Determination of Fluid Viscosity and Femto Newton Forces of *Leishmania amazonensis* Using Optical Tweezers

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ABSTRACT

The displacements of a polystyrene microsphere trapped by an optical tweezers (OT) can be used as a force transducer for mechanical measurements in life sciences such as the measurement of forces of living microorganisms or the viscosity of local fluids. The technique we used allowed us to measure forces on the 200 femto Newtons to 4 pico Newtons range of the protozoa Leishmania amazonensis, responsible for a serious tropical disease. These observations can be used to understand the infection mechanism and chemotaxis of these parasites. The same technique was used to measure viscosities of few microliters sample with agreement with known samples better than 5%. To calibrate the force as a function of the microsphere displacement we first dragged the microsphere in a fluid at known velcoity for a broad range of different optical and hydrodynamical parameters. The hydrodynamical model took into account the presence of two walls and the force depends on drag velocity, fluid viscosity and walls proximities, while the optical model in the geometric optics regime depends on the particle and fluid refractive indexes and laser power. To measure the high numerical (NA) aperture laser beam power after the objective we used an integration sphere to avoid the systematic errors of usual power meters for high NA beams. After this careful laser power measurement we obtained an almost 45 degrees straight line for the plot of the optical force (calculated by the particle horizontal displacement) versus hydrodynamic force (calculated by the drag velocity) under variation of all the parameters described below. This means that hydrodynamic models can be used to calibrate optical forces, as we have done for the parasite force measurement, or vice-versa, as we did for the viscosity measurements.

Keywords: optical tweezers, calibration, integrating sphere, force and viscosity

1. INTRODUCTION

Optical tweezers have become a versatile tool in investigating and manipulating microscopic particles on scales of micrometers. Typically, small particles in a liquid environment, like biological cells, or polystyrene microspheres, are trapped in the focus of a laser beam[1-3]. It has been used as a tool to manipulate biological material at cellular level, as well as to measure mechanical properties such as forces in femtoNewtons scale and stiffness or elasticity of membranes and single DNA macromolecule[4-7]. In the geometrical optics regime the optical force is generated by the momentum transfer due to refraction of the laser light at the boundaries of the particles that have a higher index of refraction than their surrounding medium and several different methods have been developed for trapping force strength calibration[8-10]. One method consists in dragging a microsphere at known velocities through a fluid with known viscosity and observing the microsphere displacement from the original position at null velocity. The optical force could then be accessed by the calculated hydrodynamical force. However, hydrodynamical forces also relies in complicated models that must take into account the presence of the walls, specially for such small dimensions as used in optical tweezers experiments where the particle is only a couple of hundreds of microns from the walls. Actually, depending on the knowledge of the parameters involved it is possible to use the method the other way around, that is, to use the optical force model to measure the hydrodynamical force. Therefore, we checked the geometrical optics force model against the hydrodynamical models in the vicinity of two parallel plane walls, obtaining, after careful measurement of the parameters involved, a plot of the optical versus hydrodynamic force as a straight line at 45 degrees. Among the parameters used by the models the laser power measurement has

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been the most difficult to obtain, because the usual power meters readings are not accurate for the very high numerical aperture of the beam after the objective. We solve this problem by the use of an integration sphere after the objective, to assure that optical rays in any angle are taken into account. The measurements have been performed under a broad variation of parameters such as fluid viscosity, refractive index, drag velocity, wall proximities and laser power. This provided us with enough confidence in both, optical and hydrodynamical models, to the extent that we can use hydrodynamic models to calibrate optical forces or vice-versa. Our experimental setup was able to measure forces up to a hundred of picoNewtons, which is particularly important when dealing with strong microorganisms such as spermatozoa, where the force can be as high as 50 pN[11]. Optical tweezers capable only to provide up to 10 picoNewtons forces could not hold them. With this calibration we observed the forces of polystyrene bead attached to the protozoa *Leishmania amazonensis*, responsible for a serious tropical disease called Leishmaniose. The forces ranged from 200 femto Newtons to 10 pico Newtons and show that Optical Tweezers can be used for infection mechanism and chemotaxis studies in parasites. The determination of a normal Leishmania force is the first step to study the mobility of the flagella, adhesion and sensibility from external environment stimulus as well as mechanisms of infection. Leishmania target cell for infection is the macrophage and each kind of Leishmania prefers different tissue macrophages. The *Leishmania amazonensis* is blander compared to other Leishmanias and only causes simple lesions on the skin[12-15]. The other application was to use the optical force to measure viscosities of few microliters sample. Our results show 5% accuracy measurements.

