Dark field differential dynamic microscopy enables accurate characterization of the roto-translational dynamics of bacteria and colloidal clusters

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Abstract
Micro- and nanoscale objects with anisotropic shape are key components of a variety of biological systems and inert complex materials, and represent fundamental building blocks of novel self-assembly strategies. The time scale of their thermal motion is set by their translational and rotational diffusion coefficients, whose measurement may become difficult for relatively large particles with small optical contrast. Here we show that dark field differential dynamic microscopy is the ideal tool for probing the roto-translational Brownian motion of anisotropic shaped particles. We demonstrate our approach by successful application to aqueous dispersions of non-motile bacteria and of colloidal aggregates of spherical particles.

Keywords: Brownian motion, microscopy, rotational diffusion, bacteria, aggregation

Supplementary material for this article is available online
(Some figures may appear in colour only in the online journal)

1. Introduction
Understanding and quantifying Brownian processes is relevant for soft condensed matter scientists as well as for a wider audience, ranging from biologists to economists [1–3]. As far as colloidal particles are of interest, the erratic nature of their Brownian motion is reflected in the well-known fractal appearance of their trajectories as well as in the irregular change of their orientation in time [4, 5]. In the past, rotational Brownian motion has received considerably less attention than its translational counterpart, in part because characterizing rotational Brownian motion is more challenging. Most of the characterization makes use of optical methods such as video particle tracking (VPT), dynamic light scattering (DLS), and fluorescence correlation spectroscopy [6–9]. In particular, depolarized dynamic light scattering has been shown to be a powerful tool to assess the roto-translational dynamics of an ensemble of anisotropic (by shape and/or optically) particles by analyzing the fluctuations in the depolarized scattered light intensity [6, 7, 10–12]. Very recently, it was shown that depolarized DLS experiments, usually requiring a custom laser-based optical set-up, can be performed successfully with an optical microscope [13].

This approach, called polarized-differential dynamic microscopy (p-DDM), builds on differential dynamic microscopy (DDM) that extracts scattering information from the quantitative analysis of time-lapse microscope movies [14]. While DDM in its original implementation probes the translational diffusion coefficient of a colloidal suspension observed in bright field microscopy, p-DDM also provides access to the rotational diffusion of anisotropic colloidal particles, provided that the sample is observed between suitably oriented polarizers. Compared to DLS and depolarized DLS, DDM and p-DDM offer a simpler implementation, more robust...
Both depolarized DLS and p-DDM work well with spherical particles made of a markedly birefringent material and with non-spherical (optically homogeneous) particles provided that both the shape anisotropy and the optical contrast with the dispersion medium are large enough. These requirements are not always met and, as a result, the rotational dynamics of important classes of particles cannot be probed with depolarized DLS and p-DDM. In particular, particles that are quasi-index matched with the solvent or sparse aggregates exhibit a depolarized component of the scattered light that is insufficiently large for reliably computing the temporal autocorrelation function of the scattered field (p-DDM) or intensity (depolarized DLS). A quick and reliable tool for quantitative characterization of the roto-translational dynamics of such samples is not yet available but it would be useful, not only for the great potential of anisotropic particles in the self-assembly arena [18], but also because many systems of biological interest such as bacteria and prokaryotic cells cannot be easily studied with depolarized DLS [19].

An alternative to depolarized DLS for the characterization of the rotational dynamics of shape anisotropic particles is available, at least for particles that are not too small. In fact, for an anisotropic particle whose longest dimension \( L \) is comparable or larger than the inverse scattering wave-vector (\( L \gtrsim 150 \text{ nm} \), typically), the scattered light intensity depends upon the orientation of the particle itself, and from the study of the fluctuations in the scattered light intensity one can measure the so-called dynamic form factor [20], which encodes information on the rotational dynamics of the particles, as well as on other internal degrees of freedom, if present [7].

Here we show that dark field differential dynamic microscopy (d-DDM), a recently proposed method for the characterization of the translational diffusion of colloidal particles [21], enables the effective quantification of the intensity fluctuation associated with rotational dynamics. Our key observation is that, in a conventional dark field microscope, the image of a small particle is mainly formed by light scattered at relatively large angles, while forward scattered light is not collected. This implies that the intensity associated with the dark field image of an anisotropic particle depends on its orientation. Accordingly, if the particle rotates, for example because of thermal motion, the intensity of the collected light fluctuates, leading to an image that ‘blinks’ over time. This also makes d-DDM a valuable tool for quantitative characterization of the roto-translational dynamics of colloidal particles, especially in regimes where p-DDM does not work, namely the case of micron-sized particles closely index-matched with the solvent. To demonstrate this approach we first present measurements on a dispersion of non-motile rod-shaped bacteria. The determination of the rotational and translational diffusion coefficients allows one to determine the relevant dimensions of the bacterial particles. In a second set of experiments, d-DDM is applied to a suspension of spherical colloidal articles containing a small number of aggregates. In this case d-DDM is demonstrated to be extremely sensitive to the presence of the anisotropic aggregates that lead to a significant rotational signal.

### 2. Materials and methods

#### 2.1. Sample preparation

In order to assess the potential of d-DDM in characterizing the roto-translational dynamics of anisotropic colloidal particles, we have employed two different kinds of samples that are difficult to study with other tools such as p-DDM and/or depolarized DLS. In particular, we studied aqueous dispersions of non-motile bacteria and of aggregates of spherical nanoparticles, both presenting shape anisotropy and a moderate refractive index difference with the dispersion medium (water).

##### 2.1.1. Non-motile bacteria. One of the most frequently used coli strain for routine biological cloning applications is the DH5α strain, which are non-flagellated bacteria. They are almost non-motile, less fragile to handle and easy to grow, which makes them the perfect anisotropic particles for our purposes. We grew single colonies from frozen stocks on Luria broth (LB) agar plates at 37 °C overnight. A single colony was transferred from a plate to 20 ml of liquid LB and incubated overnight (16 h) at 37 °C while shaken (for aeration) at 200 rpm. The next step was to transfer cells from LB to a minimal medium with no exogenous nutrients in order not to have bacterial reproduction and to minimize the growth rate. To this aim, the medium underwent a washing process consisting of a 2 min centrifugation (6000 rpm and 2000 g), the expulsion of the supernatant and the resuspension of the bacterial pellet in 1 ml of PBS (phosphate-buffered saline, a water-based salt solution containing sodium hydrogen phosphate and sodium chloride). This washing procedure was repeated three times (two centrifugations). After this, the system was sucked into a 0.3 × 1 × 20 mm capillary and the capillary was fixed to a glass slide with vaseline petroleum jelly, a non-toxic glue. This operation was carefully performed in order not to let air get inside the capillary that could lead to unwanted sample drifts. It was also important to carefully avoid liquid residuals between the capillary and the glass slide, which would have caused a dynamic drying front expanding during the microscope acquisition and affecting the DDM experiments.

