Chapter 3: Optical Tools for Single Cell Manipulation and Analysis

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Abstract Experiments on individual cells require a range of extremely precise tools to permit their selection, manipulation, stimulation and analysis. This is further complicated by the cells' sensitivity to their environment, meaning that such tools must also be very gentle (or at least very localised) to minimise the generation of artefacts. Optical tools provide ideal performance in a number of such roles, exhibiting high spatial and temporal selectivity while causing minimal non-specific effects. This review will focus upon the optical tools that have been developed for these purposes, ranging from optical trapping systems which provide a contact-free technique for the manipulation of micron-scale objects, through to a selection of the different optically-mediated cell membrane disruption methods available for lysis and/or delivery of material.

Optical trapping techniques provide the means to manipulate matter at approximately a 1-100 μm scale, without requiring direct contact with the cell [1]. The use of infrared wavelengths minimises the amount of light absorbed by biological targets, while a range of light-sculpting approaches are available to generate a wide array of complex beams which can be dynamically modified. This means that cells (or micro-structured probes) can be directly manipulated, either to build arrays or to perform mechanical measurements of the properties of the cell membrane.

However, to modulate or measure processes occurring beyond the cell membrane, a mechanism of controlled membrane rupture must be utilised. These can either be destructive, for selective lysis experiments, or reversible, to allow the introduction of material to stimulate responses with minimal disruption to the cell. Both approaches feature a range of modalities: for example, lysis can be induced by direct plasma formation using high-energy pulsed lasers to induce catastrophic damage to anything within a defined radius [2], or can be combined with electrical fields to provide lysis with single-cell resolution [3]. Similarly, photoporation can be accomplished directly with very high precision (although conditions must be finely tuned to minimise cell disruption), or in combination with materials with specific

absorption characteristics to deliver similar effects using much longer wavelengths and commensurately lower cell damage [4].

The theories underpinning these techniques will be discussed, and illuminated using examples of recent research to provide first-hand examples of their successful application. The advantages and disadvantages of each approach will be comprehensively debated, and directions of promising research will be presented to give insight into the tools and techniques likely to be available in the future.

3.1 Introduction

The traditional tools of biochemistry, based around the measurement of the ensemble behaviour of tens or hundreds of thousands of cells, have served faithfully for decades and provided deep insights into the structure and function of many of the proteins and pathways that underpin biological functions. However, by their nature they provide limited resolution: such averaged measurements, necessary to generate the masses and concentrations of sample required for meaningful measurements, cannot identify outliers from the main population and are unsuitable for the analysis of rare cell types. This is of particular importance in the case of circulating tumour cells, widely believed to be the vectors responsible for cancer metastases but present at such vanishingly low concentrations in blood as to be almost invisible [5]. However, it has been demonstrated that stochastic behaviour in protein transcription and regulation makes a substantial contribution to (and may even dominate) cellular responses to a number stimuli and stresses [6]. In order to fully probe these effects, a new range of tools are under development: tools which combine the extraordinary sensitivity and precision required to provide single-cell or even single-molecule resolution, with the throughput necessary to identify quantitative differences between outliers and the median behaviour of a cell population.

Optical platforms provide the ideal basis for this new generation of tools. Coupled with advances in microscopy techniques, particularly in the field of super-resolution microscopy, optical systems provide a view into the inner workings of the cell in unprecedented detail. These microscopy techniques require a textbook of their own right to do them justice and so are beyond the scope of this review, but as a starting point the interested reader is directed to a recent overview of the topic by Godin et al. [7].

3.1.1 Typical sample volumes

The development of tools to precisely handle and measure low volumes of biological material has been at the forefront of life science research almost since its inception. A typical mammalian cell has an internal volume of measured in tens of picolitres (1 picolitre = 10^{-15} m^3), surrounded by a membrane of detergent and protein that is rarely more than two molecules thick. This miniscule envelope

contains a system of dazzling complexity: the ~23,000 genes in your DNA each code for a protein that may be modified in a variety of ways post-transcription, leading to hundreds of thousands of potential forms, expressed at concentration levels spanning some six orders of magnitude. For example, membrane proteins (those studded throughout or somehow affiliated with the detergent bilayer that maintains cellular compartmentalisation) make up some 27% of the human genome and the majority of active drug targets [8], despite being constrained within a vanishingly small total volume.

In order to generate workable volumes, cell biology has often focussed upon the generation of clonal colonies of cells: cells bred from a single progenitor which should be genetically identical. Similarly, feeding and starvation cycles have been developed which co-ordinate the growth phases of cells within a culture, providing some measure of metabolic alignment between neighbouring cells. Despite these methods, wide disparities in responses to stimuli are observed amongst cells in many studies. Some of this will inevitably be statistical: delivery of bulk stimulus to a bulk population of cells will lead to a distribution of effective dosages, leading to a range of responses. However, more fundamental stochastic effects are observed in studies of cellular metabolism, effects which are transmitted throughout the cell [9]: it is hypothesised that these heterogeneities may contribute to a number of phenomena observed at the phenotypic level, such as antibiotic or chemotherapeutic drug resistance.

3.1.2 Early single-cell approaches

In order to probe these single-cell phenomena, a range of single cell techniques were developed. Microinjection techniques were an early approach: using precisely controlled pressures across micropipettes, skilled researchers could isolate, manipulate and inject material into individual cells of interest. Most famously, these techniques were applied to *in vitro* fertilisation: while the first human birth was recorded in the late 1970s [10], this was achieved using relatively crude co-incubation techniques and the first successful microinjection of human sperm was not performed in humans for another 15 years [11].

While undoubtedly a powerful tool, microinjection is challenging to apply to smaller cells, which are much harder to manipulate and experience commensurately greater damage during injection. Extensive discussion of the field of microinjection is available in chapter 7, while detailed protocols are available from Zhang [12] and a comprehensive review of the history and development of the application of the technique to *in vitro* fertilisation is provided by Neri et al. [13].

To ameliorate these issues, research into contact-free techniques has been pursued. A number of approaches have used microfluidic flow systems to sort and capture cells into corrals. Although almost infinitely configurable, such techniques can only be applied to blood-borne or artificially detached cells which have been released from their native culture, as the targets must be free-floating in suspension. A number of innovative approaches have been developed to minimise the disruption to the target cell's surface [14], this still necessitates the removal or the cell from its

surroundings, upon which it depends for a constant stream of signals that maintain or modulate its growth cycles and function. What was required for the study of epithelial tissue, however, was a platform that could provide spatial resolution by delivering the experiment to the target cell, not *vice versa*. Optical tools provide the perfect mechanism to do so, providing a gentle, sterile and contact-free system to manipulate micron-scale objects in three dimensions, or alternatively by delivering powerful pulses of light to disrupt the membranes of specific cells of interest, allowing direct access to their contents one cell at a time.

