Master in Photonics

MASTER THESIS WORK

HIGHLY STABLE HOLOGRAPHIC DUMBBELL COMBINED WITH ACCURATE FORCE MEASUREMENTS THROUGH BACK-FOCAL-PLANE INTERFEROMETRY

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Highly stable holographic dumbbell combined with accurate force measurements through back-focal-plane interferometry

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**Abstract.** Optical trapping experiments with multiple traps have been used to measure small net movements on the nanometer range, but at present more stability is demanded to measure even smaller scales which are typical of many biological motions. The main problem of these systems is that the degree traps are affected by perturbations can vary from one trap to the other due to differences in the optical path they follow. In holographic optical tweezers the traps share the same optical path and hence stability should be enhanced. However, holographic systems are weak regarding force measurement when multiple traps are used. Here, we test a method that allows measuring forces through back-focal-plane interferometry when multiple holographic traps are in use, and compare the stability of dumbbells created by a holographic system or by a two split beams configuration, which is currently the standard setup in experiments in which precision is a concern.

**Keywords:** Optical tweezers, holography, dumbbell, back-focal-plane interferometry.

1. **Introduction**

Over the last five centuries, humankind has used light as a tool to observe and study the microscopic world. During these years, microscopy has been extremely enhanced, nevertheless interaction with this world has always been limited. It was not until last decades that light could be used as a tool not just for imaging but also for manipulating particles. The invention relies on the fact that light carries linear and angular momentum that can be transmitted to the particles light interacts with and, in consequence, forces over these particles can be exerted. This phenomenon was first observed by Arthur Ashkin [1] in 1969, who was able to push and trap microspheres taking advantage of radiation pressure. However, to be able to confine particles another force was required in order to counteract radiation pressure, either gravity or the radiation pressure exerted by another contra-propagating beam. The same Arthur Ashkin developed this idea and he managed six years later to confine dielectric particles through a single sharpened beam [2]. This last invention is called optical tweezers. Optical tweezers is a tool capable of grabbing, holding and manipulating samples in the size range from tens of nanometres up to tens of micrometres [24-25].

Furthermore, optical tweezers can be calibrated and therefore forces exerted over trapped particles can be measured. Different methods of calibration for optical traps have been developed since its invention [3-7], but the most accurate one is the study of the remaining
Brownian motion of the bead, which also allows measuring forces dynamically. By means of a Fourier transformation of the bead's positions in the different time steps the power spectrum is found [7, 8]. The power spectrum of a trapped bead represents the amount of energy the bead carries for each frequency. Its shape follows a Lorentzian function that can be fitted and from its corner frequency the stiffness of the trap can be deduced [7, 8].

Available methods for studying Brownian motion are video-based methods [9, 10, 22, 35] or detection of the bead's position using a photodiode [33]. The maximum frequency that can be represented in the power spectrum, the so-called Nyquist frequency, is half of the sampling frequency [8]. Due to this maximum frequency, when calibrating a trap by means of the power spectrum using video-based methods a fast CCD camera is required. Fast CCD cameras are much more expensive and its acquisition frequencies are much slower than photodiodes. Another possibility is to find the potential of the trap from the bead's position in time [35] and from here deduce its stiffness. This could be achieved using a normal CCD camera, but the resolution of the tracking is not precise enough to find an accurate potential; even so, it could be still useful to get an easy estimation of traps stiffness [36]. On the other hand, video-based methods allow tracking more than one particle and determine its position at the same time step. 

When a photodiode is used to determine positions in time, a smart configuration is imaging on the photodiode the back-focal-plane (BFP) of a high numerical aperture lens placed as a condenser. This configuration allows back-focal-plane interferometry (BFPI) [11-15] performance. In the BFP of the lens a light pattern arises from the interference between the Gaussian wave of the laser and the spherical wave reemitted by the trapped bead. This interference pattern contains information related only to the relative positions between the bead and the trap, so that any movement of the trap within the sample does not affect calibration.

