# Optically-controlled platforms for transfection and single- and sub-cellular surgery

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## Abstract

Improving the resolution of biological research to the single- or sub-cellular level is of critical importance in a wide variety of processes and disease conditions. Most obvious are those linked to aging and cancer, many of which are dependent upon stochastic processes where individual, unpredictable failures or mutations in individual cells can lead to serious downstream conditions across the whole organism. The traditional tools of biochemistry struggle to observe such processes: the vast majority are based upon ensemble approaches analysing the properties of bulk populations, which means that the detail about individual constituents is lost. What are required, then, are tools with the precision and resolution to probe and dissect cells at the single-micron scale: the scale of the individual organelles and structures that control their function.

In this review, we highlight the use of highly-focused laser beams to create systems providing precise control and specificity at the single cell or even single micron level. The intense focal points generated can directly interact with cells and cell membranes, which in conjunction with related modalities such as optical trapping provide a broad platform for the development of single and sub-cellular surgery approaches. These highly tuneable tools have demonstrated delivery or removal of material from cells of interest, but can simultaneously excite fluorescent probes for imaging purposes or plasmonic structures for very local heating. We discuss both the history and recent applications of the field, highlighting the key findings and developments over the last 40 years of biophotonics research

## Introduction

The familiar techniques of biochemistry have provided remarkably detailed insights into the structure and function of biological tissue, and identifying the mechanistic aetiology of a wide range of disease states and malfunctions. However, they encounter something of a road-block in the study of a range of degenerative diseases and conditions based on stochastic failure or mutation of individual cells. Almost all biochemical laboratory techniques developed over the last half-century are based on ensemble measurements: tens or hundreds of thousands of cells are simultaneously treated, and the average response is recorded. If the cells' deviation is low (and in many conditions, this assumption holds true), then the response is a fair reflection of the sample; however, there is increasing and compelling evidence that small populations of cells exhibit super- or non-responsive behaviour in response to stimuli ranging from heightened oxidative stress to chemotherapeutic drug

administration, even amongst populations of supposedly clonal cells. It has long been theorised that such populations exist, implicated in phenomena such as cancer relapse after remission, but they exist is such low relative numbers that are essentially invisible to bulk cell analyses. As a response, a new generation of tools have been and are being developed, designed to probe individual cells and regions within them, dissecting their function in a manner analogous to the approaches taken by traditional techniques towards whole organism or tissue samples.

## Delivery and sampling from single cells

In order to study live cells at a single- or sub-cellular resolution, tools are required to deliver and remove material across the cell membrane, an effective barrier that has evolved to exclude exogenous substances such foreign DNA, proteins and drugs (Hearn et al 2009). Studded with transporter proteins that actively remove such materials, the membrane presents a major barrier to research in the life sciences and medicine. Introduction of foreign material to cell is vital to biological research, to probe responses to stimuli, introduce new nucleic acids or to “knock down” the activity of existing genes. This process is called transfection or injection, depending upon whether the technique is applied to nucleic acids or other molecules, and the development of optically-mediated membrane disruption has provided a step-change in terms of success rates and the viability of transfected cells.

A wide range of bench-top techniques exist for the delivery of payloads such as drug molecules or DNA plasmids. However, the vast majority of these rely upon ensemble approaches: hundreds of thousands of cells are treated simultaneously, and each receives a dosage within a normal distribution of the applied concentration, which is measured within the cell suspension as a whole rather than within the cells themselves. As a result, such methods are necessarily coarse-grained and all information regarding the detail of dose-response or cellular heterogeneity is lost. Foreign substances (particularly nucleic acids) can be introduced passively inside the cells through active processes such as endocytosis, which may be at least loosely controlled through the application of vectors such as cationic detergents and polymers, which electrostatically interact with the target cell’s lipid membrane (Le Bihan et al 2011). However, this approach is only typically successful in 20-50% of targeted cells, and the mechanisms controlling this remain the topic of some debate. Direct diffusion through the cell membrane can also be utilised to introduce small molecules, but this is not suitable for large or amphiphilic structures which are either excluded or captured by the membrane itself (Seddon et al 2009). However, to achieve precise, quantitative control of dosage and localisation, single-cell techniques must be used.

Initially, techniques centred upon microinjection, where skilled researchers would manually direct a micropipette through the membrane. While successful, this technique is very low-throughput and risks causing severe damage to the target cell; more recently, gentler approaches have attempted to make the membrane permeable optically. Optical techniques provide exquisite control of both the location and magnitude of power applied to a target cell, permitting single- and even sub-cellular experiments to be conducted routinely. When combined with a holographic control system such as a spatial light modulator (Mao et al 1992), these approaches rapidly become amenable to software control and automation, combining the throughput of traditional techniques with the precision of microinjection. If a microcapsule payload of known concentration can be optically trapped and

introduced to the pores so created, truly quantitative investigations into the fundamental mechanics of life become accessible.

## Optical trapping

A fundamental challenge in biology is to be able to directly manipulate cells and their environs while maintaining their sterility and viability. Optical trapping presents an ideal solution: a contact-free force that can be applied through the walls of a cell culture chamber via an infrared wavelength of light that is negligibly absorbed by biological tissue. When focused to a diffraction-limited spot (known as optical tweezers), objects can be trapped in three dimensions, which can then be controlled and moved relative to their environment using either the optics of the laser system or the stage and focus of a microscope.

