

## Femtosecond cellular transfection using a nondiffracting light beam

X. Tsampoula,<sup>a)</sup> V. Garcés-Chávez, M. Comrie, D. J. Stevenson, B. Agate, C. T. A. Brown, F. Gunn-Moore, and K. Dholakia

*SUPA, School of Physics and Astronomy, University of St. Andrews, St. Andrews, Fife KY16 9SS, Scotland, United Kingdom and School of Biology, University of St. Andrews, St. Andrews, Fife KY16 9TS, Scotland, United Kingdom*

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The ability to permeate selectively the cell membrane and introduce therapeutic agents is a key goal in cell biology. Optical transfection is a powerful methodology but requires exact focusing due to the required two-photon power density. The authors use a Bessel beam that obviates the need to locate precisely the cell membrane, permitting two-photon excitation along a line leading to cell transfection. Assuming a minimum efficiency of 20%, the Bessel beam offers transfection at axial distances 20 times greater than that of its Gaussian equivalent. Furthermore, the authors demonstrate cell transfection beyond obstacles due to the self-healing nature of the Bessel beam. © 2007 American Institute of Physics. [DOI: 10.1063/1.2766835]

The cell membrane is a thin (5 nm) bilayer film of hydrophobic lipids, embedded with various protein molecules at interspersed locations. The membrane encloses the cell, defines its boundaries, and maintains the essential physicochemical differences between the cytoplasm and the extracellular environment. Under normal circumstances, the lipid nature of the cell membrane acts as an impermeable barrier to the passage of most water-soluble molecules. Thus, the selective introduction of therapeutic agents to the inside of dysfunctional or diseased cells is a challenging issue. Approaches for poration include physical injection into individual cells using glass micropipettes, membrane fusion of loaded liposomes, ballistic introduction of coated gold nanospheres (gene gun), delivery of therapeutic agents encapsulated in membrane permeable shells, local permeabilization of cells via the application of pulsed electric fields, and local permeabilization of cells via the application of diagnostic ultrasound (sonoporation). The introduction of foreign DNA into cells (transfection) is a key procedure in genetic analysis and recombinant protein experiments.

Laser based photoporation and transfection is an emergent methodology that holds promise for the delivery of therapeutic agents to cells in a targeted and sterile manner. The most powerful method for implementing this has been as a two-photon process using a femtosecond light source.<sup>1-3</sup> Due to its nonlinear intensity dependence, two-photon excitation predominates solely within the focal region leading to a short axial range over which this process occurs. This brings with it stringent requirements for focusing: locating the exact position of the cell membrane for such studies is a difficult and time consuming task. For multiphoton processes in biophotonics, such as poration or ablation, it would be a major advance to achieve this without the need to locate and focus the beam exactly on a given cell membrane.

In this letter, we demonstrate an advance for photoporation whereby we use a nondiffracting light mode, a Bessel beam,<sup>4</sup> for multiphoton cell transfection. The Bessel mode obviates the need for exact focusing<sup>5</sup> and permits transfection in cell monolayer samples over large axial distances. We

compare this method with a Gaussian light mode and measure cellular transfection efficiencies for both beams. Additionally, we demonstrate that cells within a monolayer may be porated beyond an obstruction offering the potential for novel methods for multiplexing cell transfection.

The zeroth-order Bessel light beam is a solution of the scalar Helmholtz equation. It is termed “nondiffracting” or more accurately propagation invariant as the transverse profile of the beam remains unaltered during free-space propagation.<sup>6</sup> The electric field amplitude of a zeroth-order Bessel beam is given by

$$E(r, z) = A \exp(ik_z z) J_0(k_r r). \quad (1)$$

Here,  $J_0$  is the zeroth-order Bessel function and  $k_r$  and  $k_z$  are the radial and longitudinal components of the free-space wave vector  $k$  where  $k = 2\pi/\lambda$ . The central maximum of a zeroth-order Bessel beam (radius  $= r_0 = 2.405/k_r$ ) can theoretically approach the wavelength of the illuminating light. A finite approximation to a Bessel beam can be realized with a conical glass element known as an axicon.<sup>6</sup> The light is refracted to yield emergent wave vectors lying on a conical surface which is a central characteristic of the beam. This leads to the property of self-healing such that the beam reforms upon encountering an obstruction. For an axicon of opening angle  $\gamma$ , refractive index  $n$ , and illuminated with a Gaussian light beam of beam waist  $w_0$ , the propagation distance is given by