On the other hand, manipulation and creation of multiple optical tweezers can be achieved using different methods. The simplest approach would be the combination of two different laser beams or the splitting of one single beam in two different orthogonal polarizations in order to set two optical traps at the sample plane. However, these are restricted to only two traps. Another possibility for the generation of more than two traps are the methods based on time sharing [16], that can be implemented using acousto-optic deflectors, galvanometer mirrors or electro-optic deflectors. They consist in switching the trapping beam into different positions fast enough to trap at different points. Since the traps are flicking at a determinate frequency, the information collected by a photodetector from the BFP can be synchronized and thus forces exerted by the different traps can be isolated and measured. Nevertheless, manipulation through time sharing is restricted to 2D. Their direct competitors are holographic optical tweezers (HOT) [17]. HOT take advantage of digital holography incorporating a spatial light modulator (SLM) to have the desired light distribution on the sample plane [31]. HOT offer a better manipulation capacity than time-sharing methods since they are capable of introducing displacements in the axial direction [18-20] and they also allow to create optical vortices [21]. However, HOT are not able to measure forces through BFPI when multiple traps are used since all traps produced by the holographic system are permanent and indistinguishable. Hence, the interference pattern created at the BFP contains mixed information related to the different traps of the system, which cannot be separated. To overcome this problem calibration of multiple HOT has been performed by means of high-speed cameras [22, 35], though it is limited due to its lower acquisition frequency.

We present a method [23] that allows HOT to be combined with precise force measurement provided by BFPI. The method introduces an optical non-holographic trap, which is orthogonally polarized with respect to holographic traps. This additional trap is created by changing the conventional angle of the linear polarized light before being reflected on the SLM. This way, part of the light is normally modulated and follows the spatial distribution of light assigned by the hologram, leading to the creation of the desired pattern of holographic traps. The remaining light has an orthogonal polarization and is not affected by the SLM, so it is just
reflected creating a permanent trap at the centre after being focused by the microscope objective. Both types of traps are created from the same laser source and share the same path, and therefore they compose a highly stable system. They also have perpendicular polarizations, and thus positioning a polarizer before the photodiode measuring the BFP pattern allows an easy separation of light from the non-holographic trap and from the remaining holographic traps. Hence, analysis of the non-holographic trap through BFPI can be performed, allowing precise force measurements over one trap while using multiple holographic traps for dynamic manipulation of the sample. Measuring forces over a single particle is sufficient for a great amount of experiments, for instance, those related to the study of molecular motors [26-29] and single molecule research [32].

In optical trapping experiments the main problem to resolve net motion below of 1nm is the noise introduced by Brownian motion and mechanic drift [30]. Brownian motion noise, since it has a zero mean, can be easily reduced by making an average in time. However, mechanical drift is induced by perturbations over the assembly such as temperature changes or air currents. These perturbations affect to the different parts of the setup in a non-uniform way and cannot be removed by averaging.

At present, optical trapping experiments where high accuracy is demanded are mainly performed either by means of two traps split from the same beam, time-shared methods or with a single trap while the other extreme of the sample is anchored to the cover slip or it is held by a pipette. In the latter case, noise and mechanical drift affect totally different to both extremes as we will show below, making measurements certainly rough. In the first case, since samples are in suspension and held by multiple traps, drift and noise in the different traps are comparable making the system more precise. Nevertheless, beams follow different paths and hence perturbations do not affect equally to all traps. Because of this reason in [37] the optical differential path is extremely reduced to enhance the stability of the system. Finally, time-shared methods have a greater spatial stability, however their degrees of freedom for manipulation are restricted and since traps are not permanent perturbations affect slightly to the different traps. HOT introduce a better approach in this kind of experiments since all traps follow the same path and therefore discrepancies between them are minimized. For this reason, there is an increase interest in studying the performance of holographic optical tweezers in experiments where high accuracy is required. In [34] instability of HOT due to hologram fluctuations introduced by the SLM is analysed, showing that the pointing stability is comparable to that of non-holographic tweezers. Here, we complement that study by analyzing the mechanical stability of a dumbbell composed by a holographic and a non-holographic trap, generated following the method described above [23]. A HOT system was build implementing the necessary elements for the correct performance of the method. The setup was also adapted to be able of splitting the beam in two with orthogonal polarizations for a quantitative comparison of stability between both assemblies.

