This chapter describes the use of light to transiently increase the permeability of mammalian cells to allow membrane impermeable substances to cross the plasma membrane. We refer to this process as optical injection or more specifically optical transfection, when genetic material is introduced into and expressed by a cell. A brief discussion of nonphotonic transfection and injection technologies is presented. A review of the field of optical transfection and the underlying physical mechanisms associated with this process is then described.

**Key words:** optical transfection, optoporation, optoinjection, photoporation, laserfection, transfection

### 3.1 Introduction: Why Cell Transfection?

The ability to introduce exogenous DNA into a cell whether that is a prokaryotic cell (transformation) or an eukaryotic cell (transfection) has allowed molecular biologists not only to explore the molecular mechanisms within cells, but also to produce genetically engineered organisms. Földes and Trautner first coined the term transfection in 1964, when they introduced phage SP8 DNA into
B. subtilis bacteria [1]. Since then, there have been a variety of technologies developed to encourage a cell to take up and express foreign nucleic acids. These include optical transfection, lipoplex transfection, polyplex transfection, calcium precipitation, electroporation, the use of the gene gun, hydrodynamic delivery, ultrasound transfection, viral transfection, or even the simple addition of naked DNA. The word “transfection” has historically been used to mean the loading of any membrane impermeable molecule into a cell, but biologists now wish to introduce other molecules into cells such as membrane impermeable drugs, impermeable fluorophores, or even antibodies. Technically the word transfection specifically refers to the internalization and subsequent biological effects of nucleic acids, while the term optical injection is for these other substances. At present the introduction of heterologous nucleic acids into a cell, be it DNA, messenger RNA (mRNA) or small oligonucleotides (as used in iRNA, interference RNA, studies), is by far the most common reason to inject a membrane impermeable molecule.

The field of transfection is wide, and it is not the purpose here to review all of the various technologies but is rather to highlight some of the more commonly used techniques in order to give context to the difficulties associated with transfection. DNA encounters a number of physical and chemical barriers presented by the cell before finally it is able to reach the nuclear machinery required for its expression. If a solution of naked plasmid DNA is mixed with a solution containing mammalian cells, the probability of transfection taking place is very low for almost all cell types. However, exposure of naked plasmid has been demonstrated to transfect some cell types, including the liver [2], skeletal muscle [3, 4], cardiac muscle [5], lung [6], solid tumors, the epidermis, and hair follicles. It is unsurprising that this type of transfection is difficult to achieve as the DNA transfection machinery is located within the nucleus, requiring the plasmid DNA to cross the plasma membrane, a highly viscous cytoplasm, the nuclear membrane, and an even more viscous nucleoplasm. Penetrating these membranes is energetically unfavorable due to the highly hydrophobic nature of the inner lamellae of the lipid bilayers and the hydrophilic nature of the DNA molecule. A typical plasmid DNA molecule is a circular string of double-stranded nucleic acids several kilobase pairs (kbp) long, which depending on the chemical environment can exist in an uncoiled, coiled, or supercoiled state. The volume occupied by plasmid DNA, or its radius of gyration, depends on the number of bases, the extent of its coiling, and whether it has become linearized. For example, the radius of gyration of a 5.2 kbp plasmid ranges between 85 and 120 nm depending on its state of coil [7, 8]. Plasmids of this size may also be compacted into spheres as small as 20–40 nm using polyethylenimine polyplexes [9], the details of which are discussed later.

Crossing the plasma membrane is a necessary but not sufficient requirement for transfection to occur. Once plasmid DNA has crossed the plasma membrane, various physical barriers still exist. Experiments utilizing microinjection within different regions of a cell provide an insight into how these barriers affect naked plasmid DNA. Microinjection was pioneered by Capecchi in 1980, who used an extruded glass needle to inject DNA solution directly into either the nucleus or the cytoplasm of mammalian cells [10]. This simple but powerful technique has changed little since its conception, although some reports have demonstrated micromechanical systems (MEMS) microinjection needles [11]. A small 10–20 bp double-stranded oligomer when microinjected directly into the cytoplasm will rapidly diffuse throughout the cytoplasm and cross the nuclear membrane with ease [12], probably through nuclear pores. The diffusion rate is fast in spite of the viscosity of the cytoplasm. As larger strands are injected, the behavior changes: 500-bp DNA strands will not cross the nuclear membrane, but will diffuse homogenously if directly injected into either the nucleus or the cytoplasm [12]. For comparison, this behavior is also true of 500 kDa dextran spheres [13] with a diameter of 26.6 nm [14]. DNA strands greater than 2,000 bp diffuse very slowly through the highly viscous cytoplasm, at less than 1% of their diffusion rates in water [12]. This size of DNA is still much smaller than that commonly employed in transfection experiments, which tends to range from 5,000 to 10,000 bp. When plasmids of this size range are directly microinjected into the cytoplasm, it has been estimated that as little as 1 in 1,000 are able to make their way to the nucleus for subsequent expression [15]. In contrast only a few (<10) plasmids microinjected into
the nucleus are necessary for expression [15]. As well as these physical barriers, there are also chemical barriers such as nucleases present in both the extracellular and intracellular environment that can affect transfection efficiencies by degrading plasmid DNA.

In this chapter, the many advantages of optical injection/transfection will be surveyed. To place this technology within context, a brief review of nonoptical methods of transfection is introduced (section 3.2), followed by a comprehensive review of optical injection and optical transfection since its first discovery in 1984 (3.3). Finally, the physics of transport through a transiently generated photopore (3.4), along with the laser-cell interaction that leads to the generation of a photopore (3.5) are briefly discussed.

3.2 Nonoptical Methods of Transfection

To overcome the various physical and chemical barriers, numerous nano/microparticle mediated transfection techniques have been developed, and a wealth of terminology has been generated to distinguish the various technologies and chemistries from each other.

3.2.1 Lipoplex transfection

Lipoplex transfection was first demonstrated by Felgner et al [16]. Lipoplexes are comprised of cationic lipids containing a polar head and a hydrophobic tail. When mixed in an aqueous solution, these cationic lipids spontaneously aggregate into hollow aqueous nanoshells with a lipid bilayer wall, with the polar heads of one half of the bilayer facing outwards into the surrounding external solution, and the polar heads of the other half of the bilayer facing inwards into the lumen of the lipoplex nanoshell. This behavior is reminiscent of the phospholipid bilayers comprising the plasma membrane of a cell. The polar heads typically consist of amidine, but can also be guanidium or pyridinium. When plasmid DNA, which is negatively charged, is present during this spontaneous lipoplex formation the positively charged groups of the polar heads electrostatically bind to the DNA and sequester it both within the lumen and along the external wall of the lipoplex nanoshell [17]. The diameters of typical lipoplexes range from 50 nm to over a micron [17, 18]. Exposure of these lipoplexes to cell cultures causes transfection with an efficiency that varies depending on the cell type. Lipoplex mediated transfection is thought to involve a number of steps [17–19]. Briefly, DNA containing lipoplexes bind to cells and become internalized by endocytosis. From there, DNA must escape lysosomal degradation and make its way to the nucleus. The crossing of the nuclear membrane may be in part due to passive entry during division, or by some form of direct nuclear import. The latter is suggested because postmitotic cells are able to be transfected using lipoplexes, so DNA must still be able to get into the nucleus without nuclear membrane degradation.