$$Z_{\max} \approx \frac{w_0}{(n-1)\gamma}. \quad (2)$$

The central maximum propagates for several Rayleigh ranges without appreciable spreading and thus offers a focal line of light. The outer rings of the Bessel beam act to “self-heal” the central maximum and prohibit it from spreading.

We explored laser-assisted poration and transfection of chinese hamster ovary (CHO) cells using a Bessel beam and compared our data to a Gaussian beam of equivalent (central core) diameter. In a standard geometry, the Bessel mode propagates downward into the sample chamber. The experimental setup used here is shown in Fig. 1.

The experiments were all performed with a femtosecond pulsed Ti:sapphire laser emitting at 790 nm, with a pulse

<sup>a)</sup>Electronic mail: xt3@st-and.ac.uk

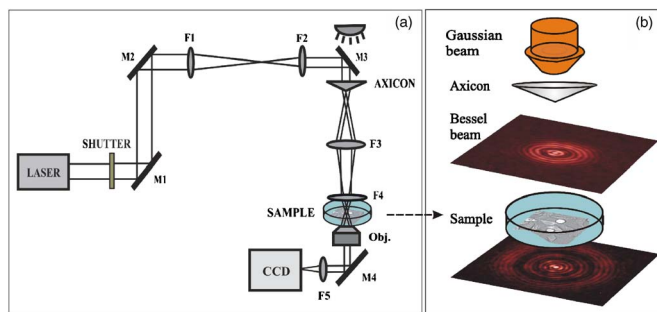


FIG. 1. (a) Bessel beam transfection apparatus. A laser beam is demagnified and is incident upon an axicon which generates the Bessel beam. A telescope demagnifying by a factor of 8, consisting of an achromat and an aspheric lens, is placed in the beam path in order to create a Bessel beam with the optimum parameters for cell transfection. (b) Image of the generated Bessel beam incident upon the sample.

duration of 100 fs and pulse repetition frequency of 80 MHz (Coherent, MIRA). To generate a Bessel beam, an axicon of opening angle of  $5^\circ$  was illuminated by a Gaussian mode from the laser at a mean power of 800 mW. By using a demagnifying telescope, a central core diameter of  $1.8 \mu\text{m}$  and a propagation distance up to  $200 \mu\text{m}$  were achieved. The optical system had a 78% power transmission efficiency yielding a Bessel beam of power 628 mW entering the sample, corresponding to a mean power of 70 mW in the central core.

For the sake of comparison, both the Gaussian and Bessel light fields (central core) were matched in diameter. A  $60\times$  Newport objective was illuminated by a Gaussian mode and rendered a central core diameter of  $2r_0=1.8 \mu\text{m}$ . The estimated confocal parameter was  $6.4 \mu\text{m}$ . The mean power fed into the Gaussian optical system was 100 mW. The efficiency of the optical system was such that the sample was treated with approximately 70 mW which matched the power within the central core of the Bessel beam. For both the Bessel and Gaussian beams, we explored the transfection rate as a function of position  $z$  in the beam propagation (axial) direction. At each position, we exposed  $N=50$  cells to the laser radiation. Cells were positioned at the laser beam focus by manipulating an  $xyz$  translation stage upon which the sample dish was placed.

For the cell transfection experiments, CHO cells were prepared using our previously described scheme.<sup>3</sup> In brief, the CHO monolayer was washed twice with OptiMEM (Invitrogen) and exposed to a  $40 \mu\text{l}$  solution of OptiMEM containing  $1.2 \mu\text{g}/\mu\text{l}$  mitoDSRed plasmid which encodes a mitochondrially targeted *Discoideum* red fluorescent protein (BD Biosciences, Oxford, UK). A 23 mm diameter type-0 coverslip (BDH, Poole, UK) was then floated on top of this  $40 \mu\text{l}$  solution and individual cells were dosed with the laser from above.