To avoid bacteria sedimentation, which impact on the dynamics, the density mismatch between the bacteria and the PBS physiological medium was compensated by adding Percoll® to the dispersion. Percoll® is routinely used for density gradient centrifugation of cells, viruses, and sub-cellular particles. It consists of colloidal silica particles of 15–30 nm diameter prepared at 23% w/w in water. The silica particles are coated with polyvinylpyrrolidone (PVP), which makes them completely non-toxic and ideal for use with biological materials. PVP is randomly bound to the silica particles as a monomolecular layer. The size of these particles is so small that the intensity of the scattered light is negligible when compared to the intensity of the light scattered by bacteria, as we checked experimentally in preliminary DDM experiments.

We found that a sample made of 20% of PBS medium with bacteria and 80% of Percoll® was stable upon centrifugation for 2 h at 2000 g, which is a longer time than the 30–40 min needed to acquire microscope videos in bright field and...
dark field. We note that for large concentrations of bacteria, bacterial aggregation was found to occur in the presence of Percoll®, an effect presumably due to depletion interactions. For this reason we worked at a low bacterial concentration (about $1 \times 10^5$ bacteria/ml, corresponding to a volume fraction of about $\phi = 2 \times 10^{-7}$), below the threshold needed to trigger appreciable aggregation during our experiments. The viscosity of the PBS-Percoll® solution has been measured by a capillary viscometer for temperatures in the range of 24–25 °C. For $T = 24$ °C, the temperature at which the experiments with bacteria have been performed, we found $\eta = (1.87 \pm 0.02) \times 10^{-3}$ Pa · s.

2.1.2. Polystyrene particles. We used spherical polystyrene particles (Spherotech (TM) SPHERO (TM) Biotin Polystyrene particles), with a certified mean diameter equal to 0.74 μm (intensity-weighted Nicomp distribution rescaled to number density). The samples were prepared by gently vortexing the bottle in order to resuspend the colloidal particles. Serial dilutions in 15 mM PBS buffer lead to a final concentration of about $10^5$ particles/ml, corresponding to a volume fraction of about $\phi = 2 \times 10^{-8}$. The solution was then sonicated for 10 min and confined to a rectangular glass capillary (VITROCOM, internal size: $0.3 \times 1 \times 20$ mm) for microscopy observations. The capillaries were sealed on both sides with UV glue (UV30-20, Loxeal s.r.l., Cesano Maderno, Italy), cured for 10 min under UV light (VL-6.M, Vilbert Lourmat, Marne la Vallée, France) and successively loaded on the microscope. The particles’ concentration was low enough to ensure that single particles could be identified and tracked when observed under the microscope, which allowed a direct-space based characterization of the particles’ trajectories, in addition to DDM. The viscosity of the PBS solution at the temperature $T = 24$ °C at which the experiments have been performed has been estimated by using the literature value for pure water at the same temperature ($\eta = (0.91 \pm 0.01) \times 10^{-3}$ Pa · s).

2.2. Differential dynamic microscopy

Microscopy measurements were performed with a Nikon Eclipse Ti-E commercial microscope equipped with a Hamamatsu Orca Flash 4.0 v2 camera (pixel size $d_{\text{pix}} = 6.45$ μm). Dark field images were collected with a 10X standard microscopy objective ($\text{NA}_d = 0.15$), while the sample was illuminated with a condenser stage ($\text{NA}_c = 0.4$) coupled with a PH3 phase-contrast ring mask. Bright field images were also collected with the same objective lens and bright field illumination, by using the same condenser stage with a standard diaphragm. For bacteria we also used a 40X phase-contrast objective ($\text{NA}_d = 0.6$), the sample being illuminated through the same condenser stage used in the previous cases, using a proper ring mask in order to achieve the phase-contrast condition. Each acquisition typically corresponds to a sequence of $N = 50\,000$ images $I(x, t)$ acquired with a frame rate $1/\Delta t_0$ equal to 100 fps in the case of the bacterial suspension and to 20 fps for the latex particles. Movies acquired in dark field microscopy exhibit a characteristic blinking due to the rotation of anisotropic particles, which is not present in the bright field or phase contrast movies (see supplementary movies SM00 and SM01, respectively (stacks.iop.org/JPhysCM/30/025901/mmedia)). To extract quantitative information from these movies we analyzed them by using the standard DDM algorithm [14, 16, 22], which is based on calculating the difference $I(x, t_0 + \Delta t) - I(x, t_0)$ between two images acquired at times $t_0$ and $t_0 + \Delta t$. Once this quantity is performed, its spatial Fourier power spectrum is computed by using a fast Fourier transform (FFT) routine and, in the presence of stationary or quasi-stationary statistical processes, an average over power spectra with the same $\Delta t$ but different reference time $t_0$ is obtained, which increases the statistical accuracy of the data. This leads to the so-called image structure function (ISF)

$$d(q, \Delta t) = \langle |\text{FFT}[d_I(x, t_0, \Delta t)]|^2 \rangle_{t_0}$$

(1)

that captures the dynamics of the sample as a function of the two-dimensional scattering wave-vector $q$ and of the delay time $\Delta t$. The ISF is connected to the (normalized) intermediate scattering function $f(q, \Delta t)$ [7] by the relation

$$d(q, \Delta t) = 2A(q)|1 - f(q, \Delta t)| + 2B(q)$$

(2)

where $B(q)$ is a term that accounts for the camera noise, $A(q)$ is an amplitude term that contains information about the static scattering from the sample and details about the imaging system [16]. The form of the intermediate scattering function for a sample observed under dark field imaging, undergoing both translational and rotational Brownian motion will be discussed below in section 3.1.

In general, the two-dimensional nature of the ISF provides a powerful means to probe the sample dynamics along different directions in the $q$ plane which may be of particular interest for the problem under study [23]. However, whenever the ISF bears a circular symmetry, as for all the experiment described here, azimuthal averaging of $d(q, \Delta t)$ is often used to obtain the one-dimensional function $d(q, \Delta t)$ of the radial wave-vector $q = \sqrt{q_x^2 + q_y^2}$ [14, 24–29].

In order to obtain a reliable determination of the sample dynamics, which is encoded in the intermediate scattering function, a robust procedure for estimating the amplitude $A(q)$ and the noise contribution $B(q)$ in equation (2) is required. Dark field microscopy experiments are very sensitive to non-idealities such as dust particles and scratches on the optical elements and/or on the sample cell, which can give a significant background signal on top of which the signal from the particle is superimposed. This background signal appears as an additive, positive term to the image intensity distribution. The intensity distribution $I(x, t)$ associated with each image can be thus written as the sum of three independent terms:

$$I(x, t) = I_0(x) + I_L(x, t) + I_N(x, t)$$

(3)