3.2.2 Polyplex transfection

Polyplex nanoshells are commonly made of cationic polymers such as poly-L-lysine [20] or polyethylenimine [15, 21]. Unlike liposomes, polyplexes are entirely hydrophilic in nature. Delivery of DNA to the nucleus follows a similar route to that of liposomes, with some key differences, including the mechanism of endosomal escape [18], where it has been suggested that there is endosomal swelling and bursting, due to a Cl⁻ influx brought on by a decrease in pH within the endosome [21]. Polyethylenimine polyplexes also clearly increase the ability of DNA to cross the nuclear membrane [15]. In a variation of polyplex transfection, “magnetofection” uses PEI complexed
to superparamagnetic nanoparticles, and high transfection efficiencies have been demonstrated in the presence of high magnetic fields [22].

3.2.3 Gene gun transfection

The gene gun method accelerates micron sized tungsten particles through the cell wall and cell membrane [23]. Although the original incarnations of gene guns used gunpowder, modern versions tend to use high-pressure helium gas [24, 25]. Reasonable injection/transfection efficiencies have also recently been demonstrated using a gene gun without a nano or microparticle carrier: the solution becomes aerosolized and enters a cell culture medium at high velocity [25]. Gene guns come in many architectures, including a vacuum chamber and handheld [26], but the most interesting in terms of direct comparison with optical injection is that of the pneumatic capillary gun. This miniature gene gun is capable of delivering highly localized lateral (150 μm) transfection in *Hirudo medicinalis* leech embryos. Notably, the axial resolution is 15 μm, and the penetration depth is variable between 0–50 μm depending on the pressure of the helium.

3.2.4 Ultrasound transfection

Ultrasound is transmitted as a periodic compressive [longitudinal] wave that is characterized by frequencies typically within the range 20 kHz–20 MHz. Interestingly, the biological effects of such waves were recognized almost from the outset of ultrasound technologies during the 1920s, long before imaging applications emerged [27]. Such bioeffects, including uptake, are a function of the ultrasound field parameters of frequency, amplitude and pulse duration, as well as the transmission (and absorption) character of the medium. For sustained application of ultrasound, heating effects typically dominate, and this is the basis of the emerging therapeutic approach of high intensity focused ultrasound. When short pulses of ultrasound are applied to cells (both in suspension and as plated monolayers), it has been found that molecules can be taken up from the locale. Such studies have measured the size dependent uptake from a spectrum of lower molecular weight species [29, 30] as well as intermediate to high molecular weight species, including peptides and protein [28], as well as genetic material such as DNA [29, 30]. A key clue to elucidating the mechanism of uptake arose when insonated cells were inspected by scanning electron microscopy: here, Tachibana and coworkers provided the first evidence that the cell membrane is physically compromised by the process [31]. They illustrated that insonated cells appear to be peppered with pore-like structures and this was later confirmed by high resolution microscopy observations using also confocal [30] and scanning probe microscopy [32]. The process is termed “sonoporation” in recognition of the apparent requirement for membrane disruption to accompany uptake. Notably, while the application of ultrasound could by itself lead to uptake of molecules, when insonation was performed in the presence of microscopic (typically 5 μm in diameter) bubbles (microbubbles), then these effects could be markedly enhanced. Microbubbles thus act to increase the probability of sonoporation [35–37].

3.2.5 Electroporation

Electroporation mediated mammalian cell transfection was first described by Neuman et al [33–35]. The cell membrane can be transiently permeabilized by the application of electrical pulses and because the DNA is charged, the technique has the added advantage that it forces DNA to interact with the cell membrane. In a typical experiment, high concentrations of naked DNA (many 10s of μg/ml) are added to the medium with the cells sitting between two electrodes which are then exposed to an electric field. A typical dose would be 10 pulses of a 0.8 kV/cm field, each 0.1–5.0 ms in duration [36]. Within 3 ms of field application, the sides of the cell facing both the cathode and the anode become permeabilized. Pores in the cell appear volcano-shaped, with the tip of the
Volcanoes facing the inside of the cell [37]. These pores range in size from 20–120 nm [37]. Almost immediately, aggregations of negatively charged plasmid DNA can be seen to adhere to the cathode facing side of the cell in “spots” or “islands” on the membrane [36]. The association of the DNA-plasma membrane is strong, and cannot be disrupted by subsequently electroporating the cells with a reversed polarity [36]. Within 10s of seconds, the pores start to disappear [37]. Within 30 min of electroporation, fluorescently labelled DNA can be seen to diffuse into the cytoplasm [36]. It is noteworthy that while electroporation has not been traditionally able to transfect individual cells, single cell electroporation has recently been realized in a microfluidic platform [38].

3.3 Review of Optical Injection and Transfection

Figure 3.1 shows a typical optical injection setup, and Table 3.2 summarizes the optical injection and transfection experiments performed in the literature to date. In a typical optical injection experiment, a solution of membrane impermeable substance such as a fluorophore, macromolecule, or nucleic acid is first exposed to the cells of interest. A laser is then used to transiently permeabilize the cell membrane, during which time the substance of interest either passively diffuses into the cell or is sucked into the cell due to volume exchange resulting from osmotic pressure differences. The mechanism of how the laser permeabilizes the plasma membrane depends on the laser wavelength, whether or not it is pulsed, its pulse duration, the diameter of the focused beam, and how the laser is applied to the cell or cell population.

The use of light to inject or transfect cells enjoys a number of advantages in comparison with the previously described techniques. In comparison with its main alternative for single cell treatment, microinjection, optical injection offers unrivalled simplicity in the ability to target cells in a sterile environment. Microinjection suffers from the requirement of an open solution; optical injection can be performed through a sterile coverslip. Furthermore, the efficiency and viability of optically transfected cells compare favorably with other nonviral technologies. Finally, it is straightforward to integrate an optical injection setup with other microscopic techniques, such as confocal laser scanning microscopy, optical tweezers, and microfluidic systems [39]. Broadly speaking, there are two categories of optical transfection – targeted and untargeted. In targeted transfection, the focal point of the laser is aimed on the plasma membrane, and a tiny hole or pore is generated. Only the targeted cell is transfected; neighboring cells remain completely unaffected. In targeted transfection, an acute reaction to the exposure of the laser is often observed under bright field microscopy. This reaction depends on the dose of exposure, the laser source, and in some cases the presence of chemical absorbers placed in the medium [44, 45]. Below a threshold dose, no reaction to the laser will occur. Above a certain dose, acute morphological changes in the cell occur, including an increase in granularity, “blebbing,” and loss of membrane integrity. In an ideal scenario, a therapeutic dose would exist somewhere between the under- and overdose, where the cell membrane permeabilization is transient and the cell recovers within seconds to minutes. In reality, often the therapeutic dose and the overdose slightly overlap, and the postdose viability is <100% [46, 47]. This overlap is not uncommon in other transfection technologies. A more thorough description of the reaction of the cell to laser irradiation, including a detailed description of the biophysical characteristics of the “photopore” generated, is given in the final section of this document and in the comprehensive reviews of Vogel et al [48, 49].

Typical laser sources for targeted optical injection and transfection include the 800 nm femtosecond (fs) pulsed titanium sapphire laser (Figure 26.2a-d) [13, 50–61], and continuous wave (cw) sources such as 405 nm [40] (Figure 26.2e,f) and 488 nm [41–43]. 1064 nm nanosecond (ns) pulsed Nd:YAG lasers have also been reported in the literature to produce a targeted transfection [44–46].
FIGURE 3.1: A typical optical injection/optical transfection setup. A pulsed (femtosecond or nanosecond) or continuous wave laser is passed through a Neutral Density (ND) filter to attenuate the power (typically 10s of mW at focus) and a shutter to provide the correct dose (typically 10s of ms). It is then passed through a steering mirror and a lens relay telescope. The lens relay telescope ensures that the image at the steering mirror is relayed to the back aperture of the microscope objective such that rotation of the steering mirror results in movement of the diffraction limited spot at focus without “clipping” the edge of the back aperture, or beam “walkoff.” A filter in front of the CCD camera blocks the beam wavelength but not light in the visible range, allowing the sample but not the laser to be imaged.