Ashkin's pioneering work on lasers provided the first clues to the phenomenon, when he observed that micron-sized particles were accelerated by radiation pressure alone (Ashkin 1970). This effect arises from the change of momentum of a photon as it is scattered by an object: this in turn creates an equal and opposite force pushing back towards the most intense region of the laser beam. This force is called the gradient force which, as the name implies, depends in the gradient of the intensity of the trapping beam. Another force, called the scattering force, pushes the particle along the direction of the beam propagation and depends on the intensity of the beam (see Figure 1). This photon pressure is the same effect used by solar sails, such as the recent successful demonstration of the IKAROS probe (Tsuda et al 2012): however, in optical trapping the beam shape is controlled to provide a single energy well that is able to overcome the Brownian motion of the particle, and with which an object can be manipulated.

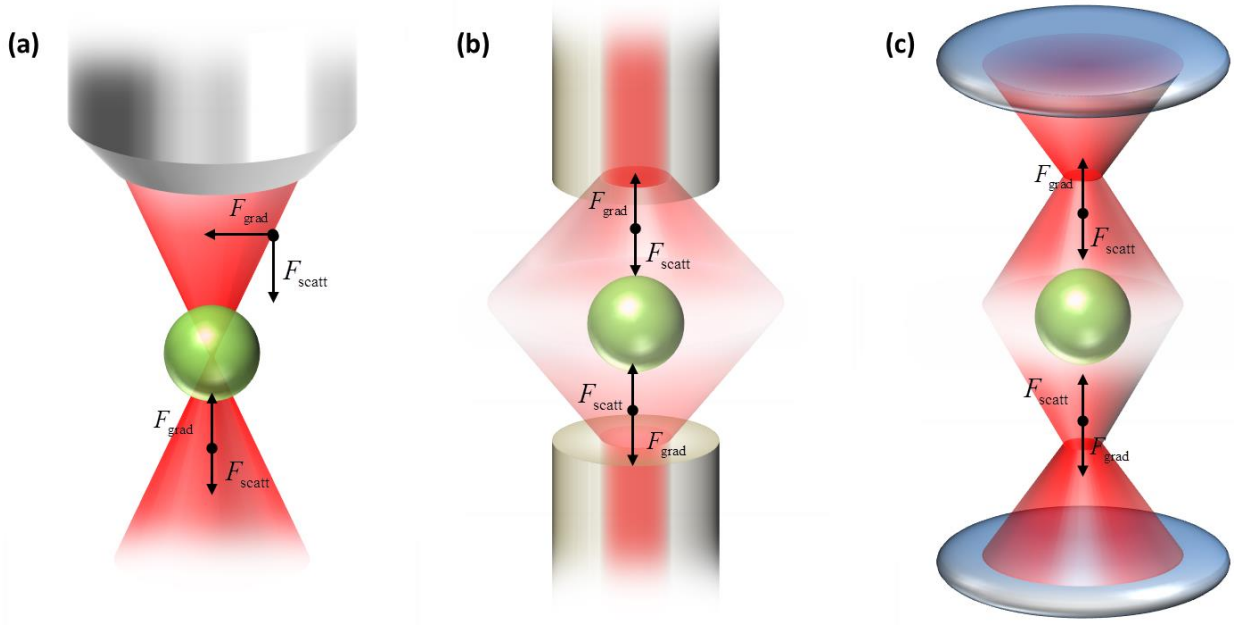


Figure 1. Schematic diagram of different trapping geometries. (a) Tightly focused single beam trap and counter-propagating beam traps using (b) fibres and (c) generalised phase contrast (GPC) method (only final lens shown). The arrows indicate the direction of the forces.

Optical trapping is categorized depending on the size of the trapped particle relative to the wavelength of the trapping beam. In the Mie regime where the radius $a$ of the particle is much larger than the wavelength ($a \gg \lambda$), ray optics are sufficient to describe the strength and direction of the optical

forces. In the Rayleigh regime ($a = \lambda$) the trapped particle can be treated as a dipole. Mathematically, the forces in this regime are given by

$$F_{grad} = \frac{2\pi\alpha}{cn_m}\nabla I_0 \quad (1)$$

$$F_{scatt} = \frac{I_0\sigma n_m}{c} \quad (2)$$

where $I_0$ is the intensity of the trapping beam, $c$ is the speed of light in vacuum, $n_m$ is the index of refraction of the medium, $\alpha$ is polarizability of the particle and $\sigma$ is the scattering cross section. Both the polarizability and scattering cross section depends on the ratio of the index of refraction of the particle $n_p$ and the medium. Between them lies the so-called intermediate regime where the particle size is comparable to the wavelength ($0.1 - 10\lambda$). Most biological samples lie in this range. In this regime, a more elaborate electromagnetic field theory must be used to calculate the forces (Neuman and Block 2004; Zhang and Liu 2008). Force calculation in optical traps is discussed in the work of Rohrbach and Stelzer (2002) and a computational toolbox is presented by Nieminen et al (2007).

A common approach to create single beam gradient force trap is to use high numerical aperture (NA) objective lenses. These lenses generate a tight cone of light forming a beam waist: for a beam with a Gaussian profile, the maximum intensity (and thus maximum trapping force) is to be found at this waist. The versatility of optical tweezers has progressed with the advent of spatial light modulator (SLM) (Mao et al 1992). These systems are generally (although not exclusively) rapid switching liquid crystal (LCD) screens, which may be programmed to display patterns to diffract incoming laser beams into tight foci. Multiple patterns can be generated on the same SLM and the diffracting patterns rapidly recalculated, generating large numbers of individually addressable traps which can be dynamically altered in three dimensions (Hossack et al 2003; Lanigan et al 2012).

