Single cell optical transfection

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The plasma membrane of a eukaryotic cell is impermeable to most hydrophilic substances, yet the insertion of these materials into cells is an extremely important and universal requirement for the cell biologist. To address this need, many transfection techniques have been developed including viral, lipoplex, polyplex, capillary microinjection, gene gun and electroporation. The current discussion explores a procedure called optical injection, where a laser field transiently increases the membrane permeability to allow species to be internalized. If the internalized substance is a nucleic acid, such as DNA, RNA or small interfering RNA (siRNA), then the process is called optical transfection. This contactless, aseptic, single cell transfection method provides a key nanosurgical tool to the microscopist—the intracellular delivery of reagents and single nanoscopic objects. The experimental possibilities enabled by this technology are only beginning to be realized. A review of optical transfection is presented, along with a forecast of future applications of this rapidly developing and exciting technology.

Keywords: optical injection; optical transfection; optoinjection; phototransfection; optoporation; photoporation

1. INTRODUCTION

Light is incredible. It can be harnessed to image both the tiny and the enormous, spanning the scale from the microscopic to astronomical. It is now 345 years since the microscopic illumination of single cells by Robert Hooke, and the light microscope has proven a reliable workhorse in our understanding of many fundamental life processes. However, with the advent of the laser, there was a distinct paradigm shift in how light could be used in microscopy. Not only could light actively manipulate cells—ablating, moving, cutting, stretching and spinning with elegant dexterity (Guck et al. 2001; Kohli et al. 2005b; Fisher et al. 2007; Brown et al. 2008; Uchugonova et al. 2008a). As the technology continues to move from physics laboratories into mainstream biology, the power of single cell nanosurgery and micro-environmental control is being realized. Using a focused laser, DNA, RNA and other nanoscopic particles can be placed directly inside a cell.

The injection of a nucleic acid species by laser microbeam irradiation has many names, but we refer to it as optical transfection (see the electronic supplementary material, nomenclature). Why is transfection important? As the relationship between DNA, RNA and protein was being unravelled in the mid-1900s in the form of the now famous ‘central dogma of molecular biology’ (Crick 1970), molecular biologists began using bacterial plasmid DNA or other cloning vectors to alter the genetic makeup of organisms or cells. Today, there are numerous applications of recombinant DNA including agricultural biotechnology, biopharmaceuticals, genetic therapy and cell biology. In agricultural biotechnology, genes are used to increase the yields of plants grown on semi-arable land, allowing the cultivation of food in arid, wet or highly saline conditions. New generations of crops containing higher nutritional values are being developed. In medicine, recombinant proteins can replace their traditionally animal-derived equivalents. For example, recombinant insulin used in the treatment of diabetes mellitus type 1 was approved by the Food and Drug Administration in 1982 and today largely replaces bovine- or porcine-derived sources. In genetic therapy, there is the prospect of using recombinant DNA to ameliorate or cure a variety of diseases by using the body’s own expression system to restore correct cellular function (Gao et al. 2007). In cell biology, a protein encoded for by a gene-of-interest may be tagged with

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a fluorescent product such as green fluorescent protein (GFP). Protein-tagged chimeras (i.e., a protein-GFP hybrid) may then be used to assess the spatial location of the protein-of-interest, its interaction with other fluorescently tagged proteins via fluorescence resonance energy transfer analysis or the change in spatial location in response to chemical or environmental stimulation. In fact, it would be difficult to imagine the study of a single disease that has not benefited from DNA recombinant technology.

In the cell biology genre, a gene of interest is typically isolated, inserted into a plasmid DNA vector (immediately adjacent to a GFP encoding sequence), amplified in a bacterial system, harvested, purified and finally transfected into a cell. In this final step, using a highly focused laser to inject DNA offers a number of advantages over existing transfection technologies. It is highly compatible with standard microscopy optics; the most focused point of the laser is aligned with the image plane so that the operator may very easily observe the cell during transfection. It is a non-contact and aseptic method. The cell culture configuration may therefore remain ‘closed’ to the external environment during dosing. In contrast, it is challenging to maintain an aseptic environment on a microscopy platform with an ‘open-top’ tissue culture configuration; this configuration is often necessary for competing techniques employing micromanipulator arms such as capillary microinjection or single cell electroporation (Hewapathirane & Haas 2008). Most importantly, optical transfection is compatible with the ubiquitous sandwiched-coverslip configuration used in standard microscope geometries including microfluidic, lab-on-a-chip applications. Finally, although competing technologies can in principle be adapted for single cell reagent delivery, these systems are often significantly more complicated to use. Virions or single nanoparticle capsules could be optically tweezed to a single cell (Ashkin & Dziedzic 1987; Sun & Chiu 2004), or single cells could also be isolated prior to dosing. The true value of optical injection lies in its simplicity of operation compared with these systems.