3.2 Optical trapping

Optical trapping (sometimes referred to as optical tweezers) is a technique based around the combination of light's classical and quantum mechanical properties. Quantum mechanics dictates that a photon has a momentum linked to its wavelength by Equation 1. As light is scattered by a dielectric particle there must therefore be an associated momentum transfer.

$$p = \frac{\lambda}{h} = mc$$

Equation 1

Conservation of momentum requires that every action has an equal and opposite reaction, meaning that as a photon is scattered away from its incident path, a force acts on the scattering object to force it back in the opposite direction. If targeted by an Gaussian beam like a laser, this has the effect of driving the target away from the light source along the centre of the incident beam. The photon intensity is highest in the centre of the beam, meaning that less light is scattered around the edges: the net effect is that the target is trapped and pulled back towards the centre, but flung forwards through photon pressure. This phenomenon is exactly the one used by the IKAROS project in their recent demonstration of solar sail technology, using sunlight upon a 20 m-diameter gold sail to assist in driving a small satellite from Earth to Venus [15].

While this presents an interesting effect, in this form it is of limited use as a propulsion system: on Earth, these effects of light are normally negligible next to gravity and air resistance. However, by using a tightly focused beam, the region of maximum intensity can be restricted into a small three-dimensional space, the beam waist – discussed in detail, along with the mechanics of the trapping force, in section 3.2.2. This creates a far more useful tool, a trap in all three planes, and one that can overcome the Brownian forces experienced by objects of approximately cellular diameters and densities in solution, restricting them within the focal volume. This force directing particles back to the beam waist can be precisely calculated or measured, meaning that tools so captured can be not only be precisely manipulated but also used as picoNewton- or femtoNewton-sensitive force probes for investigating the topography and mechanical behaviour of microscale biological structures.

Practically, this can be achieved using a high numerical aperture (NA) objective lens fitted to an inverted microscope, meaning that in the apparatus' simplest form the microscope's focal range provides *z*-axis control while trapped objects may be moved relative to their surroundings by the use of a stage control. This configuration allows the imaging of the trapped particle utilising the same apparatus used to maintain its position. Remarkably little power is required to exhibit effective trapping: a 5 mW continuous-wave Helium-Neon (HeNe) source emitting at 633 nm may be effectively used to trap polystyrene beads of 5-10 μm diameter in a safe and visually very impressive demonstration of the technique. However, it is important to remember that within the focal volume, photon intensities can be intense: the HeNe laser in the above example would, if focused to a relatively-easily-achieved 5 μm diameter spot, reach an intensity of some 2.5×10^8 W/m^2, or some 200,000 times more intense than sunlight at the Earth's surface. As a result, a number of effects may be observed in some samples due to heating or direct photon damage, and it is typically wise to choose a laser wavelength that features a low or negligible extinction coefficient in the target material.

3.2.1 History and development

Modern optical trapping has developed as a result of the work undertaken by Ashkin in the 1970s during his experiments exploring the nature of radiation pressure [16]. He observed the effects of the scattering and gradient forces which act on a small refractive particle in a beam of light. Light exerts a force on all objects that refract or reflect light due to the change of the incident photons' momentum, which is transferred when they collide with or are diverted by a target, as described in section 3.2. However, for macroscopic objects, these forces are so small that they are negligible compared to other forces acting on the object such as air pressure or turbulence. Ashkin's breakthrough experiments came when he attempted the manipulation of micron sized particles in liquids and gases using a continuous-wave, visible laser light in order to observe the effects of these forces.

His initial experiments used microscopic transparent latex spheres freely suspended in water. An argon laser was focused horizontally through a glass cell and manipulated to focus on single spheres. A schematic cross-section through the experiment set-up is shown in Figure 1.

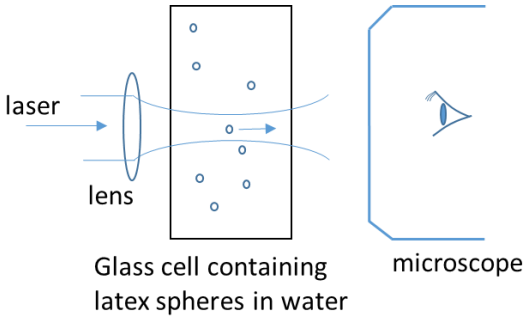

Figure 1: Schematic cross-section of Ashkin's experiment set-up during the discovery of optical trapping [16]

The effects of the laser on the sphere were observed with a microscope. As the beam hit a sphere off-centre the sphere was observed being drawn into the beam axis whilst simultaneously being accelerated in the direction of light from the beam. It moved in this direction until it hits the cell wall where it then remained trapped in the laser beam. If the beam is blocked then the sphere moves away, randomly driven by Brownian motion. More powerful lasers are required as the diameter of the spheres increase relative to that of the beam. By introducing a second beam operating in the opposing direction, Ashkin was able to produce what he termed 'a stable optical well', as shown in the sketch in Figure 2.

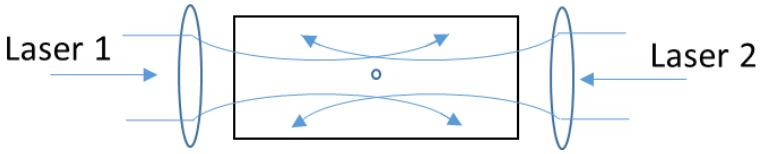

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Figure 2: Plan view of a 'stable optical well', as described by Ashkin [16]

Light can cause temperature gradients across the medium surrounding the particle under investigation. These temperature gradients can produce thermal forces much larger than the radiation pressure, obscuring its effects. This had historically been a limiting factor in exploring the phenomenon. However, Ashkin eliminated these obscuring effects in his experiments through using particles and surrounding medium with low refractive indices, i.e. relatively transparent materials, which minimised the absorption of photons.

Ashkin's experiments initially focused on manipulation of particles ranging in diameter from 0.59 to 2.68 μm as the traps appeared to be most stable in this range. However, results from his experiments suggested that tuneable lasers could selectively accelerate, trap or separate larger particles including single molecules [17].

Further exploration has resulted in the development of optical trap instrumentation enabling the trapping and manipulation of single molecules with nanometre precision along with measurement of the forces acting on the particles. It is this ability for precise measurement that has led to the use of optical traps in biological applications [18].

3.2.2 Theory and optical physics

When light hits an object, some of the light will be reflected by the object and some of it will be refracted. The amount refracted or reflected will depend upon the refractive index of the object. This change in direction of the photons also changes their momentum: the magnitude of the effective forces exerted on the light rays will depend upon the size of the object, the wavelength of the light and the refractive index of the medium. This change in momentum leads to an equal and opposite force in the opposing direction, back towards the centre of the incident beam. For microscopic particles these forces can be significant enough to move the particle. Optical tweezers, as optical traps used in biological applications are often referred, consist of a single laser beam strongly focused through a lens. Infrared trapping beams are commonly used for biological processes as tissue is effectively transparent at these wavelengths meaning little is absorbed by the tissue, minimising optical damage [17].

