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-cell 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 details of 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 which provide 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-cell and sub-cellular surgery approaches. These highly tuneable tools have been demonstrated to deliver or remove material from cells of interest, and they 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.

Keywords Optical trapping · Optoporation · Transfection · Single-cell science

Introduction

The familiar techniques of biochemistry have provided remarkably detailed insights into the structure and function of biological tissue, leading to the identification of 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 the 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 that they exist in such low relative numbers that they are essentially invisible to bulk cell analyses. In response, a new generation of tools have been and are being developed with the aim to probe individual cells and regions within them, dissecting their function in a manner analogous to the approaches taken by traditional techniques towards the whole organism or tissue samples.

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Delivery and sampling from single cells

In order to study live cells at the single-cell 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 as 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. The introduction of foreign material into cell is vital to biological research – to probe responses to stimuli, introduce new nucleic acids or to “knock down” the activity of existing genes, among other applications. This process is called transfection or injection, depending upon whether the technique is applied to nucleic acids or to 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 are currently available 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 details of dose-response or cellular heterogeneity is lost. Foreign substances (particularly nucleic acids) can be introduced passively into cells through active processes such as endocytosis, which can 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, as a general rule, this approach is only successful in 20–50 % of targeted cells, and the mechanisms controlling this remain a topic of active debate. Direct diffusion through the cell membrane can also be utilised as a means to introduce small molecules into the cell, but this approach is not suitable for large or amphiphilic structures which are either excluded or captured by the membrane itself (Seddon et al. 2009). Thus, to achieve precise, quantitative control of dosage and localisation, single-cell techniques must be used.

Microinjection was the first technique used to deliver material into a single cell, where skilled researchers would manually direct a micropipette through the membrane. Although successful, microinjection methods are very low-throughput and associated with the risk of causing severe damage to the target cell. This has led

to the more recent development of less aggressive approaches such as optoporation. Optical techniques provide exquisite control of both the location and magnitude of power applied to a target cell, permitting single-cell 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 are within reach.

Optical trapping

A fundamental challenge in biology is to be able to directly manipulate cells and their environs while maintaining cell viability and the sterility of the system. 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 (3D), 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 of optical trapping, when he observed that micron-sized particles were accelerated by radiation pressure alone (Ashkin 1970). This effect arises from the change in 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 on the gradient of the intensity of the trapping beam. A second force, called the scattering force, pushes the particle along the direction of the beam propagation and depends on the intensity of the beam (see Fig. 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.

Optical trapping is categorised according to 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

Fig. 1 Schematic diagram of different trapping geometries. **a** Tightly focused single beam trap; counter-propagating beam traps using fibres (**b**) and the generalised phase contrast (GPC) method (**c**) (only final lens shown). Arrows Direction of the forces

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

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

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

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, α is polarisability of the particle and σ is the scattering cross section. Both the polarisability and the scattering cross section depend on the ratio of the index of refraction of the particle n_p and that of the medium. Between these values 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). For more detail on these topics, 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 used to create a single-beam gradient force trap is to use high numerical aperture (NA) objective lenses. These lenses generate a tight cone of light to form a beam waist: for a beam with a Gaussian profile, the maximum

intensity (and thus maximum trapping force) can be found at this waist. The versatility of optical tweezers has progressed with the advent of the spatial light modulator (SLM) (Mao et al 1992). These systems generally (although not exclusively) consist of rapid-switching liquid crystal (LCD) screens, which may be programmed to display the diffraction patterns of incoming laser beams as tight foci. Multiple patterns can be generated on the same SLM, and the diffracting patterns can be rapidly recalculated, generating large numbers of individually addressable traps which can be dynamically altered in 3D (Hossack et al. 2003; Lanigan et al. 2012).

However, high NA lenses do impose limitations on the system. A tight cone angle imposes a short working distance ($<250 \mu\text{m}$) and typically requires an oil or water immersion lens. These are impractical conditions for many on-chip applications, while experiments requiring elevated temperatures become problematic as heat is conducted through the body of the objective, 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 relatively long-range trapping and sorting experiments. The discussion of Bessel beams and related light-sculpting is beyond the scope of this review, but the interested reader is directed to comprehensive reviews by McGloin and Dholakia (2005) and Woerdemann et al. (2013). 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 and also permits the simple organisation of microparticles into ordered patterns and geometries (Xin et al. 2012). Another possible trapping configuration is the application of 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 which provides 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 for use with larger objects. The less stringent requirement for a tight focus in CP beams also prevents photodamage to live samples (Thalhammer et al. 2011).

