D1.1: Demonstration of TIRF based optical tweezers system suitable for cell mechanics investigations

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Lead Beneficiary:	UNIVDUN		
Author(s):	Valentina Ferro and David McGloin		
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1 Introduction

Optical tweezers represent a powerful tool in the study of cell biology. One class of techniques makes use of them as a localised probe used to exert forces on a biological sample. The reaction of the sample is usually recorded using bright field or fluorescence microscopy. However, these standard techniques often lack of the resolution required to explore the phenomena occurring inside cells, especially in the axial direction. Modern high-resolution microscopy techniques represent a more complete approach to the study of the field. Combining optical manipulation techniques with such imaging would therefore appear to be advantageous for future imaging of mechanosensing and mechanotransduction studies.

One such high-resolution technique that has proved very successful, while being relatively simple to implement is Total Internal Reflection Fluorescence Microscopy (TIRFM). The high resolution is obtained by confining the excitation region in the sample to a thin axial plane, allowing just few fluorophores to emit and to be imaged. The excitation region is illuminated by the evanescent field created at the sample surface when total internal reflection occurs, making use of a powerful high numerical aperture microscope lens.

Here we demonstrate the first step along the path to probe cellular response to mechanical stimuli making use of high strength optical tweezers probes. Our system demonstrates the integration of an optical tweezers with a TRIF microscope, using a technique that is suitable for integration into commercially assembled systems. We refer to this as “TIRPh” (Total Internal Reflection fluorescence microscopy with integrated PHotonic tweezers). We demonstrate the feasibility of the system, exploring both advantages and disadvantages of such an integrated tool. After a brief introduction on the basic concept about optical tweezers and about TIRF microscopy, we discuss the implementation carried out in the laboratory, together with the experiments used to characterize the system and some preliminary results. In the discussion section, we examine possible future developments for the system.

1.1 Principles of optical tweezers

The general principle behind optical trapping is the transfer of momentum from an optical field, typically in the form of a laser beam, to a micron-sized particle such as a dielectric bead or a cell. For convenience, the force generated by the interaction between the field and the particle can be described separating the trapping force in two components. The first,

called scattering force, pushes the bead away from the laser: the incident light is scattered by the micro-sphere resulting in a net momentum transfer in the direction of the beam propagation. For the microsphere to be trapped, we need the second force component, called the gradient force, that pulls the bead toward the laser, to at least balance the scattering force. The laser induces dipoles in the microsphere; when it presents a steep intensity gradient; those dipoles, immersed in an inhomogeneous field, experience a force in the direction of the gradient.

For optical trapping, the gradient force must exceed the scattering force; focusing a Gaussian shaped laser through a high numerical aperture (NA) microscope objective creates the required steep gradient to trap a bead close to the focal point.

1.2 Principles of TIRF microscopy

As previously stated, TIRF microscopy is based on the presence of an evanescent field when a laser is totally internally reflected at a boundary. A critical angle exists above which any reflected light is no longer transmitted, but it is totally reflected. Although no bulk transmission occurs, an exponentially decaying transmitted field occurs at the boundary. The extent of this field is only few hundreds of nanometres, with intensity decaying exponentially according to eq. (1), where I(z) is the intensity at different height, I(0) is the intensity at the interface and d varies with the incident angle (θi) and the wavelength of the used light in vacuum (λ0) (as in (2)).

$$I(z) = I(0)e^{\frac{-z}{d}} \tag{1}$$

$$d = \frac{\lambda_0}{4\pi}\sqrt{n_1^2 \sin^2\theta_i - n_2^2} \tag{2}$$

If a biological sample that has been tagged with fluorophores is illuminated by such a field, only the fluorophores in the highly localised evanescent field region will be excited and imaged. This pre-selection of the excited region results in an increased resolution than normal epi-fluorescence microscopy. In epi-fluorescence microscopy, not only the light from the focal point is collected, but also all the light coming from above and below that region with the result of a much blurred image.

Many ways exist to implement TIRF microscopy, but all can be grouped in two families according to the approach used:

1. Prism based approach: a prism is used to set the incident angle of incidence of a laser beam on top of a coverslip sample. The sample is then imaged from below using a normal MO.
2. High NA approach (see Figure 1): a high numerical aperture microscope objective is used to produce the evanescent field. Focussing a laser near the edge of the back aperture of a MO results in a collimated beam coming out from the front aperture at an angle great enough to obtain total internal fluorescence between the coverslip and the sample chamber. The higher the NA, the higher the range of angles that can be used for the TIRF.

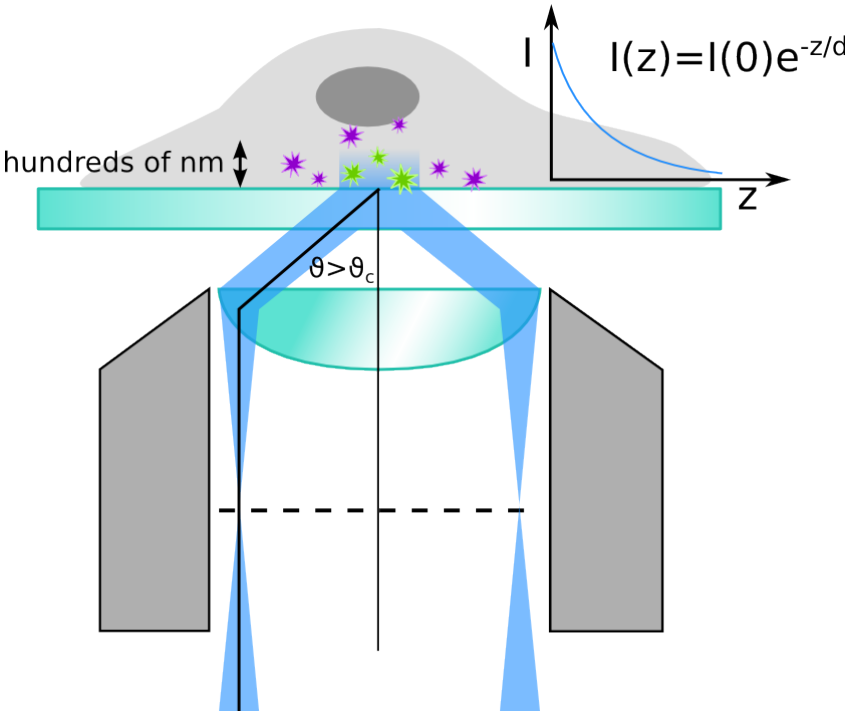

Figure 1: Microscope Objective based TIRF - *scheme of the principle of TIRF. An exciting laser is focused on one side of the back focal plane of the microscope objective (MO). At the exit from the MO, the beam is collimated and if the numerical aperture of the MO is high enough, it will be incident on the biological sample at an angle greater than the critical one, creating a thin evanescent field to excite the fluorophores on the first few hundreds nanometres.*

Because in the second approach, the TIRF MO has a high NA, it can be used to focus a beam for optical tweezers.