where $I_0(x)$ is a background image (i.e. the intensity distribution that would be observed in the absence of the sample), $I_L(x, t) \geq 0$ is the contribution to the image from the particles and $I_N(x, t)$ is the camera noise. In our case, the main contribution to the camera noise is from the shot noise and we can safely assume that it has zero average: $\langle I_N \rangle = 0$ and that it is delta-correlated in both space and time:
\( \langle I_0(x + \Delta x, t + \Delta t) I_0(x, t) \rangle = \langle I_0^2 \rangle \delta(\Delta x) \delta(\Delta t) \). If the amplitude of the noise is small compared with the background intensity \( \sqrt{\langle I_0^2 \rangle} \ll \langle I_0 \rangle \) and if the density of the scatterers is low enough to ensure that only a finite fraction of the image is covered by the particles, an accurate reconstruction of the background image can be obtained as \( I_b(x) = \min_t \{ I(x, t) \} \). In fact, picking up (for each pixel) the lowest intensity value registered during a suitably large time window—larger than the diffusion time of a single particle over its image—allows one to minimize the additive contribution from the single particle itselfs. Once an estimate for \( I_b(x) \) has been obtained, an estimate of the total amplitude of the fluctuating parts can be extracted from \( C(q) = \langle |FFT[I(x, t) - I_b(x)]|^2 \rangle = A(q) + B(q) \), where we have made use of equation (3). Equation (2) can be thus rewritten in terms of \( C(q) \) as

\[
d(q, \Delta t) = 2 [C(q) - B(q)][1 - f(q, \Delta t)] + 2B(q). \tag{4}
\]

\( B(q) \) can be estimated with high accuracy as the intercept for \( \Delta t \to 0 \) of \( d(q, \Delta t) \). This could be obtained in practice by fitting \( d(q, \Delta t) \) over a small interval \([0, \Delta t]\) to a polynomial function and by taking the 0th order coefficient. This procedure sets the value \( C(q) - B(q) \) of the amplitude of the first term, allowing thus a reliable estimate of the relaxation times in \( f(q, \Delta t) \) even if they exceed the width of the acquisition window.

3. Theory

In this section we will first provide a brief summary of the scattering theory from optically anisotropic particles [7]. In the second part we will describe the features of a dark field imaging system and the imaging process of anisotropic particles.

3.1. Scattering by anisotropic particles

3.1.1. Small anisotropic particles: Rayleigh scattering. The description of the scattering of light by a particle much smaller than the wavelength is usually based on the so-called Rayleigh approximation [30]. For simplicity, we will restrict our discussion to the case of uniaxial particles, whose polarizability tensor admits a diagonal form with diagonal elements \( \alpha_1, \alpha_2, \alpha_3 \), where \( \alpha_2 = \alpha_3 \). For these types of particles, the anisotropy parameter is defined as \( \beta = \alpha_1 - \alpha_2 \) and the average (excess) polarizability as \( \alpha = \frac{2\alpha_2 + \alpha_0}{3} - V (n_i^2 - 1) \). Here \( V \) is the particle volume and \( n_i \) is the refractive index of the dispersion medium. If a plane wave electric field \( A_0(z) = E_0 e^{-jkz} \) of wave-number \( k \) impinges on such particle, the latter emits a scattered field that, in addition to the component \( E_VH \) that is parallel to \( E_0 \), also bears a perpendicular component \( E_WH \).

One has [30]

\[
E_{VV} = S_{VV} \frac{e^{-jkx + jkz}}{jk r} A_0(z) \tag{5}
\]

\[
E_{WH} = S_{WH} A_0(z) \]

where \( j \) is the imaginary unit, \( r \) is the distance from the particle, and \( S_{VV} \) and \( S_{WH} \) are dimensionless amplitudes that depend on the scattering angle \( \theta \), measured with respect to the direction \( z \) of the incident radiation, and on the orientation \( (\theta, \phi) \) of the particle. For small scattering angles, the scattering amplitudes \( S_{VV} \) and \( S_{WH} \) are given by the following expression [7]

\[
S_{VV} = jk^2 \alpha + jk^3 \beta \sqrt{\frac{16\pi}{45}} Y_{2,0}(\theta, \phi), \tag{7}
\]

\[
S_{WH} = jk^3 \beta \sqrt{\frac{2\pi}{15}} [Y_{2,-1}(\theta, \phi) + Y_{2,1}(\theta, \phi)]. \tag{8}
\]

Here \( Y_{lm}(\theta, \phi) \) is the spherical harmonic function of order \( l \), \( m \).

Given the shape and the refractive index distribution within a particle, the calculation of its polarizability tensor is not in general a trivial task. Even in the Rayleigh regime, closed-form expressions can be obtained only in the presence of particularly simple geometries. In order to better elucidate how the scattering properties of a particle depend on its shape and refractive index, it is worth considering a specific model system for which a simple analytical description is available, namely a homogeneous ellipsoid of revolution (spheroid) of semi-axes \( [R, R, R] \). In this case, the diagonal elements \( \alpha_1 \) of the (excess) polarizability tensor are given by \( \frac{V}{2\pi n_i^2} = L_a + \frac{m - 1}{n_i} \), where \( m = n_p/n_\infty \) is the ratio between the refractive index \( n_p \) of the particle and the refractive index \( n_\infty \) of the solvent and \( L_{2,3} = L, L_1 = 1 - 2L, \) with \( L = e \int_0^\infty \frac{dw}{2(w+1)(w+e^2)^{1/2}} \). \( L \) is a parameter ranging from 0 (flat elliptical disk) to \( \frac{1}{4} \) (infinitely long ellipsoid). The value \( L = \frac{1}{4} \) corresponds to the isotropic case. We can also define an anisotropy factor \( a = 2(3L - 1) \) in such a way that \( a = 0 \) corresponds to a sphere, while one has \( a = 1 \) for an infinitely long ellipsoid. If we also define \( \delta = m^2 - 1 \), we obtain the following simple expression for the amplitude depolarization ratio [7]

\[
\frac{\beta}{\alpha} = \frac{3\delta a}{6 + \delta (2 - a)} \approx \frac{1}{2} \delta a \tag{9}
\]

which in fact is the ratio between the amplitudes of the orientation-dependent component and the orientation-independent component of the scattered field in equations (6) and (8). It is clear from equation (9) that the contrast in the fluctuation of the depolarized scattering component depends both on the anisotropy of the particle and on the refractive index mismatch with respect to the solvent. Even a strongly anisotropic particle (\( a \simeq 1 \)) cannot produce a significant depolarized signal if it is quasi index-matched with the solvent (\( \delta \simeq 0 \)). We note that, as far as the Rayleigh approximation is satisfied, the result expressed by equation (9) holds independently of the particle size.