As the laser beam passes through the microscope lens, the rays converge to a focal point. However, each ray is refracted at the surface of the particle resulting in the change of path and momentum as shown in the schematic in Figure 3. In return the rays of light exert an equal and opposite force on the particle effectively pulling it to in to the centre of the beam and towards its focal point, as shown. The off-axis components of these forces cancel each other out resulting in a restoring force F directed towards the focal point of the laser beam.

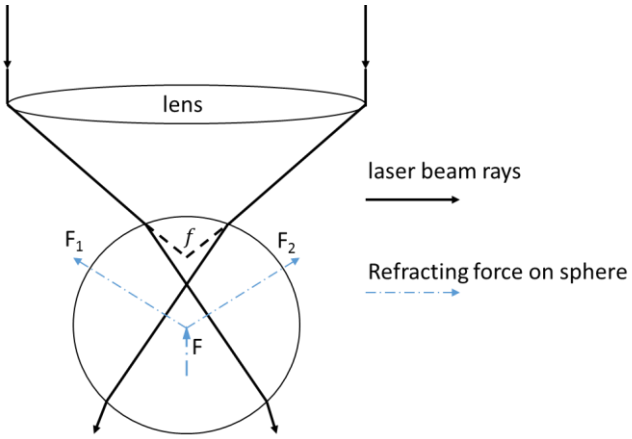

Figure 3: Refracting forces on a particle in an optical trap

The scattering (or pushing) force is a result of the light reflecting off the target particle and acts in the direction of the light propagation and is proportional to the light's intensity as shown in Figure 4. It is the balancing of the reflecting and refracting forces that facilitates trapping of the particle [19].

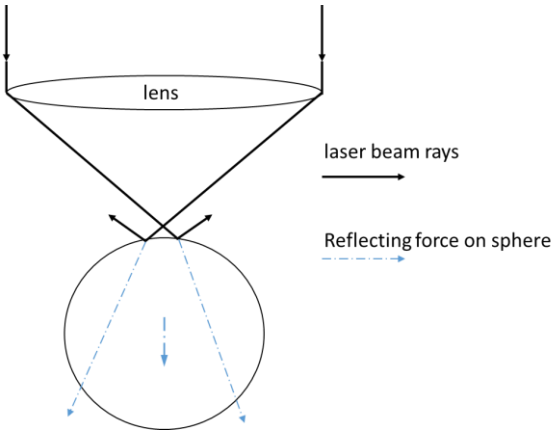

Figure 4: Reflecting forces on a particle in an optical trap

As the size of the particle changes relative to the laser wavelength, the trapping regime also changes. The interaction between light and particles can be explained

by two different theories, Rayleigh or Mie, depending upon the particle size. The Rayleigh theory relates to particles which are much smaller than the wavelength of the incident light. As the particle size approaches that of the light wavelength then the interaction becomes more complicated and can be determined using the Mie theory [20]. Biological applications usually require manipulation of particles in the Mie regime.

The change in direction of the laser beam rays due to refraction at the particle surface will depend upon its angle of incidence along with the refractive indices of the particle and the surrounding medium. This change in direction can be determined using Snell's law (Equation 2, [18]), which states that where n_1 and n_2 are the refractive indices of the particle and of the surrounding medium (generally air, water or oil) respectively, θ_1 is the angle of incidence of the ray with respect to line perpendicular to the particle surface and θ_2 is the angle with respect to the same line at which the ray propagates within the sphere their relationship can be characterised thus:

$$NA = n_1 \sin\theta_1 = n_2 \sin\theta_2$$

Equation 2

In order to trap a particle, the forces that produce scattering of the particles, such as those due to reflection, must be overcome. To overcome this the trap requires a high trapping force which in turn requires a microscope with a high numerical aperture (NA). The numerical aperture of the lens describes its ability to gather the light from the beam, but essentially dictates the focal length of the lens and thus its maximum working distance. Also, in order to obtain a trapping beam with a high convergence angle, the input aperture needs to be adequately filled by the beam.

Optical traps are characterised as having a dimensionless quality factor, Q. The quality factor depends upon the type of trap, the NA of the microscope lens and the target particle size. Ashkin has suggested that Q can be as high as 0.3 for trapping forces acting perpendicular to the direction of the laser beam. The trapping force, F, can be determined from Equation 3 where P is the incident power of the laser beam and $\frac{n_1 P}{c}$ is the incident momentum per second in a medium of refractive index n_1.

$$F = Q\left(\frac{n_1 P}{c}\right)$$

Equation 3

If particles, such as micron sized polystyrene beads can be attached to single molecules then by manipulating the bead and hence the molecule using an optical trap, then forces acting on the molecule can be determined. Through this, biological interactions can be investigated if the bead is attached to, for example, a DNA molecule [18].

3.2.1.1 Spatial Light Modulators

Spatial light modulators (SLMs) are screens or masks which can manipulate the shape of light as it is reflected, refracted or passed through the device. They come with varying degrees of sophistication: an over-head projector is a spatial light modulator, as is a cinema projector. However, those based upon remotely addressable screens can display rapidly-updating patterns, allowing the shaping of incident light into complex arrangements. In optical trapping applications, the screens can be used to generate holographic patterns of concentric rings, effectively a sinusoidal wave in two dimensions. These diffract light into the Fourier transform of the display, providing a highly-focused point of illumination which makes an excellent optical trap.

These screens can be mechanically, optically or electronically controlled to alter their patterns, providing dynamic control over the properties of the transmitted or reflected light. This means that arrays of traps can be created and individually controlled, or that a range of alternative trap geometries can be explored to induce or alleviate the effects of optical aberrations. Early devices were based upon relatively slow-updating nematic liquid crystal displays [21]. In order to maintain several traps at once, multiple holograms had to be calculated and spatially multiplexed simultaneously, which led to a high computational cost. More rapidly updating systems such as ferroelectric liquid crystal systems with millisecond response times mean that a range of much simpler holograms can be generated and overlaid temporally rather than spatially [22]. A similar effect can be achieved with mechanically-actuated mirrors, but the very rapid switching of an SLM means that many more traps can be implemented and controlled, while the system's outputs are infinitely more flexible than a mirror of fixed geometry.