2 The TIR-PH system

The experimental set-up used is illustrated in Figure 2.

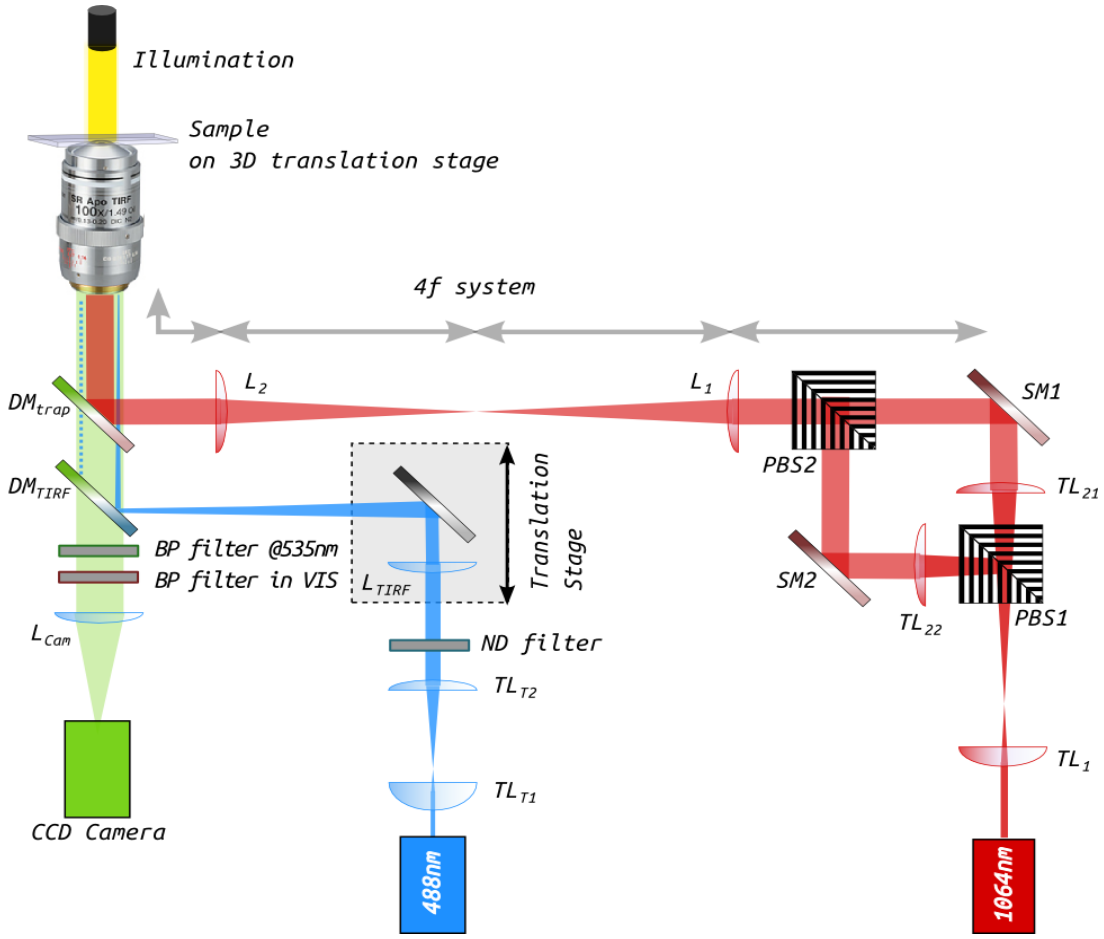

Figure 2: Experimental set-up of TIR-PH v1.0 – *In red, the 1064nm laser used for the tweezers. The laser is split and then re-combined it in S and P components with a couple of PBS. Using TL_1 and $TL2_{1,2}$ the laser is expanded in order to slightly overfill the back aperture of the microscope objective (MO). The mirrors SM1 and SM2 collocated between the PBS are steering mirrors for the two independent traps and the first components of two overlapping 4f systems that make them conjugate with the back focal plane of the MO. In blue, the exciting laser for the TIRFM. L_{TIRF} is used to focus the beam at the back focal plane of MO, while the mirror on the translation stage is used to switch between epi-fluorescence and TIRF. For the IR laser and for the blue laser, two appropriate dichroic mirrors are used, to reflect the lasers and transmitting the emission light. In green is the imaging path. The emission light is collected by the MO and focused onto a CCD camera, after being filtered by two band pass (BP) filters. The illumination used is a simple critical system illumination.*

The TIRPH is constituted by two laser paths being collected by the same microscope objective (MO). The trapping laser is an IPG Photonics ytterbium doped fibre laser emitting in the infrared (IR) with a 1064nm wavelength. The laser has a tuneable power up to 10W and a waist diameter of about 5mm. The IR laser beam is first reduced by use of a 3:2

telescope (of lenses with 75mm and 50mm focal lengths) in order to match the aperture of an Isowave optical isolator (I-106T-L); it is then split with a halfwave (HW) polarising plate followed by a polarising beam splitter (PBS) to enable the power to be controlled while maintaining a stable power output (not in picture). The laser then passes through onto the first lens of an expansion telescope (Telescope Lens TL1 of focal length 75mm) and then split again using a HW plate and a second PBS to produce two independent beams, with *s*- and *p*- polarisations. Two further lenses, TL_{21} and TL_{22} both of 200mm focal length, are used to complete two expanding telescopes together with the first lens TL_1. The telescopes expand the beams to a diameter of about 9mm, in order to slightly overfill the back plane aperture of the MO. The two beams illuminate two different IR mirrors that constitute two independent steering mirrors for the traps and are the first components of the indicated 4f systems; after being reflected by the mirrors, the beams are collected by a third PBS that recombine the *s*- and *p*- components of the beam. A lens L_1 of focal length f=150mm is located after the second PBS at a distance of 150mm from the two steering mirrors. Another lens L_2 of the same focal length f is located at a distance of 2f from the latter and at a distance f from a dichroic mirror (DM_{trap}) that points the laser upward to the MO. The DM_{trap} is a hot mirror with 50% transmission at 700nm.