3.1.2. Weakly scattering anisotropic particles of larger size: Rayleigh–Gans–Debye description. The case of a homogeneous, quasi-index-matched particle can be adequately
described within the Rayleigh–Gans–Debye (RGD) approximation if the overall phase delay associated with the particle is small: $|\delta|kd \ll 1$ [30]. In this approximation, the amplitude of the wave scattered by the particle can be calculated as the sum of independent contributions from each portion of particle. In the RGD approximation the amplitude of the depolarized scattering is negligible and the scattering amplitude entails only a polarized component

$$S_{VV}(q) = \frac{jk^3}{4\pi} V \cdot F(q)$$  \hspace{1cm} (10)$$

where

$$F(q) = \frac{1}{V} \int_V d^3x e^{-i\mathbf{q} \cdot \mathbf{x}}$$  \hspace{1cm} (11)$$
is the so-called form factor amplitude and $\mathbf{q} = (\mathbf{k}_i - \mathbf{k}_s)$ is the transferred momentum, i.e. the difference between the scattering wave-vector $\mathbf{k}_s$ and the wave-vector $\mathbf{k}_i$ of the incident light. For a particle of a given shape, $F(q)$ has in general an implicit dependence on the particle orientation. Within the RGD approximation, the scattering in the forward direction ($\mathbf{q} = 0$) does not depend on the details of the particle shape or orientation, as $F(0) = 1$. A non-trivial dependence of the scattered amplitude on the particle orientation can be observed only for $q > 0$ and if the size of the particle is not too small compared with the wavelength of light [7].

For a spheroidal particle, such as the one considered in the previous paragraph, the form factor amplitude can be calculated explicitly:

$$F_{\text{ell}}(q) = 3 \left[ \frac{\sin(qR_\gamma) - qR_\gamma \cos(qR_\gamma)}{(qR_\gamma)^3} \right]$$  \hspace{1cm} (12)$$

where $R_\gamma = R \sqrt{\sin^2 \theta_0 + e^2 \cos^2 \theta_0} = R \sqrt{1 + (e^2 - 1) |\mathbf{q} \cdot \mathbf{n}|^2/q^2}$. Here $\mathbf{n}$ is the unit vector oriented along the particle axis and $\theta_0$ the angle between $\mathbf{n}$ and the transferred momentum $\mathbf{q}$. In contrast to the forward depolarized scattering in the Rayleigh regime, here the relative amplitude of the fluctuation in the scattered intensity due to a rotation of the particle does not depend at all on the optical contrast, while it is strongly influenced by the overall particle size and by the collection angle, which in turn determines the transferred momentum $q$.

This property, namely the fact that the fluctuation in the intensity of the light scattered at a large angle by an ensemble of anisotropic colloidal particles reflects also its rotational dynamics, has been exploited in DLS for accessing its rotational-translational dynamics, as a complementary approach with respect to low angle depolarized scattering measurements [20, 31, 32]. Equation (12) takes a particularly simple form if the particle is not too large compared to the inverse transferred momentum. In fact, for a relatively small uniaxial anisotropic particle (for which $qL \lesssim 1$, where $L$ is its longer dimension), the expression in equation (11), can be expanded in $(qL)$, leading to:

$$F \simeq 1 - \frac{q^2}{V} \left[ I_{zz} + 2 (I_{xx} - I_{zz}) \cos^2 \theta_0 \right]$$  \hspace{1cm} (13)$$
or, to the same order in $(qL)$, to

$$P = |F|^2 \simeq 1 - \frac{2q^2}{V} \left[ I_{zz} + 2 (I_{xx} - I_{zz}) \cos^2 \theta_0 \right],$$  \hspace{1cm} (14)$$

where $I_{xx} = \int_V d^3x (x^2 + z^2)$ and $I_{zz} = 2 \int_V d^3x x^2$ are the diagonal elements of the particle tensor of inertia [7], and $\theta_0$ is the angle between the axis of the particle and the transferred momentum $\mathbf{q}$. The simple harmonic dependence of the scattered intensity on $\theta_0$ described by equation (14) will be used in the following paragraphs to link the correlations of the scattered intensity to the statistical properties of the rotational motion of the particle.

3.2. Dark field microscopy

3.2.1. Dark field imaging. In the context of optical microscopy, the term dark field (DF) indicates a family of microscopy configurations characterized by the fact that the transmitted illumination beam is not collected by the imaging optics and thus only the light scattered from the sample contributes to the image. In practice, this can be obtained in a number of different ways, for example by using dedicated illumination stages. A common implementation, which is compatible with most commercial inverted microscopes is the one reported in figure 1(a), where a schematic representation of a microscope with Koehler illumination is shown. In this configuration, a circular aperture (1) or radius $R_{\text{ring}}$ carved into an opaque mask is placed in the back focal plane of the condenser lens (2), of focal length $(FL)_{\text{cond}}$. In this condition the object plane (3) is illuminated only by rays forming an angle $\theta \simeq \arcsin(NA)_c$, where $(NA)_c = \arctan \left[ R_{\text{ring}} / (FL)_{\text{cond}} \right]$. The dark field condition is achieved by using an objective lens...
(4.5) with numerical aperture \((\text{NA})_o < (\text{NA})_c\), so that the transmitted beam is not collected by the objective. In practice, this is commonly achieved by coupling a high numerical aperture phase contrast ring with a low power objective. In this configuration, the illumination beam can be thought of as the incoherent superposition of many plane waves propagating at angles \(\theta \simeq \arcsin (\text{NA})_c\), with respect to the optical axis i.e. the sample is illuminated by a collection of uncorrelated coherent patches of transverse size \(\Delta_o \simeq \lambda/(\text{NA})_o\). Since \((\text{NA})_o < (\text{NA})_c\), the size of these coherent patches is smaller than the transverse size \(\Delta_o \simeq \lambda/(\text{NA})_o\) of the objective PSF. For this reason, the imaging process is, to all effects, incoherent, as interference effects between different scattering centers are negligible. For this reason, we refer to this configuration as incoherent dark field microscopy (IDFM).

According to the nomenclature introduced in [24], IDFM is thus a linear imaging system and can be, therefore, profitably used for digital Fourier microscopy experiments, where a linear space-invariant imaging system turns out to be beneficial [16]. We note that, as pointed out in [21], dark field imaging can, in some cases, lead to a non-homogeneous illumination pattern, which violates the space-invariant assumption. In our optical set-up this effect was negligible, in that the illumination was found to be uniform across the entire field of view.

Clearly, other implementations of dark field imaging, alternative to IDFM, exist, but not all of them are linear space-invariant and thus suitable for digital Fourier microscopy. An explicit example along this line is presented in appendix B.

### 3.2.2. The incoherent dark field microscope as a fixed, non-zero angle scattering set-up

In a dark field microscope the main contribution to the particle image intensity comes from light scattered at a non-zero angle, roughly corresponding to the illumination numerical aperture. As a consequence, if the particle is anisotropic, its reorientation produces a modulation in the image intensity. This property opens up the possibility of exploiting IDFM to study the rotational dynamics of anisotropic particles. The main elements of typical dark field microscopy set-up are shown in the simplified scheme in figure 1. The elementary scattering process contributing to the image formation is shown in figure 1(b), where \(\mathbf{k}_i\) denotes the incident wave-vector, \(\mathbf{k}_s\) is the scattering wave-vector and \(\mathbf{q} = \mathbf{k}_s - \mathbf{k}_i\) is the transferred momentum. By introducing \(\mathbf{k}_0\) as the wave-vector oriented along the optical axis, having the same amplitude of \(\mathbf{k}_i\) and \(\mathbf{k}_s\), we can express the transferred wave-vector as \(\mathbf{q} = \mathbf{q}_s - \mathbf{q}_i\), where \(\mathbf{q}_s = \mathbf{k}_s - \mathbf{k}_0\) and \(\mathbf{q}_i = \mathbf{k}_i - \mathbf{k}_0\). In the image plane, the total intensity \(I\) associated with the particle’s image can be calculated as the (incoherent) sum of all such processes

\[
I_p = V \int dq_i \int dq_s I_0(q_0) P(q_s - q_i) T(q_i) \tag{15}
\]

where the function \(I_0(q_0)\), representative of the angular distribution of the illumination beam, weights the contribution of each incoming plane wave, the particle form factor \(P(q)\) accounts for the scattering properties of the sample, and the incoherent transfer function \(T(q_i)\) quantifies the collection efficiency of the objective lens.