Optical transfection has been demonstrated using a variety of laser sources and the laser–cell interaction differs depending on the laser source used. With continuous wave (CW) lasers, the mechanism is probably based on a localized heating of the plasma membrane. With pulsed sources, the reaction of the cell depends strongly on the pulse duration. For very short pulse durations (femtoseconds—fs) and high repetition rates, free electrons generated at the cell surface cause a low density plasma to open a single pore. Application of kilohertz laser repetition rates introduces more than an order of magnitude increase in pulse energies and here one relies on thermoelastically induced formation of very small transient cavities. Longer pulse durations (nanoseconds—ns) generate free electrons but also demonstrate heating, bubble formation and thermoelastic stresses (Vogel et al. 2005). Fs lasers are therefore highly suitable for targeting single cells. In contrast, the use of ns lasing for transfection has not been reliably demonstrated to target single cells, but rather has typically created therapeutic shockwaves that target many dozens of cells at a time. As the field stands high repetition rate, near infrared fs-pulsed lasers are the present source of choice for single cell transfection. A comparison of the various sources and their efficiencies is shown in table 1. For completeness, we briefly highlight ns and CW transfection prior to concentrating on fs lasers, but the interested reader is referred to a more detailed recent review on this subject (Stevenson et al. 2010).

2. OPTICAL TRANFECTION WITH NS-PULSED SOURCES: MULTI-CELL TRANSFECTION

The optical transfection of plasmid DNA into a cell using a focused laser was first demonstrated by Tsukakoshi et al. (1984). In their seminal work, a ns-pulsed laser at 355 nm was focused to a 0.5 μm diameter spot on the plasma membrane of normal rat kidney cells which were bathed in a solution containing plasmid DNA. Days later, the cells had been transfected. The age of optical transfection had begun. As previously mentioned, the mechanism of ns-pulsed poration is thought to involve shockwave generation (Venugopalan et al. 2002) which can span many cell widths in distance from the target (Soughayer et al. 2000). This large therapeutic zone can either be doughnut-shaped (often dubbed ‘optoporation’; Krasieva et al. 1998; Soughayer et al. 2000; Venugopalan et al. 2002; Rhodes et al. 2007) or circular (dubbed ‘laserfection’; Rhodes et al. 2007; see the electronic supplementary material, nomenclature). Early reports of transfection efficiencies were less than 14 per cent (Tsukakoshi et al. 1984; Tao et al. 1987; Gao et al. 1995; Shirahata et al. 2001; Knoll et al. 2004; Badr et al. 2005). Note that we are careful here to distinguish between injection (of non-nucleic acids) and transfection (of nucleic acids) efficiencies. More recent reports indicate high injection efficiencies of 85–100% while retaining up to 80 per cent viability (Clark et al. 2006). Although transfection efficiency is not stated in the latter study, it is probably much greater than earlier reports; the optical transfection of small interfering RNA, for example, results in stronger protein knockdown than traditional lipoplex-mediated delivery (Clark et al. 2006; Rhodes et al. 2007).

3. OPTICAL TRANFECTION WITH CW SOURCES: TARGETED SINGLE CELL TRANSFECTION

CW sources are largely reliant upon heating to cause the membrane to be more receptive to foreign material. Including the absorbing chemical phenol in the cell culture medium during dosing is a common practice (Palumbo et al. 1996; Schneckenburger et al. 2002; Nikolskaya et al. 2006). CW transfection is a single cell technology. The therapeutic zone is limited to the plasma membrane of the cell, and neighbouring cells are unaffected. Although CW sources offer high post-irradiation viability (Schneckenburger et al. 2002;
Table 1. Laser sources used for optical injection, probable mechanisms of action and typical efficiencies. (CW, continuous wave; fs, femtosecond pulsed; ns, nanosecond pulsed.)