High NA lenses impose their limitations, however. A tight cone angle imposes a short working distance (< 250 μm) and typically requires an oil or water immersion lens. This is impractical for many on-chip applications, while experiments requiring elevated temperatures become problematic as heat is conducted through the objective body, resulting in both optical aberrations and extended equilibration times. As a result, SLM beam shaping has been used to generate a number of other beam profiles. For example, Bessel beams comprising concentric rings of light are (for the purposes of microscopy and trapping) non-diffracting, leading to their application in longer-range trapping and sorting experiments. The discussion of Bessel beams and related light-sculpting is beyond the scope of this article, but the interested reader is directed to comprehensive reviews by McGloin and Dholakia (2005) and Woerdemann et al (2012). However, the applicability of such exotic beam-shaping to trapping and micromanipulation applications was recently demonstrated by Brzobohatý et al (2013), who used interference effects between two angled, polarised lasers to generate a long-range “tractor beam”.

A number of other modalities for extending or altering trapping geometries also exist: for example, a trapping effect similar to that of a high-NA lens can be generated using a tapered optical fibre, which provides both a simple technique for the introduction of a beam orthogonal to the viewing plane but also permits the simple organisation of microparticles into ordered patterns and geometries (Xin et al 2012). Another possible trapping configuration is using counter-propagating (CP) beams. This has been also demonstrated for optical fibres (Constable et al 1993) and generalised phase contrast (GPC) -

based trapping beams (Rodrigo et al 2006). CP beams can also be used with lower NA objective lenses. The long working distance offered by these types of microscope objectives allows a side imaging configuration providing a more intuitive optical trapping in 3D (Ulriksen et al 2008). CP beams use the scattering force for trapping, and the axial movement of the trap is controlled by varying the ratio of the intensities of the beams. Because 3D optical traps become less stable as particle size increases, CP beams are more suited to larger objects. The less stringent requirement for a tight focus in CP beams also prevents photodamage for live samples (Thalhammer et al 2011).

Optical trapping provides a way to isolate and manipulate cells of interest but also allows the precise direction of microtools in the region, adding an extra layer of spatial control. Trapping either the cell membrane itself, or (as in most experiments) beads or probes coupled to it by means of a covalent or biotin linker, provides a platform for the performance of mechanical measurements such as a membrane's tensile strength (Neuman and Nagy 2008) or the non-destructive sampling of its constituent material (Lanigan et al 2009). However, such tools on their own only provide access to a cell's outer surfaces: the next step is to penetrate the cell membrane in order to introduce foreign material or gain access to the cell's inner structure. A range of optical techniques are also capable of achieving these steps, and may be delivered co-axially with a trapping beam meaning that all steps in the process may in principle be conducted on the same instrument.

## Direct light-induced membrane disruption

In the majority of trapping applications, light absorption by the target cell or microtool is to be minimised: even laser powers in the tens of milliwatts reach ferocious intensities when focused to an area of just a few square microns. As a result, the majority of trapping systems use lasers in near-infrared (NIR) wavelengths, which experience little to negligible absorption by biological tissue. However, even these contribute heating effects which must be considered: Liu et al (1995) report Chinese hamster ovary cells heating by ≈ 1.15 °C / 100 mW applied, measured by Laurdan fluorescence.

Such effects are normally deleterious, but they have been successfully applied as a tool to promote cell poration in transfection experiments: the excellent review on the topic by Stevenson et al (2010) features a comprehensive list of studies exploiting the phenomenon. Tsukakoshi et al (1984) first demonstrated that nanosecond UV pulses can create self-healing holes in cell membranes that can serve as entry point for genetic material in the cell medium. The reported rate was $10^3$ cells per minute and the success rate was far greater than previous methods. This technique was named optoporation by Palumbo et al (1996): in their work they used a continuous wave (CW) Argon laser operating at 488 nm with 2 W input power and were able to generate holes of similar dimensions to the light spot, although they also demonstrated that exposure greater than 0.5 s killed the cells.

Many of these direct optoporation techniques operate at short visible or ultra violet (UV) wavelengths, which are strongly absorbed by tissue (exemplified in Figure 2). For example, Paterson et al (2005) demonstrated that a 0.3 mW CW violet laser diode was sufficient to induce poration, a decrease of some six orders of magnitude on the powers required using pulsed laser systems. However, these photons are rarely absorbed by the lipids of the membrane itself (which are largely UV transparent except at very low wavelengths), instead being absorbed by the electron-rich, conjugated bonds amongst the cell's proteins and nucleic acids. This introduces the potential for inducing photochemical damage to either the target cells or indeed the plasmid DNA in solution. Optoporation using UV is

therefore typically limited to membranes that are distant from the nucleus to minimise DNA damage. As a result, the majority of such studies report transfection efficiencies of around 30%, which is insufficient to form the basis of a single cell transfection system with any meaningful throughput. There is also significant scattering of light at these wavelengths, and thus they are not suitable for deep tissue penetration.