A system developed in laboratories at Imperial College utilised this technique to generate up to 24 individually addressable traps based on a single ferroelectric SLM [23]. This approach used each of the red-green-blue colour channels in 8 bit-planes to generate an individual trap, as the SLM output was by its nature monochrome making the intensities equal. By interlacing these relatively simple holograms onto the SLM at 60 Hz, each trap could be manipulated without interfering with the others, and by programming the system in OpenGL the entire process could be conducted using a graphical processing unit designed for gaming, thus minimising data transfer across the system and the load upon the controlling computer's CPU. This system was driven through a point-and-click interface written in the LabVIEW programming environment, but more intuitive and flexible systems have been developed such as the multi-touch platform pioneered at the University of Bristol which allows trapping control using an interface similar to an Android or Apple tablet [24].

3.2.2 Practical implementation

The installation of a single optical trap on an inverted microscope is a remarkably straightforward exercise, and effective trapping can be achieved at low powers (≈ 5 mW) making the system a powerful demonstration of optical physics for the

classroom or outreach activities as well as a tool in research. A comprehensive guide to the construction of such a system and its optimisation for force measurement is provided by [25], while the theory behind such measurements is discussed in section 3.2.3.2. For simple trapping, however, all that is required is a laser source, some form of alignment mirror system such as a periscope arrangement and a beam-expander to fill the back aperture of a high numerical aperture microscope objective (see Figure 5, below). A steering lens or mirror arrangement may be fitted to the path to provide a measure of additional control over a trapped object, but is not essential.

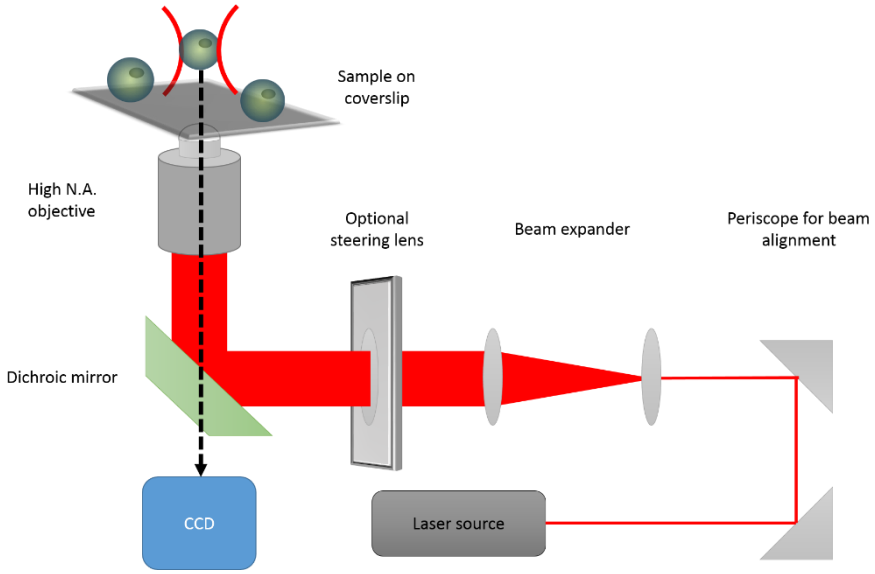

Figure 5: Schematic of a simple single-trap system built into an inverted microscope. Trapping of 5-10 µm polystyrene particles can be easily achieved with laser powers as low as 5 mW. N.A.: Numerical aperture; CCD: charge-coupled diode; digital camera. The captured image is shown using the black dashed arrow.

More complex systems with more traps can be produced through the use of either a beam-splitter to produce a dual-trap system, or the introduction of an SLM after or instead of the alignment mirrors to generate multiple, individually addressable holographic traps. An alternative technique was also recently developed, using multiple static microlenses to create a geometric pattern of stable traps, allowing the assembly of an optically confined microarray of individual cells without the use of complex optics or software [26].

A trapping system even simpler than that described in Figure 5 can be produced using a tapered optical fibre to produce the tight focal volume required. Such a fine

point can be relatively simply produced using chemical etching or microforge apparatus such as that manufactured by the Narashige Group (Japan), used routinely for the production of micro-pipettes. These shaped fibres produce a trap at a defined distance from their point that may be simply steered by moving the fibre itself using a micromanipulator or piezoelectric stage, allowing the rapid organisation of microscopic objects at angles and geometries that are not always available using conventional optical tweezers [27]. More recently, unusual behaviour has been observed showing the ability of these systems to trap multiple objects end-to-end, providing a regular spacing in one or two dimensions, controlled by the cone angle of the tip [28, 29]. This relatively simple set-up provides lower spatial precision than a conventional system, but provides a range of analogous and sometime complementary characteristics that may be explored for limited investment of time or resources.

3.2.2.1 Effects of trapping on biological systems and structures

When designing an optical trapping system, it is vital to consider the effects of the incident light upon what are often extremely delicate samples. Photons absorbed by the sample are very likely to release their energy as heat, and the tightly confined focal volumes mean that light intensities in can easily reach levels in excess of 10^9 W m^{-2}, even when using low laser powers such as those outlined above. As such, even a small extinction coefficient in the sample will lead to rapid heating and damage.

In order to minimise this, it is normal to use micron-scale wavelengths when applying optical trapping for the study of biological systems. In this window, absorbance by most biological tissue is almost zero, meaning that samples can in theory be handled indefinitely without ill-effect. Such wavelengths are conveniently accessible using Nd:YAG (neodymium-doped yttrium aluminium garnet) and Yb-fibre lasers, which are commonplace is trapping systems both for this reason and because they can provide high power densities capable of maintaining multiple traps simultaneously. However, even at these wavelengths heating has been observed in cell-sized vesicles [30]; while the majority of cells and their components may be unaffected, some cell types, metalloproteins and organelles exhibit different absorption spectra to the median. These tissues will be selectively heated and thus damaged by the trapping beam, although the average temperature profile of the target may only rise by a degree or two. Optical trapping has also been observed to cause changes in cellular behaviour, inhibiting growth and division in bacteria even at low powers [31]. Such effects must be carefully considered when assessing the applicability of trapping techniques to an experimental problem.

3.2.2.2 Safety

Safety must be a prime consideration when building or using trapping systems, particularly bespoke or home-build instruments which may lack the safety features and robustness of off-the-shelf models. Micron-scale wavelengths such as the Nd:YAG's primary output at 1064 nm are in the infrared, and as such are invisible

to human vision. This means that a user working on a poorly-aligned or -shielded system will have little or no indication of exposure unless beam visualising cameras or other tools are available.

The majority of trapping lasers in research use are Class 4, meaning that they are capable of causing significant damage to exposed tissue, and may cause permanent and catastrophic eye damage even from indirect reflections. As a result, the most significant risks are experienced during assembly and alignment, when the beam is imperfectly positioned and beam tubes and other shielding are likely to have been removed to improve access to the instrument. Alignment must be conducted at the lowest possible laser power visible through the available imaging equipment, and users must wear goggles suitable for filtering the wavelengths and powers at hand, as well as removing all reflective items from their person such as rings, watches etc. Practically, a major source of risk can be eliminated if the system can be aligned by using the microscope back-light rather than the laser itself: this light should propagate down the same path as the laser if no sample is present on the stage to block its path, and the white light sources generally used (typically filament bulbs or LEDs) pose little or no risk, particularly when compared to the laser systems. Once assembled, trapping systems should be interlocked and key-switched to prevent unauthorised use or access to the beam path.