<table>
<thead>
<tr>
<th>laser source, wavelength, pulse duration and repetition rate</th>
<th>reference</th>
<th>likely mechanism of action</th>
<th>transfection efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>193 nm/308 nm, 6 ns, 200 Hz</td>
<td>Badr et al. (2005)</td>
<td>heating, thermoelastic stress</td>
<td>0.5%</td>
</tr>
<tr>
<td>355 nm, ns, Hz</td>
<td>Tsukakoshi et al. (1984), Tao et al. (1987), Gao et al. (1995) and Shirahata et al. (2001)</td>
<td>heating, thermoelastic stress</td>
<td>0.3–38%</td>
</tr>
<tr>
<td>405/488 nm, CW</td>
<td>Palumbo et al. (1996), Schneckenburger et al. (2002), Paterson et al. (2005) and Nikolskaya et al. (2006)</td>
<td>heating or photochemical; typically requires chemical absorbers in the medium such as phenol red</td>
<td>less than 30%</td>
</tr>
<tr>
<td>532 nm, ns, kHz</td>
<td>Soughayer et al. (2000) and Clark et al. (2006)</td>
<td>heating, thermoelastic stress</td>
<td>30–80%</td>
</tr>
<tr>
<td>approx. 800 nm, fs, greater than 1 MHz</td>
<td>Tirlapur et al. (2001), Tirlapur &amp; Konig (2002), Zeira et al. (2003), Kohli et al. (2005a), Stracce et al. (2005), Barrett et al. (2006), Stevenson et al. (2006), Zohdy et al. (2006), Peng et al. (2007), Tsampoula et al. (2007), Baumgart et al. (2008), Brown et al. (2008), Lei et al. (2008), Tsampoula et al. (2008), Uchugonova et al. (2008b) and Yamaguchi et al. (2008)</td>
<td>multi-photon effect; generation of a low density free electron plasma cloud</td>
<td>often greater than 90%</td>
</tr>
<tr>
<td>1064 nm, ns, Hz</td>
<td>Mohanty et al. (2003) and Knoll et al. (2004)</td>
<td>heating, thermoelastic stress</td>
<td>less than 2%</td>
</tr>
<tr>
<td>1554 nm, fs, MHz</td>
<td>He et al. (2008)</td>
<td>multi-photon effect; generation of a low density free electron plasma cloud</td>
<td>77%</td>
</tr>
<tr>
<td>2080 nm, a few hertz</td>
<td>Sagi et al. (2003)</td>
<td>not known</td>
<td>41%</td>
</tr>
</tbody>
</table>

Nikolskaya et al. (2006), they have to date demonstrated low transfection efficiencies of less than 30 per cent (Schneckenburger et al. 2002; Nikolskaya et al. 2006). Both stable (Paterson et al. 2005) and transient transfection (Palumbo et al. 1996; Schneckenburger et al. 2002; Nikolskaya et al. 2006) have been reported using these sources.

4. OPTICAL TRANSFECION WITH FS-PULSED SOURCES: TARGETED SINGLE CELL TRANSFECION

An approximately 800 nm laser source, with short pulse durations <200 fs and thus very high peak powers, has emerged as the most popular laser for single cell optical transfection and is the focus of the remainder of this discussion. The delicacy of pore control demonstrated by fs lasers remains unrivalled. During irradiation, a tiny, submicrometre-sized pore, lasting fractions of a second, is generated on the plasma membrane. Cell viabilities greater than or equal to 90 per cent are often reported in the literature (Tirlapur & Konig 2002; Kohli et al. 2005a; Peng et al. 2007; Baumgart et al. 2008; Lei et al. 2008; Uchugonova et al. 2008b) to plasmid DNA (Tirlapur & Konig 2002; Zeira et al. 2003; Stracce et al. 2005; Stevenson et al. 2006; Tsampoula et al. 2007, 2008; Baumgart et al. 2008; Uchugonova et al. 2008b), and mRNA (Barrett et al. 2006; Sul et al. 2009). Recently, our own laboratory has even shown that a single 100 nm gold particle can be optically tweezeed and subsequently injected into a cell (McDougall et al. 2009; as discussed in more detail later).