The laser used for the TIRF microscope system is a 488nm Coherent Sapphire laser with a fixed output power of 200mW and a waist of 1mm in diameter. The laser is expanded using a telescope with 1:4 ratio and lowered in power to ~50mW with a neutral density filter. Then the laser is incident on a movable and removable lens L_{tirf} of focal length 200mm, situated onto a 1D Thorlabs translational stage together with a silver mirror that deviates the laser onto a dichroic mirror (DM_{tirf}), that directs the laser upward to the MO. The DM_{tirf} is a Chroma ZT488rdc-UF1, with a sharp transition at 500nm (reflection at 488nm>98%).

Both the lasers are collected by a Nikon Apochromat 100x oil microscope objective. The MO has a working distance of 0.120 mm and a NA of 1.49. Such a large NA makes it easier to reach the TIR condition, and it can be beneficial to tightly focus the trapping laser.

The microscope illumination system makes use of a fibre illuminator and just a simple lens with a focal length of 25.4mm, and illuminates the microscope sample just above a 3D Newport ultra high precision translational stage (562-XYZ) which is used to move a sample holder above the MO. Below the dichroic mirrors is a a silver mirror which collects the light into a Hamamatsu Orca C4742 digital camera. In the camera path, two additional filters are allocated to block the back-reflected light from the 488nm (band pass BP filter centred at

535nm, 30nm wide) and the 1064nm laser (BP filter 315-710nm). The filters let the emission light pass and reach the camera, as well as a generous amount of white light for bright field imaging. The sample preparation involves the deposition of 200ul of a medium in which the cells or the beads are dispersed in a chamber created by placing a spacer between a 50x22mm coverslip and a 22x22mm coverslip of thickness No. 1 from VWR.

The location of the lens and the mirror on the translation stage and the possibility of removing the lens are useful to switch from TIRF mode to epi-fluorescence mode. When the lens is not mounted, and the translation stage is in the reference position, the laser reaches the back aperture of the MO collimated and it is focused in the sample, resulting in epi-fluorescence illumination. When the lens is mounted, it can be moved in the axial direction of the laser, until the laser is focused on the back focal plane of the MO. Because the laser is focusing at the back aperture, it does not meet the conditions for TIR at the sample.

2.1 Optical Tweezers characterisation

The characterisation of the trapping system consists of:

- Characterisation of the laser shape, to guarantee a Gaussian shape (mode TEM_{00})
- Characterisation of the power stability of the laser
- Characterisation of the splitting PBS
- Characterisation of the transmission properties of the optical elements in the system
- Calibration of the camera
- Evaluation of the trapping forces.

First the IR laser has been characterised using a Thorlabs power meter (PM100D). In Figure 3, the average data from the knife edge measurement are reported and fitted with an error function; the first derivative of the data is presented on the right and fitted with a Gaussian. An average diameter of 5.76 ± 0.04mm is measured. The power stability of the laser was measured monitoring the output power variation during a period of one hour, with the same power meter. The relative change in power does not exceed 0.9% of the measured power.

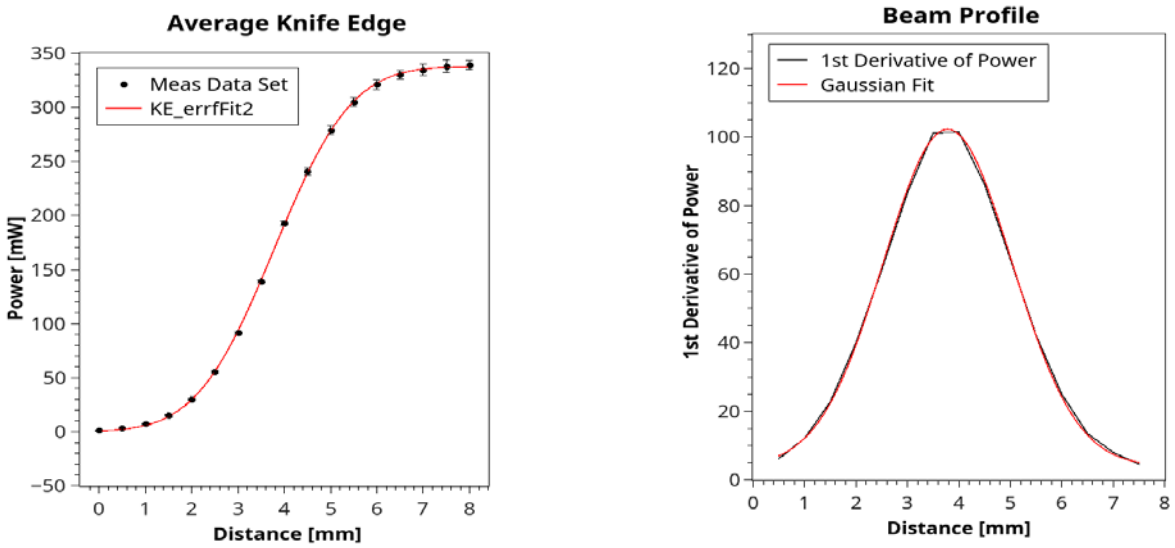

Figure 3: Knife Edge result for 1064nm laser – *Result of the Knife Edge measurement for the 1064nm laser fitted with a custom written Error Function (left) and its derivative fitted with a Gaussian (right).*

Using the same power meter, the two PBS have been characterised, measuring the transmitted and the reflected power each rotation of 5 degree of the HW. An appropriate polarisation angle was chosen for the beamsplitter to split the trapping laser in almost equal components.