Optical trapping provides a method to isolate and manipulate cells of interest and has the additional advantage of allowing the microtools in the region to be precisely directed, 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 mechanical measurements, such as measurement of 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 aims and may be delivered co-axially with a trapping beam, with the result that all of 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 wavelengths contribute

heating effects which must be considered: Liu et al. (1995) report that Chinese hamster ovary cells were heated by $\approx 1.15^\circ\text{C}/100\text{ mW}$ 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 ultraviolet (UV) pulses can create self-healing holes in cell membranes that can serve as entry points for genetic material in the cell medium. The rate reported by these authors was 10^3 cells per minute, and the success rate was far greater than that obtained with previous methods. This technique was named optoporation by Palumbo et al. (1996) who used a continuous wave (CW) Argon laser operating at 488 nm with 2 W input power to generate holes of similar dimensions to the light spot, although they also demonstrated that an exposure duration of $>0.5\text{ s}$ killed the cells.

Many of these direct optoporation techniques operate at short visible or UV wavelengths, which are strongly absorbed by tissue (as shown in Fig. 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 compared to the power 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) and are instead 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. Therefore, to limit damage to DNA, optoporation using UV is typically limited to membranes that are distant from the nucleus. 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.

NIR laser pulses have the advantage of low absorption in living cells, except within the focal volume where multiphoton absorption occurs. This provides a very precise “nanoscalpel” that can penetrate depths of $>100\text{ }\mu\text{m}$. König et al. (1999) demonstrated the laser-mediated knockout of nanometer-sized regions within the nuclei of living cells, as well as the dissection of chromosomes. However, multiphoton absorption is a relatively rare event; consequently, these authors required extremely bright laser pulses and intensities of up to 10^{12} W/cm^2 ; 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 were performed by switching the mode

Fig. 2 Perforation of normal rat kidney cells using ultraviolet (UV) nanosecond pulses. **a** Before irradiation. **b–d** After irradiation (**b**), a hole is produced, as indicated by the *yellow 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 SBM. **e** A UV laser diode operating in continuous wave is also used in optoporation

of Chinese hamster ovary cells. **f, g** Green fluorescent protein (GFP) fluorescence (**f**) and expression of red fluorescent 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

of a 780-nm Ti:sapphire laser from continuous to pulsed mode, initially to manipulate a yeast cell and subsequently to rupture its membrane, thereby 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

Two major difficulties facing life scientists are the extreme complexity of the environment in which biological 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) laser pulses 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). Approaches involving the use of shorter wavelengths (both CW and pulsed) will inevitably cause substantial local heating due to their high extinction coefficients, and UV techniques are likely to cause a large degree of molecular disruption as well.

Many studies report that the pores formed were similar in size to that 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) attempted to characterise the size of the pores produced by 800-nm fs laser pulses using fluorescein-labelled dextran macromolecules with a diameter of ~ 13.3 nm: 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) reported that pore size and acute recovery time are excellent predictors of long-term cell viability, and in their study they 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 >1 μ m. In addition, success rates were low. More 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 open to discussion (Casey et al. 2015).

Laser interaction with a secondary target

An alternative to direct light-induced membrane disruption involves the use of 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 throughout 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, as discussed in more depth in the following section (Gu et al. 2012). A laser beam can then be used to induce breakdown or 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 Fig. 3a, b; Arita et al. 2014).

A related technique has been developed to provide temperature-controlled drug delivery (Fong et al. 2010). The authors incorporated hydrophobic gold nanorods (GNRs) within a liquid crystalline matrix and then remotely heated the GNRs by NIR laser. This plasmonic effect provided extremely localised heating to temperatures 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 Fig. 3c). Although not immediately applicable to single-cell 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 microfluidic systems presents the first opportunities to conduct entire biochemical assays from delivery to sampling and analysis at the single-cell level, as recently exemplified by grab-and-drop techniques reported by 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

Fig. 3 Laser-induced breakdown of trapped gold nanoparticles can form expanding cavitation bubbles. **a** Application of this method for the transfection of Mito-DsRed plasmids into Chinese hamster ovary (transfected cells indicated by arrows). **b** Fluorescence image 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) were irradiated with an infrared laser to induced localised heating. At different temperatures, the liquid crystalline matrix under went 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

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 was 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 Fig. 4).

Optical tools in combination with orthogonal techniques

In addition to optical trapping, optoelectronic tweezers (OET) have also been used for parallel manipulation of

micro- and nanoscopic particles. The setup is quite different from that of 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, thereby inducing localised electric field gradients. The particles then experience dielectrophoretic (DEP) force. Since light is only used for illumination purposes, the required optical power density for OET is around 100,000-fold smaller than that needed for optical tweezers (Valley et al. 2008). Simple light sources and spatial light modulators, such as a projector, can be used to create patterned illumination, enabling parallel manipulation. In this case, the trapping chamber for the OET is sandwiched between two glass substrates coated with indium tin oxide (ITO), with the bottom substrate additionally coated with a photosensitive film