Beams which are diffracted using an SLM or other technique must dump unwanted energy into a specialist beam dump to prevent unwanted reflections and heat build-up. Similarly, it is good practice to for a beam-stopper or other shield above the plane of the microscope stage, as trapping beams will typically be entering from underneath the sample and otherwise unwanted reflections may cause injury.

3.2.3 Applications in single cell studies

3.2.3.1 Cell manipulation

The most obvious application for optical trapping in single cell studies lies in the manipulation of the cells themselves. Optical traps provide a gentle, sterile and contact-free method to arrange cells within a chip or microfluidic system, isolating them by dragging them through a labyrinth or narrow channel for independent analysis [32], or arranging them into arrays for parallel screening. Most cells are too large to be wholly trapped (typical mammalian cells have diameters in excess of 20 μm, while a typical trap focus is below 2 μm), although bacteria and viruses can be effectively captured [33]. However, one or more traps can be effectively used to capture a region of membrane in a detached cell, which normally provides sufficient purchase to move it through solution. Alternatively, silica or polystyrene beads which trap strongly due to their strong scattering of light can be functionalised with a biochemical handle such as an antibody or biotin, which may be used as an anchor point for cells engineered to express a membrane associated avidin protein [34].

However, the majority of optical traps are based around infrared lasers, which operate at wavelengths at which the cytosol is essentially transparent. This means that organelles with different optical properties such as mitochondria may be

trapped and handled while within the cell body [35], while in small systems such as zebrafish embryos, cells and other small objects may be moved and probed *in vivo* [36].

3.2.3.2 Single cell force measurements

The precise and contact-free trapping mechanism afforded by optical tweezing provides the idea tool for the quantitative measurement of membrane stiffness and environment viscosity, in a manner similar to atomic force microscopy (AFM) but with several major advantages. Most obviously, the optical trapping techniques offer the opportunity to measure in three dimensions, as opposed to AFM which measures almost exclusively in the *z*-direction. A micron-scale sphere or other probe can be captured and the magnitude and rate of its Brownian motion analysed in real time (or close to it), which when coupled with information about the trapping force, can give great insight into the environment of the probe and the barriers and heterogeneities within it. One valuable property of optical traps is that the trapping force obeys Hooke's Law, i.e. the restorative force increases linearly with distance as the particle strays or is pushed from the centre of the trap. This makes the calculation of the resistance exerted by the target object relatively simple to calculate.

Currently, such data are best acquired through the use of a quadrant detector [37], which provides extremely fast response rates and nanometre resolution by breaking the region of interest into four separate zones, effectively quadrupling the frame rate and providing extremely high sensitivity to movements of an object around the centre point where the four quadrants meet. Particle tracking algorithms can then follow the probe, typically a high refractive index and/or albedo particle such as silica, through its random walk about the focal volume and identify any deviations from the norm. Although this approach is only really applicable in the *x* and *y* directions on a typical inverted microscope, as the combination of short focal distance and non-linear response in the *z* direction (in which a particle typically experiences the weakest trapping forces) limits its applicability, for most viscosity measurements the environment can be considered isotropic. In membrane (or other microstructure) rigidity or elastic modulus measurements, this weakness can be ameliorated by approaching the target from all angles: unlike AFM, an optical probe can be brought into contact with the object of study from any side. An important practical note to remember, however, is that binding between many probe materials and biological structures is commonplace, and that optical trapping requires a defined scattering edge to operate effectively. As such, care must be taken to "block" the probe's surface, either by way of material choice or through the use of some agent like bovine serine albumen.

This and related techniques have been successfully applied in investigations including the measurement of kinesin stepping [38, 39], and analysis of the mechanical properties of membrane lipids in giant vesicles, where the response of the trapped particles could be directly measured as a function of membrane composition and the protein content of the surrounding medium [40].

A more direct approach to measuring the structural properties of biological microsystems has been developed in the laboratories of Jesper Glückstad, who has utilised two-photon polymerisation techniques to develop a range of micro-probes with a designed, three-dimensional structure comprising three or more handles connected by a chassis attached to a probe-tip. The rigid body of the ~40 µm tools, combined with the additional stability conferred through the application of multiple traps, may be manipulated in all axes to provide direct force measurements. However, the group has also incorporated a microscopic waveguide into their designs, permitting the same tool to be used for spectroscopic analysis of the target at extremely high precision and in a plane orthogonal to normal microscope geometry [41].

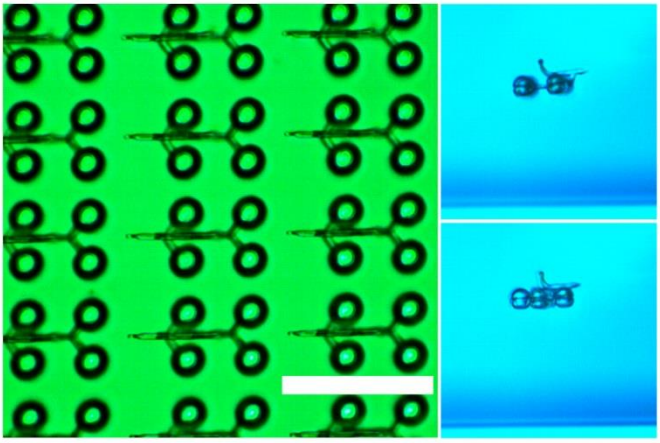

Figure 6: Optically-guided probes created by two-photon polymerisation on glass. Each may be manipulated in true 3D by gripping each bead with an individually addressable optical trap, providing the means for both direct force measurements and, through the incorporation of a waveguide into the probe tip, the ability to introduce light to a sample in the x-y plane (scale bar = 40 µm). The inset pictures show side-view image of these probes being manipulated in solution [41]. *Figure used with permission from the Optical Society of America.*

The binding of microtools to biological materials can itself be utilised, however. Coupling biological polymers to silica or polystyrene beads provides a handle or handles by which their spring constants or affinities for a target can be measured. The pico- or femto-Newton forces applied through trapping can, with care, be used to pull individual proteins from membranes or ligands from receptors in such a way as to resolve individual events within the unbinding process. Typically, many such recordings are required to statistically eliminate the inevitable noise resulting from thermal fluctuations, but the technique in nonetheless a powerful one. In an intriguing recent demonstration, such tools were linked to force-feedback controllers allowing the user to experience a physical measure of the resistance felt by the trap as interpreted by the system software [42].