Finally, measuring the power at the exit of the laser and at the focal point of the MO, a transmission of just about 4.6% was measured for the optical train. The MO causes a drop in the power of about 70%; the massive dropping in power may be explained considering that the MO is an apochromat objective, therefore composed by a greater number of color corrective lenses than Plan MO. In addition, overfilling the back aperture of a TIRF lens results in a further drop of power, due to the fact that the laser incident on the objective close to the edge of the aperture will not be focused by the MO, going instead in total internal reflection.

Finally a calibration of the camera pixel values to length was obtained making use of a 1mm grid printed on glass with 10um minimal division from Thorlabs (shown in Figure 4). A conversion 1um:9.15pixel is found.

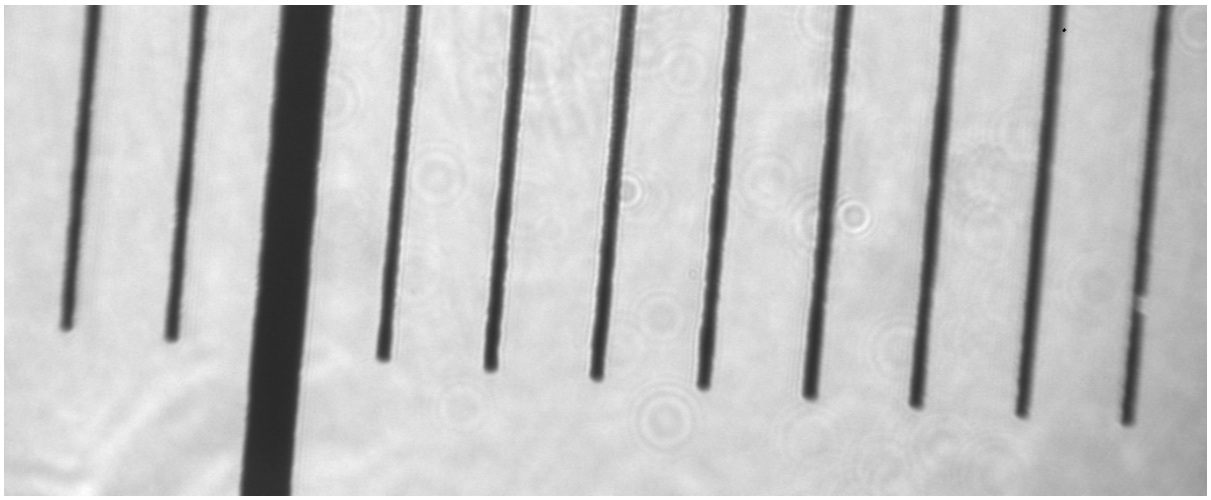

Figure 4: Camera calibration grid – *Image of the scale used for the camera calibration. Given that each division measures 10um, it is found that 1 um is equal to ~9.15px*

2.2 Trapping Force Evaluation

The trapping force was measured using the Escape Force method. The Escape Force measurement relays on the fact that, being the bead a spherical object immersed in a fluid of known Stoke's coefficient, it is easy to extrapolate the force acting on the bead when it moves in the medium. A trapped bead escapes from the trap when the Stokes force is equal to the trapping force:

$$F_{trap} = F_{Stoke} = 6\pi\mu r v_e \tag{3}$$

Where μ is the drag coefficient, r the radius of the trapped bead and v_e the escape velocity.

Since moving a bead in a viscous medium, or moving the medium while the bead is trapped result in the same Stoke's force, for the measurement the sample is translated by the use of a Newport New Step N12 motor with an increasing velocity. The velocity at which the trapped bead falls from the trap is called escape velocity and is used to evaluate the Stoke's force acting on the bead at that moment.

The escaping velocity is obtained for each measurement recording the position of another bead stuck on the coverslip imaged contemporary to the trapped one (see Figure 5). In Table 1 the measured forces are reported, for different powers, for beads of about 2.56um in diameter.

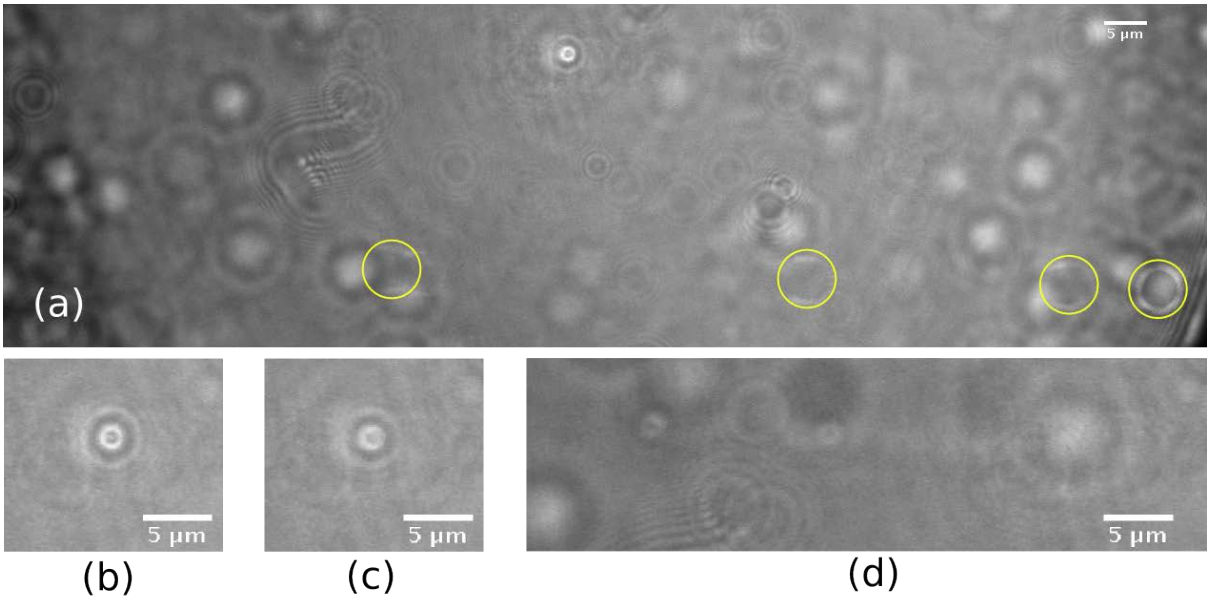

Figure 5: Escape force evaluation – *Time frames overlapping (a) showing the different positions of a bead stuck on the coverslip while moving the stage. In (b), (c) and (d), frames showing the trapped bead falling from the trap. For this picture, the trapping laser in the focus was ~180mW and the measured force for this bead was 83pN. The round objects in background come from the BP filter @535.*