However, this laser is more often used for untargeted optical transfection. Figure 26.2a is a fluorescent image showing the optical injection of the membrane impermeable dye, propidium iodide [53]. The image was taken immediately after optical injection. At this stage, the postinjection viability is unknown. 90 minutes later (Figure 26.2b), the same cells were re-stained with propidium iodide. All cells remained viable except one (white arrow), whose membrane was permanently compromised. This demonstrates nicely the dual use of a membrane impermeable dye in optical injection experiments – it can determine both optical injection efficiency and optical injection viability. Figure 26.2c is a fluorescent image showing the optical transfection of a Chinese Hamster Ovary (CHO) cell with plasmid DNA encoding for Green Fluorescent Protein (GFP). The image was taken 48 h after treatment, and cell was co-stained with the blue nuclear dye DAPI. Figure 26.2d is a fluorescent image showing the optical transfection of a primary rat hippocampal neuron with mRNA encoding for Elk1-GFP [54]. The image was taken 30 min after treatment. Note typical timings of each event – optical injection occurs immediately, optical transfection of DNA occurs > 24 h after dosing, and optical transfection of mRNA occurs > 30 min after dosing. Finally, Figure 26.2e-f is an example of targeted optical transfection by cw laser. CHO cells were exposed to plasmid DNA encoding for DsRed-Mito and an antibiotic resistance gene and individual cells were dosed with a focused 405 nm laser [40]. After culturing in the antibiotic, only cells that had been optically transfected survived; a stable culture had therefore been established.

In untargeted transfection, groups of 10s to 1000s of cells are membrane permeabilized by direct or indirect laser dosing. This may be achieved directly by simply raster scanning a region of interest with a highly focused laser [4]; directly by dosing a large population with an unfocused nanosecond pulsed beam [47, 48]; or indirectly by creating a laser induced shock wave [49] (Figure 26.3). In shockwave mediated transfection, a laser induced pressure gradient transiently permeabilizes the plasma [50, 51] and/or nuclear membrane [52]. This pressure gradient is formed by hitting
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a membrane target [50], or the glass-solution interface of the culture dish upon with the cells are sitting [49]. In this respect, it has more in common with the ultrasound genre of transfection.

In the case of targeted optical injection using either cw wavelengths or lasers with a very short pulse-duration, a diverse array of molecules has been loaded into single cells. 800 nm fs pulsed sources have been used to optically inject ethidium bromide [55], sucrose [56], propidium iodide [53, 57–59], lucifer yellow [53, 54, 57], Trypan Blue [39], cascade blue [13], 500 kDa FITC labelled dextran [13], plasmid DNA encoding for GFP [4, 13, 53, 55, 60, 61], luciferase, β-galactosidase, murine erythropoietin [4], and DsRed-Mito [56, 72]. One case in the literature also demonstrated the targeted transfection and translation of a number of mRNA species [54]. 405 nm and 488 nm cw sources have been used to transfect plasmid DNA encoding for GFP [45, 62, 63], beta-galactosidase, and chloramphenicol acetyltransferase [41]. In cases where plasmid DNA was optically injected, both stable and transient transfection have been observed with these targeted laser sources.

Because an optical injection setup is also often capable of optically tweezing, it is also possible to individually manipulate nonadherent cells using a Ti:sapphire laser in cw mode before and after optical injection [39]. Figure 26.4 outlines an experiment demonstrating this. Figure 26.4a-i are screenshots from a movie (available online via reference [39]) where an HL60 cell is optically tweezed into a capillary tube containing a membrane impermeable dye, optically injected, and tweezed back out of the tube once again. Figure 26.4j shows a schematic diagram of the path of the cell. The cell begins its journey by being optically tweezed in the cw regime from the bottom of a microchamber surface (Figure 26.4a), up in z approximately 30 μm (Figure 26.4b), and into the opening of a square Trypan Blue filled capillary tube (Figure 26.4c). It is then positioned next to a blue cell that had previously been fixed, permeabilized, and placed into the capillary tube by tweezing (Figure 26.4d). The cell was then optically injected using the same laser, perturbed to induce a modelocked operation. This resulted in a transient pore (Figure 26.4e, white arrow), and allowed Trypan Blue to stain the cell after six minutes. In the next image (Figure 26.4f), it can be seen that Trypan Blue has entered the cell nucleus. The laser was then unblocked, and in the cw regime the optically injected cell was tweezeed out of the capillary tube and placed next to an untreated cell (Figure 26.4g-i).

It is reasonable to assume that almost any membrane impermeable fluorophore, macromolecule, or nucleic acid, below a certain size, is able to be optically injected using a Ti:sapphire 800 nm fs laser source. The utility of targeted optical injection is left to the imagination of the reader. Literally thousands of membrane impermeable fluorophores have been developed or discovered over the years that bind to specific intracellular biochemical targets [62]. These fluorophores are largely useless in investigations involving live cells; the traditional way to load these membrane impermeable substances into cells involves fixation with formaldehyde, followed by permeabilization with Triton-X 100. Another traditional way to overcome this problem is to alter the chemistry of the fluorophore. Smaller organic fluorophores can often be chemically modified to generate an inactive but membrane permeable product, upon which entering the cell undergoes esterase cleavage, activation, and fluorescence [74]. However, this is simply not an option for many larger biological constructs: plasmid DNA, mRNA, siRNA, and antibodies are notable examples. Considering that many commercially available microscopy laboratories, especially those performing multiphoton microscopy, already have an 800 nm titanium sapphire source, we believe that it is only a matter of time before this optical injection is more widely adopted.

In the case of untargeted optical injection with a 1064 ns pulsed Nd:YAG source, a similarly diverse array of molecules have been transfected into small populations of cells. These include Ca2+, Zn2+, Sytox Green, Sytox Blue, tetramethylrhodamine-dextran (MW 3000), Cdc42 binding domain of WASP conjugated to an I-SO dye (55 KDa), quantum dots, siRNA [46], merocyanin 540 [45], Texas Red-glycine, Texas Red-dextran (3kD, 10kD, 40 kD), fluorescein labelled protein kinase C [49], and plasmid DNA encoding for GFP [45, 48, 63], pSV2-neo [64, 76], pAB6 [64] and Eco-gpt [65]. Where plasmid DNA was used, both stable and transient transfection have been observed with this laser source.
FIGURE 3.2: (See in color after page 572.) Examples of targeted (a,b) optical injection and targeted (b,c) optical transfection using a femtosecond (fs) pulsed Ti:sapphire laser or by (e,f) 405 nm continuous wave (cw) laser. See text for details. Images a and b reprinted with permission (Optical Society of America) [53]. Image d reprinted by permission from Macmillan Publishers Ltd: Nature Methods [54], copyright 2006. Images e and f reprinted with permission (Optical Society of America) [40].
A variety of cell-types have been optically transfected, including Chinese Hamster Ovary cells [13, 47, 51, 55, 60, 62, 63], NIH3T3 [66], BAEC [67], HeLa, SU-DLH-4, NTERA-2, MO-2058, PFSK-1, 184-A1, CEM, NIH/3T3, 293T, HepG2, primary rat cardiomyocytes, embryonic C166 [46], GFSHR-17 granulosa, MTH53a canine mammary [53], rat cardiac neonatal [43], SK-Mel 28, NG108-15, T47D clone 11 [13], HT1080-6TG [44], transitional cell carcinoma [48], normal rat kidney [65], tibial mouse muscle (in vivo) [4], MCF-7 [45], human salivary gland stem cells, human pancreatic stem cells [55], Madin-Darby Canine Kidney cells [56], PC12, primary rat astrocytes [59], spisulsa solidissima oocytes [67], PtK2 [57], rat basophilic leukemia [49], Triticum aestivum L. cultivar Giza 164 (wheat) [64], and MatLu rat dorsal prostate adenocarcinoma [47]. However, with specific regard to plasmid DNA transfection, to our knowledge there have been no reports in the literature of a differentiated postmitotic primary cell being optically transfected. The majority of cells transfected by DNA are in fact rapidly dividing established cell-lines, which may highlight the need for the nuclear membrane to dissolve as a requirement for this technique to be successful. It should be re-emphasized however that the optical transfection of mRNA species in postmitotic cells (primary neurons) has been demonstrated [54].