Fig. 4 Integration with microfluidic 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). PDMS Polydimethylsiloxane, EGFP enhanced GFP. **b** To test the effectiveness of the protocol, the SDMs were labeled with rhodamine. **c** The decrease in fluorescence indicates successful unloading of the cargo. Images are reproduced in part from

Schrems et al. (2014) with permission from the Royal Society of Chemistry. **d, e** Rare cell types, such as circulating tumor cells, are important specimens in proteomics studies. Integration of optical trapping in the microfluidic system allows isolation of the cell of interest (**d**) and subsequent lysis for analysis (**e**). **f** Anti-GFP antibody spot image by total internal reflection fluorescence (TIRF). Scale bar: 25 μm. Images are reproduced in part from Salehi-Reyhani et al. (2011) with permission from the Royal Society of Chemistry

made of hydrogenated amorphous silicon (a-Si:H). An AC bias potential is applied between the two substrates. The OET system can switch between manipulation and electroporation (Valley et al. 2009) and electrolysis (Kremer et al. 2014) depending on the applied bias potential. Reported cell survival rates are around 91 %, and genome-editing success rates of 73 % have been reported (Kaneko et al. 2014). Examples of OET in experimental situations are shown in Fig. 5. As with optoporation, the mechanism of cell-membrane permeation by electroporation is relatively poorly understood. One proposed theory is that of electromechanical instability caused by the balance of the electrostatic compressing force and the elastic force of the membrane. Another is the molecular reorientation theory, in which the interfacial polarisation 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).

Interactions between traps and cells – multipurpose tools

As previously discussed, irradiated cells experience a rise in temperature even within the IR “biological window” of laser wavelengths. Neuman et al. (1999) characterised the photodamage caused by a trapping laser of wavelengths 790–1064 nm in an experimental setup involving *Escherichia coli*. These authors found that the photodamage to the cells was minimal at wavelengths of between 830 and 970 nm but that it increased at wavelengths of 879–930 nm. However, damage was reduced to background levels under anaerobic conditions, which indicates that oxygen plays a role in photodamage, likely via the medium of its highly reactive singlet state, and photodamage was linear with intensity, suggesting a single-photon process.

Aside from detrimental side effects, there are laser-induced phenomena that function as valuable research tools. For example, cell fusion has been demonstrated with the aid of UV pulses and IR optical tweezers in the so-called laser cell fusion

Fig. 5 Dielectrophoretic forces can be used to trap biological samples. Electroporation can occur by increasing the bias voltage. *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 use electric field gradients to perform trapping which is also affected by the shape of the sample. **c** Selective electrolysis can also be performed by increasing the bias voltage. *RBC* Red blood cell, *WBC* white blood cell. Reproduced in part from Kremer et al. (2014) with permission from John Wiley and Sons

Fig. 6 **a** Wave-guided optical waveguide (WOW) are two-photon polymerisation (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 2PP three-dimensional (3D) microstructures for fluorescence enhancement. Scale bars: 5 μm (**c**) and 1 μm (**d**). 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. Numbers in circles 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 $15 \times 15 \mu\text{m}$. Reproduced in part from Olsen et al. (2013) under Creative Commons Attribution 3.0 Unported Licence

trap, where the IR laser was used to bring two cells together and UV pulses were then 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, as has been demonstrated in symbiotic efficiency studies, such as in the study of Leitz et al. (2003) with the nitrogen-fixing soil bacterium *Frankia* and woody dicotyledonous plants. These authors separated the bacterium from the nodule for subsequent PCR amplification.

In some applications, such as in developmental biology, complete photoablation of subcellular organelles is required, as shown by Berns et al. (1969, 1981) who achieved selective gene deactivation by irradiation with light pulses from an argon-ion laser. Intracellular surgery has been demonstrated in living one-cell stage *Caenorhabditis 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 minimise photodamage and improve 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. Colombelli et al. (2005) selectively isolated microtubules from a rat kangaroo (PtK2) cell by using UV laser pulses. This technique has become a standard 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), who quantified the viscoelastic properties of the fibres.

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

Custom-fabricated microtools for specialised 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 μm into attached tissue culture; a similar size range is seen for human mesenchymal stem cells, and an egg cell can reach $\geq 100 \mu\text{m}$. 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 by 3D-light printing on polymer using two-photon polymerisation (2PP). Some of these tools are presented in Fig. 6.

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, i.e., the tip can be used for sensing light and passing it through the 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 optical hardware (Villangca et al. 2014). Furthermore, functionalising 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 probes, as demonstrated by Phillips et al. (2012), who were able to measure the 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 the level of the micron, 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.

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Compliance with ethical standards

Conflict of interest Mark Villangca declares that he has no conflict of interest.

Duncan Casey declares that he has no conflict of interest.

Jesper Glückstad declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human or animal subjects performed by the authors.

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