z$, discussed in Section II. The results are summarized in Table I: for each diameter the theoretical diffusion coefficient calculated from Eq.(7) is compared to the experimental asymptotical coefficient $D_0$ supplied by fitting the data to Eq. (8).

By comparing the perpendicular diffusion coefficient $D_z$ to Eq. (9) and keeping the same $D_0$ and $z_0$, we can independently calibrate the penetration depth $\zeta$ of the evanescent wave. Experimentally we find $\zeta \sim 215nm$, which is consistent with the value estimated in section III A.

All the $D_x(z)$ and $D_z(z)$ are plotted simultaneously (Fig.7) as a function of the position parameter $z/r$ (sphere-surface distance / bead radius). For each bead size, the diffusion coefficient is normalized to their bulk values $D_0$. The continuous lines show normalized diffusion coefficients according to Eq.(8) and Eq.(9), which are in good agreement with the experimental data.
FIG. 7: Parallel (empty marks) and perpendicular (filled marks) diffusion coefficient as a function of the bead-surface distance parameter $z/r$ (distance/bead radius). The diffusion coefficient is normalized to the diffusion far from the surface $D_0$; it been measured for beads of 200 ($\square$), 300 ($\circ$) and 500 ($\triangle$) nanometers.

IV. CONCLUSION

We have introduced a simple, versatile and powerful technique for tracking submicrometer objects: the Traveling Wave Tracking. The TWT allows us to follow the particle position along two directions, with a lateral resolution of 2-3 Angströms, and a time resolution in the microsecond range. Extension to three dimensions is in principle straightforward. We probed this technique by measuring the brownian motion of a polystyrene bead in a confined environment.

Considering the time-space resolution achieved by the TWT, we expect this technique to be a useful tool for investigating biological objects, moving on the sub-nanometer scale.

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