2. THEORY

The hydrodynamic force is not the simple Stokes Law force due to the presence of two parallel plane walls, the cover slip and the bottom of the chamber. The complete expression can be found in reference 16 that used an infinite series of images and the hydrodynamic force reads as follows, neglecting terms of order higher than $(a/l)^3$:

$$F = \frac{6\pi\eta a u}{1 - A\left(\frac{a}{l}\right) + B\left(\frac{a}{l}\right)^3 + \ldots} \quad (1)$$

Here, u and *a* are the sphere velocity and its radius, η is the fluid viscosity and *l* is the distance from the center of the sphere to the bottom of the chamber. The constants A and B are complicated numerical integrals that depend on *l* and b, where b is the distance from the center of the sphere to the cover slip that can be evaluated using packets such as *Mathematica*.

The model for the optical force in a geometrical optics approximation[1] consists in calculating the geometrical path for each ray of a conical beam incident on the sphere. Accuracy is increased by considering not only the refractions but also the reflections, as shown in Figure 1.

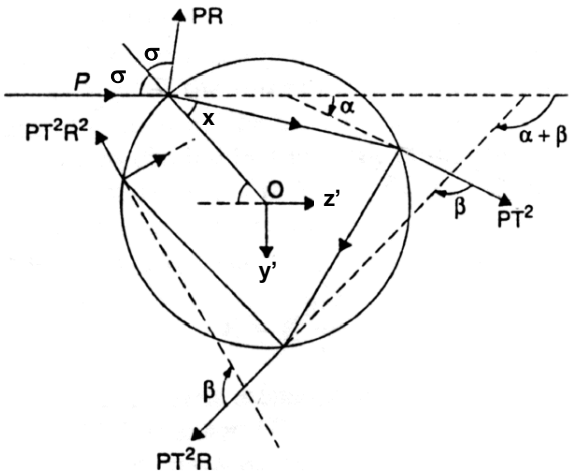

Figure 1 Path of a ray of a beam, considering refractions and reflections in the sphere. Coordinates Origin is in the center of the sphere and the z' axis is in the incident ray.

The forces for each direction are given by:

$$F_{z'} = \frac{n_1 P}{c}\left[1 - R\ cos(\pi + 2\sigma) + \sum_{n=0}^{\infty} T^2\ R^n\ cos(\alpha + n\beta)\right]$$

$$F_{y'} = \frac{n_1 P}{c}\left[-R\ sin(\pi + 2\sigma) + \sum_{n=0}^{\infty} T^2\ R^n\ sin(\alpha + n\beta)\right] \quad (2)$$

The trick to perform the sums is to change to the complex plane by the transformation $F_c = F_{z'} + i\,F_{y'}$, so the series become geometric series, and the result for F_c is:

$$F_c = \frac{n_1 P}{c}\left(1 + R\ exp(2\sigma i) - T^2\ exp(i\alpha)\left[\frac{1}{1 - Re\ xp(i\beta)}\right]\right) \quad (3)$$

Where $\alpha = 2(\sigma - x)$, $\beta = (\pi - 2x)$ and $\sin x = n \sin\sigma$. The variable σ is the incident angle, $n = n_1/n_2$ is the relative refraction index between the fluid (n_1) and the sphere (n_2), c is the light velocity and P is the laser power. The variables $R = (\tan(\sigma - x)/\tan(\sigma + x))^2$ and $T = 1 - R$ are the reflectance and transmittance, respectively, for a linear polarized ray. This expression depends on the value of the incidence angle σ and the relative refractive index.