In the case of plasmid DNA transfection, a useful parameter for optimizing the laser dose is that of transfection efficiency. It was mentioned previously that the efficiency and viability of optically transfected cells compare favorably with other nonviral technologies. In this particular field of research, there have been inconsistencies in the methodology used to express transfection efficiency, and as such caution should be used in the interpretation of reported transfection efficiency highlighted in Table 3.2. It is therefore appropriate to highlight some key points about transfection efficiency. There has been a strong trend on this topic to have the following definition of transfection efficiency:

\[ N_{\text{transf}} = \frac{E}{D} \times 100, \]  
(3.1)
FIGURE 3.4: Example of the targeted optical injection of a nonadherent HL60 cell using a femtosecond (fs) pulsed Ti:Sapphire laser. The same laser was used in cw mode to optically tweeze the injected cell (laser modes are indicated per image). Time is noted in min:sec. Asterisk indicates cell of interest, and color of asterisk indicates solution surrounding the cell (White: RPMI 1640. Blue: 0.4% Trypan Blue). See text for details. Image and caption reprinted with permission from [39]. Original reference also contains a supplementary movie of this experiment online.
where $N_{un}$ is the uncorrected transfection efficiency (%), $D$ is the number of cells dosed on a given experiment in the presence of a plasmid containing a reporter gene, and $E$ is the number of cells transiently expressing the plasmid DNA after a suitable amount of time has passed. The amount of time it takes for transient transfection to occur is on the order of 24–96 hours. Equation 3.1 does not take into account two parameters: 1) some of the dosed cells lose viability; 2) all cells, including those transfected, divide during the time between dosing and analysis. A corrected form of this equation was proposed over 20 years ago by the first group to perform optical transfection, Tsukakoshi et al. [68]:

$$N_{cor} = ((E/D) \cdot 100)/XD,$$

(3.2)

where $N_{cor}$ is the population corrected transfection efficiency, $D$ is the number of cells dosed on a given experiment, $E$ is the number of cells transiently expressing the plasmid DNA after a suitable amount of time has passed, and $XD$ is the ratio of proliferation that has occurred by the dosed cells between dosing and the measurement of expression. Although it is difficult to quantify $XD$ directly without performing long-term image analysis, $XD$ can be approximated by assuming that the population doubling time of the dosed cells is the same as those of all other cells in the culture dish. It is therefore simply the ratio of confluency on the day of analysis to that of the confluency on the day of dosing. To be clear, confluency is the percentage of the area of a culture dish that cells cover, and can be easily obtained by image analysis or measuring total protein by the Lowry assay [69] or equivalent.

Finally, another extremely important but often overlooked parameter is the postirradiation percentage of viability, $V$, which for simplicity can be defined as:

$$V = (S/D) \cdot 100,$$

(3.3)

where $S$ is the number of cells surviving about an hour after dosing and $D$ is the number of cells dosed. Survival can be measured by a variety of membrane impermeable dyes, such as Trypan Blue [61].

The importance of correcting transfection efficiency for cell proliferation is highlighted in the hypothetical example shown on Table 3.1. Both researchers could report an uncorrected transfection efficiency of 100%. This would disguise the fact that the second researcher had twice the postdose survival rate, and twice the (corrected) transfection efficiency. We propose that both corrected transfection efficiency (Eq. (3.2)) and postirradiation viability (Eq (3.3)) should be the minimum acceptable standard of reporting future optical transfection optimization studies.

It should be stressed that in spite of these minor inconsistencies, optical injection has established itself as a key nonviral transfection technology, the competitiveness of which is not limited by efficiency or viability.

### 3.4 Physics of Species Transport through a Photopore

The question as to the quantity of substance that enters a cell has been addressed both experimentally and theoretically in the literature. Consider a single, targeted, transiently generated photopore on the surface of a cell. Species transport through this pore will be dominated by two factors: 1) the volumetric changes a cell undergoes in response to the pore being created and 2) the passive diffusion of a species through the pore as it remains open.
**TABLE 3.1:** Hypothetical example of parameters measured by two typical researchers during a typical optical transfection experiment.

<table>
<thead>
<tr>
<th>Experimental Details</th>
<th>Researcher 1</th>
<th>Researcher 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells dosed (D)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Number of cells found to be expressing GFP (E)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Time in hours between dosing and analysis of expression</td>
<td>96</td>
<td>24</td>
</tr>
<tr>
<td>Ratio of confluency at time of analysis to that of confluency at time of dosing (X_D)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Number of cells surviving 1 hour after dosing (by simple dye exclusion)</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Uncorrected transfection efficiency calculated by Eq. (3.1) (N_m)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Corrected transfection efficiency calculated by Eq. (3.2) (N_cor)</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>% viability calculated using Eq. (3.3) (V)</td>
<td>25</td>
<td>50</td>
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<table>
<thead>
<tr>
<th>λ</th>
<th>Ref.</th>
<th>Category of transfection or optical injection</th>
<th>Laser details</th>
<th>Dose</th>
<th>Viability</th>
<th>Obj/NA</th>
<th>Cell type</th>
<th>Transfected item (concentration)</th>
<th>Transfection efficiency</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>193/308</td>
<td>[72]</td>
<td>Targeted stable ns DNA</td>
<td>Eximer, 6 ns, 200 Hz</td>
<td>2–4 mJ</td>
<td>No mechanical damage or malformation 39% 1 h post-irradiation, progressively decreasing to 16% 12 h, &gt; 60% apoptosis (Annexin V Alexa488)</td>
<td>37× –</td>
<td><em>Triticum aestivum</em> L. cultivar Giza 164 (plant: wheat)</td>
<td>pAB6 (50 μg/ml)</td>
<td>0.5%</td>
<td>Targeted, high throughput (600,000 cells/hour) wheat transformation. See 770 nm entry. Irradiation with fs is less toxic than ns pulses.</td>
</tr>
<tr>
<td>337</td>
<td>[50]</td>
<td>Targeted ns toxicity assay</td>
<td>N2 laser, 3 ns</td>
<td>1 pulse</td>
<td>–</td>
<td>100 ×/1.3 1.3</td>
<td>NG108</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>355</td>
<td>[71]</td>
<td>Targeted ns transient and stable DNA</td>
<td>Frequency tripled Nd:YAG</td>
<td>Single pulse at 0.91–1.10 μJ/pulse</td>
<td>–</td>
<td>–</td>
<td>HuH-7 (highly differentiated hepatocellular carcinoma cells) (transient and stable transfection achieved) NIH/3T3 mouse BALB/C fibroblasts (stable transfection achieved), stimulatory protein 2 (SP2) mouse (BALB/C) myeloma cells (stable transfection achieved)</td>
<td>pEGFP-N1 (20 μg/ml)</td>
<td>HuH-7: 9.4% at 24 h, 13% at 48 h. NIH/3T3: 10.1% at 24 h</td>
<td>Uncorrected transfection efficiency formula used. First example in literature of nonadherent cells being transfected, by employing a separate optical tweezer to hold cell <em>in situ</em> during dosing.</td>
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Table 3.2 – continued from previous page

