Fibre based cellular transfection

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Abstract: Optically assisted transfection is emerging as a powerful and versatile method for the delivery of foreign therapeutic agents to cells at will. In particular the use of ultrashort pulse lasers has proved an important route to transiently permeating the cell membrane through a multiphoton process. Though optical transfection has been gaining wider usage to date, all incarnations of this technique have employed free space light beams. In this paper we demonstrate the first system to use fibre delivery for the optical transfection of cells. We engineer a standard optical fibre to generate an axicon tip with an enhanced intensity of the remote output field that delivers ultrashort (~ 800 fs) pulses without requiring the fibre to be placed in very close proximity to the cell sample. A theoretical model is also developed in order to predict the light propagation from axicon tipped and bare fibres, in both air and water environments. The model proves to be in good agreement with the experimental findings and can be used to establish the optimum fibre parameters for successful cellular transfection. We readily obtain efficiencies of up to 57% which are comparable with free space transfection. This advance paves the way for optical transfection of tissue samples and endoscopic embodiments of this technique.

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1. Introduction

Laser initiated delivery of foreign DNA and therapeutic agents into living mammalian cells (laser-assisted cellular transfection) has proved to be a significant method for studying and elucidating basic cellular processes and genotypic changes inside cells. The ability to permeate the lipid cell membrane in a sterile and non-invasive manner without compromising the cell viability would be a powerful tool especially in fields such as applied drug discovery and gene therapy.

Traditional transfection methods include the application of electrical pulses, direct cell microinjection as well as chemical techniques to transfer a gene of interest inside a cell. However, the invasive nature of these methods may raise associated problems concerning cell viability and delivery efficiency. In comparison to these methods, laser methods offer single cell selectivity and are sterile. Continuous wave lasers may be used for transfection [1] but in particular the use of tightly focused infrared femtosecond laser pulses has been the most powerful laser transfection technique to date. It leads to a sterile and ultra-precise technique for cellular transfection by means of creating a self-healing pore (photoporation) on the cell membrane [2-7].

Femtosecond photoporation is a highly localized effect due to the non-linear nature of the cell membrane-laser interaction solely within the focal region, leading to minimal collateral damage. In addition, it offers the ability for single cell transfection within a large cell population. Previous studies have successfully demonstrated femtosecond cellular transfection, using a tightly focused Gaussian laser beam [2-5, 7] as well as implementing a “non-diffracting” light beam which eliminates the need for precisely locating the cell membrane [8]. These studies have all been restricted to free space transfection. A significant step forward would be to implement this technology in a robust fibre geometry that has the potential compatibility with endoscopic techniques, microfluidics and fibre multiplexing. By combining an optical fibre and endoscopic technology, fs-laser transfection may offer real advantages over standard techniques, especially the high efficiency and spatial selectivity to be used in vivo. In contrast, viral transfection and electroporation will very likely remain standard techniques in vitro, due to their ease of use and high throughput.

In this paper, we demonstrate the first fibre based transfection scheme and perform photoporation of Chinese Hamster Ovary (CHO) cells by means of an axicon tipped optical fibre [9-11].

2. The axicon tipped optical fibre fabrication and theoretical modelling.

Optical fibres offer robust tools in research areas of biophotonics. They can be integrated into complex microfluidic environments as well as substituted for expensive optical setups, hence offering significant flexibility in performing a variety of demanding photoporation tasks. A key point that we address is the fact that the output of a standard optical fibre would be naturally divergent. This introduces a significant limitation to the available working distance between the fibre and cell sample because of the high intensity laser light needed for the multiphoton process to initiate cell transfection. One option to increase the intensity at a given distance from the fibre and so obtain a reasonable working distance, could be to use a micro-lens tipped fibre [10, 11]. However, the process of their manufacture can be highly complicated. As an alternative, selective chemical etching, when applied to the optical fibre, offers a very simple inexpensive route to obtain an intensity enhancement. Applying this method to a standard single mode optical fibre forms a conical lens (axicon) on the fibre facet.
This is due to the differing etching rates of the core and the cladding of the fibre, since the slower etched fibre core is only gradually exposed to the solution [9]. The presence of an axicon on the fibre tip modifies the output such that the subsequent enhanced intensity is sufficient for multiphoton excitation. Considering the very small diameter of the Gaussian beam (~5 μm) illuminating the axicon tip, the axicon tipped fibre does not produce a Bessel mode, rather just an elongated focus. This results in successful cell transfection using the fibre at a relatively remote position to the cell sample, thereby obviating any potential physical contact between the tip and cell sample [8].