Our choice of a thin phase contrast ring of numerical aperture \((\text{NA})_c\), makes the evaluation of the integral on the right-hand side of equation (15) particularly simple, as \(I_0(q_0) \simeq I_0[\theta(q_0) - q^*]\), where \(q^* = 2k_0 \sin \left(\frac{\theta}{2}\right) \simeq k_0 \sin(\theta_c) = k_0(\text{NA})_c\). In general, \(T(q)\) is an azimuthally symmetric function of width \(\sim k_0(\text{NA})_o < k_0(\text{NA})_o < k_0(\text{NA})_c\) centered around \(q = 0\). If we take the (rather crude) approximation: \(T(q) \simeq \delta(q)\), corresponding to considering only the light scattered along the optical axis, we obtain the following simple result, where the total intensity of the particle’s image is written as an azimuthal average of the particle form factor performed for \(|q| = q^*\):

\[
I_p \propto \int dq \delta(|q| - q^*) P(q) = \int dq P \left( q^* \cos \alpha, q^* \sin \alpha, q^u \right),
\]

where \(q^u \parallel q^*\) and \(q^u \perp q^*\) are the projections of the transferred momentum in the direction perpendicular and parallel to the optical axis, respectively. If we assume for the form factor \(P\) the expression given in equation (14), \(I_p\) can be easily integrated leading to:

\[
I_p \propto 1 - \frac{2q^2}{V} \left[ I_{xx} + (I_{zz} - I_{xx}) \sin^2(\theta) \right] \tag{16}
\]

or, equivalently

\[
I_p = I_{p,0} [1 + c Y_{20}(\theta)] \tag{17}
\]

where \(Y_{20}(\theta) = \sqrt{\frac{5}{16\pi}} (3 \cos^2 \theta - 1)\) is the spherical harmonic of order 2, \(\theta\) is the angle between the axis of the particle and the optical axis, \(I_{p,0}\) is a constant amplitude and \(c\) is a constant. Equation (17) shows that the intensity signal due to an anisotropic particle depends on the particle orientation with respect to the optical axis, which explains why a particle that undergoes a rotational Brownian motion appears as a randomly blinking object. We note that considering a finite value for the objective numerical aperture does not affect this result. In fact, in that case \(I_p\) is still given by the integral of \(P(q)\) over an annular region of radius \(q^*\), but with a finite width \(\sim k_0(\text{NA})_o\), which does not affect the angular dependence of \(I_p\) in equation (17), but only the prefactor \(c\). We note that equation (17) holds under the same hypotheses under which equation (14) is valid, namely that the \(Lq^* \lesssim 1\), where \(L\) is the longest dimension of the particle. For very large particles a more complex expression is expected, involving higher order spherical harmonics.

### 3.2.3. Dark field DDM probes the roto-translational dynamics of anisotropic particles

The fact that the image intensity corresponding to one particle depends on the particle orientation can be, in principle, used to assess the rotational dynamics by studying the characteristic time of blinking in movies obtained by IDFM (see supplementary movies SM01 and SM02). For the case of interest in this work, i.e. when the particle undergoes a rotational Brownian motion, its rotational diffusion coefficient \(D_\alpha\) can be obtained by calculating the temporal auto-correlation function of the intensity given in Equation (17)

\[
C_p(\Delta t) = \langle I_p(t + \Delta t)I_p(t) \rangle = I_{p,0}^2 \left( 1 + c^2 e^{-6D_\alpha \Delta t} \right) \tag{18}
\]
whose characteristic time \((6R_D)^{-1}\) would give immediate access to the rotational diffusion coefficient \(D_R\) [7]. However, measuring \(C_p(\Delta t)\) is usually quite difficult: for instance, particles can disappear from the image when they exit the focal region or two particles might superimpose along the optical axis and give rise to spurious effects. These difficulties can be bypassed by working in the wave-vector space, as in dark field differential dynamic microscopy (d-DDM) [21]. The image intensity distribution of a collection of identical particles observed in IDFM is given by

\[
I_t(x, t) = \sum_n I_t^{(n)}(t)\psi(x - x_n)
\]

where \(\psi(x)\) is a function that describes the intensity distribution associated with the image of an individual scatterer in real space. If \(\hat{I}_t(q, t)\) is the spatial Fourier transform of \(I_t(x, t)\), an alternative quantification of the particle dynamics is provided by the intermediate scattering function

\[
\langle \hat{I}_t(q, t + \Delta t)\hat{I}_s(q, t) \rangle = N|\psi(q)|^2 C_p(\Delta t)e^{-D_R q^2 \Delta t}
\]

where \(D_T\) is the translational diffusion coefficient of the particles. Also using equation (18), Equation (20) can be rewritten by introducing the normalized intermediate scattering function [7]

\[
f(q, \Delta t) = \frac{\langle \hat{I}_t(q, t + \Delta t)\hat{I}_s(q, t) \rangle}{(|\hat{I}(q, t)|^2)} = \alpha e^{-\Gamma_1(q)\Delta t} + (1 - \alpha)e^{-\Gamma_2(q)\Delta t},
\]

where the contribution of the two relaxation modes with decay rates \(\Gamma_1 = 6D_R + D_T q^2\) and \(\Gamma_2 = D_T q^2\) can be appreciated. This form for the intermediate scattering function is pretty common, as is found also in depolarized DLS and p-DDM experiments [6, 13].

4. Results and discussion

In this section, we demonstrate d-DDM as an effective tool for the simultaneous determination of translational and rotational dynamics by presenting results obtained with two representative samples: a suspension of quasi-index matched anisotropic colloidal particles (non-motile rod-shaped bacteria) and a suspension of quasi-monodisperse spherical colloids both in a non-aggregated state and forming small clusters.

4.1. Bacteria

A first set of measurements was performed on a suspension of density-matched non-motile coliform bacteria, prepared as described in the material and methods section. Movies of the sample are acquired with dark field, bright field and phase contrast microscopy. A representative dark field image of the sample is shown in figure 2 (see also the supplementary movie SM01): the bacterial particles appear as bright spots diffusing on a dark background and whose intensity fluctuates with a characteristic time of about 1 s. We will show that these two processes, namely, the concentration fluctuation due to the translational motion of the particles and the intensity fluctuations caused by the rotational dynamics of single particles, are well captured by d-DDM analysis.