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<th>λ</th>
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<th>Laser details</th>
<th>Dose</th>
<th>Viability</th>
<th>Obj/NA</th>
<th>Spot (μm)</th>
<th>Cell type</th>
<th>Transfected item (concentration)</th>
<th>Transfection efficiency</th>
<th>Notes</th>
</tr>
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<tbody>
<tr>
<td>355</td>
<td>[76]</td>
<td>Targeted ns transient and stable DNA</td>
<td>Frequency tripled Nd:YAG 5 ns, 10 Hz</td>
<td>1 mJ</td>
<td>–</td>
<td>32×0.4</td>
<td>0.5 μm</td>
<td>Normal rat kidney</td>
<td>Eco-gpt gene cloned in pBR-322 (pSV-2 gpt)</td>
<td>38.8%</td>
<td>for uncorrected transient transfection. See Notes. The first case in the literature of laser mediated transfection. Highest transient transfection efficiency was obtained when nucleus, not cytoplasm, was targeted. Transfection efficiency in the original paper was stated as 10.2%, but this was corrected for population doubling. The uncorrected efficiency of 38.8% is stated here for comparison with the current literature, where correcting for population doubling is not commonplace.</td>
</tr>
</tbody>
</table>

| 355 | [44] | Targeted ns DNA                             | Frequency tripled Nd:YAG Single 10 ns pulse, 23-67 μJ | –     | 32×2      | HT1080-6TG (a hypoxanthine phosphoribosyltransferase deficient human fibrosarcoma) | pSV2-neo (12 μg/ml) | ≤ 0.3% | Only stable transfection observed; no transient. |