Power [mW]	Average Escape Force [pN]
18.4	9.8±3.6
46	17.6±0.3
92	23.9±1.1
184	85.6±0.8

Table 1: Escape Force Measurement – *Result of the escape force measurements for 2.56um beads, at several laser power in the focal point*

2.3 TIRF characterisation

The knife edge measurement was performed for the 488nm laser too. The graph is omitted for brevity. The knife edge showed a laser diameter of 0.78 ± 0.01 mm.

To characterise the TIRF imaging system, an experiment to confirm the presence of an evanescent field was carried out. A micron-sized bead in the evanescent field is subjected to the scattering component of the light force. Therefore, such a bead is pushed away from the field and it moves in its direction of all its length. If the field is uniform, the bead moves in a straight line. The experiment carried showed a fairly good TIRF condition. An example is

given in Figure 6.

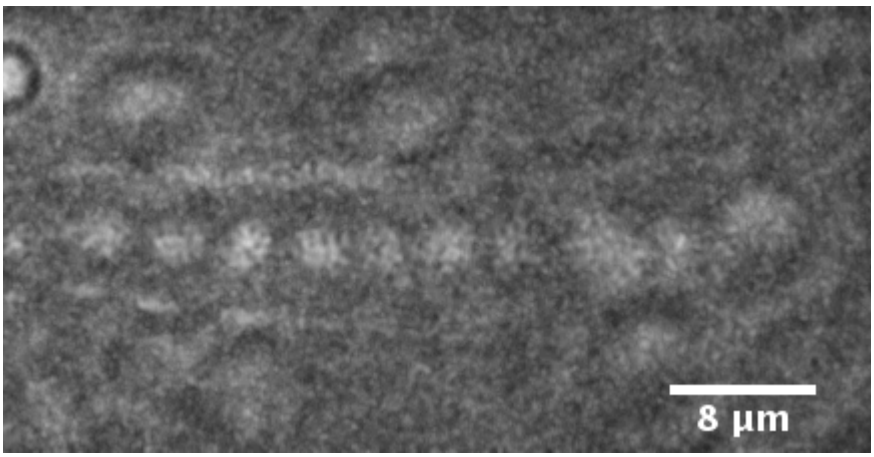

Figure 6: Scattering force exerted from the TIRF field – *overlapping time frames of a bead being pushed horizontally at constant height from the TIRF field.*

However, this measurement has to be considered purely qualitatively. For the experiments presented in the following section, in fact, a slightly different position of the 1D translational stage was used to optimise the imaging, changing the properties of the evanescent field.

3 Preliminary Results

After the characterisation stage, the system was tested making use of biological samples. Experiments with biological samples further confirmed the presence of a reasonably good TIRF system. More importantly, the use of the TIRF imaging system and the tweezers was tested at this stage.

The biological sample used for these measurements are

1. Dictyostelium cells with GFP Myosin Knock-In. The experiment with these cells was carried out in collaboration with Prof. Kees Weijer and Dr. Gail Singer.
2. Drosophila S2 cells. This experiment was carried out in collaboration with Dr. Eric Griffis.

3.1 Experiments with Dictyostelium with Myosin KI cells

Dictyostelium is a highly motile amoeba. It is a robust organism that is commonly studied to examine the mechanisms used by cells to crawl on substrates, and more interestingly to obtain information about cells collective behaviour and tissue dynamics. A crawling cell is subjected to three different and correlated mechanisms: the protrusion of the cellular membrane in the direction of the motion thanks to the polymerisation of actin bundles that push the membrane; the adhesion of the cell body on the substrate which allows the polymerisation forces to be translated into traction forces; finally, the retraction, or contraction, mechanism at the rear of the cell obtained thanks to myosin pulling the back of the cell.

For the purpose of this prototype, we focused just on the contraction mechanism, studying the signal of myosin.

Genetically modified Dictyostelium with the knock-In of the fluorophore GFP into the myosin II heavy chain gene is used to image the Myosin filaments during the retraction. In this experiment simple Dictyostelium cells dispersed in KK2 have been used, provided enough time to let the cells settle and attach on the bottom coverslip before to close the sample chamber with a 22x22mm coverslip.

Micron-sized beads dispersed in KK2 can be easily added to the chamber. Optical tweezers can be used to trap a dispersed bead that it turn can be used to exerts forces on a cell. It has been observed that if a Dictyostelium cell is pulled with a micropipette, it counter reacts

contracting itself in that area. We replicated this result by pulling the cell with a trapped bead.

First, Dictyostelium cells have been used to test the quality of the TIRF microscope. For this purpose a sample with Dictyostelium in KK2 have been imaged both by a Nikon commercial TIRF microscope and by the here presented home built TIRF.

Dictyostelium cells move less the more are exposed to high levels of excitation light, to high light exposure results in cells rounding up and starting to die.

Nevertheless, interesting results have been obtained from this first experiment. The images in Figure 7 show cells in the same “vegetative” status obtained by the commercial TIRF and the home-built system. The images look similar. Although the visible signals probably come more from auto-fluorescence than from Myosin, the images are helpful to determine that home-built TIRF has a resolution comparable to the commercial one, therefore enabling its use for future experiments.