The fibre used in this experiment was a commercially available single mode fibre of mode field diameter 5.6 μm, cladding diameter 125 μm, and an operating wavelength of 830 nm (Thorlabs, P1-830A-FC-2). The fibre was chemically etched with a solution comprising hydrofluoric acid (HF, 48%-51%) and ammonium fluoride (NH₄F, 40%). The volume ratio of NH₄F to HF was 2.1:1 which provides an axicon cone angle of approximately 118°. The cleaved end of the fibre was immersed in the etching solution for 60 minutes at 23° room temperature and subsequently rinsed with water for 1 minute. The desired cladding and core diameter can be finely determined by controlling the etching time. The final cladding diameter was measured to be 65.8 μm which proved to be sufficient for achieving fibre stability inside the liquid environment of the cell sample.

The fibre output light was analyzed from a series of its lateral cross-sections obtained in both air and water medium by using either a long working distance objective (x100 MITUTOYO, Japan) or a water immersion objective (x60 Olympus UPlanSApo) respectively, in conjunction with a CCD camera (Basler piA640-210gm). Using a motorized actuator (Newport CMA-12) the fibre facet was displaced from the objective lens in steps of 1.25 μm and at each position an image of the beam cross-section was captured (see Fig. 1). For understanding and optimising the fibre properties we developed an algorithm based on the scalar and paraxial free-space propagation approach, where the field in the initial plane on the fibre facet (the fibre mode) is decomposed into a spectrum of plane-waves (spatial spectrum) by Fourier transformation. The spatial spectrum for any plane behind the fibre is obtained by assigning a correct phase shift to each of the plane wave components and the field is retrieved by inverse Fourier transformation. Since there is no azimuthal dependency on the geometry, we could efficiently use a Hankel transformation instead of Fourier transformation thus reducing the problem from 2-D to 1-D [12].

In the case of the axicon-tipped fibre, one can assume that the axicon tip is a thin high-index transparent element modifying only the wave-front of the field. However, we found that this approach does not produce sufficiently accurate data for sharper axicons. Therefore, the evaluation of the light propagation inside the axicon was done in a sequence of small axial steps. For each of the steps the field was split in two parts, inside and outside the axicon. Both components were propagated over the step size taking into account the refractive index of the corresponding medium. The composition of these two resulting fields was used as the initial field for the next step. This procedure is related to the Babinet's principle and naturally reducing the step size gives better results as the deviations caused by the refractive index difference gets smaller. We set the step size to be λ/20 as a good compromise between the approximation quality and the cumulative numerical error (rounding numbers) that grows with amount of steps in the computation.

Figures 1(a) and 1(b) show the theoretical and experimental results of the azimuthally averaged intensity profiles of the beam as a function of the distance from the fibre facet, in air and water respectively. The experimental findings are in good agreement with the theoretical predictions.
Due to the fixed properties of the fibre, the only parameter that can be varied in the model is the axicon tip angle. As we shall explain later, there are strong experimental limitations in the working distance i.e. the distance between fibre end and the cell layer. Therefore it is important to know which axicon tip angle should be used to deliver the highest intensity at the given distance behind the fibre. The results of this study (see in Fig. 2(a) and 2(b)) indicate that with the increasing working distance, the optimal axicon tip angle grows, whilst the maximal attainable intensity falls. However in any experiment, one has to compromise the working distance (that based on the simulation should be as small as possible) with other factors such as the risk of contamination and physical contact between the cell and the fibre.