2. Instrument design

2.1 HOT system

For the construction of the HOT system a commercial inverted microscope (Nikon TE2000-E) was used. The microscope incorporates two different epifluorescence ports. The upper port is used to introduce the laser beam into the objective while the other port can be employed for normal epifluorescence performance. The laser beam is generated by an Ytterbium fibre laser source (IPG, mod: YLM-5-1064-LP). The laser radiation is linearly polarized and it has a wavelength of 1064 nm, which is extremely useful for the correct manipulation of biological samples [39]. The laser power can be shifted on the source command and in a range from 0 to 5 W. Nevertheless, when power is changed, the laser needs some minutes to be stabilized. For this reason, an external intensity control was implemented, composed by a half-wave plate, a polarizing beam-splitter and a beam-blocker. Since the laser source is linearly polarized, the
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polarization angle can be set with a half-wave plate. This allows controlling the intensity of light transmitted by the polarizing beam-splitter. After the beam-splitter, another half-wave plate is placed in order to control the incident polarization angle on the SLM.

Before being reflected on the SLM, the beam is expanded with a Keplerian telescope (L1, L2, see figure 1(a)) with the purpose of filling the whole modulator's screen (16x12mm). Then, the beam is reflected on a mirror and directed towards the SLM. The SLM (Hamamatsu LCoS - SLM X10468-03) reflects the beam pointing to the back aperture of the upper epifluorescence port. Finally, the beam is reduced again with a second telescope to fit the entrance pupil of the objective. This second telescope is composed by a lens situated before the back aperture of the epifluorescence port (L3) and by a second lens placed in a stage for an epifluorescence filter inside the microscope (L4). The same stage contains a dichroic mirror that reflects the infrared laser light to the oil-immersion objective. This objective (Nikon 100x, 1.30 N.A, 0.2mm working distance) is used both to focus the beam onto the sample and also to form the image of the sample, which passes through the dichroic mirror and ends onto one of the imaging ports. The desired port can be selected by an external display. A CCD camera (Qimaging, QCAM 12-bit Mono Fast 1394 Cooled, mod: QIC-F-M-12-C) and a high-speed CCD camera (Andor Technology, iXon mod: Du-860D-CSO-#BV) were placed on the ports. All the assembly was supported on an optical table isolator (Thorlabs, PTS502).

![Figure 1. (a) General sketch of the optical instrument, both the HOT setup (left) and the dual beam setup (in brackets). (b) 3D modelling of the force measurement system. (c) 3D modelling of the HOT setup outside the microscope.](image)

During the construction of the HOT system, to assure proper alignment the path of the beam was visualized using an external CCD camera (Ikegami, ICD-47E) and the different elements were placed checking the shape of the reflection of the traps on a cover slip and achieving a symmetric intensity distribution under displacements of the focus.

2.2 Force measurement system and calibration

For the correct performance of BFPI the condenser of the microscope was removed and replaced by a high numerical aperture oil-immersion lens (1.4 N.A, 10.5mm focal distance). A dichroic mirror was placed after this lens to reflect the beam onto the QPD (Thorlabs, mod: PDQ80A) while light from the illumination system was transmitted through. To image the BFPI pattern created by the lens onto the QPD an auxiliary lens is needed, with the proper demagnification to fit the pattern onto the QPD. In between, a polarizer was added to analyse light with the desired polarization and discard unwanted information, as described in the introduction. Also, different filters were set to have the right intensity of light onto the QPD to
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make it work under 1mw. Over this power the QPD behaves extremely non-linearly under a displacement of the pattern [8]. The force measurement system is shown in figure 1(b).