The incidence angle varies for each ray so it is necessary to changed the origin of the coordinates to a fix position which we choose as the focus of the beam as shown in Figure 2. By written the incidence angle σ as function of the angle γ and the vectors δ and r, the final expression of the force will depend only on the displacement r vector, connecting the beam focus to the center of the sphere, the convergence angle of the beam (numerical aperture), the sphere radius, beam power (supposed to be equal for each ray) and the relative refraction index, which are all measurable or known parameters. Using cosine law for vector, this transformation is given by: $\sigma = arccos(1 + d^2 - (r/a)^2/2d)$, where $d = ||\vec{\delta}||$ and the displacement $\vec{r} = (r\ sin\gamma,\ 0,\ r\ cos\gamma)$.

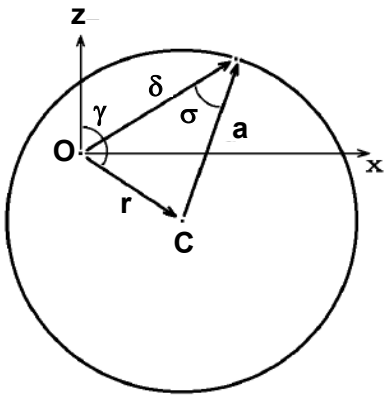

Figure 2. Change in the coordinates origin and definition of the angle and vectors used.

The force for the whole conic beam is obtained by integration $F = \int \vec{F}\, dA \Big/ \int dA$, where the area element is given by $dA = sin\theta\ cos\theta\ d\theta\ d\varphi$ obtained using the Abbe sine condition, the angle θ varies from $0 \le \theta \le \theta_{max}$ the maximum-convergence-objective-angle, the azimuthal angle varies from $0 \le \varphi \le 2\pi$. Both, the expression for the hydrodynamic and optical force were numerically obtained by using the software *Mathematica* (Wolfram Research).

3. EXPERIMENT

Our optical tweezers consisted of a Nd:YAG laser (model 3800S, Spectra Physics Lasers) focused through a 100× oil immersion objective (SPlan) of an Olympus microscope (BH2, Olympus Optical CO., Ltd.), as shown in figure 3, after passing by a telescope used to capture the particle in the focal plane of the microscope. The images of the microspheres were registered using a camera (TK1085-U, JVC – Victor Company of Japan, Ltd.) connected to the optical tweezers microscope. The velocity and movement of the microspheres and cells were controlled by a translation stage, Prior Scientific, coupled to the microscope.

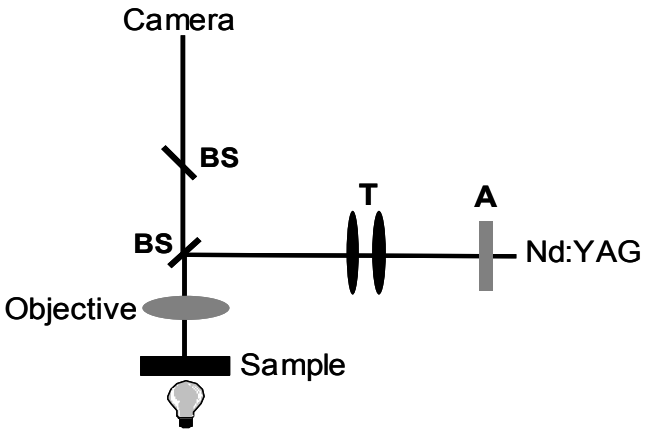

Figure 3. Setup of the Optical Tweezers, BS is a beam splitter, T is the telescope and A is an attenuator.