Individual CHO cells were treated with three exposures each of 40 ms duration by use of a laser shutter and at power levels of 70 mW at focus. During laser irradiation, no visual response was observed by using a simple homemade bright field illumination system,  $100\times$  long working distance objective (Mitutoyo, numerical aperture of 0.7,  $100\times$ ) and a charge coupled device (CCD) camera. After laser treatment, the coverslip was removed, the monolayer of cells was bathed in 90% modified eagles medium (MEM)/10% foetal calf serum (FCS), and the culture dish was returned to the incubator. 48 h after treatment, cells were viewed by fluores-

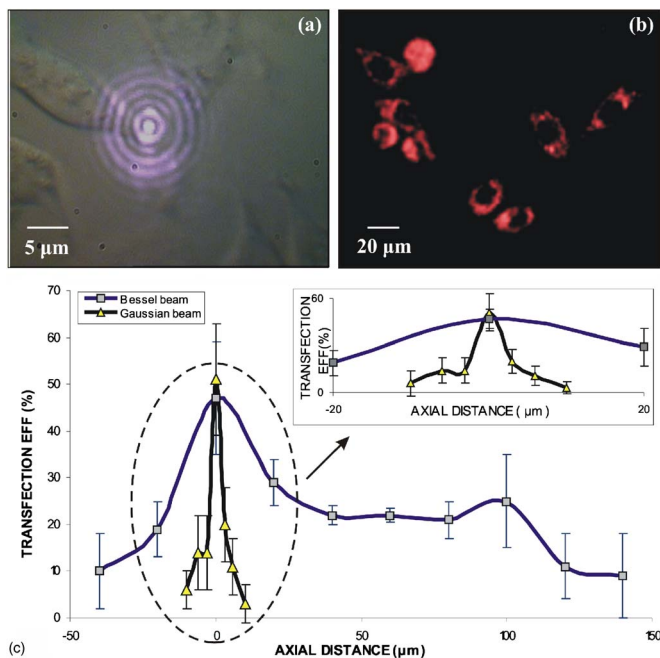


FIG. 2. (a) Bessel beam “focus” is positioned on the cell plane. (b) Upon successful transfection, the cells express the red fluorescent protein and fluorescein red. (c) Bessel beam transfection vs Gaussian beam transfection. We achieve Bessel beam transfection of CHO cells for up to  $180 \mu\text{m}$  along the propagation axis of the beam. Each data point corresponds to the average transfection efficiencies obtained at a specific axial position and includes the number of spontaneously transfected cells, which varies between 0 and 5 for each sample dish. The magnified image of the area around  $z=0$  can be viewed on the top right part of the main graph. As can be seen, the successful transfection of CHO cells can occur over 20 times the axial distance when using a Bessel beam, as compared to the Gaussian beam (when considering the threshold of efficiency to be 20%).

cent microscopy for red fluorescent protein (RFP) expression, allowing the transfection efficiency to be quantified. Images were captured by a CCD camera (Pulnix) and a personal computer equipped with a frame-grabber board. The transfection efficiency was calculated by dividing the number of cells expressing RFP at 48 h by the total number of cells that were treated by the laser in a particular region of interest at 0 h. Cells destroyed or irreversibly damaged as a result of the laser action were included in the data in order to provide a real and representative figure for the transfection efficiency of the femtosecond optical transfection technique.<sup>3</sup> Fluorescent microscopy showed that transient RFP expression had been achieved and that cells expressing RFP are viable and display normal morphology, thereby confirming that successful optical transfection has occurred. Figure 2(a) shows the CHO cells in the presence of Bessel beam prior to photoporation and in Fig. 2(b) the cells are shown expressing the red fluorescent protein after laser treatment.