Once the system was built, the traps were checked by calibrating their stiffness in function of power. Forces that appear on the bead depend linearly on the number of photons that reach the particle. Since laser power is linearly related to the number of photons, force should increase linearly with power and hence stiffness as well, as we show in figure 2.

![Figure 2](image)

Figure 2. (a) Power spectral densities of a trapped bead in the non-holographic trap obtained at the different powers displayed in the legend. (b) Relation between the corner frequencies obtained from the fitting of the power spectra shown in (a) and the sum channel of the QPD (proportional to the laser power).

The fitting of the power spectra was realized by means of the free distributed code [44] in MatLab® [40] and described in [45]. This software is very suitable since it takes into account various effects such as hydrodynamics, aliasing, cross-talk and the characterization of the photodetector as a filter.

After this test, the force measurement system was used to check the method described in [23]. The essay was performed setting a holographic and a non-holographic trap separated 10um from each other. The polarizer was rotated to select a certain polarization and different configurations of trapped beads were analyzed.

![Figure 3](image)

Figure 3. (a) Power spectra obtained from different configurations explained in the text. (b) Trapped beads distribution.

In figure 3(a) we see four different power spectra (acquired at 15kHz) corresponding to different configurations of trapped beads. In three of them only light from the non-modulated trap is allowed to pass through the analysing polarizer. The fourth case (green) shows the power spectral density obtained when both traps are trapping and light from both beads reaches the QPD. Regarding the cases where only non-modulated light is selected and analysed, the first configuration (black line) shows the power spectrum achieved when only the non-holographic trap is trapping. The red line shows the power spectrum obtained when both traps are trapping. Finally, the blue line presents the power spectrum when only the holographic trap holds a bead;
we see that, even if there is a trapped bead, the corresponding power spectrum does not follow a Lorentzian function. This is the type of power spectrum obtained when only light from the laser is collected and it does not interfere with the spherical wave reemitted by a particle. Concerning the remaining cases, either if just the non-holographic trap holds a bead or both traps are trapping, the same power spectrum is found. From here we can deduce that this power spectrum is only related to the non-modulated trapped bead. Now, if we take a look at the green line we can see the power spectrum when the polarizer allows both polarizations to pass through. In this case, the power spectrum does not exactly fit a Lorentzian function and it is different from the previous cases, as information for both trapped beads is mixed together.

This procedure has been repeated for the same configurations of trapped beads but selecting only the holographic light component. The same results and conclusions were obtained.

2.3 Non-holographic split optical tweezers setup

With the aim of comparing the instabilities of the system, the setup was adapted to include an independent trap split from the original beam. This beam follows a different path, which was reduced at the minimum distance allowed by the disposition of the different elements (around 30 cm). This modification is shown in figure 1(a) right. The first beam-splitter of the modified assembly does not separate the light by polarization. Due to this, along the alternative path another half-wave plate is located to rotate the polarization of the beam 90 degrees. After that the beam is reflected on two mirrors and coupled again with a polarizing beam splitter. The second beam splitter is set on a micrometric stage with three degrees of freedom that allow making displacements of the trap location in the sample plane.

3. Stability analysis methodology

Here we explain the methodology used to check the stability of the holographic dumbbell. During the performance of the essays several procedures have been tested and modified to get an optimal execution of the experiments. The essays were realized by trapping beads and recoding its positions with a CCD camera. The positions of the beads were tracked using the free software Video Spot Tracker [41]. The recollected data was treated with self-made software realized in MatLab®. Alternatively, the same procedure was performed by tracking the reflections of the traps on the cover slip. However, the tracking was not representative of the movement of the traps due to irregular variations in intensity and shape of the reflected beams.