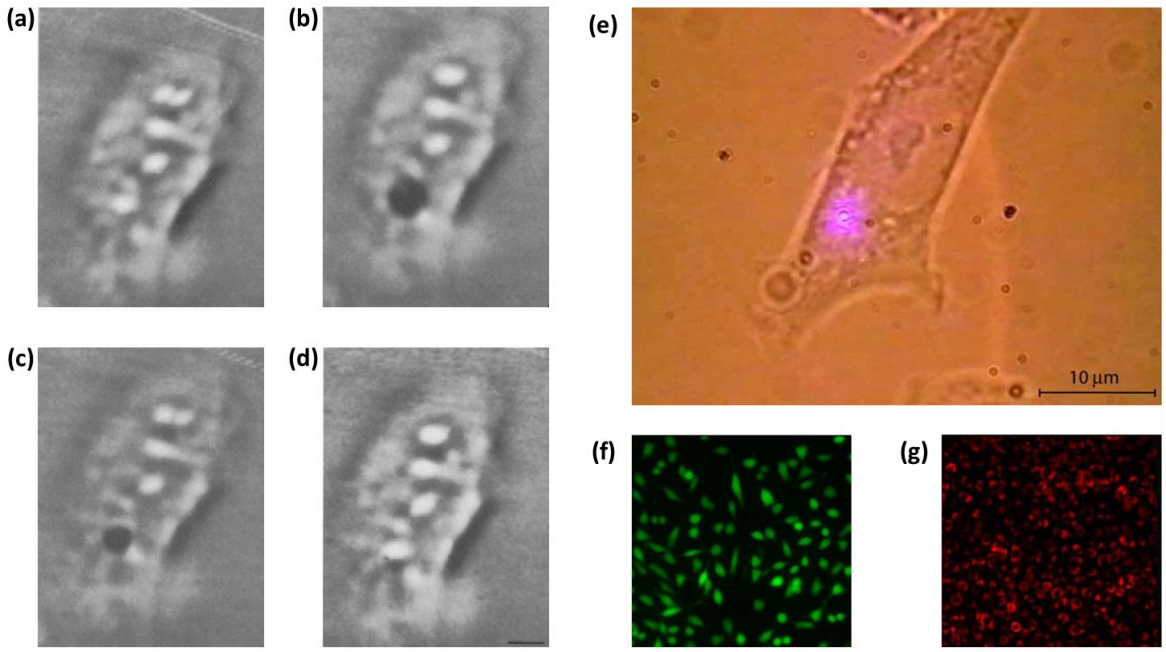


Figure 2. Perforation of normal rat kidney cells using UV nanosecond pulses before irradiation (a); after irradiation (b), a hole is produced as indicated by the arrows and begins to self-heal (c) until it completely disappears (d). Image is reproduced in part from (Tsukakoshi et al 1984) with permission from Springer. A UV laser diode operating in CW is also used in optoporation of Chinese hamster ovary cells (e). GFP fluorescence (f) and expression of red fluorescence protein in mitochondria (g) as well as antibiotic resistance indicates successful transfection. Images are reproduced in part from Paterson et al (2005) with permission from The Optical Society.

Near infrared laser pulses has the advantage of low absorption in living cells except at the focal volume where multiphoton absorption occurs. This provides a very precise “nanoscalpel” that can penetrate depths of more than 100 microns. An experiment demonstrating the laser-mediated knock-out of nanometer-sized regions within the nucleus of living cells and even the dissection of chromosomes has been reported by König et al (1999). However, multiphoton absorption is a relatively rare event meaning that extremely bright laser pulses are required: intensities of up to $10^{12}$ W/cm$^2$ were needed, and power levels slightly in excess of that required were observed to cause catastrophic out-of-focus damage, mediated by the shockwaves generated by plasma cavitation bubbles.

A clever implementation on nanosurgery has also been demonstrated by Ando et al (2008). In their work, optical trapping and surgery was performed by switching the mode of a 780 nm Ti:sapphire laser from continuous to pulsed mode, initially to manipulate a yeast cell and then to rupture its membrane, releasing its intracellular contents. For further examples, the interested reader is directed to an extensive review of the mechanisms of femtosecond nanosurgery of cells and tissue by Vogel et al (2005).

## Mechanism(s) of poration

One of the major difficulties facing life scientists is the extreme complexity of the environment in which the processes operate, and the instability of cellular components when isolated from their native environment. This means that despite the advances described above, the dynamics and mechanisms of pore formation, transfection and membrane resealing remain poorly understood and the focus of some debate. High flux NIR systems such as femtosecond (fs) pulsed lasers are thought to induce a low-density electron plasma through multiphoton effects, causing significant local disruption to the membrane at both the structural and molecular scales (Davis et al 2013). Shorter-wavelength approaches (both CW and pulsed) will inevitably cause substantial local heating due to their high extinction coefficients, although UV techniques are likely to cause a large degree of molecular disruption as well.

Many studies report that the pores formed were similar to the size of the irradiated area. However, it is quite plausible that this is an artefact brought about by the limitations of optical microscopy and the wavelengths of light typically used, as dynamic structures much smaller than ≈ 500 nm are typically very difficult to resolve accurately. Stracke et al (2005) made an attempt to characterise the size of the pores produced via 800 nm fs-pulse irradiation using fluorescein-labelled dextran macromolecules of approx. 13.3 nm diameter: while uptake of these species was observed, this did not cast a great deal of light on the situation given the earlier reports of micron-scale defects. Davis et al (2013) report that pore size and acute recovery time are excellent predictors of long-term cell viability, and used initial fluorescence decay behaviour to measure pore sizes. The pores were found to scale in size with increasing energy, but with a wide spread: cells were observed with pores of < 100 nm and of over 1 µm; still, success rates were low. Recently, our own work has identified optothermally-produced pores with diameters of ≤ 10 nm measured using quantum dots, but the mechanism behind their formation remains a topic of debate (Casey et al 2015).

# Laser interaction with a secondary target

An alternative to direct-light induced membrane disruption uses a secondary target such as gold (Arita et al 2014) or carbon (Gu et al 2011) nanoparticles. These particles can be optically trapped and positioned near the cells, or simply allowed to diffuse freely in the culture medium with only those in contact with the cell membrane at the site of interest receiving irradiation. Alternatively, orthogonal techniques may be employed, such as the creation of multifunctional materials that may be delivered via magnetic or dielectrophoretic trapping techniques, discussed in more depth below (Gu et al 2012). A laser beam can then be used to induce breakdown or other heating of the trapped particle, creating a cavitation bubble that will disrupt nearby cell membranes and facilitate transfection. Secondary targets for laser-induced breakdown (LIB) provide greater spatial control compared to related techniques such as sonoporation (which uses microbubbles formed by acoustic cavitation). Furthermore, the extent of cavitation can be controlled by changing parameters such as the material and particle size in a way that is not easily possible in sonoporation, where bubble size is a function of frequency and thus transducer dimensions. It has also been shown that LIB of gold nanoparticle provides a gentler cavitation than LIB of the liquid medium, thus maximising cell viability (see Figure 3a-b, Arita et al 2014).