3.2.3.3 Sub-cellular sampling and biopsy

As alluded to in the chapter introduction and elsewhere within this book, in the ideal case it should be possible to repeatedly monitor and quantify the individual responses of large numbers of cells from a population, while causing minimum perturbation to those specimens in the process. Through this approach, it will be possible to build up a solid statistical model of both the bulk tissue response and the heterogeneity of reaction amongst cells to a given stimulus. This is of critical importance in the study and treatment of diseases showing high resistance to treatment or persistent relapses, implying some reservoir of infection or "stem-cell"-like behaviour that complicates treatment [43].

Again, optical tools provide the ideal combination of precision and lightness of touch to repeatedly and non-destructively sample specific regions of a cell – particularly its surface, the home of >30% of its presently druggable targets [44]. Tools for sub-cellular sampling have typically revolved around delicate microinjection or electrowetting techniques requiring a direct puncture of the cell membrane using a piezoelectric actuator [45]. However, we have recently published a protocol in collaboration with the University of Natural Resources and Life Sciences in Vienna, Austria utilising optically trapped, micron-diameter probes surrounded by a fusogenic detergent bilayer to directly extract functional protein from the membrane of a cell and deposit it into a supported planar membrane for analysis [46]. This technique samples using detergent solubilisation techniques alone, meaning that an intact membrane is present at all times and repeated samples could be removed without damaging the cell.

This and other techniques promise the first genuinely scalable single-cell measures of heterogeneity of response: information which will help identify the differences between high- and low-responding cells under treatment. Such approaches can and must be simply and rapidly automated, to provide huge data-sets from each individual cell of study across a broad population. This is of value in situations where rare cells can be of crucial importance, such as leukaemia and circulating tumour cells in cancer metastases, but also in such cases as bacterial resistance to therapy where rapidly-changing phenotype amongst a small population of cells can determine the outcome of a broadly applied treatment.

Sampling and measurement approaches are insufficient to conquer such issues alone, however. The volumes of data generated using these and related approaches are such that in practice, as much thought and preparation must be applied to its management and analysis as is devoted to its initial generation.

3.2.4 Alternative beam profiles

While simple trapping systems described above can provide a useful tool, they are limited by a number of factors such as their short working distances and the fixed position of the trap generated. It is possible to shift the centre of a trapping beam using manual controls such as a steering lens or adjustable mirrors, although

early designs simply maintained a static trap and used the movement of the microscope stage to achieve relative motion. Similarly, multiple individually addressable traps can be generated using a beam splitter and mirrors in a 4-f arrangement [47]. However, manual control of traps is extremely cumbersome in real experiments, particularly one in which cells or samples must be moved through a complex environment. As such, the development of optical components which allow the rapid and automated sculpting of an incident laser beam greatly expanded the applicability of optical trapping to micromanipulation experiments. Early approaches centred upon the use of fast-scanning lasers, directed through actuated mirrors which shared the beam between a number of sites, and relying on the fluid viscosity of the medium to retain target localisation between cycles [48]. However, there is a limit to how rapidly such a system can scan and as such a limit on the number of stable traps that can be generated, and a more elegant solution was presented by the development of spatial light modulators.

3.2.4.1 Bessel beams and related wave masks

One of the major limitations of optical trapping as a technique comes from the relatively short working distances available (generally below 250 μm). Optical trapping depends upon an extremely narrow focal volume to generate its forces, with a typical diameter of only 1-2 wavelengths of light: the spread of a Gaussian light source, typically characterised by the Rayleigh range Z_R which denotes the distance over which the beam area doubles, depends upon the wavelength λ and the beam waist radius w_0 according to Equation 4 [49].

$$Z_R = \frac{\pi w_0^2}{\lambda}$$

Equation 4

It can therefore be seen that maintaining a tight focus in a situation where $\lambda \approx w_0$ requires a tight cone angle, thus a high numerical aperture lens and a commensurately short working distance. These high numerical aperture lenses normally require water or oil immersion and thus thermal contact with the target coverslip or chip. This can lead to serious experimental issues, as these distances are often incompatible with cell culture or microfluidic assemblies. Similarly, thermal gradients through the objective assembly can generate optical aberrations which may distort the traps or forces produced, although they can in some cases be avoided by allowing the entire microscope to reach thermal equilibrium. However, the wavelengths used for trapping are normally invisible to the human eye and filtered out by the microscope optics, meaning that identifying and ameliorating such aberrations can be a frustrating and time-consuming task.

One approach that has been developed to extend the range of trapping effects is through the use of Bessel (or at least approximations of Bessel) beams, light shaped to form a series of concentric rings which are effectively non-diffracting over the range of normal microscopy experiments [50]. Although these structures possess no

beam waist and thus cannot form a true three-dimensional trap, they can be used to direct material in a given direction across relatively long ranges, corral objects with a defined long axis such as many bacteria and also have applications in trapping low-refractive-index materials which would be repelled by a normal Gaussian trap, sequestering them in the dark regions between light rings.

Figure 7: A typical Bessel beam profile. The white regions (indicating laser beam) show negligible diffraction over the working range of a microscope, meaning they can be used over longer distances than a normal Gaussian trap.

The scope and complexity of the field is beyond the scope of a broad, practical guide such as this, but the interested reader is directed to comprehensive reviews elsewhere [51, 52]. However, a related approach using interfering plane waves has been recently developed that allows the generation of long-range trapping forces: the first demonstration of a genuine tractor beam. This approach allows micron-scale spheres to be directed via control of the incident wave's polarisation rather than gradient forces normally used for trapping, greatly extending its effective range [53].

3.3 Optoporation and optically-controlled cell lysis

Experiments to manipulate and sample cells are extremely valuable in watching their response to stimuli, either in terms of their cell-level structural and motile behaviour or even in the expression of markers at a molecular level. However, for truly quantitative studies, such observations must be tied to a known dose of said stimulus and this is another area in which traditional techniques are weak. In normal biochemical experiments from drug-receptor binding assays to genetic modification, payloads are dissolved or dispersed throughout the bulk cell medium to a known concentration. This payload is then taken up by the cells: either passively via diffusive processes governed broadly by Overton's Rule [54, 55]; actively in the case of specific nutrients or biomimetic substances such as the chronic myeloid leukaemia drug imatinib [56]; using delivery vectors such as cationic lipids or

polymers to trigger endocytosis [57] or finally via direct disruption of the membrane through applied voltage, ultrasound or heating [58]. Each of these approaches is generally successful when applied in the correct context, but each delivers a distribution of material to the target cells based upon their position within the well or culture dish, the chemistry of payload and state of the cell at the time of administration. As a result, biochemical dose-response data are famously noisy, and all information is lost about the cell heterogeneity that contributes a substantial proportion of the effect.