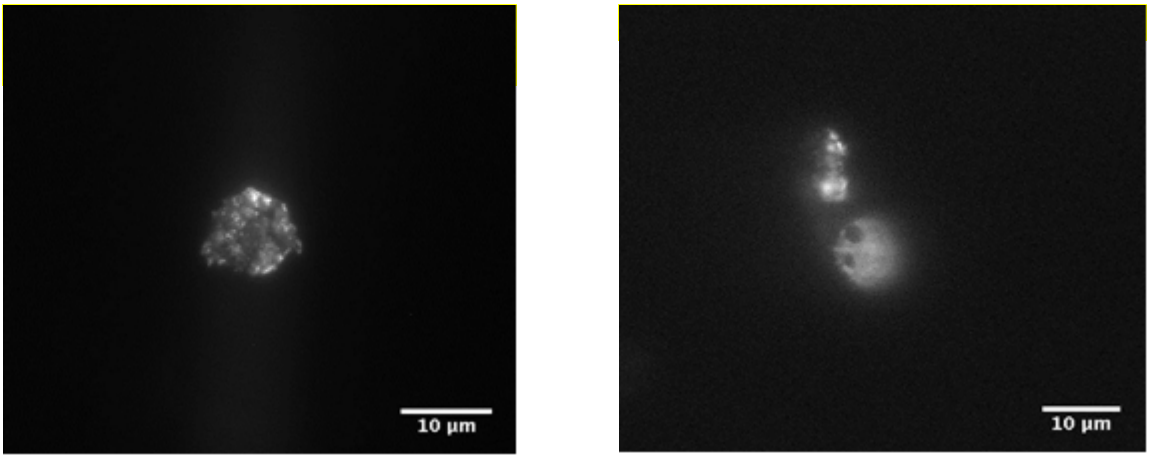

Figure 7: TIR-PH resolution – *Dyctostelium cells in vegetative state laying on a substrate imaged with the home-made TIRFM of TIRPH (left) and with a Nikon commercial microscope (right). Both the microscopes were using the same MO and the same excitation laser. Nevertheless, the images are obtained using different camera and different software.*

The simultaneous use of optical tweezers and TIRF microscope resulted in a successful implementation, but a few issues have been encountered:

- The quick degrading of the cells because of the continuous exposure to the exciting laser light does not allow long time measurement.
- The IR filter makes it impossible to visualise the trapped bead during the TIRF imaging. It is then necessary to continuously switch between bright field and

fluorescence imaging. The time spent to switch often results in a change in the z-position of the sample. An adjustment in the z-position changes the relative position of the trap to the sample, interrupting any interaction if present.

Some images obtained with this system, using a power of ~380mW in the focal trapping point, are presented in Figure 8 and in Figure 9.

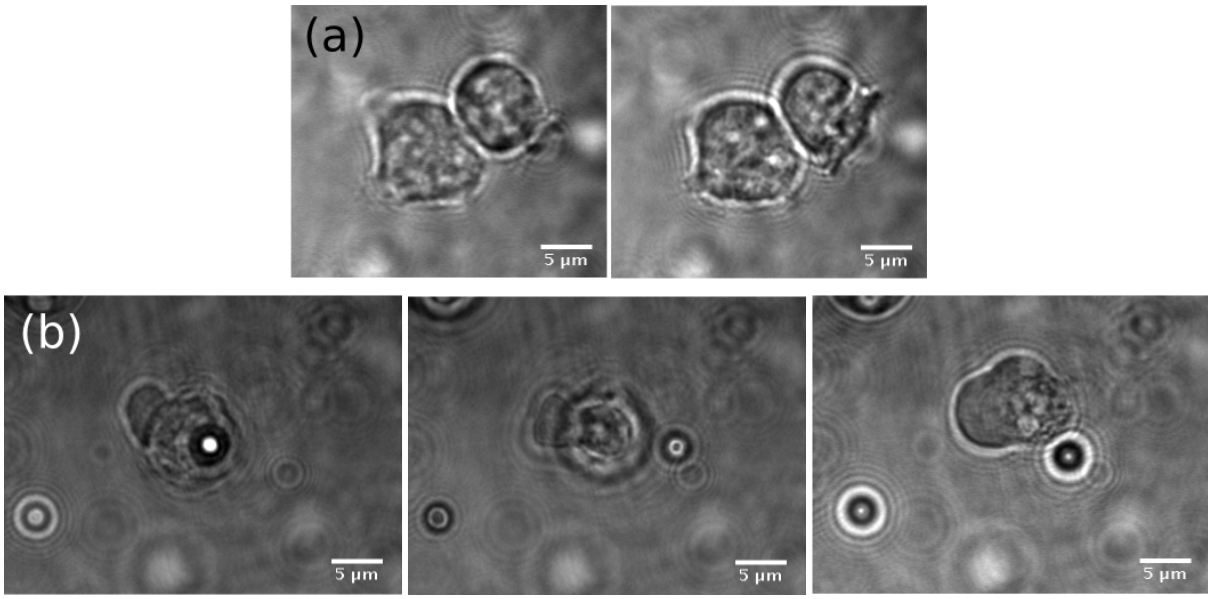

Figure 8: Effect of TIRF and trapping laser on Dictyostelium – *In (a) two frames showing how cells start to round after few second of exposure to the TIRF field. They become less and less motile, and eventually start to detach from the substrate. In (b) a time sequence showing the interaction between a cell and trapped bead; the cell seems to "escape" the laser trapping light; in fact, not being driven by chemotaxis to contrast the trapped bead that is interacting with its membrane, it seems to moves on the opposite direction.*