Continued on next page
### Table 3.2 – continued from previous page

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<th>λ (nm)</th>
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<tbody>
<tr>
<td>355</td>
<td>[78]</td>
<td>Targeted transient and stable ns DNA and fluorophore</td>
<td>Frequency tripled Nd:YAG 15 ns, 10 Hz</td>
<td>12000 pulses over 20 min period at 0.2–1.0 mJ</td>
<td>–</td>
<td>40×</td>
<td>1</td>
<td>Embryonic calli of <em>Oryza sativa</em> L. cv. Japonica (plant: rice)</td>
<td>pBI221 (encoding for bacterial β-glucuronidase) (50 μg/ml) and other plasmids (including one encoding for hygromycin phosphotransferase), calcein (5 mM)</td>
<td>0.48%</td>
<td>Excellent example of the influence of tonicity on the cell response to photoporation</td>
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<tr>
<td>405</td>
<td>[40]</td>
<td>Targeted cw stable DNA</td>
<td>cw violet diode 0.3 mW for 40 ms</td>
<td>No acute morphological signs of injury</td>
<td>100×</td>
<td>2</td>
<td>Chinese hamster ovary cells K1</td>
<td>pEGFP-N3, DsRed-Mito (3 μg/ml)</td>
<td>–</td>
<td>Only stable transfection observed; no transient.</td>
<td></td>
</tr>
<tr>
<td>488</td>
<td>[42]</td>
<td>Targeted cw transient DNA</td>
<td>cw argon ion 1.0–2.0 MW/cm² for 1–2.5 s. Optimum dose: 1.0 MJ/cm².</td>
<td>69 ± 19% of control colony formation at optimum dose. Similar recovery rates of control and irradiated cells</td>
<td>40×/0.60 1.0 or 0.7 63×/0.90</td>
<td>CHO</td>
<td>GFP (8.3 μg/ml)</td>
<td>29 ± 10%</td>
<td>Notably, one of the few cases in the literature to observe efflux of a fluorophore (calcein AM) upon irradiation.</td>
<td></td>
<td></td>
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</tbody>
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<tr>
<td>488</td>
<td>[43]</td>
<td>Targeted cw transient DNA</td>
<td>cw argon ion</td>
<td>17 mW for 2 seconds (with medium containing 40 mg/L phenol red) or 10 seconds (with 15 mg/L phenol red)</td>
<td>94.4 ± 3.6% (2 second dose), 27.7 ± 10.5% (10 second dose)</td>
<td>10×/0.45</td>
<td>4</td>
<td>Cardiac neonatal rat cells</td>
<td>GFP (5 μg/ml)</td>
<td>5.1 ± 3.5% (2 second dose), 3.25 ± 2.4% (10 second dose)</td>
<td>Used phenol red and FM 1-43 to increase absorption of laser by plasma membrane. Employed the use of Cyclosporin A, SIN-1, and SNAP to protect against reactive oxygen species generated by laser. Employed the use of 1000x antioxidant supplement, CaCl₂, and Pluronic-F68 to promote membrane rescaling after dosing. Employs the use of phenol red solution (15mg/l) to increase laser absorption, which was necessary for the generation of photopores at these doses.</td>
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<tr>
<td>488</td>
<td>[41]</td>
<td>Targeted cw transient (36h) DNA</td>
<td>cw argon ion</td>
<td>2·10⁶ W/cm² for 0.25 s</td>
<td>No acute morphological signs of injury</td>
<td>100×/1.0</td>
<td>5–8</td>
<td>–</td>
<td>Lac-Z (beta-galactosidase) (1.5 μg/ml) or CAT (chloramphenicol acetyltransferase) (8 μg/ml)</td>
<td>–</td>
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<tr>
<td>532</td>
<td>[65]</td>
<td>Untargeted ns transient fluorophore, macro-molecule, and peptide</td>
<td>Frequency doubled Nd:YAG 5 ns</td>
<td>Single pulse at 10 μJ</td>
<td>Target area was glass/medium interface on monolayer of adherent cells. Central zone (0–30 μm from the center): 0% viability. Pericentral zone (41–50 μm): &gt; 90% viability. Distal zone (&gt; 50 μm): 100% viability</td>
<td>100×/1.3 0.3–0.4</td>
<td>Rat basophilic leukemia</td>
<td>Texas red-glycine (800 D), Texas red-dextran (3 kD, 10kD, 40 kD), fluorescein labelled protein kinase C pseudosubstrate region (RFARKGSLRQ KNV-fluorescein)</td>
<td>30–80%</td>
<td>Up to 40 kD dextran species can be loaded into cells, but loading efficiency at this size is 10x less than for 800 D glycine species. This suggests that passive diffusion through pores may be a mechanism</td>
<td></td>
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<tr>
<td>532</td>
<td>[46]</td>
<td>Targeted or untargeted ns multiple macro-molecule</td>
<td>Frequency doubled Nd:YAG 0.5 ns, 2 kHz</td>
<td>Optimum dose ~60 nJ/μm²</td>
<td>–</td>
<td>–</td>
<td>HeLa, SU-DLH-4, NTERA-2, MO-2058, PFSK-1, 184-A1, CEM, NIH/3T3, 293T, HepG2, primary rat cardiomyocytes, embryonic C166</td>
<td>C2⁺, Zn²⁺, Sytox Green, Sytox Blue, tetramethylrhodamine-dextran (MW 3000), Cdc42 binding domain of WASP conjugated to an I-SO dye (55 kDa), quantum dots, SiRNA</td>
<td>–</td>
<td>Pore seals in approximately 30 seconds</td>
<td></td>
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<tr>
<td>λ (nm)</td>
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<td>770</td>
<td>[50]</td>
<td>Targeted fs vs targeted ns toxicity assay</td>
<td>Ti:sapphire, 110 fs, 80 MHz</td>
<td>150 mW or 190 mW for 5 ms</td>
<td>79% viability 12 h post-irradiation by MitoTracker Red, ethidium bromide, Annexin V, and morphology</td>
<td>100×/1.3 1.3</td>
<td>NG108</td>
<td>–</td>
<td>–</td>
<td>Compared toxicity of ns pulsed laser at 337 nm with fs pulsed laser at 770 nm. Fs laser treatment resulted in higher viability. See 337 nm entry.</td>
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<tr>
<td>780</td>
<td>[51]</td>
<td>Targeted fs single 100 nm particle</td>
<td>Ti:sapphire, 100 fs, 83 MHz</td>
<td>25–70 mW at focus</td>
<td>–</td>
<td>100×/1.4 Diffraction-limited</td>
<td>CHO</td>
<td>Single 100 nm gold nanoparticle</td>
<td>–</td>
<td>The first case in the literature a group has optically tweezed a single nanoscopic object to the surface of a cell prior to optical injection.</td>
<td></td>
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<tr>
<td>780</td>
<td>[4]</td>
<td>Untargeted (raster scanning) transient and stable DNA in vivo</td>
<td>Ti:sapphire, 200 fs, 76 MHz</td>
<td>20 mW raster scan of 95 × 95 µm² over the course of 5 s (for optimal dose) at a tissue depth of 2 mm</td>
<td>Morphology: rare fibers observed at 48 h but not 24 h or 70 h post-irradiation. Creatine phosphokinase levels not significantly higher than non-irradiated control 2h post-irradiation. No apoptosis by TUNEL assay observed 7d after irradiation.</td>
<td>50×/0.5 1</td>
<td>Tibial mouse muscle in vivo</td>
<td>GFP, luciferase, β-galactosidase, murine erythropoietin (333 µg/ml in 30 µl (i.e. 10 µg total) directly injected into muscle)</td>
<td>About half of the efficiency of electroporation.</td>
<td>Toxicity of electrotransfection is more pronounced than phototransfection. Electroporated cells had significantly increased levels of creatine phosphokinase, higher levels of apoptosis, and demonstrated extensive and irreversible damage by histological observation.</td>
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<tr>
<td>790</td>
<td>[52]</td>
<td>Targeted fs transient DNA</td>
<td>Ti:sapphire, 120 fs, 100 MHz</td>
<td>3X doses of 40 ms of 70 mW at central core of Bessel beam</td>
<td>–</td>
<td>60×/1.8</td>
<td>1.8 CHO</td>
<td>DsRed-Mito (1.2 μg/ml)</td>
<td>&gt;20% over an axial distance of 100 μm. Peak efficiency was ∼47%.</td>
<td>The first incidence in the literature to use a Bessel beam, which has a beam profile akin to a rod of light. This allows targeted fs DNA transfection without the need for focusing, and opens the technique up to automation.</td>
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<tr>
<td>790</td>
<td>[69]</td>
<td>Targeted fs transient DNA</td>
<td>Ti:sapphire, 800 fs, 100 MHz</td>
<td>3X doses of 80 ms of 110 mW at focus</td>
<td>–</td>
<td>see Notes</td>
<td>~5 CHO</td>
<td>DsRed-Mito (3 μg/ml)</td>
<td>25–57%</td>
<td>The first incidence in the literature to use a fiber to deliver targeted optical transfection. Fiber delivery, with custom axicon tip producing a focused beam.</td>
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<tr>
<td>792</td>
<td>[54]</td>
<td>Targeted fs transient DNA</td>
<td>Ti:sapphire, 12 fs, 75 MHz</td>
<td>50–100 ms, 5–7 mW at focus</td>
<td>100%</td>
<td>40×/1.3, 63×/1.25</td>
<td>Human salivary gland stem cells (hSGSC), human pancreatic stem cells (hPSC), CHO</td>
<td>Ethidium bromide, pEGFP-N1 (0.4 μg/ml)</td>
<td>hPSC: 75%, hSGCS: 80%, CHO: 90%</td>
<td>&lt;20 fs pulse duration measured at the focus.</td>
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<td>793</td>
<td>[53]</td>
<td>Targeted fs viability study</td>
<td>Ti:sapphire, 100 fs, 3.8 kHz, regeneratively amplified</td>
<td>5–9 J/cm² (low), 14–23 J/cm² (medium), and 41–55 J/cm², 10–10000 pulses</td>
<td>LD50 (by Trypan Blue exclusion) for 5–9 J/cm² is ∼100 pulses</td>
<td>40×/0.6</td>
<td>1.6 CHO</td>
<td>–</td>
<td>–</td>
<td>A lower LD50 was observed in cell populations dosed in their cytoplasm compared with those hit in their nuclei.</td>