Optical tools, either alone or in combination with other modalities, provide a complementary technique to these studies. In general, the high photon flux required means that optical techniques operate at the low-throughput, single-cell level, but in doing so provide a level of precision, control and quantitation far in excess of that available to traditional experiments. Optical cell poration typically operates via one of two mechanisms: either a short-duration pulsed laser is used to generate free electrons at the cell surface which create a localised plasma whose shockwave can mechanically disrupt nearby membranes; alternatively a continuous-wave (CW) system may be used to cause sufficient heating to destabilise the hydrophobic bonds between lipids within a defined volume, triggering defect formation. The narrow three-dimensional focal volume of an optical trapping system makes it the ideal CW photoporation system, turning the cell manipulation tool developed earlier in the chapter into an all-purpose single-cell biology workstation.

Both pulsed and CW approaches have advantages and disadvantages, and both are capable of causing significant damage to the target membrane and wider cell if intensity or exposure time is too great. These destructive effects are also of value, however, permitting the approach to be utilised as a selective lysis tool, rupturing individual cells of interest to allow the analysis of their contents or even therapeutically as a precision ablation tool.

As with other topics in this chapter, an in-depth analysis of optically-mediated membrane disruption and its applications could fill a textbook in its own right and so this should only be taken as an introduction to the topic. The interested reader is, however, directed to the review of Stevenson et al. for a concise, thorough review of the state of the field [59].

3.3.1 Pulsed laser poration

The poration of a cell membrane and its subsequent transfection using laser light was originally demonstrated by Tsukakoshi et al. [60]: their use of a nanosecond-pulsed UV laser ($\lambda = 355$ nm) was the first to generate microscopically observable membrane holes which would heal over the course of 1-2 seconds after the cessation of the light pulse. Their technique showed relatively low success rates of DNA transfection, but these were ameliorated by a throughput of thousands of cells per minute, meaning that the technique could be used to generate statistically valid populations of test subjects. However, the reported success rates were strongly coupled to the portion of cell illuminated: cytoplasmic irradiation showed $<1\%$ efficacy, whilst nuclear targeting yielded $\approx 10\%$ transfection under ideal conditions.

This was one of the first indications of the mechanisms underpinning the heterogeneity of response described in section 3.1: the region of the cell that a penetrating payload encounters may have a strong influence upon the degree of response it triggers. Such information is irretrievably lost in bulk studies.

The high extinction coefficients experienced by short-wavelength light in biological tissue means that extremely low-powered lasers can induce effective poration, with powers as low as 0.3 mW sufficient to induce observable defects [61]. However, the lipids of the cell membrane are nearly transparent in UV and visible wavelengths, meaning that the absorption must be elsewhere in the cell. The majority of biological macromolecules such as proteins or DNA will strongly absorb in these regions, but this in itself poses a problem: the energies of the photons involved are of the order of chemical bonds, meaning they are sufficient to trigger substantial photochemistry which is likely to irreparably damage the affected proteins or nucleic acids. This strong absorbance also means that cells other than those on the surface closest to the laser are difficult or impossible to target, and certainly may not be probed without causing catastrophic damage to any intervening tissue.

Two-photon effects can ameliorate some of these shortcomings, however. Under the right conditions, two identical photons can sum their energies to form one of twice the energy (and thus half the wavelength) [62]. This effect requires very high light intensities from pulsed laser sources, as the two photons must arrive at the chromophore at almost precisely the same instant and in phase with each other. This means that the effective absorption coefficients observed are both nonlinear and many orders of magnitude below those experienced using normal illumination. However, the effect means that pulsed infrared lasers may be used to achieve similar effects to UV sources, eliminating many of the off-target problems and providing scope for investigations through several layers of cells, as photons arriving individually or in regions of lower intensity (outside the focal volume) experience little or no interactions with the tissue through which they pass. Such a technique is routinely applied to selectively excite short-wavelength-absorbing fluorophores in tissue without interacting with those around it [63]; photoporation requires significantly higher intensities but proceeds via the same process.

Different pulse intensities and durations trigger different effects: femtosecond-pulses will typically generate micron or sub-micron scale pores within an individual cell, which can be targeted with remarkable precision. In an elegant recent demonstration, Waleed et al. utilised the difference in focal length of two different laser wavelengths through the same optics to deliver optoporation pulses precisely to the membrane of a target cell, using an optically trapped microbead as a gun-sight and subsequently as a delivery vehicle for plasmids once the pore had been established (Figure 8; [64]).

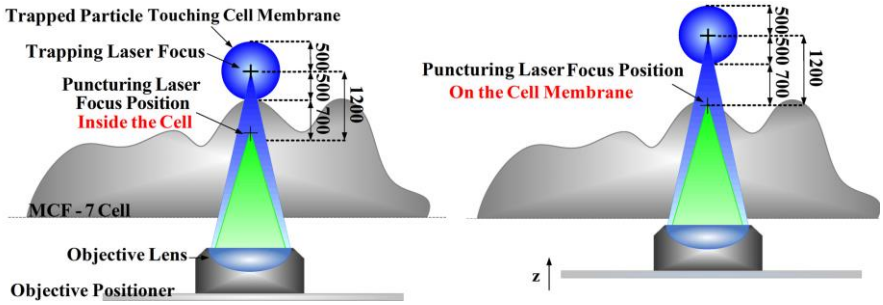

Figure 8: Waleed et al. used the intrinsic difference in focal lengths of two different laser wavelengths to provide extremely precise targeting of a cell membrane by means of an optically trapped microbead [64]. *The bead was held in a λ = 1064 nm trapping beam (blue), which was found by calculation and measurement to have a focal length of 1.2 μm longer than a pulsed poration beam of λ = 800 nm (shown in green). Precise alignment of the other optical components meant that the bead could be used as a sight for the poration pulse, while functionalisation of the bead with plasmids meant it could be used to directly deliver nucleic acid through the pore created. Figure used with permission of the Optical Society of America.*

Longer duration pulses of the order of picoseconds or nanoseconds will generate bubbles whose shockwave can lyse, damage or porate many cells across a range of ≈100 μm. The effects of these bubbles decrease with distance from the laser focus, meaning that different degrees of disruption can be inflicted upon a range of cells in the same chamber [2, 65].

3.3.1.1 Secondary target pulsed laser techniques

An alternative approach to using two-photon absorption to avoid cell damage comes from the use of secondary targets in the cell culture medium, materials which strongly absorb infrared wavelengths and transduce the laser energy into another form. This is typically an optothermal approach, although plasma formation using the right conditions. These materials can either be incorporated into the cell chamber structure itself, or can be introduced in the form or micro- or nanoparticles that offer opportunities for precision delivery via an alternative (ideally orthogonal) manipulation strategy. Care must be taken to ensure that the target material does not itself induce artefacts in the cells under study, and materials which are not wholly biocompatible and/or biodegradable are likely to accumulate over repeated treatments, exacerbating effects, but used carefully this tool can provide a pathway to the poration of even the most sensitive of cells and membranes.