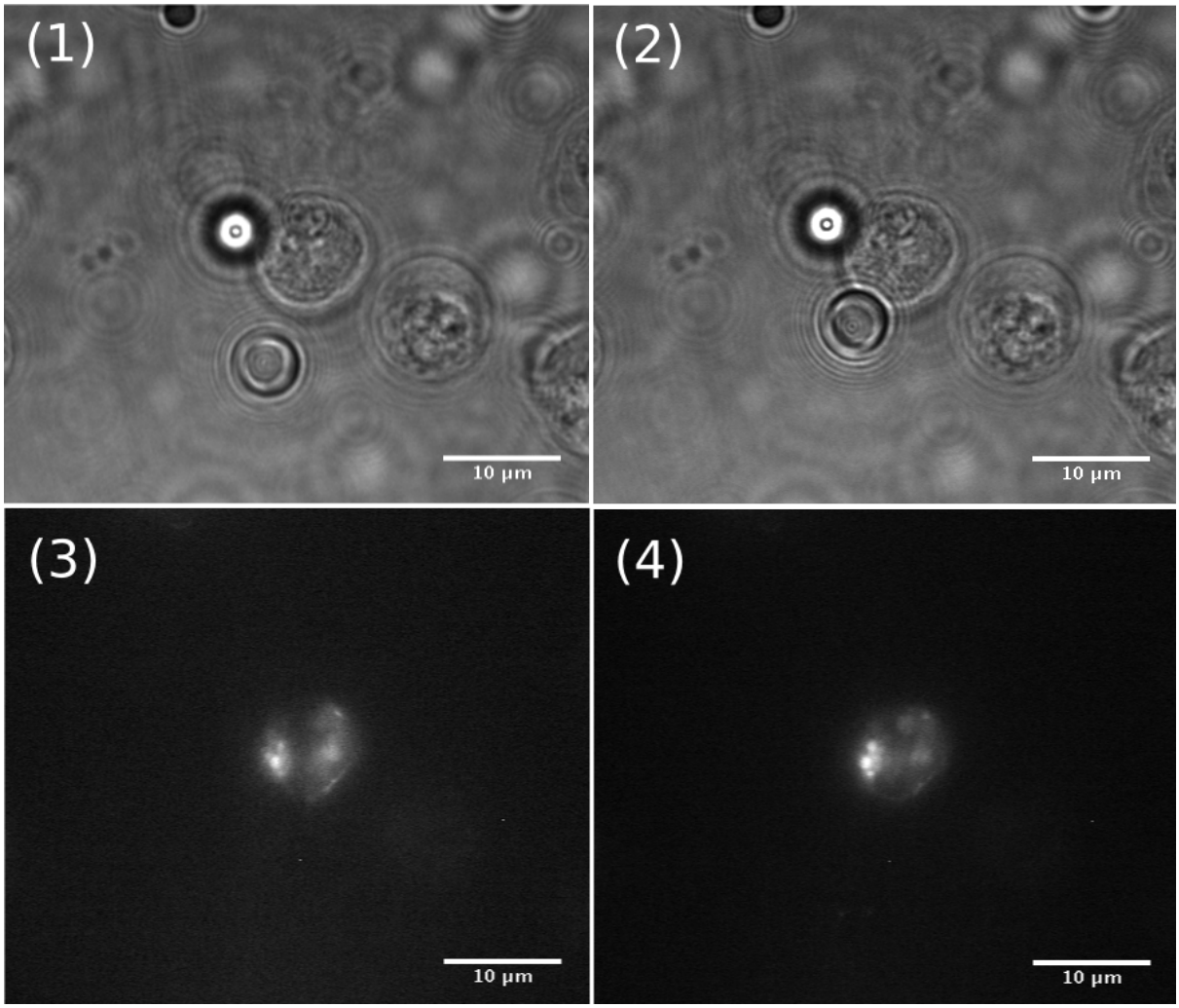

Figure 9: Interaction bead-cell in TIRF – *Time sequence of cell-bead interaction. The cell is slightly deformed in (2), being pulled by the attached bead. In (3) and (4), a weak TIRF signal shows the same kind of interaction (same movement of the bead repeated after having switched off the bright field).*

Due to the strong influence of the two lasers on the normal behaviour of Dictyostelium (Figure 8), it is difficult to give a satisfactory interpretation of Figure 9. In fact, although it is shown that the cell interacts with the bead and that it can be deformed, such a deformation may be due to the fact that the cell is not in normal condition and it is, indeed, dying and detaching from the substrate. It is in fact unlikely that the forces exerted by the bead could move a healthy attached cell. For the same reason, for the TIRF images, we neglect to indicate any Myosin signal due to the pulling action of the bead.

3.2 Experiments with Drosophila S2 cells

For these experiments Drosophila S2 cells with GFP labelled actin bundles have been used. The cells where attached to a substrate in order to image the cell cortex in TIRF. In addition

2.56µm beads were added to cells; before running the experiment, sufficient time was allowed to enable the cells to phagocytose the beads. Some time sequences from the experiment are presented in Figure 10.

Remarkably, the tweezers were able to move the beads inside the cells themselves, proving the feasibility of in vivo experiments. However, no significant change was observed in the TIRF signal of the cell cortex. It is highly probable that the beads inside the cells settled too far from the cellular cortex, and by moving them with the tweezers, no interaction could be visible. A useful further experiment would involve the functionalisation of some beads to let them attach onto the cell. In this way, we aim to image the tension applied to the cellular cortex by the external side of the cell.