The beads used were melamine resin microspheres (Fluka, 2.19 um) contained in a solution of distilled water and enclosed in a micro-chamber. The samples were dissolved trying to have as few beads as possible. This not just decreased the interruption of the experiments due to involuntary simultaneous trapping of beads in a single trap, but also reduced the presence of microspheres at planes different from that of the trapped samples, which could affect the measures (even if not detected by eye in the images taken by the camera). When beads are introduced in trapping experiments Brownian motion noise is introduced as well. Since Brownian motion of a bead has a zero mean [30], the noise can be reduced by averaging its positions in time. For this reason, positions of the beads were averaged at each second. In order to average more data points and thus reduce Brownian noise even more, a high-speed CCD camera was used. Although the acquisition was faster, the resolution of this camera was lower than the regular CCD camera and therefore the analysis of the data was not improved at normal acquisition rates. Nevertheless, we could optimize the performance of the fast camera to reach acquisition rates higher than 2kHz. This was done by reducing the active area of the CCD chip. Each pixel of the CCD chip collects information and then it is transmitted pixel by pixel until all data is recollected in a line. The information transmitted pixel by pixel always follows the same direction. In order to increase the acquisition rate of the camera, the active area of the CCD chip has to be reduced in the same direction in which information is addressed. In our case, both cameras were aligned to have the same axis of the sample stage. In consequence, the active area
of the CCD was reduced in the y-direction while reductions in the x-component did not affect speed acquirement. Therefore, to have the minimum active area in the y-direction, the hologram displayed on the SLM screen was changed in order to have both beads trapped in the same y-coordinate and separated 10um in the x-component. The holograms were created using self-made software in LabVIEW® [43], which allowed an interactive control of the trap distribution.

The essays were carried out during approximately 70 seconds. The acquisition time was not rigorously controlled since the fixed parameter was the number of acquired frames and the frame rate could slightly differ in the different essays. Since the recording rate was considerably increased, the software delivered by the camera was not able to record that amount of data. Due to this, RAM memories of the computer had to be increased and data had to be stored in a specific way. The information was saved in three different consecutive files that had to be converted to video format by means of an external program (ImageJ [42]).

The beads were trapped 10um away from the surface of the cover slip to avoid viscosity issues [38] and not much deeper to minimize spherical aberration introduced by oil-immersion objectives [46]. Intensity of light for each polarization was adjusted in order to have traps with similar stiffness. Stiffness differences between traps led to discrepancies in the relative positions of the beads due to irregularities in the amplitude of the oscillations. Also, maximum power of the laser was supplied to have less Brownian motion.

The motors of the cooling system of the cameras were switched off during the acquisition time. These motors were a noise source that affected the movement of the sample and mainly in the same axis the motor was oriented. The experiments were realized at room temperature but the essays were also repeated with an induced thermal drift. Drift is mainly related to temperature fluctuations and loud sounds [14]. Because of this reason the effects of thermal fluctuations were magnified using a heater in such a way that the response of the system could be evaluated. In [14], the system had a temperature control in order to avoid these effects and the room was isolated achieving a noise criterion 30 (NC30) sound level. In our laboratory, the sound level was measured while the essays were developed and the reading was of 68dB in normal conditions, mainly due to the laser source.

4. Results and discussion

Before presenting the main results of stability of the two systems explained above we present experimental evidences of the instability when only one optical trap is used while a pipette holds the other extreme of the sample. This pipette in the best case is anchored somehow to the sample stage. Hence, here we determine the movement the sample stage is affected by:

![Figure 4](image.png)

Figure 4. Tracking of a stack bead on the lower cover slip of a micro-chamber. The acquisition was performed at 2kHz and data averaged at each second.

Figure 4 shows the tracking of a bead that has been stack to the lower cover slip of the micro-chamber. Once the bead is fixed, its movement is representative of the drift of the sample plane, which is mainly related to displacements of the sample stage and is totally independent of the drift affecting the optical traps. This mechanical drift is also essentially related to temperature
fluctuations and although it affects both extremes of the sample, its effect is not comparable since one of the extremes is suspended in a fluid. The perturbations noticed by the suspended bead are the drag forces exerted over that particle due to the remaining amplitude of the movement the fluid has at the trapping depth. However, the other extreme is completely affected by the drift of the sample stage. Furthermore, the trapped bead will be affected by the drift introduced by the trap as well, while the stack bead will be not. In this way, relative distances between sample's extremes are not constant due to these uncontrolled displacements. This situation is totally representative of the trapping experiments realized using a pipette. In [30] this effect is reduced by replacing several parts of the microscope, but the remaining drift is still an important error source.