Figure 2(c) shows the comparison between the on-axis intensity produced by the manufactured fibre with an axicon-tip angle of 118° and the maximal attainable on-axis intensity from Fig. 2(b). It is clearly seen that optimizing the axicon-tip angle plays the most important role in the vicinity of the fibre facet, but further from the fibre it does not bring significant enhancement. Since the minimal practical working distance of the experiment was 13 µm, we concluded that the manufactured fibre brings sufficient field enhancement for performing cellular transfection. Using an axicon tip of about 75° would produce higher peak intensity at the specific distance of 13 µm, but the intensity will fall off much faster at this larger working distance when compared to that of the 118° axicon fibre (in other words, the elongation of the focus is shorter). Therefore choosing 118° for an axicon tip angle leads to a better performance over a wider selection of possible working distances. In addition, we remark that 118° was also chosen due to practical limitations of the fibre etching process for the present results and in future studies it would be worth exploring photoporation for a wider range of cone angles.
3. Experimental procedure

Cell transfection was instigated by a femtosecond Ti: Sapphire laser emitting at 790 nm, with output pulse duration of ~100fs and a pulse repetition frequency of 80 MHz (Coherent, MIRA). At the exit of the axicon tipped fibre, the pulses undergo stretching due to non-linear phenomena occurring inside the fibre such as self-phase modulation (SPM) and group velocity dispersion (GVD), giving an overall pulse duration of approximately 800 fs, as measured using a home built autocorrelator [13].

The experimental setup used was built around a NIKON microscope (model TE-2000U) as shown in Fig. 3(a). A magnifying telescope (x1.6) expanded the incoming laser beam which was subsequently coupled to the 35 cm long axicon tipped fibre, using a fibre collimator (THORLABS, F810FC-780). The coupling efficiency of the fibre collimator-optical fibre system was 27%. The fibre output power was adjusted by means of a neutral density (N.D.) filter wheel appropriately placed in the beam path. During photoporation the average power of the beam was kept equal to 110 mW, with peak power per pulse equal to 1.3 kW. A mechanical shutter was positioned close to the laser output and controlled the time duration of the laser dosage on the cell membrane. Each cell was irradiated with three laser doses. The duration of each dose was experimentally determined to be 80 ms. The number of the laser doses was experimentally determined to be the most successful number of doses in conjunction with the dose duration of 80 ms. While irradiating the cells, a slight adjustment of the laser focus was taking place to ensure that we achieve maximum power delivery on the cell membrane. Three doses were used ensuring consistency of irradiation for all cells and indeed this is aligned with the number of doses used in previous work [8].

The fibre was mounted on a three axis (X-Y-Z) translation stage and was carefully inserted into the medium, as shown in Fig. 3(b). Due to restrictions imposed by the geometry of the hosting microscope, the fibre could not be accommodated vertically between the sample stage and the microscope condenser, as the imaging path was disrupted.

A protractor enabled the adjustment of the fibre orientation with respect to the lateral plane. When the fibre was positioned at angles below 50° with respect to the lateral plane, the beam could not reach the cell membrane and permit successful transfection. At a 60° angle, the laser beam could only reach and perforate the most rounded shaped cells, whereas the cells which appeared more flat remained out of reach. At angles of 80° and 90° the fibre obstructed the imaging path, hence preventing the direct observation of the photoporation process. The optimum angle for photoporation using an axicon tipped fibre was established to be 70° for all cells regardless of their shape. Additionally the minimum successful distance that was physically achieved between the fibre and the cells, without the fibre cladding coming in contact with the sample dish bottom was 13 μm. We note that reducing the fibre cladding diameter by use of the etching procedure brings a significant benefit here, since such a short working distance would not be possible with the original bare fibre using this geometry. Considering the limitations emerging from the positioning of the beam at 70 degrees with respect to the bottom of the dish, from geometric calculations the axial distance along the beam propagation that reaches the bottom of the sample is 13 μm. For a round cell we assume thickness of approximately 4 μm so we adjusted the z-actuator of the (x, y, z) translation stage by approximately 4 μm, in order to position the cell at 13 μm with respect to the fibre tip. When the cell is flat and we photoporate near the edge and assume a thickness of approximately 2 μm and as such we adjusted the z-actuator by 2 μm from its initial position.