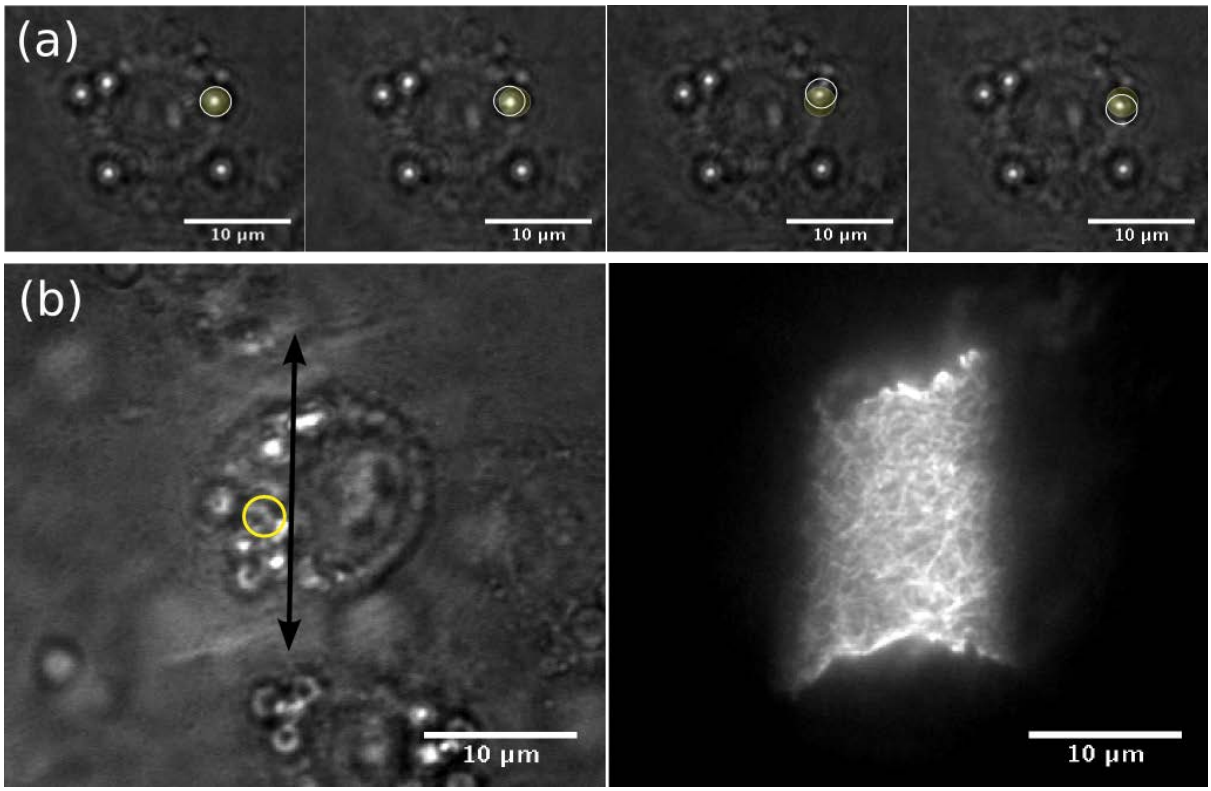

Figure 10: Drosophila S2 - *Time sequence of a trapped bead moved by the tweezers inside the cell (a); the yellow filled circle highlight the initial position of the bead, while the white empty circle indicates the position of the bead in the frame. In (b), bright field (left) of a cell and TIRF picture (right) of the cellular cortex for the same cell; note that cellular cortex extend more than the visible cell in bright field (the black arrow indicates the approximate extension of the cellular cortex as measured in TIRF), being too thin to have enough contrast to be imaged. In TIRF instead (b, right) the actin bundles network is resolvable. The yellow circle indicated the position of a trapped bead. Moving the beads in TIRF did not produced any significant change in the TIRF imaging, and it is therefore not shown here.*

4 Discussion of results and future development

The system showed several interesting results, as well as several issues that need to be addressed. In this section, I will highlight them both, providing ideas on how to improve the system to achieve the desired results.

4.1 The TIRF microscope

The microscope showed a really good resolution, comparable to the resolution of one of the commercial system available in the Imaging group of Life Science department in Dundee. Both fluorescence signals from Dictyostelium and actin bundles signals in the cellular cortex of Drosophila S2 have been satisfactorily recorded.

However long imaging times (or at least under laser illumination) are not suitable for this system. Dictyostelium cells start to die very soon when exposed to the exciting laser, and intense bleaching of the fluorophores has been observed as well. Adding other ND filter to reduce the laser intensity is not advisable, since they also add distortions and back reflection in the system. Suggested improvements involve instead

1. the addition a HW plate and a PBS at the exit of the laser beam, sensible for 488nm laser light. These elements would allow to tune the laser power in a continuous way.
2. the addition of a shutter that can be triggered with micro-manager (an open source microscope control software). This would permit illumination of the sample for a limited amount of time, reducing photo-damage due to the continuous exposition to the excitation light.

In addition it has been observed a small relaxation in z of the sample stage holder. This causes the images to slowly defocus when in TIRF. The addition of a z-controlled MO mount may solve this issue.

4.2 The trapping system

The trapping system represented, up till know, just a partial success. The possibility to obtain stable tweezers, even with such a little transmission from the MO, and to interact with biological sample represents a great achievement. Nevertheless, no change in the TIRF signal has been observed when the trapped beads were interacting with the cells. This issue may be caused by several factors in the current experiments (the use of phagocytised beads

instead of external interacting beads in the Drosophila S2 cell and Dictyostelium experiments, the position of the beads being too high compared to the TIRF field, the weak trapping force.

Several improvements can be made to TIR-PH experiments:

1. For the preparation of the sample, it is suggested to
    - functionalise the beads and repeat the experiment with the Drosophila
    - work with starving Dictyostelium cells or cells that are induced to move by chemotaxis.
2. To improve the position of the trap, there are two different approaches that can be taken
    - Using a spatial light modulator (SLM), to change the height of the focus.
    - Changing the position of the focus by moving the second telescope lens of the trap (TL_{2i})

    Both approaches have side effects. The SLM causes an important loss in power, usually resulting in weak traps. Moving the lens, on the other end, causes the beam to expand or shrink instead of being collimated, adding aberration in the focal point, and again resulting in a weak trap. An extensive study and comparison of the two methods will be carried out to decide which approach is more convenient for the case. Finally it is important to mention that the addition of a z-controlled MO mount may be beneficial for the trapping too. In fact when the stage *z*-position changes because of the stage holder relaxation, the relative distance between the trapped bead and the biological sample also changes.
3. Finally to improve the trap quality, it is possible for the TIRF objective, to slightly underfill the back aperture. This could save a reasonable amount of power that can reach the trap and being used to exert forces. The use of high strength photonic tweezers is also under consideration.

To conclude, we have implemented a simple optical tweezers system combined with a TIRF microscope system, using commercial components. Our system has demonstrated that there two techniques are compatible and importantly we have implemented our system making use of interesting biological samples. This work is a precursor of measurements of biological relevance, and an indication of how cellular mechanical probing can be carried out within this project in the future. It is very likely that the suggested improvements will lead to a new version of the system, capable of a wide range of studies.