<td>continued on next page</td>
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<tr>
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<tr>
<td>800</td>
<td>[55]</td>
<td>Targeted fs DNA</td>
<td>Ti:sapphire, 120 fs, 80 MHz</td>
<td>30–225 mW for 10–250 ms. Optimum dose: 1.2 μJ/cm²</td>
<td>70 ± 8% by Trypan Blue exclusion 2 h after irradiation.</td>
<td>60 × 0.85</td>
<td>1</td>
<td>CHO-K1</td>
<td>pEGFP-N2</td>
<td>50 ± 10% at optimum dose</td>
<td>It is worth reading Brown et al (2008) to put this work within context [39].</td>
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<tr>
<td>800</td>
<td>[39]</td>
<td>Targeted fs macromolecule</td>
<td>Ti:sapphire, 100 fs, 82 MHz</td>
<td>Series of &lt; 100 ms doses</td>
<td>No blebbing or acute morphological changes post-irradiation</td>
<td>100 × 1.4 0.8</td>
<td>HL60</td>
<td>Trypan Blue (0.4%)</td>
<td>–</td>
<td>–</td>
<td>Employed the same laser source to optically tweeze (in a cw regime) and optically inject (in a mode-locked regime) an impermeable substance. See Figure 3.4.</td>
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<tr>
<td>800</td>
<td>[56]</td>
<td>Targeted fs transient DNA</td>
<td>Ti:sapphire, 140 fs (~210 fs at sample), 90 MHz</td>
<td>40 ms, 0.9 nJ per pulse (optimum dose)</td>
<td>90% viability by propidium iodide exclusion.</td>
<td>/0.8</td>
<td>–</td>
<td>GFSHR-17 granulosaMTH53a canine mammary</td>
<td>pEGFP-C1-HMGB1, pEGFP-C1 (50 μg/ml), propidium iodide (1.5 μM), lucifer yellow (100–1000 μM)</td>
<td>70%</td>
<td>Quantified experimentally the relative volume exchange of about 0.4 during photopore opening.</td>
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<tr>
<td>800</td>
<td>[57]</td>
<td>Targeted fs transient macro-molecule</td>
<td>Ti:sapphire, sub-10-fs, 80 MHz</td>
<td>&lt; 10 ms</td>
<td>91.5 ± 8% (by when 0.2 M sucrose employed)</td>
<td>/0.95</td>
<td>&lt; 1</td>
<td>Madin-Darby canine kidney cells</td>
<td>0.2–0.5 M sucrose (1.6 nm diameter)</td>
<td>For 0.2 M sucrose, a theoretical loading efficiency of 72.3% was obtained</td>
<td>The first case in the literature to critically examine the relationship between osmolarity and the amount of species able to be optically injected into a cell. Experiments performed at 4°C.</td>
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<td>800</td>
<td>[58]</td>
<td>Targeted transient fluorophore</td>
<td>Ti:sapphire, 12 fs, 75 MHz</td>
<td>100 ms, 20 mW at focus</td>
<td>91% (PC12), 100% (primary astrocytes)</td>
<td>25 × 0.4 2</td>
<td>PC12, primary rat astrocytes</td>
<td>Propidium iodide (5 μg/ml)</td>
<td>–</td>
<td>–</td>
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<tr>
<td>800</td>
<td>[75]</td>
<td>Targeted fs fluorophore</td>
<td>Ti:sapphire, 130 fs (~200 fs at sample), 82 MHz, 800 fs</td>
<td>0–400 mW for 20 seconds, 4–33 W/cm²</td>
<td>100% for doses ≤ 12 · 10¹²</td>
<td>40×/1.3</td>
<td>0.308</td>
<td>BAEC, Spisula Solidissima Oocytes</td>
<td>Lucifer yellow, propidium iodide</td>
<td>~100% at doses &gt; 4.0 · 10¹² W/cm²</td>
<td>–</td>
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<tr>
<td>800</td>
<td>[13]</td>
<td>Targeted fs transient DNA and fluorophore</td>
<td>Ti:sapphire, 170 fs, 90 MHz</td>
<td>49 or 65 mW for 17 ms</td>
<td>CHO and SK-Mel 28 cells showed good morphology after irradiation; NG108-15 often apoptotic</td>
<td>40×/1.3</td>
<td>–</td>
<td>CHO, SK-Mel 28, NG108-15, T47D clone 11</td>
<td>FITC labelled dextran (MW 500 kDa, which corresponds to a diameter of 26.6 nm [73]), eGFP-N1 (4.7 kB), cascade blue</td>
<td>–</td>
<td>An interesting example of how treating two cells with the same dose can result in the immediate loss of viability of one cell but not the other, highlighting the inherent variability of a cellular response to laser irradiation.</td>
</tr>
<tr>
<td>800</td>
<td>[61]</td>
<td>Scanning fs fluorophore</td>
<td>Ti:sapphire, fs, 80 MHz</td>
<td>7 mW</td>
<td>–</td>
<td>40×/1.3</td>
<td>–</td>
<td>PtK2</td>
<td>–</td>
<td>–</td>
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<tr>
<td>800</td>
<td>[60]</td>
<td>Targeted fs transient DNA</td>
<td>Ti:sapphire, fs, 80 MHz</td>
<td>50–100 mW for 16 ms</td>
<td>&quot;high NA&quot;</td>
<td>–</td>
<td>CHO</td>
<td>pEGFP-N1 (0.4 μg/ml)</td>
<td>100%</td>
<td>Timelapse microscopy indicates no detrimental effects on growth or division.</td>
<td></td>
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<tr>
<td>800</td>
<td>[74]</td>
<td>Targeted fs microbead</td>
<td>Ti:sapphire, 150 fs, 125 Hz, regeneratively amplified</td>
<td>20 pulses of 10 nJ/pulse</td>
<td>100×/1.251</td>
<td>NIH 3T3</td>
<td>200 nm polystyrene</td>
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<tr>
<td>840 [62]</td>
<td>Targeted fs mRNA or fluorophore</td>
<td>Ti:sapphire, 100 fs, 80 MHz</td>
<td>8–16 regions on the plasma membrane dosed for 1–5 ms with 24 mW at focus</td>
<td>Transfection with GFP mRNA remained viable for at least 24 h</td>
<td>40×/0.8</td>
<td>Diffraction limited</td>
<td>Primary rat neuron</td>
<td>Lucifer yellow, Elk1-GFP mRNA, Elk1 mRNA, Elk1-ETS mRNA, c-fos mRNA, DS-RED, Venus fluorescent protein, pTRI-Xef (all 10–15 μg/ml)</td>
<td>–</td>
<td>mRNA transfected into the dendrite, but not the cell body, of a primary neuron results in the death of the cell. An excellent example of the power of optical transfection in its ability to transfect specific regions of a cell</td>
<td></td>
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<tr>
<td>840 [63]</td>
<td>Targeted fs mRNA</td>
<td>Ti:sapphire, 100 fs, 80 MHz</td>
<td>16 regions on the plasma membrane dosed for 5 ms with 30–35 mW at the back aperture</td>
<td>Repeated transfection with mRNA over seven day period resulted in high viability over the subsequent four week period</td>
<td>40×/0.8</td>
<td>Diffraction limited</td>
<td>Primary rat neuron</td>
<td>200 μg/ml astrocyte mRNA transcriptome</td>
<td>–</td>
<td>Transfection of astrocyte transcriptome into primary neurons results in phenotype remodelling (i.e. conversion of neurons into astrocytes). First demonstration of multiple rounds of transfection</td>
<td></td>
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<tr>
<td>1064 [45]</td>
<td>Targeted ns transient DNA or fluorophore</td>
<td>Q-switched Nd:YAG, 17 ns, 10 Hz</td>
<td>3–4 J/cm$^2$</td>
<td>–</td>
<td>100×/</td>
<td>–</td>
<td>MCF-7</td>
<td>mercocyanin 540 (7.5 μg/ml), par pEGFPN1 (5 μg/ml)</td>
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<th>Category of transfection or optical injection</th>
<th>Laser details</th>
<th>Dose</th>
<th>Viability</th>
<th>Obj/NA</th>
<th>Spot (μm)</th>
<th>Cell type</th>
<th>Transfected item (concentration)</th>
<th>Transfection efficiency</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1064</td>
<td>[64]</td>
<td>Untargeted transient DNA</td>
<td>Ho:YAG and Nd:YAG, 10 Hz</td>
<td>Nd:YAG: 150 mJ, dosed with 1000, 2000, or 2500 pulses. Ho:YAG: ideal dose was 750 pules of 2000 mJ</td>
<td>–</td>
<td>See notes</td>
<td>–</td>
<td>Transitional cell carcinoma</td>
<td>pEGFP-N1 (200 μg/ml)</td>
<td>21.50%</td>
<td>Beam delivered unfocused to cells with 200 μm diameter fiber. The exclusion by cells of Propidium Iodide 10 s after dosing indicates that photopores seal in less than 10 s. DNA transfection efficiency calculated taking proliferation into account.</td>
</tr>
<tr>
<td>1554</td>
<td>[79]</td>
<td>Targeted transient fluorophore and DNA</td>
<td>20 MHz, 170 fs</td>
<td>7 s, 10^{12} W cm^{-2}</td>
<td>Mitochondrial depolarisation (as measured by JC-1) did not occur in cells 1.5h after exposure.</td>
<td>40×/1.0</td>
<td>2</td>
<td>HepG2</td>
<td>Propidium Iodide (1.5 μg/ml). pEGFP-C1 (20 μg/ml)</td>
<td>77.3% (corrected)</td>
<td>The Ho:YAG mechanism may be thermally mediated, as there is strong absorption at this wavelength. Cells increase their temperature to 45–55 °C during treatment, which is why the protocol calls for cells to be placed on ice during irradiation. Beam delivered unfocused to cells with 220 μm diameter fiber.</td>
</tr>
<tr>
<td>2080</td>
<td>[47]</td>
<td>Untargeted transient DNA</td>
<td>Ho:YAG and Nd:YAG, 10 Hz</td>
<td>2000 mJ, 200 pulses</td>
<td>See notes</td>
<td>–</td>
<td>Mad. u rat dorsal prostate adenocarcinoma</td>
<td>pEGFP-N1 (300 μg/ml)</td>
<td>41.3%</td>
<td>Beam delivered unfocused to cells</td>
<td></td>
</tr>
</tbody>
</table>
Volumetric changes to a cell occurring during photoporation are mediated largely by the tonicity of its environment. A cell photoporated in a hypertonic environment will undergo solution influx and volumetric increase, in a hypotonic environment solution efflux and volumetric decrease, and in an isotonic environment no net solution flux will occur.