The CHO cells were grown to sub-confluence in a 30 mm diameter glass-bottomed culture dish (made by the World Precision Instruments, Stevenage, UK) in 2ml of culturing cell media (MEM), in a humidified atmosphere of 5% CO₂ / 95% air at 37 ºC. Prior to the experiment, the cell monolayer was washed twice with OptiMEM (Invitrogen) and the sample was bathed in 500 μl solution which comprised of 10 μl mitoDSRED plasmid (concentration 0.3 μg/μl) encoding a mitochondrially targeted Discoidium Red Fluorescent protein (BD Biosciences, Oxford, UK) and 490 μl of OptiMEM. No coverslip was used.
Fig. 3. (a). The microscope based photoporation apparatus using an axicon tipped fibre. The laser beam generated by the mode locked Ti: Sapphire laser was sent through a x1.6 demagnifying telescope and was subsequently coupled into the axicon tipped fibre which was rigidly mounted on a xyz translation stage. Each irradiated cell experienced three laser doses, each of 80 ms duration. (b). The distance of the fibre with respect to the cell membrane was the minimum possible distance, approximately 13 μm, as dictated by the geometry of the setup. The fibre is optimally positioned at 70 degrees with respect to the cell monolayer regardless of the cell shape. The working distance is determined to be the distance between the axicon tip and the cell membrane.

During laser irradiation no visual response was observed. After the laser treatment, the cell monolayer was bathed in 90% modified eagles medium (MEM) fortified with 10% foetal calf serum (FCS), 18 units/ml of penicillin, 18 μg/ml of streptomycin, 1.8 mM of L-Glutamine and the culture dish was returned in the incubator. 48 hours later, the sample was viewed under a fluorescent microscope, where successfully transfected cells expressed the red fluorescent protein.

5. Results

Figures 4(a) and 4(b) show the axicon tipped fibre inside the cell sample prior to photoporation and the successfully transfected fluorescent cells respectively. As in our previous studies, the transfection efficiency was defined as the number of cells expressing the correctly targeted red fluorescent protein, divided by the total number of cells that were laser treated in a particular area of interest. In order to monitor for potentially spontaneous transfected cells, each photoporated sample dish was accompanied by a control sample dish in which cells were cultured, bathed in plasmid DNA solution and experienced the fibre presence in the absence of laser radiation. In the course of this experiment we irradiated 6 sample dishes (N=6), and a total number of 150 cells. The number of spontaneously transfected cells varied between 0-3 cells for each sample dish. The average transfection efficiency was calculated ranged from 25% to 57% between the treated dishes which compares very well with data for free space transfection [4].
Photoporation was also attempted by means of a cleaved single mode fibre (Thorlabs, P1-830A-FC-2). The experimental setup was kept the same as in the case of the axicon tipped fibre, with the cleaved fibre inserted inside the sample dish at 70° with respect to the cell monolayer. The cladding diameter was 125 μm which determined the minimum distance between the fibre and the cells to be approximately 23 μm. As shown in Fig. 2(c), the beam intensity at 23 μm is significantly lower than that of the axicon tipped fibre; as expected no transfection was obtained using this type of fibre.

6. Summary

In conclusion, we have demonstrated optically assisted transfection of cells using an axicon tipped fibre with results that compare very favourably to free space transfection studies. This fibre is very simple to manufacture and allows for “remote” delivery of femtosecond light for transfection to a cell sample. Future studies could involve more sophisticated multiple fibre bundles that may treat larger numbers of cells in a single dose. Overall we envisage that this fibre technique will simplify this process of laser transfection and mean that one can implement this methodology readily on any microscope. The method decouples the observation by the microscope’s optical system from the ultra-short pulse delivery. Additionally our technique paves the way for potential laser transfection of tissue samples and potential for incorporating a fibre within a microfluidic channel for transfecting cells within a flow.

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