A recent electrophysiology-based study, combined with quantitative fluorescence microscopy, provides some interesting insights into the response of the cell to fs irradiation (Baumgart et al. 2008). Figure 1 demonstrates the set-up employed. Using patch clamping, the authors calculate a volume exchange of (0.4 × cell volume) of the surrounding solution. For example, if the extracellular concentration of a fluorophore is 1000 μM, upon irradiation the intracellular concentration will be 400 μM. They further verified their findings by experimentally assessing the fluorochrome of injected Lucifer Yellow, a membrane impermeable dye, and found an average volume exchange of approximately 0.37 × cell volume. Another study investigated the size of the photopore generated. Transmission electron microscopy images of fixed cells irradiated at threshold doses for pore formation indicate a pore diameter between 0.5 and 1.0 μm (Niioka et al. 2008).

The value of optical injection in the future will most probably lie in its application to single cell analysis.
especially in combination with a range of other single cell modalities. There is a general trend of assay miniaturization occurring in molecular and cell biology. Traditional microlitre volume experimentation using pipettes and Eppendorf tubes is being replaced with automated microfluidic systems allowing nano- and picolitre reagent handling. A number of macroscopic assays traditionally developed for many hundreds to millions of cells are now being demonstrated on single cell systems. Examples include polymerase chain reaction (Lee et al. 2006; Kumaresan et al. 2008; Sul et al. 2009), gene expression profiling by microarray (Bontoux et al. 2008; Sul et al. 2009), ionization Q-time of flight mass spectrometry (Tsuyama et al. 2008), capillary electrophoresis (Yang et al. 2008), electrical physiometry (Werdich et al. 2004) and Raman spectroscopy (Jess et al. 2006). In combination with traditional fluorescence microscopy, these new technologies will aid the study of subjects as far reaching as cell–cell communication, the role of the microenvironment in stem cell differentiation (Lii et al. 2008), stem cell lineage commitment (Takano et al. 2004), cancer metastasis and nerve regeneration.

Optical injection adds value to this miniaturization trend by enabling a simple and convenient method to ‘point-and-inject’ material into a cell observed under the microscope.

5. POINT AND INJECT

To realize the goal of a point-and-click optical transfection with an fs laser, a major engineering challenge needed to be solved, i.e. the precise axial alignment and positioning of the beam on the plasma membrane is crucial for the technique to work. The time it takes to manually align a focused laser onto the plasma membrane of a cell represents the rate limiting step in moving from a targeted single cell injection regime to an automatic multi-cell injection regime. A standard Gaussian beam focused through an objective has a very narrow axial therapeutic zone owing to the small depth of focus; a misfocus of as little as 3 μm results in a greater than 50 per cent reduction in transfection efficiency (Tsampoula et al. 2007). To overcome this problem in 2007, the use of a ‘non-diffracting’ Bessel beam was demonstrated. The therapeutic zone of this beam is a rod of light over 100 μm in length (Tsampoula et al. 2007). A concept diagram of this is shown in figure 2. Using this configuration, the authors demonstrated the ability to transfect CHO cells over a 100 μm axial range. In a follow-on study in 2008, the ability to generate multiple arrays of Bessel beams using a dynamically addressable array of liquid crystals, the so-called spatial light modulator, was demonstrated. With this device, point-and-click Bessel beam shaping and steering for transfection was demonstrated (figure 3; Čizmár et al. 2008). As many cells may be dosed per field of view, automatic and high throughput transfection of entire populations is now potentially achievable.