In figure 3 we show for some representative values of the wave-vector \(q\), logarithmically spaced in the range \([0.075, 2.3]\) \(\mu m^{-1}\), the intermediate scattering functions obtained with d-DDM analysis. As expected from equation (21), the observed relaxation exhibits two distinct decays. This is particularly evident in the low-q regime, where the time-scale separation between the two decays is more pronounced (see figure 3(b)). The intermediate scattering functions are well described by a sum of two simple-exponential decays (continuous lines in figure 3). Fitting the obtained curves to equation (21) thus provides an estimate for the two \(q\)-dependent relaxation rates \(\Gamma_1(q)\) and \(\Gamma_2(q)\). The so-obtained \(\Gamma_1\) and \(\Gamma_2\) are shown in figure 4 together with the best fitting curves: \(\Gamma_1(q) = D_T q^2 + 6D_R\), and \(\Gamma_2(q) = D_T q^2\). From this last fit we obtain an estimate for the translational and the rotational diffusion coefficient of the bacteria: \(D_T = (1.57 \pm 0.02) \cdot 10^{-11} \ mu m^2 \ s^{-1}\) and \(D_R = (1.5 \pm 0.1) \cdot 10^{-11} \ mu m^2 \ s^{-1}\).

The simultaneous measurement of both \(D_T\) and \(D_R\) allows one to estimate the size of the bacterial particles. We consider two simple models, for which analytical expressions for the diffusion coefficients are available, namely a spheroid of semi-axes \(a, b, c\), with \(b = c\) [33] and a cylinder of length \(L\) and radius \(r\) [34, 35]. Explicit expressions for \(D_T\) and \(D_R\) in the two cases are reported in appendix A and can be inverted numerically to determine the best estimates for the geometrical parameters in the two cases. In particular, it is convenient to parametrize the spheroid by expressing the length of the semi-axes \([a, b, c]\) in terms of the radius \(R\) of the sphere with equal volume and of the eccentricity parameter \(\epsilon = \frac{a}{R}\), as \([a, b, c] = R\epsilon^{-1/3}\ [1, 1, 1]\). We obtain: \(R = 0.70 \pm 0.02 \mu m\), \(\epsilon = 2.3 \pm 0.3\) for the spheroid and \(L = 1.6 \pm 0.2 \mu m\), \(r = 0.6 \pm 0.2 \mu m\) for the cylinder. We note that both models provide meaningful results, that are in good mutual agreement and are fully compatible with both the values in the literature.
1.58

1.57

vectors.

characterized by a visible depression at small scattering wave-

\( q \), the optical transfer function for the bright field experiments is
dispersion. While the dark field amplitude is a decreasing function
squares) and dark field (blue circles) movies of the same bacterial
same microscope objective from DDM analysis of bright field (red
curve not shown). Inset: static amplitude
dash-dotted and the continuous lines are the best fits to the curves
microscopy experiments on a dilute bacterial dispersion. The
field (orange crosses) and in phase-contrast (purple circles)
obtained in dark field (open blue circles and squares), in bright
obtained from the fit of the intermediate scattering functions
Decorrelation rates as a function of the wave-vector
Figure 3. Symbols: intermediate scattering function \( f(q, \Delta t) \) obtained for different wave vectors \( q = 0.075, 0.11, 0.19, 0.30, 0.44, 0.65, 0.98, 1.5, 2.3 \text{ \mu m}^{-1} \) in d-DDM experiments on a dilute bacterial dispersion. Continuous lines: best fitting curves with the model function given in equation (21).

Figure 4. Decorrelation rates as a function of the wave-vector \( q \) obtained from the fit of the intermediate scattering functions obtained in dark field (open blue circles and squares), in bright field (orange crosses) and in phase-contrast (purple circles) microscopy experiments on a dilute bacterial dispersion. The dash-dotted and the continuous lines are the best fits to the curves \( \Gamma_1(q) = 6D_R + D_T q^2 \) and \( \Gamma_2(q) = D_T q^2 \), respectively, which lead to the estimates \( D_T = (1.57 \pm 0.02) \cdot 10^{-1} \text{ \mu m}^2 \text{ s}^{-1} \) and \( D_R = (1.5 \pm 0.1) \cdot 10^{-1} \text{ s}^{-1} \). Bright field and phase-contrast data provided the value \( D_T = (1.58 \pm 0.01) \cdot 10^{-1} \text{ \mu m}^2 \text{ s}^{-1} \), obtained by fitting the experimental data to the curve \( \Gamma_1(q) = D_T q^2 \) (fitting curve not shown). Inset: static amplitude \( A(q) \) obtained with the same microscope objective from DDM analysis of bright field (red squares) and dark field (blue circles) movies of the same bacterial dispersion. While the dark field amplitude is a decreasing function \( q \), the optical transfer function for the bright field experiments is characterized by a visible depression at small scattering wave-vectors.

[36, 37] and direct, high magnification microscopy observations (see figure 2).

As a consistency check, we also analyzed bright field and phase-contrast movies of the same sample. In both cases, the obtained intermediate scattering functions show a single decay that is very well fitted by a simple exponential function (data not shown). The corresponding \( q \)-dependent relaxation rates are reported in figure 4 and exhibit very clean \( q^2 \) scaling, and the estimated translational diffusion coefficient \( D_T = (1.58 \pm 0.01) \cdot 10^{-1} \text{ \mu m}^2 \text{ s}^{-1} \) is in excellent agreement with the results of d-DDM. Of note, the wave-vector range over which a reliable estimate of the dynamics can be obtained is not the same for all the imaging conditions. In particular, we observe that, although dark field and bright field measurements are performed with the same objective, frame rate and duration of the acquisition, dark field DDM is much more effective in probing the dynamics in the low-\( q \) regime. This can explained by inspecting the static scattering amplitudes \( A(q) \) obtained from the DDM analysis in the two datasets shown in the inset of figure 4, which outlines the effect of the different transfer functions of the two methods. The bright field amplitude shows a characteristic depression at low \( q \), reflecting the fact that bacteria, being quasi-index matched with the solvent, behave as phase objects [24]. In contrast, the dark field amplitude is a monotonically decreasing function of \( q \). The phase contrast data, being obtained with a different microscope objective, with larger magnification and numerical aperture (40 versus 10 and 0.6 versus 0.15), cover a \( q \) range shifted by approximately half a decade to larger wave-vectors.