We experimentally checked our models results using a 9 μm diameter polystyrene spheres (Polysciences). At null velocity there are no optical or hydrodynamic forces actuating on the sphere, except for a small force due to the reflections, which do not change its lateral position, so the centre of the sphere coincides with the laser focus. On the other hand, at a constant velocity, the optical force will equilibrate the hydrodynamic one through a change in the centre position of the sphere that is no more in the beam focus. This displacement can be easily measured by superimposing the images before and after the dragging and measuring the distance between the centers as shown in figure 4. The movement of the microspheres was recorded in tapes, then digitalized using a video capture card and the displacement was measured with the software *Image-Pro Plus* (Media Cybernetics). Because the movement is in the x direction we assume the γ angle to be 90 degrees. The depth l of 15μm, 25μm, 50μm, 75μm and 85 μm were chosen and measured using the microscope micrometer, while b is obtained by difference with the total depth of 100 μm of our Neubauer chamber. A microcomputer controls translation stage drag velocities that we set to 150μm/s, 200μm/s, 250μm/s, 300μm/s and 350μm/s. The optical power was measured after the microscope objective using a spectralon integrating sphere (Labsphere). The fluid refraction indexes and viscosities were varied together by using solutions of water and sugar at 14%, 20% and 28% concentrations. We checked the handbook values of the solution with an Ostwald viscometer and an Abbe Refractometer.

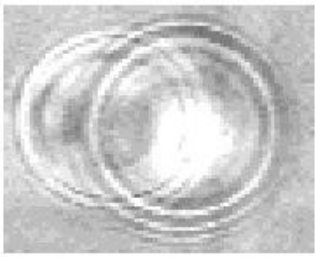

Figure 4. Experimental displacement of a sphere at null and constant velocities.

4. RESULTS AND DISCUSSION

With this experimental scheme we have been able to change, for a given optical power the following parameters: the dragging velocity, the depth *l*, the viscosity η and the relative refractive index n. Knowing all the parameters we were able to calculate both, the hydrodynamic and the optical forces, and we could compare both forces for a large range of different parameters. Figure 5 shows a plot of the calculated optical vs. hydrodynamic forces for more than 30 points. The slope of the straight line corresponds to 47 degrees, very close to the expected 45 degrees and the R^2 value is higher than 0.9. This small difference can easily be explained by systematic error of the polystyrene sphere refractive index provided by the manufacturer. This plot shows the ability of our procedure to measure forces in the hundreds of picoNewtons scale.

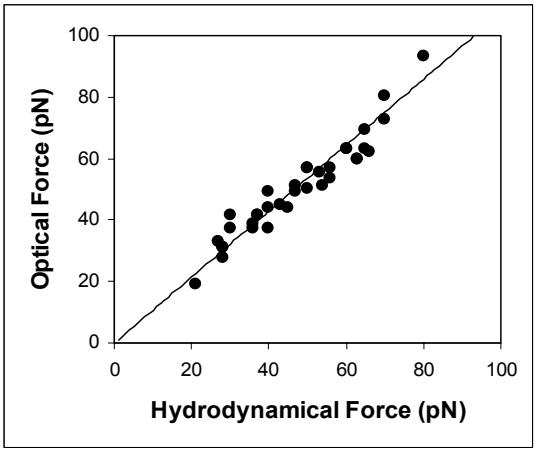

Figure 5. Plot of the Optical versus Hydrodynamic force for a wide range of the parameters.

We also dragged the spheres at the same velocities in a solution of 20% and 32% glycerol in water to measure its viscosity, as shown in Table 1 and compared our result with the Handbook of Chemistry and Physics[17] values. These values confirm again the precision of our models. We only needed 10 μl of the fluid to measure these viscosities using optical tweezers, showing the ability of this technique to measure local viscosity of very small amounts of biological fluids.

Table 1. Fluid viscosities tabled in the Handbook of Chemistry and Physics and measured by optical tweezers.