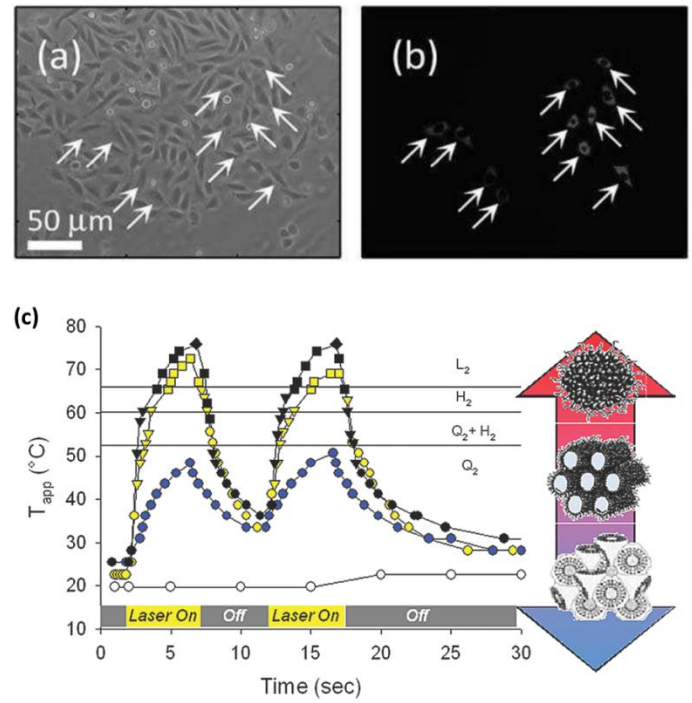


Figure 3. Laser-induced breakdown of trapped gold nanoparticles can form expanding cavitation bubbles. The method has been applied in the transfection of Mito-DsRed plasmids into Chinese hamster ovary (a). Fluorescence image (b) indicates successful transfection (reproduced in part from Arita et al (2014) with permission from SPIE). (c) Gold nanorods (GNR) embedded in liquid crystalline matrix can act as a temperature-controlled drug delivery vehicle. Different concentrations of GNR (white: 0 nM, blue: 0.3 nM, yellow: 1.5 nM, black: 3 nM ) are irradiated with infrared laser to induced localized heating. At different temperatures, the liquid crystalline matrix undergoes phase transitions (indicated by the different symbols corresponding to the rightmost graphics). Reproduced with permission from Fong et al (2010). Copyright 2010 American Chemical Society

A related technique has been developed to provide temperature-controlled drug delivery (Fong et al 2010). Hydrophobic gold nanorods (GNRs) were incorporated within a liquid crystalline matrix and remotely heated by NIR laser. The plasmonic effect provides extremely localised heating to well above biological conditions, flipping the phase of the lipid and releasing the payload, while unintentional drug release was prevented through the choice of lipid composition and its subsequent bearing upon transition temperature (see Figure 3c). Although not immediately applicable to single- or sub-cellular experiments, one can imagine how related technologies could one day be used for targeted chemotherapy or tumour ablation in deep tissue.

The combination of optical manipulation with microfluidics presents the first opportunities to conduct entire biochemical assays from delivery to sampling and analysis at the single cell level, exemplified by the recently-published grab-and-drop techniques of Schrems et al. (2014). In this case, cell membrane fragments were captured from the surface of modified donor cells using modified optically trapped smart droplet microtools (SDMs). These cell membrane fragments were then unloaded to a defined region in solid supported lipid bilayers. The processes of extraction and unloading of membrane fragments were performed in separate chambers in a microfluidic system and the movement of the SDMs from one chamber to the next facilitated by optical traps. When coupled with other emerging technologies such as surface-immobilised immunological assays and TIRF microscopy, (Salehi-Reyhani et al 2011), the potential now exists for truly quantitative dose-response experiments at the single-cell level (see Figure 4).