4.2. Spherical colloids

A second set of d-DDM measurements was performed on a suspension of monodisperse spherical latex particles. A first sample (sample 1) was prepared with a 5 min sonication stage before the measurement, as described in the materials and methods section. Surprisingly, we found that d-DDM analysis showed two distinct decays \( \Gamma_1(q) \) and \( \Gamma_2(q) \) for the ISF (5), as previously found for the bacteria. This finding contrasted our expectation to observe a single relaxation mode due to translational diffusion of the particles. In order to better understand the reason for such unexpected behavior we thus performed a bright field DDM experiment on the same sample: as expected for a reasonably monodisperse sample, the experimental
Figure 5. Decorrelation rates obtained from the fit of the intermediate scattering functions obtained in dark field (open blue circles and squares) and in bright field (orange dots) microscopy experiments on a partially aggregated suspension of the same microscopy experiments on a non-aggregated suspension of the same sample (sample 1). The dash-dotted and the dashed lines are the best fits to the curves $\Gamma(q) = 6D_{R} + D_{\text{BF}} q^{2}$ and $\Gamma_2(q) = D_{\text{BF}} q^{2}$, respectively, which lead to the estimates $D_{\text{BF}} = 0.49 \pm 0.02 \mu m^2 s^{-1}$ and $D_{R} = 1.30 \pm 0.05 s^{-1}$. Bright field data provided the value $D_{\text{BF}} = 0.475 \pm 0.05 \mu m^2 s^{-1}$, obtained by fitting the experimental data to the curve $\Gamma_2(q) = D_{\text{BF}} q^{2}$ (fitting curve not shown). Upward purple triangles correspond to the single decorrelation rate $\Gamma(q)$ measured in a dark field microscopy experiment on a non-aggregated suspension of the same particles (sample 2). The continuous line is the best fit to the curve $\Gamma(q) = D_{T} q^{2}$, leading to the estimate $D_{T} = 0.695 \pm 0.01 \mu m^2 s^{-1}$. ISFs were well fitted to a single exponential decay and the so-obtained relaxation rate $\Gamma(q)$ was well fitted to the function $\Gamma(q) = D_{T} q^{2}$ (figure 5). The estimate $D_{\text{BF}} = 0.475 \pm 0.05 \mu m^2 s^{-1}$ for the translational diffusion coefficient of the particles is fully compatible with the value found in dark field experiments, which gave $D_{\text{BF}} = 0.49 \pm 0.01 \mu m^2 s^{-1}$ and $D_{R} = 1.30 \pm 0.05 s^{-1}$ for the translational and rotational diffusion coefficients, respectively. However, none of them was found to be compatible with the value $R_{\text{H}} = 0.37 \pm 0.02 \mu m$, certified by the producer for the hydrodynamic radius of the particles. Both values $R_{\text{H}} = 0.503 \pm 0.005 \mu m$ and $R_{R} = 0.49 \pm 0.01 \mu m$, obtained from $D_{\text{BF}}$ and $D_{R}$, respectively, via the Stokes–Einstein relation are about 30% larger than the nominal value. We interpreted all these results as consequences of the presence of anisotropic particles in suspension, originating from aggregation of the spherical particles. Careful inspection of the dark field movies (see supplementary movie SM02) further supported this hypothesis in that it pointed to the presence of a small number of blinking particles. 

To obtain final confirmation, we performed measurements on a second sample (sample 2) that was carefully prepared from the same batch of particles by using a longer sonication stage (30 min instead of 5). With sample 2, the results of DDM analysis of bright field and dark field movies (see supplementary movie SM03) confirmed the absence of aggregates. In both cases, the ISFs exhibited a single exponential decay, with a relaxation rate displaying a clean quadratic scaling with $q$, from which we obtain the estimate $D_{T} = 0.695 \pm 0.01 \mu m^2 s^{-1}$ for the translational diffusion coefficient (figure 5). The extracted hydrodynamic radius $R_{H} = 0.35 \pm 0.01 \mu m$ is now fully compatible with the nominal size of the particle, as certified by the producer. We could therefore safely conclude that sample 1 contained aggregated particles and that d-DDM provides a very powerful means to spot the presence of small aggregates in colloidal dispersions of spherical particles. In particular, when sizing unknown samples, it is recommended to complement bright field DDM experiments with dark field ones, to check whether a rotational diffusion decay mode due to aggregates is present or not.

We then turned to assessing how far can d-DDM be brought in obtaining information on the aggregates. To this aim we evaluated the values expected for the translational and rotational diffusion coefficients of small clusters of particles (dimers, trimers, tetramers, etc) [38]. In particular, the value $D_{\text{trans}} = 0.65 \mu m^2 s^{-1}$, corresponding to the hydrodynamic radius certified by the producer, gives the following values for small clusters: $D_{\text{dim}} = 0.48 \mu m^2 s^{-1}$ (dimers), $D_{\text{tr}} = 0.41 \mu m^2 s^{-1}$ (trimers), and $D_{\text{tet}} = 0.38 \mu m^2 s^{-1}$ (tetramers). We note that these values are very close to each other, which explains why translational diffusion does not discriminate very effectively between these contributions. This is confirmed by our d-DDM results, as we found that the translational diffusion coefficient extracted with d-DDM exhibited an intermediate value between the expectation for monomers and for the small clusters. By contrast, the rotational diffusion coefficient is not sensitive to the presence of monomers. Consistently, the value obtained with d-DDM ($D_{R} = 1.30 \pm 0.05 s^{-1}$) was found to be within the range of the rotational diffusion coefficient expected for small clusters: $D_{\text{dim}} = 1.39 s^{-1}$ (dimers), $D_{\text{tr}} = 0.87 s^{-1}$ (trimers), and $D_{\text{tet}} = 0.67 s^{-1}$ (tetramers).

In order to check the consistency of this picture, we performed a detailed VPT analysis on the same movies, which is very delicate and time-consuming but provides more detailed information about the sub-populations of which the sample is composed. Particles’ trajectories are obtained with the Particle Tracker plugin, included in the Mosaic suite for ImageJ. With a custom software written in Matlab, we extracted from each trajectory the particles’ mean-squared displacement and, by fitting the resulting curve as a function of the time delay we obtain and estimate for the translational diffusion coefficient $D_{T}$ of each single particle. By rejecting trajectories shorter than 200 time steps, we obtained $D_{T}$ for about 1000 particles for each sample. In figure 6, we report the histograms representing the distributions of the values of $D_{T}$ obtained for each of the two samples. For sample 2, the distribution of $D_{T}$ is fairly symmetric and it is well described by a Gaussian function with mean value $D_{T} = 0.69 \mu m^2 s^{-1}$ and standard deviation $\sigma_{D_{T}} = 0.1 \mu m^2 s^{-1}$. This result is compatible with a moderately dispersed distribution peaked around a value that is in excellent agreement with estimated $D_{T} = 0.695 \pm 0.01 \mu m^2 s^{-1}$ obtained for the translational diffusion coefficient from the d-DDM analysis. For sample 1, the same SPT-based analysis provides a completely different result. The distribution of $D_{T}$ is broader and, beside a peak centered

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about a value compatible with $D_T$, a secondary peak for $D_T \simeq 0.4 \mu m^2 s^{-1}$ is also clearly visible. As shown in figure 6, the range covered by this secondary peak is compatible with the translational diffusion coefficients expected for small clusters of monomers, assuming $D_T$ as the diffusion coefficient of a monomer. The vertical dashed-dotted, dashed and dotted lines correspond to the expected values for dimers, trimers and tetramers, respectively.