	20%	32%
Handbook's values	1.73cP	2.63cP
Our values	1.83cP ±5%	2.76cP±5%

After these consistent results we applied the technique to study the movements and forces of the *Leishmania amazonensis*. Our results show that our system is able to measure the force of protozoa in the femtoNewtons scale. The promastigotas were collected from skin lesions of rats Balb/c and cultivated in RPMI (Sigma) complemented with anti-biotic, glutamine and serum fetal bovine (10% - Cultilab) in temperature of 26°C

in a stove. The proliferation and growing of the cultures were followed by counts in Neubauer chamber. We diluted a concentration of 10^6 protozoa in the culture medium until we could capture a single Leishmania without interference of the movement of the others. We also added some microspheres to the diluted protozoa solution. The optical tweezers captured a single sphere that was adhered to the Leishmania by simple contact. Then we decreased the power of the laser using an attenuator until the Leishmania was able to move the sphere horizontally. We observed that the 4.5 μm and 6 μm spheres (Polysciences) sizes were the better sizes because they were easily displaced by the Leishmania and this displacement was easily quantified by our system. Knowing the power, the displacement, the sphere and culture medium refraction indexes we calculated the forces of the normal *Leishmania amazonensis*. Our results show that these forces are between 0.19 – 3.6 pN, with an average of 1.49 pN. We measured the forces of approximated 30 protozoa (figure 6). Usually, the Leishmania moves with its flagella ahead, but we verified that captured Leishmania tends to move with its flagella to back. One important point in our measurement as that there is an instantaneous equilibrium between the optical and hydrodynamic forces. This means that we do not consider the inertia, kinetic energy, which is true in a low Reynolds number regime habitated by microorganisms[18].

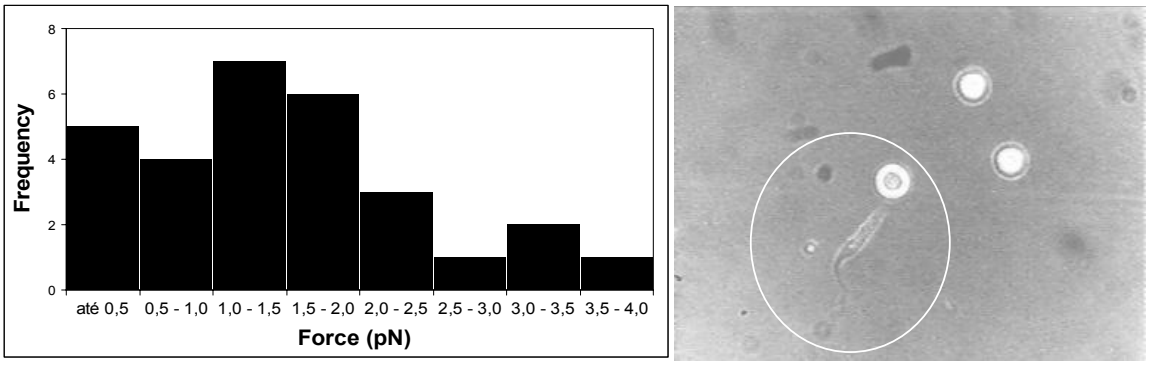

Figure 6. a) Leishmania coupled to a sphere captured by an optical tweezers. b) Distribution of the intensities of the forces of the *Leishmania amazonensis*.

Its important to estimated the influence of Brownian motion on the captured microsphere for such small forces as 200 fN. Considering the optical tweezers as a harmonic oscillator. Then,

$$\left\langle \frac{K\delta x^2}{2} \right\rangle = \frac{k_B T}{2}, \quad \delta x = \sqrt{\frac{k_B T}{K}} \quad (4)$$

where the forces due the Brownian motion are $F(flut) = K\ \delta x = \sqrt{K k_B T}$, K is the spring constant, T is the temperature, estimated as 25°C, k_B is the Boltzmann constant and δx the displacement caused by the Brownian motion. For this measurement the spring constant K is in the scale of 10^{-7} N/m, then $F(flut) \approx 0.02\,pN$. Because of that, we can omitted corrections caused by the Brownian motion in the value of the forces obtained since them are at least ten times bigger than $F(flut)$.

5. CONCLUSION

In conclusion we have shown that we can have an accurate calibration of the optical tweezers using a geometric optics and hydrodynamic models. We also show the importance of the use of integrating sphere to measure the laser power of a convergent beam. Our system was able to measure viscosities from very small quantities of fluid, has sensitiveness to observe forces in the femto Newtons scale and potential to study the movement, chemotaxis and infection process of protozoa like *Leishmania amazonensis.*

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