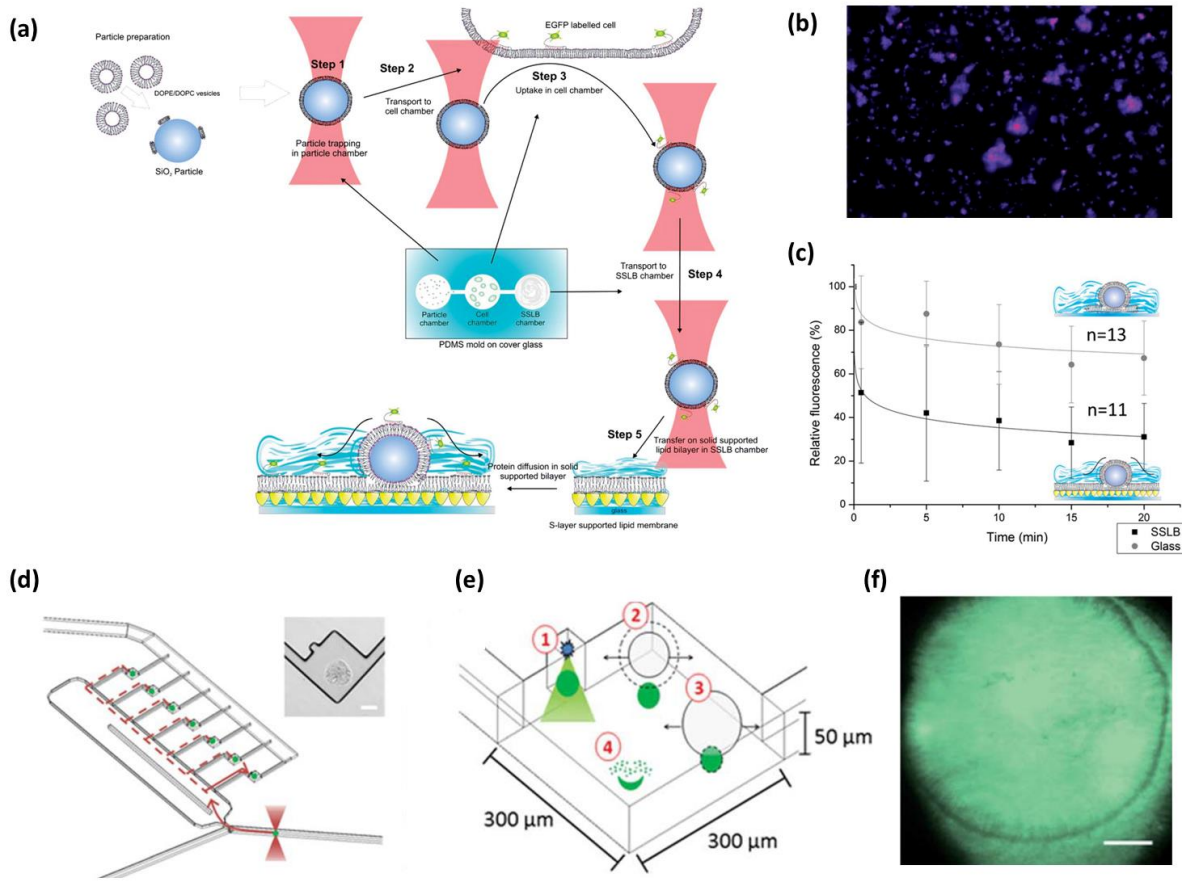


Figure 4. Integration with microfluidics systems allows downstream analysis of biological samples. (a) Grab-and-drop protocol has been shown to load membrane fragments from a cell chamber to smart droplet microtools (SDMs) and unload them at another chamber containing solid-supported lipid bilayer (SSLB). To test the effectiveness of the protocol, the SDMs are labeled with rhodamine (b). The decrease in fluorescence indicates successful unloading of the cargo (c). Images are reproduced in part from Schrems et al (2014) with permission from the Royal Society of Chemistry. Rare cell types such as circulating tumor cells are important specimens in proteomics studies. Integration of optical trapping in microfluidics system allows isolation of cell of interest (d) and subsequent lysis for analysis (e). (f) Anti-GFP antibody spot image by TIRF. Scale bar: 25 µm. Images are reproduced in part from Salehi-Reyhani et al (2011) with permission from the Royal Society of Chemistry.

## Optical tools in combination with orthogonal techniques

Aside from optical trapping, optoelectronic tweezers (OET) have also been used for parallel manipulation of micro- and nanoscopic particles. The setup is quite different from the tightly focused optical tweezers in the sense that the input light is only used to create transient electrical pseudo-terminals in an otherwise resistive layer, inducing localized electric field gradients. The particles then experience dielectrophoretic (DEP) force. Since light is only used as illumination, the required optical power density for OET is around 100,000x smaller than optical tweezers (Valley et al 2008). Simple light sources and SLMs such as a projector can be used to create patterned illumination enabling parallel manipulation. In this instance, the trapping chamber for OET is sandwiched between two glass substrates coated with indium tin oxide (ITO) where the bottom substrate is further coated with a photosensitive film made of hydrogenated amorphous silicon (a-Si:H). An AC bias potential is applied between the two substrates. Depending on the applied bias potential, OET can switch between manipulation and electroporation (Valley et al 2009) and electrolysis (Kremer et al 2014). Reported cell survival rates are around 91% and genome-editing success rates of 73% have been reported (Kaneko et al 2014). Some OET experimental demonstrations are shown in Figure 5. As with optoporation, the mechanism of cell membrane permeation by electroporation is relatively poorly

understood. Proposed theories include electromechanical instability caused by the balance of the electrostatic compressing force and the elastic force of the membrane. Another is the molecular reorientation theory, which indicates that the interfacial polarization leads to a strong trans-membrane field and eventually causes major structural rearrangements. Further discussions on the mechanism(s) of electroporation can be found in the review article by Ho and Mittal (1996).