One might reasonably ask the question to what extent the exposure of out-of-focus light during optical injection affects other non-plasma membrane regions of the cell, especially as the outer rings of the Bessel beam seem to envelop the entire cell during dosing (Tsampoula et al. 2007). It is important to note with the use of a Bessel beam that only the central core has a high enough fluence to elicit the destructive multi-photon reaction necessary for free electron generation (Mazilu et al. in press). This is why the central rod-of-light, but not the rings, is visible in figure 2, for example. In comparison, out-of-focus light from a Gaussian beam, at fs-pulsed modes, only results in a small, approximately 1 × 3 μm, destructive confocal volume (Niioka et al. 2008). In CW mode, out-of-focus light can be dangerous to the cell especially at ultraviolet wavelengths (Hockberger et al. 1999). Finally,
regardless of the beam configuration, any longer pulse duration beam (ns, picosecond, etc.) can result in destructive shockwaves that extend beyond the single cell.

6. IN VIVO OPTICAL TRANSFECTION

Engineering developments continue to be made to allow targeted optical transfection in vivo. A noteworthy experiment realized the stable optical transfection of tibial mouse muscle (Zeira et al. 2003). The authors injected 10 μg of GFP, luciferase or murine erythropoietin protein directly into a mouse tibial muscle and raster scanned a 95 × 95 μm² at a tissue depth of 2 mm. They found that in comparison with electroporation, optically transfected cells had a significantly higher viability, lower levels of apoptosis and better morphology under histological staining. The penetration of a focused laser at infrared wavelengths is approximately 2 mm, which limits certain in vivo applications. However, there have been significant advances in fibre-based optical transfection systems. Tsampoula et al. (2008) micro-etched an axicon onto the tip of an optical fibre, allowing the focused delivery of laser to single cells (Tsampoula et al. 2008). With this configuration, transfection efficiencies of up to 57 per cent were demonstrated. This paves the way for future in vivo endoscopic embodiments of the technology.

Another excellent in vivo application has been the transfection of embryonic zebrafish (Danio rerio) cells (Kohli et al. 2007). Zebrafish embryos have proven to be a good developmental biology model and have gained a great deal of attention recently in their ability to be continuously imaged using digital scanned laser light sheet fluorescent microscopy for over 24 h post-fertilization (Keller et al. 2008). In the work of Kohli et al. (2007), single zebrafish blastomere cells at the two-, four-, eight- and 16-cell stage were optically transfected with GFP plasmid using an 800 nm fs-pulsed source, and a wide distribution of the protein was observed 24 h post-fertilization in the resulting larva. The optical injection of fluorescein isothiocyanate and streptavidin-coated quantum nanodots was also successful, with a high loading efficiency (greater than or equal to 78%) and embryonic survival rate (greater than or equal to 89%). Later studies found no significant morphological differences in optically injected animals, after incubation for 2 or 7 days, relative to untreated controls (Kohli & Elezzabi 2008, 2009). These are the first studies showing the effects of optical transfection in embryonic cells, and demonstrate that...
optically transfected embryos can develop into fully functional animals.

7. SINGLE CELL ANALYSIS

The miniaturization of analysis to the single cell level is a driving goal for many lab-on-a-chip innovations. Combining optical injection with other single cell technologies has begun to unveil new and exciting experimental opportunities.

The apparatus used to inject cells optically simply consists of a laser carefully aligned into the back aperture of a microscope objective. With a few minor alterations to the system, the same apparatus can be used as optical tweezers, allowing both the physical micromanipulation and the optical injection of a cell with the same laser source. The laser can be toggled between the passive CW regime (for tweezing) and the pulsed (therapeutically) active permeabilization mode for optical injection. A broad range of miniaturized assays can therefore be performed. This combination was recently demonstrated by Brown et al. (2008). Using an 800 nm laser in CW mode, a single cell was optically tweezed into a micro-chamber containing trypan blue. The same laser was then blocked, mode-locked and used to optically inject the cell, after which time it was removed from the micro-chamber. This paper demonstrated two important concepts: (i) single cells may be physically positioned and optically injected with the same laser and (ii) single cells may be dosed with a number of reagents in situ without the need for fluid flow by tweezing the cell between chambers loaded with different chemistries. In essence, almost any assay can be miniaturized to the level of a single cell using optical tweezers, optical injector and commercially available square capillary tubes.