Arita et al. have demonstrated this technique using an optical trap to isolate an individual gold nanoparticle, triggering its laser-induced breakdown with a nanosecond laser pulse at a different wavelength using some three orders of magnitude less energy than that required to achieve the same effect in water alone [4]. Fan et al. have demonstrated that a similar effect can be mediated by a thin layer of amorphous silicon irradiated using an infrared source with microsecond pulses [66], while Wu et al. have recently expanded the utility of the technique by making

it possible to apply secondary-target techniques to large numbers of cells in batches of ~100,000 over the course of approximately a minute [67]. In this experiment, the researchers developed a multi-layer cell culture and poration chip incorporating micron-scale regions of TiO_2 only 100 nm thick. This titanium dioxide film absorbs the nanosecond-duration, λ = 532 nm pulses applied to generate cavitation microbubbles in the manner described above, which in turn create membrane pores of around 3µm in diameter allowing the introduction to the target cells of relatively large biomolecules. This provides a substantial improvement over previous techniques to increase throughput, which relied upon moving the cells through a laser focus via microfluidic flow focusing and thus required that the cells were detached from their native culture before treatment [68].

3.3.2 Continuous-wave poration and lysis

In contrast to pulsed laser sources, the intensity of CW lasers is typically many orders of magnitude lower and so lasers of infrared wavelengths are insufficient to generate the same plasmas. As a result, CW lasers almost always operate via an optothermal mechanism with a secondary target, similar to that described above. As in the case of pulsed systems, orthogonal target steering methods can be used to selectively porate or lyse cells of interest. Gu et al. recently demonstrated this technique using iron-doped carbon nanoparticles which could be localised in target regions by application of a small magnet, before irradiation with an infrared source [69]. Opto-electronic tweezers are a variation on this theme, using focused, low intensity CW light to facilitate precisely controlled dielectrophoresis. Such techniques are well-covered elsewhere in this book and will not be covered further here, although research by the groups of Jon Cooper and Steven Neale in Glasgow have recently demonstrated that such a platform can be utilised to electroporate cells selecting both spatially and for cell morphology, based upon the "electrochemical shadow" that the cells cast upon the amorphous silicon surface [3, 70]. Amorphous silicon layers can be used to directly facilitate optoporation, however: the microsecond-pulse experiments of Fan et al. [66, 71] and continuous-wave experiments in our own laboratories [72] have demonstrated that hot-spot generation upon infrared irradiation of thin-layer silicon is sufficient to cause single- or even sub-cellular poration (see Figure 9, below).

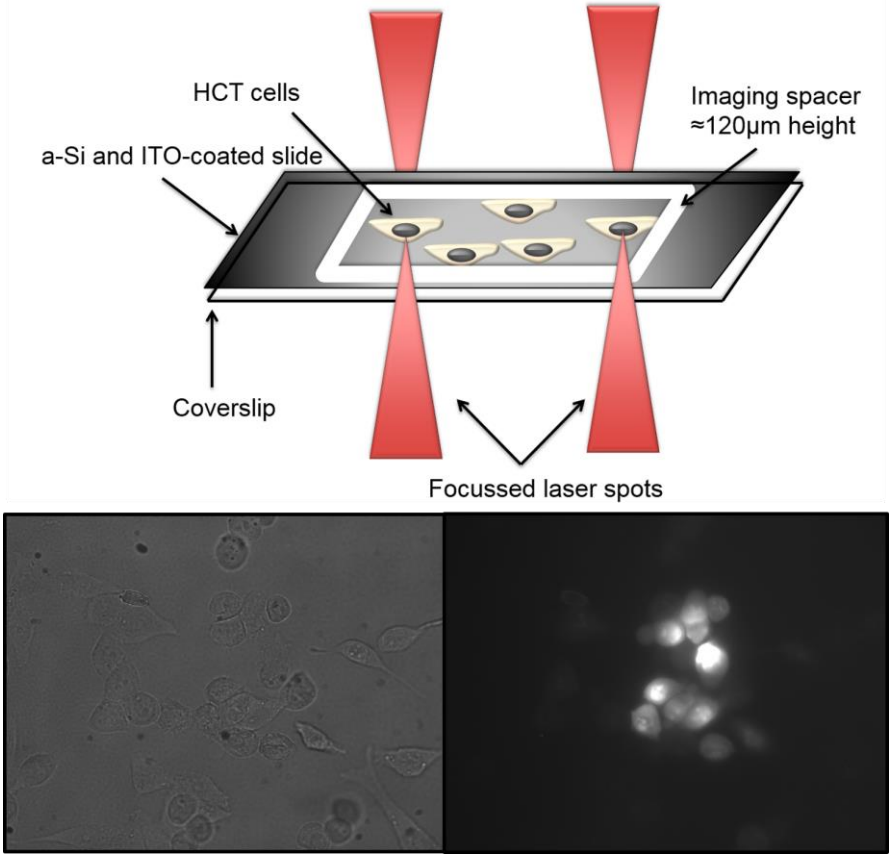

Figure 9: (Above) a schematic of the experimental apparatus used in our laboratories for continuous wave optoporation (λ = 1064 nm). HCT116 cells [73] *were cultured directly onto thin-layer amorphous silicon and assembled into a closed chip, then inverted so as to hang pendant. (Below) Bright-field and fluorescence images showing selective poration of HCT cells, illustrated using propidium iodide* [72].

If tissue lysis or ablation is the aim, CW sources become the method of choice: infrared lasers with powers of 5-50 W are routinely used in medicine for highly localised tumour ablation, particularly in cases where small tumours are scattered across a region of tissue such as is often the case in secondary cancers such as small hepatocellular carcinoma [74]. In such cases, the technique's applicability is greatly strengthened by the surgeon's ability to steer the light using a flexible, narrow fibre optic cable and the light's relatively short penetration length of around 12-15 mm, minimising damage to healthy tissue.

3.4 Conclusions

Optical tools provide an almost universal tool for single cell biology experiments, although what is gained in flexibility and precision is often lost in

throughput. The sterile, contact-free manipulation techniques provided by optical trapping allows extremely gentle spatial control in three dimensions between multiple microscopic objects simultaneously, while coupling trapping with either co-axial high intensity sources or the introduction of carefully chosen secondary targets allows the delivery of specific payloads to a specific cell or cells, all with sub-micron accuracy.

At present, such techniques are generally confined to the research laboratory as throughput is too low and optical path-lengths through tissue too short to be of immediate clinical significance. However, tissue ablation through fibre-coupled lasers, a process requiring less finesse of power and exposure time, is already a routine surgical tool, and as control systems develop in line with endoscopic delivery tools it is likely that optical manipulations and payload delivery platforms will become increasingly important in medicine as well as fundamental research.

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