When experiments are performed with two suspended beads held by optical traps, the so-called dumbbell configuration, stability is highly increased. This is due to both traps are affected by the same type of drift, which is somehow comparable. Nevertheless, differences in the optical path followed by the beams make drift in the different traps independent and hence relative distances between traps vary in time.

![Figure 5](image1.png)

**Figure 5.** Tracking of the trapped beads (red and green) in the dumbbell composed by two independent beams and relative distance between both beads (blue). The acquisition was performed at 2kHz and averaged at each second. All the data has been centred in reference to its mean value. (a) x-channel distribution. (b) y-channel distribution.

We can see in figure 5 that the movement followed by the two beads is correlated, however the amplitude of the displacements is not equal and hence the relative distance is affected. Nevertheless this configuration constitutes a higher stable system than the previous one just explained above. In [37] this differential path was reduced to avoid this kind of instabilities, however it exists a physical limitation in its construction that will always affect its performance. By using the holographic system, the differential path is annulated and therefore stability is improved as shown in figure 6.

![Figure 6](image2.png)

**Figure 6.** Tracking of the trapped beads (red and green) in the dumbbell composed by the holographic system and relative distance between both beads (blue). The acquisition was performed at 2kHz and averaged at each second. All the data has been centred in reference to its mean value. (a) x-channel distribution. (b) y-channel distribution.
Figure 6 shows the same experiment and with the same conditions as shown for the dual beam optical trapping experiment but with the HOT system. We can observe stability is extremely enhanced. Also, one could notice the stability of one axis is much better than the other, a fact which also could be observed for the dual beam configuration. This is due to an undetermined noise source that came out along the course of the experiments. In figure 7 we show some results obtained before this noise source appeared. In this case, an induced thermal drift was exerted and the fan of the cooling system of the fast camera was operative. This fan generates an oscillation along x-axis, following the orientation of the fan. We can see this effect in the graph of figure 7, in which the relative distances between both extremes are mainly affected in the x-component. It can also be observed that the y-component is much more stable than in the experiment shown in figure 8(b).

In all the cases, the induced thermal drift mainly affected to the y-component of the traps distribution. This was due to the mechanical composition of the different optical elements. However, the effects of the induced thermal drift were not well controlled. Although the position, orientation and exposure time were intended to be the same, it was impossible to have the same drift results. The method was still useful to magnify the displacements of the traps. In figure 7(b) a thermal drift of approximately 35 nm affecting both traps is shown, while the relative distance between the traps remains constant.

5. Conclusion

In this work we have shown that the stability of the dumbbell configuration has been further increased by using the holographic system, compared to the dumbbell created by two orthogonally-polarized split beams. Although an undetermined noise source appeared, the results show that the stability of both channels in a holographic dumbbell configuration is below 0.5 nm (standard deviation). Probably, solidness of the system goes beyond this limit, however the measurements are still being affected by residiary Brownian noise, stiffness differences and the resolution of the tracking. In any case, these discrepancies are due to the differential path followed by the beams, which was the main instability source of the previous system. Furthermore, even in the case where the measurements in the y-channel are affected by the unclear noise, since the experiment was oriented in the x-axis the relative distance between the traps was mainly affected by the stability along the x-axis, obtaining a variation in absolute distance of 0.36 nm (standard deviation). This fact is really interesting since the orientation of the experiment could always be set in the direction where discrepancies are lower. It has also been shown that perturbations in the different axes are strongly determined by the assembly's geometry. This geometry can be modified in order to achieve one axis with the highest accuracy.
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possible and experiments could be performed along this axis, thus precision of measurements could be further enhanced.

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