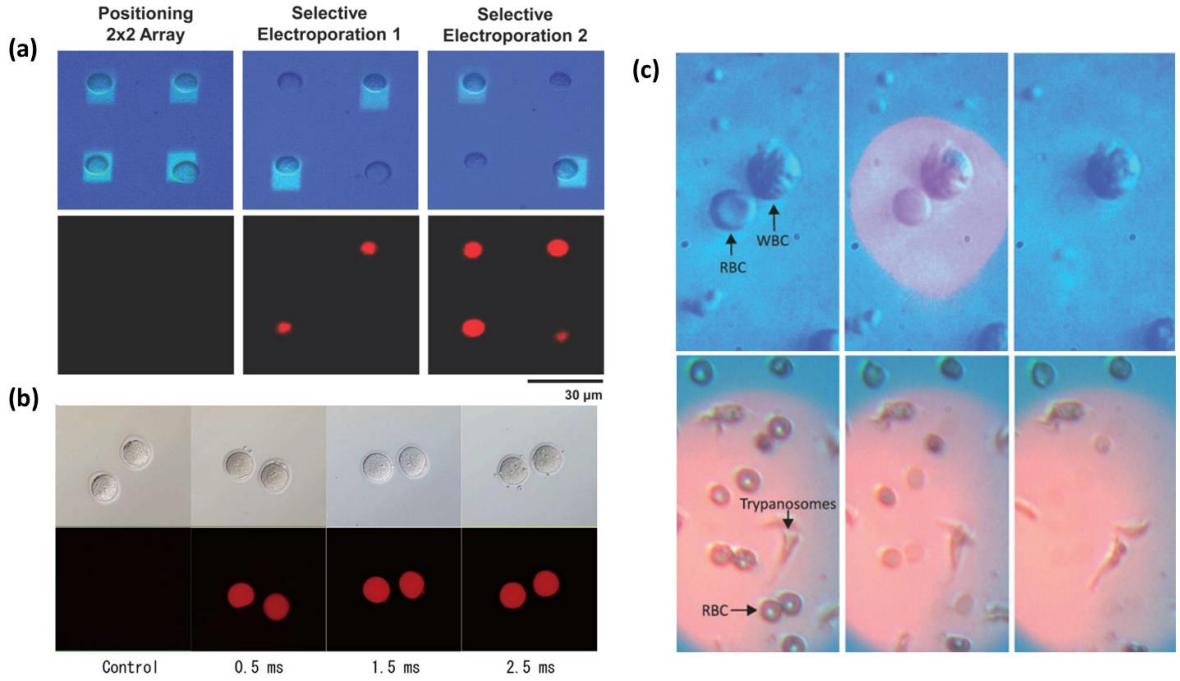


Figure 5. Dielectrophoretic forces can be used to trap biological samples. By increasing the bias voltage electroporation can occur. Fluorescence images of the samples indicate successful electroporation. (a) Electroporation of HeLa cells. Reproduced from Valley et al (2009) with permission from the Royal Society of Chemistry. (b) Electroporation of intact rat embryos. Reproduced from Kaneko et al (2014) under Creative Commons Attribution 4.0 International License. Optoelectronic tweezers uses electric field gradients to perform trapping which is also affected by the shape of the sample. By increasing the bias voltage, selective electrolysis can also be performed (c). Reproduced in part from Kremer et al (2014) with permission from John Wiley and Sons.

## Interactions between traps and cells – multipurpose tools

As previously discussed, irradiated cells experience a rise in temperature even within the infrared "biological window" of laser wavelengths. An experiment was performed by Neuman et al (1999) on *Escherichia coli* which characterized the photodamage caused by a trapping laser in a range of wavelengths from 790 nm to 1064 nm. They found that the photodamage is minimal between 830 and 970 nm but was increased from 879 to 930 nm. However, damage was reduced to background levels under anaerobic conditions which indicates that oxygen plays a role, likely via the medium of its highly reactive singlet state, and that photodamage is linear with intensity suggesting a single photon process.

Aside from detrimental side effects, there are laser-induced phenomena that are valuable for research. For example, fusion of cells has been demonstrated with the aid of UV pulses and infrared optical tweezers in the so-called laser cell fusion trap. The infrared laser was used to bring two cells together and then UV pulses used to cut the common wall between them to allow fusion (Steubing et al 1991). Alternatively, a laser can be used as a scalpel to isolate individual cells from their binding matrix. This has been performed in symbiotic efficiency studies, such as for the nitrogen-fixing soil

bacterium *Frankia* and woody dicotyledonous plants. The bacterium was separated from the nodule for subsequent polymerase chain reaction (PCR) amplification (Leitz et al 2003).

In some applications such as in developmental biology, complete photoablation of subcellular organelles is required. This has been shown in selective gene deactivation by irradiation with light pulses from an argon-ion laser (Berns et al 1969; Berns et al 1981). Intracellular surgery has been demonstrated in living one-cell stage *C. elegans* embryos by ablating the centrosome with UV pulses (Colombelli et al 2004). The use of optical vortices in nanosurgery of living cells has been shown to minimize photodamage and improved the functionality of transported subcellular organelles.

The structural integrity of cells is based upon the actin cytoskeleton in the cytoplasm, a cross-linked network of polymers which maintains its 3D form. One key structure formed by the cytoskeleton is the microtubule, which were selectively isolated from a rat kangaroo (PtK2) cell by Colombelli et al in 2005 using UV laser pulses. This presented a new protocol for measuring shrinkage, growth rate, rescue frequency and their effects on actin stress fibres, which are responsible for cell-cell and cell-matrix adhesions. A similar study on these fibres was performed by Kumar et al (2006), which quantified the viscoelastic properties of the fibres.

Optical guiding is another example of trap-cell interaction. Growth directionality of neurons can be controlled using optical traps: either by direct weak irradiation that generates sufficient gradient force to bias actin polymerization-driven lamellipodia extension (Ehrlicher et al 2002), or indirectly by trapping birefringent spheres with circularly polarized light to generate microfluidic flow that causes shear force against the neuron growth cone (Wu et al 2011).

## Custom fabricated microtools for specialized applications

In exploring biological samples, a micro-to-nano coupling approach is relevant given that nanoscale biological processes must be understood in the context of the living cells where they occur, which are orders of magnitude larger. A typical mammalian cell is tens of microns in diameter and can spread up to 50 microns into attached tissue culture; a similar size range is seen for human mesenchymal stem cells; and an egg cell can reach 100 µm or larger. It is therefore necessary to retain a relatively wide field of view, both transversely and axially, and together have ample space to accommodate more elaborately designed operations with relevant biochemical instrumentation for sustaining live-cell experiments. Unfortunately, the sub-micron imaging depth-of-field and sub-millimetre working distance of high-NA optical tweezers do not support these needs, and so we propose a structure-mediated approach. The next generation of tools for optical trapping builds upon the widely used bead structures exploited for force measurements (Kuo and Sheetz 1993) and material delivery (Kress et al 2009). These new microtools can be custom-made using UV lithography and reactive ion-etching of fused silica or 3D-light printing on polymer using two-photon polymerization (2PP). Some of these tools are presented in Figure 6.