Figure 6. Histograms of the distribution of the translational diffusion coefficient for sample 1 (large blue bars) and for sample 2 (thin orange bars) as determined from VPT. The continuous vertical line corresponds to $D_T = 0.69 \mu m^2 s^{-1}$, obtained by fitting the histogram obtained from sample 2 to a Gaussian function (continuous curve). The histogram for sample 1 is well described as the sum of two Gaussian functions (thick dashed-dotted curve) centered in $D_T \simeq 0.7 \mu m^2 s^{-1}$ and $D_T \simeq 0.4 \mu m^2 s^{-1}$, respectively. This second value falls within the range of the translational diffusion coefficients expected for small clusters of monomers, assuming $D_T$ as the diffusion coefficient of a monomer. The vertical dashed-dotted, dashed and dotted lines correspond to the expected values for dimers, trimers and tetramers, respectively.

5. Conclusions

In this work, we have shown that the recently introduced dark field differential dynamic microscopy [21] is a simple and powerful tool for the simultaneous determination of the rotational-dynamics of anisotropic microparticles in suspension. Our experiments with bacterial suspensions showed that d-DDM is somehow complementary to the recently proposed polarized-DDM [13], in that the latter may fail with particles characterized by moderately shaped anisotropy and good refractive index-matching with the dispersion medium.

We also showed that the peculiar nature of the dark field signal associated with rotational dynamics makes d-DDM very effective in spotting and quantifying the presence of anisotropic aggregates of isotropic particles, even though whenever the particles are large enough, particle tracking may provide a better tool for a detailed analysis of aggregated samples in which clusters are present. On the other hand, d-DDM analysis is statistically more robust and does not require the intervention of an experienced user for the fine-tuning of the parameters involved in the image processing procedure.

These promising results open up the possibility of using d-DDM to study more complex and challenging systems. For example, it is known that, for motile bacteria, the rotational dynamics is very different from a purely Brownian one [39], and that a non-trivial interplay exists between rotational and translational degrees of freedom [40]. d-DDM could allow simple, high-throughput, characterization of this complex dynamics, possibly in combination with other quantitative microscopy methods, such as, for example, standard phase contrast DDM [41] or the so-called dark field flicker microscopy [42], that has been used for monitoring the rapid periodic fluctuation associated with the beating of flagella.

Another field of potential application is the optical characterization of the mechanical properties of soft materials, the realm of microrheology [43, 44]. Passive microrheology, in particular, exploits the thermally excited positional fluctuations of immersed tracer particles to probe the viscoelastic moduli of the hosting fluid [45]. Very recently, DDM was demonstrated to be a reliable route to microrheology, enabling accurate, tracking-free determination of the mean-squared displacement of probe particles in a variety of imaging conditions [46, 47].

In view of the results presented in this work, we expect that d-DDM, in combination with calibrated anisotropic tracers, could provide the ideal ground to extend these ideas also to the rotational degree of freedom, enabling the simultaneous execution of translational and rotational microrheology experiments [48, 49]. While this combination could appear to be redundant in the case of a perfectly homogeneous fluid—where the two approaches are expected to give equivalent results—it could be quite valuable in the presence of sources of non-ideality in the system (e.g. inhomogeneity of the matrix or specific tracer-fluid interactions altering the boundary conditions at the surface of the particles). Since these effects, that can seriously compromise the reliability of microrheology results, are expected to have a different impact on rotational and translational degrees of freedom, the execution of a combined experiment could allow one to spot them effectively.

Moreover, since d-DDM is very sensitive in detecting the presence of anisotropic particles, it can be exploited as a quality control step during the preparation or the execution of experiments involving allegedly spherical colloidal particles, or for real-time monitoring of aggregation processes and self-assembly. Another interesting application of the method could be in the on-line monitoring of water quality [50]. In this case, the ability of d-DDM to spot the presence of particles in solution and, by providing an estimate of their dimensions and optical contrast, to discriminate between them, could make it a useful screening tool for the automatic identification of particularly important classes of contaminants, in primis bacteria.
**Appendix A. Roto-translational diffusion of spheroids and cylinders**

In this appendix we report analytic expressions available in the literature for the rotational and translational diffusion coefficient for a spheroidal particle [33] and for a cylinder [34, 35].

**A.1. Spheroid**

The rotational and translational diffusion coefficients are given by, respectively,

\[
D_R = \frac{3k_B T}{32\pi\eta} \left( \frac{2a^2 - b^2}{a^4} \right) S - 2a, \\
D_T = \frac{k_B T}{12\pi\eta} S,
\]

where

\[
S = (2/k) \ln \left( \frac{\sqrt{a^2 + b^2}}{b} \right), \quad k_B = \text{the Boltzmann constant}, \quad T = \text{the absolute temperature} \quad \text{and} \quad \eta = \text{the solvent viscosity.}
\]

The spheroid is assumed to be prolate with semi-axes \([a, b, c]\), with \(a > b = c\).

**A.2. Cylinder**

The rotational and translational diffusion coefficients are given by, respectively:

\[
D_R = \frac{3D_0}{\pi L^2} \left( \ln p - 0.662 + 0.917/p - 0.050/p^2 \right), \\
D_T = \left( D^\perp + 2D^\parallel \right)/3,
\]

where \(D^\perp\) and \(D^\parallel\) are the translational diffusion coefficients along the direction perpendicular and parallel to the axis of the particle, respectively:

\[
D^\perp = \frac{D_0}{4\pi} \left( \ln p + 0.839 + 0.185/p + 0.233/p^2 \right), \\
D^\parallel = \frac{D_0}{2\pi} \left( \ln p - 0.207 + 0.980/p - 0.133/p^2 \right).
\]

In the above expression \(D_0 = k_B T/\eta L, \quad p = L/\sigma, \quad \sigma \) and \(L\) are, respectively, the diameter and the length of the cylinder. All other quantities are defined as in case of the spheroid.

**Appendix B. Coherent dark field microscopy**

In this appendix we briefly discuss a dark field imaging system alternative to the incoherent dark field microscopy (IDFM) scheme discussed in the main text. The optical arrangement is schematically shown in figure B1(a), and it is very similar to the one adopted in homodyne imaging or near-field scattering set-ups [15, 51].

Sample (3) is illuminated by a highly spatially coherent beam (obtained for example by placing a pin hole (1) in the front focal plane of the condenser lens (2)) and the transmitted light is blocked by a small opaque patch placed in the back focal plane (5) of the objective lens (4). We call this configuration coherent dark field microscopy (CDFM), since the sample is ideally illuminated by a single plane wave propagating along the optical axis and the light scattered by different points of the sample always bears a well-defined relative phase. The superposition of the scattering patterns from different points of the sample thus takes place on a coherent basis, since interference effects cannot be neglected. In other terms, the image formation process is linear in the complex amplitude rather than in the intensity, as it is for IDFM [52]. As a consequence, IDFM is a linear imaging system, while CDFM is not, in that the image intensity recorded in presence of two particles is not given by the sum of the intensities associated with the two particles imaged separately. For this reason, IDFM is to be preferred for digital Fourier microscopy experiments, where a linear space-invariant imaging system turns out to beneficial [16].


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