Figure 2(c) shows the transfection efficiency and compares the Gaussian and Bessel light modes. If we define a threshold for transfection of 20%, for example, from these data we see that the Bessel beam gives us transfection over 20 times the axial transfection length compared to the Gaussian beam. Note that in this case the Bessel beam propagates approximately 30 times the confocal parameter of the Gaussian mode. Previously, we have used the expression of green fluorescent protein as a marker for transfection;<sup>3,7</sup> however, here we chose to use a plasmid which encodes for the mitochondrially targeted *Discoideum* red fluorescent protein. This

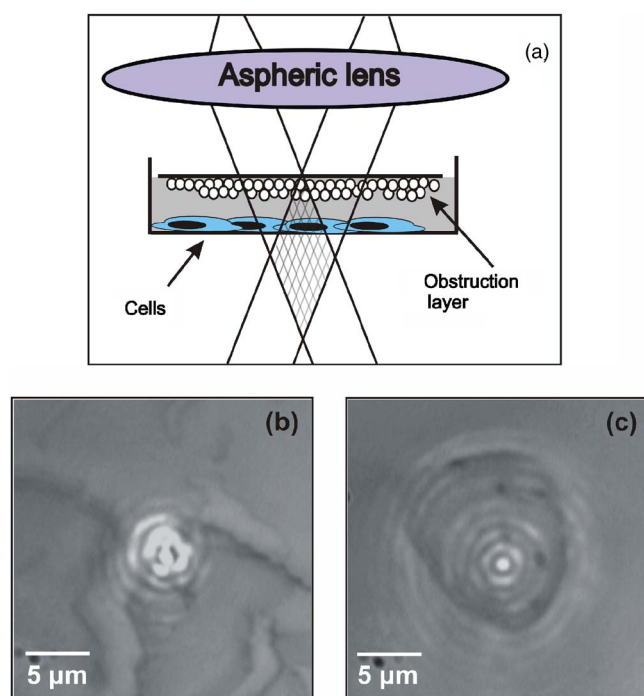


FIG. 3. (a) Bessel beam passes through the obstructive layer of microspheres and suffers distortion. Due to the self-healing property of the Bessel beam, at 50–80  $\mu\text{m}$  away from the layer the beam profile is reformed and reaches the cell plane where photoporation will occur. (b) The Bessel beam profile is distorted after passing through the microsphere obstructive layer. (c) The Bessel beam is reconstructed after 50–80  $\mu\text{m}$  (depending on the sample) subsequently imaged on the cell membrane and photoporation takes place.

additionally proved that the cells were unharmed by the laser as the RFP protein after synthesis was targeted correctly to the mitochondria.

We exploited the self-healing property of the Bessel beam<sup>8</sup> to show transfection through a “turbid” medium. The obstruction consisted of a 23 mm diameter type-0 coverslip (thickness of 80–120  $\mu\text{m}$ ) covered in concentrated polymer colloid solution and spun at 5000 rpm on a photoresist spinner before being baked at 100 °C on a hot plate for 4 min. The diameter of the colloids was 0.9–1.0  $\mu\text{m}$ . This provided a thin layer of randomly distributed dried colloids lightly adhered across the coverslip surface.

Figures 3(a)–3(c), show the experiment and Bessel beam reformation after propagating beyond the obstacle. By adjusting the imaging objective lens by 50–80  $\mu\text{m}$  along the propagation distance, we obtained the reformed section of the beam on the cell membrane and initiated successful transfection at a rate of  $24 \pm (1\%)$ . The observed beam reconstruction distance varied, as expected from sample to sample, as the obstructive layers may vary slightly in thickness from each other and due to the random distribution of the microspheres over the coverslip surface.

We believe that our experiments provide two advancements which will be beneficial for biological applications. Firstly, the need for exact focusing of the laser beam upon the membrane surface has been alleviated, enabling the automation of this technique, as exact determination of the cell surface position will not be required. The second advancement is that the self-healing properties of the beam have been shown to permit transfection through a distorting medium. Thus one can envisage *multiple* cell monolayer planes that allow much higher throughput for laser transfection combined with a laser beam multiplexing method in the lateral direction.

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