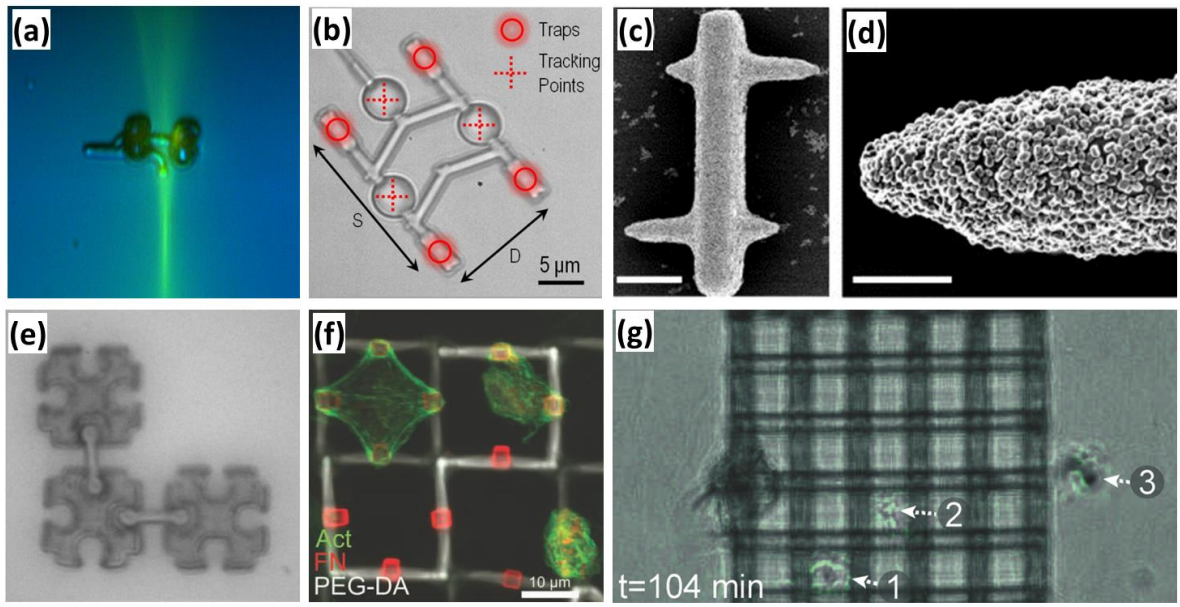


Figure 6. (a) Wave-guided optical waveguide (WOW) are 2PP-fabricated microstructures for light delivery and sensing applications. The image shows coupling of a 532 nm focused beam. Reproduced from Villangca et al (2014) with permission from The Optical Society. (b) Probe for surface topography. The handles for the trapping beams are designed to generate trapping stiffness anisotropy. This allows high sensitivity in the sensing axis. Reproduced in part from Phillips et al (2012) with permission from The Optical Society. (c)-(d) Gold-coated two-photon polymerized 3D microstructures for fluorescence enhancement. Scale bars: 5 um and 1 um respectively. Reproduced in part from Aekbote et al (2014) with permission from Elsevier. (e) Optically-assembled structures for biomedical studies. Reproduced in part from Rodrigo et al (2009) with permission from The Optical Society. (f) Tailored 3D scaffolds to control extracellular matrix distribution. The image shows f-actin (green) from chicken fibroblast and two types of polymers namely Ormocomp (red) and PEG-DA (white). Reproduced in part from Klein et al (2011) with permission from John Wiley and Sons. (g) Woodpile structure for cell migration studies. Numerical labels indicate migrating human dendritic cells across the structure starting from the right. This image is taken after 104 min. The pore size of the woodpile construct is 15 µm × 15 µm. Reproduced in part from Olsen et al (2013) under Creative Commons Attribution 3.0 Unported Licence.

One example would be our own fabricated free-floating waveguides with handles for optical trapping, coined as wave-guided optical waveguides (WOWs). With these tools it is possible to send light from low NA objectives whilst maintaining a tight light confinement at the tip of the WOWs. The WOW has also been demonstrated to work in the opposite direction, meaning the tip can be used for sensing light and pass it through waveguide back to the viewing objective lens (Palima et al 2012). Using these structures in tandem with holography allows genuine 3D light-delivery without requiring substantial investment in bespoke optical hardware (Villangca et al 2014). Furthermore, functionalizing these structures such as coating them with gold nanoparticles has been shown to enhance fluorescence (Aekbote et al 2014). Aside from light delivery, sensing and field enhancement, such microtools can function as mechanical probe as demonstrated by Phillips et al (2012), who demonstrated they are able to measure surface topography with nanometre precision. More passive structures can be used for force measurement (Klein et al 2010), to control extracellular matrix distribution (Klein et al 2011) and for cell migration studies (Olsen et al 2013).

By bringing macroscopic tools and concepts down to micron levels, exciting and new functionalities can be developed for single cell studies which are directly analogous to the familiar experiments and techniques of biochemistry. However, their increased resolution and ability to identify small sub-populations of rare or abnormally responding cells amongst a large background provides the platform required to probe the mechanisms of a range of critically important disease states. As the throughput

of these techniques increases, through a combination of innovative material design and holographic optical techniques, it seems likely that these approaches and their successors will come to dominate both life science research, and eventually clinical diagnoses in everyday screening.

## Acknowledgements

M.V. was supported by Enhanced Spatial Light Control in Advanced Optical Fibres (e-space), a project financed by Innovation Fund Denmark.

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