The combination of optical tweezers and injection has also been used for the intracellular placement of single nanosensors. Surface-enhanced Raman spectroscopy can be used to identify cellular biochemistry within close proximity (nanometres) of a metallic object such as a gold nanoparticle. A recent study showed that it is possible to optically tweeze a single 100 nm gold particle and inject it into a mammalian cell with micrometre spatial accuracy (McDougall et al. 2009). The targeted intracellular placement of single biosensors means that different regions of a cell may be spectroscopically analysed in real time.

The field is at an exciting juncture with the publication of two seminal papers from the Eberwine group (Barrett et al. 2006; Sul et al. 2009). Here, the focus of study was not the technique of optical injection itself, but the application of the technique to biological questions difficult to answer using other methods. The authors demonstrate a number of new concepts in single cell optical transfection, including (i) the first instance of the optical transfection of mRNA in a post-mitotic cell type, (ii) the first instance of optical transfection in combination with single cell polymerase chain reaction, and (iii) the first instance of optical transfection in combination with gene expression (microarray) analysis. Figure 4 shows an example of a primary rat hippocampal neuron injected with Lucifer Yellow or transfected with GFP mRNA (Barrett et al. 2006).

There is an emerging concept that mRNA translation within dendrites (the branched projections of neurons) may orchestrate cellular events differently from mRNA translated in the soma (Barrett et al. 2006). Testing this hypothesis in the past has relied on invasive techniques; the soma is physically removed from the dendritic branches by micropipette aspiration, and the dendrites are then transfected using classical lipoplex cocktails (Wu et al. 2007). While simple, this method prevents further imaging or electrophysiological analysis of the dying cell. Barrett et al. (2006) optically transfected Elk1 mRNA into primary rat neuron dendrites and compared the reaction of the cell when Elk1 mRNA was transfected into the cell body. Dendritic Elk1 transfection resulted in cell death which was blocked with the translation inhibitor anisomycin. In contrast, cell body Elk1 transfection resulted in no cell death. This has ramifications in the developing field of subcellular neuropharmacology, where drugs are targeted not only to a specific cell type but a specific region within a cell (such as a dendrite; Miyashiro et al. 2009). The aseptic, repeated delivery of cellular reagents highlights a valuable application of the technology.

A second groundbreaking application was recently published by members of the same group (Sul et al. 2009). The authors employed a phototransfection method where an 800 nm laser (100 MHz, 100 fs) at a power of 35 mW (prior to entering the objective) was used to sequentially generate 16 photopores on the membrane of a cell. In this study, the entire transcriptome (i.e. the sum of all mRNA species within the cell) was extracted from primary astrocyte cells and transplanted, using a series of optical injection steps.
over many days, into individual primary rat hippocampal neurons. The single neurons were harvested at various times after transfection and assessed for astrocytic markers by single cell nested reverse transcriptase polymerase chain reaction, immunocytochemistry and gene microarray. Remarkably, a proportion of the treated neurons had phenotypically remodelled themselves into astrocytes. Evidence for this included the up-regulation of a number of astrocyte-specific mRNA transcripts, the presence of astrocyte-specific proteins such as glial fibrillary acidic protein and fibronectin, morphological analysis and an astrocyte-specific physiological response to drug stimulation. Many of these traits were observed four weeks after the final round of phototransfection. Because the turnover of mRNA is of the order of hours, this indicates the injected mRNA milieu had truly generated a range of de novo gene expression. Further phenotypic remodelling experiments, enabled by optical injection, will help unravel whether other differentiated cell types can be inter-converted and will shed light on the extent to which cells may be re-programmed.

8. CONCLUSION

Optical transfection with fs-pulsed sources is poised to be a key player in future single cell experimentation. One can envision a combination of on-chip modalities incorporating long-term single cell culturing, single cell manipulation with optical tweezers and single cell biochemical analysis. Biochemical endpoints assessing DNA, RNA and protein levels in response to growth, metabolism and microenvironment will help unravel a wide range of fundamental cell and disease processes. Phenotypically converting a cell from one type to another has far reaching consequences in the field of bioengineering, tissue engineering and stem cell therapy.

Technologists will continue to push the field by developing high-throughput, more compact and cheaper optical injection systems. Our understanding of the mechanism of injection will no doubt be furthered. As these developments continue to be embraced by the biological community, the true power of optical injection will be realized.

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