In the case of a hypertonic environment, intracellular water will flow through the plasma membrane out of the cell, resulting in a shrunken cell state prior to dosing. Once a hole in its plasma membrane is generated, the cell will rapidly (< 1 second) expand back to its equilibrium volume \( V_{\text{equil}} \) as solute and solvent flow through the pore. This effect has been demonstrated in Madin-Darby Canine Kidney cells. When suspended in a 1.0 M hypertonic sucrose environment, they shrink to an equilibrium volume of \( V/V_{\text{equil}} = 0.578 \pm 0.085 \). Upon targeted photoporation using a femtosecond laser system, the cells return to a \( V/V_{\text{equil}} = 1.000 \pm 0.055 \) [56]. Theoretical calculations of loading efficiency based on empirical observations of the volume changes of kidney cells exposed to 0.2 M sucrose solution indicate loading efficiencies of 72.3% [56]. The pre-treatment of cells in a hypertonic environment has also been used in the optical transfection of the embryonic calli of Oryza sativa L. cv. Japonica (rice) cells [73]. The amount of species entering the cell in this situation can be readily estimated by assuming that the expansion of the cell is due entirely to the volume of the incoming solution.

Conversely, a cell exposed to a hypotonic environment will expand due to the influx of water through the plasma membrane. Once porated, the volume of this cell will shrink to its original volume as solute and solvent rapidly flow out of the cell. A hypotonic environment may therefore be disadvantageous in the loading membrane impermeable species into cells by photoporation.

In the case of an isotonic environment, during the time the pore remains open species external to the cell will passively diffuse through the pore until such time as the pore either closes, or the concentration of the species in the intracellular and extracellular environments equal each other. Negligible volumetric changes are observed in cells photoporated in isotonic medium, so the volume equilibration mechanism of species transport does not play a role in this situation. In this case, the flux (number of species travelling through the pore per second) may be calculated according using Fick’s first law [75]:

\[
\text{flux} = -\frac{D \cdot \pi \cdot R^2 \cdot \Delta c \cdot N_A}{L} \times \text{kcorr} \tag{3.4}
\]

Where \( D \) is the diffusion coefficient \( (\text{m}^2 \text{s}^{-1}) \), \( R \) is the radius of the photopore \( (\text{m}) \), \( \Delta c \) is the difference extracellular and intracellular concentration of the solute \( (\text{mol} \cdot \text{m}^{-3}) \), \( N_A \) is Avogadro’s number \((6.0221415 \times 10^{23} \text{ molecules-mol}^{-1})\), \( L \) is the channel length of the pore \( (\text{m}) \), and \( \text{kcorr} = (1 - r/R)^2 \) is a correction term that extends the validity of Fick’s law from point-like objects to objects with the Stokes radius \( r \) \( (\text{m}) \). \( D \) may be calculated by the formula:

\[
D = \frac{kT}{6\pi \eta r} \tag{3.5}
\]

Where \( k \) is the Boltzmann constant \( (1.3806503 \times 10^{-23} \text{ m}^2 \text{kg s}^{-2} \text{ K}^{-1}) \), \( T \) is the temperature \( (\text{K}) \), \( \eta \) is the viscosity of the medium \( (\text{kg m}^{-1} \text{s}^{-1}) \).

The majority of optical injection experiments have been performed in isotonic conditions. Two experiments in particular quantify the amount of species that has been loaded into an optoinjected cell. Stracke et al. (2005) demonstrated that a cell, after being photoporated in a solution of 10 \( \mu \text{M} \) FITC labelled dextran (MW: 500 kDa, diameter 26.6 nm) ended up with a cytoplasmic concentration of approximately 0.7 \( \mu \text{M} \) [13]. This represents a final cytoplasmic concentration of 7% of that which the surrounding medium contained. Baumgart et al. (2008) elegantly investigated this question using patch pipette techniques [53]. This group manually injected known concentrations of lucifer yellow into canine mammary cells to generate a standard curve of fluorescence. They then used this to calculate the intracellular concentration of lucifer yellow that had been opto-injected using...
the optimum dose noted in Table 3.2. This, in combination with the changes in membrane potential measured by patch clamping, showed that a final cytoplasmic concentration of about 40% of the surrounding medium was occurring, i.e., if the initial concentration of external fluorophore was 600 μM, one would expect the final intracellular concentration to be 240 μM.

The final section of this review now covers the underlying physical mechanisms associated with the photoporation process.

### 3.5 Physics of the Laser-Cell Interaction

The mechanisms that govern the technique of optical injection differ based upon the type of laser field employed. This is also an area of much ongoing research and as such we will outline here the main findings. In particular we shall concentrate on the mechanisms governing short pulse laser transfection which presently has proven to be the most effective. In the case of continuous wave light, we have described the use of short wavelength light, notably in the violet-blue range of the spectrum to initiate transfection. Absorption appears to play a key role here. Phenol red, a pH indicator ubiquitously added to cell culture medium, has a molar extinction coefficient of around 10,000 M⁻¹ at 488 nm [41]. Concentrations up to 40 mg/L have been added to culture medium during photoporation; using lower concentrations of phenol red necessitates longer laser irradiation times in order to obtain the same optical injection effect [43].

In the case of short pulse sources we distinguish between targeted and untargeted transfection schemes. For untargeted transfection the literature shows that ns pulses have been successfully used to create shock waves in the liquid medium that in turn cause microbubble formation that ultimately leads to cell transfection. While successful, this method is not usually suitable for targeted cell studies.

In the case of targeted cell transfection we concentrate upon the use of femtosecond pulses that have come to the fore. We note however that ns pulses for targeted transfection are now emerging as a viable method that offers a less expensive alternative [44–46]. In the femtosecond domain one has to begin by considering the breakdown processes in water [76].

The numerical studies performed to date elucidate how femtosecond poration may work (Figure 3.5). If we firstly consider the train of ultrashort pulses as having a repetition rate >1 MHz. In this regime we may consider applying average powers and energies such that we are well below the threshold required for bubble formation within the liquid. In this instance – which is the case for the main studies to date in fs transfection – heating effects or thermoelastic stress are not major considerations. However we do see the accumulation of numerous free electrons due to the application of the laser pulses that then react photochemically with the cell membrane causing transient pores to be generated [76–78]. The excitement here is that the fs beams can accumulatively generate enough free electrons without any bubble formation that can “gently” perforate the lipid bilayer and initiate transfection, in essence creating a low density plasma. If we now turn to the instance where a much lower repetition rate is used (<1 MHz) we see the pulse energies are actually much higher and the thermoelastic stress component becomes important – very small cavities (microbubbles) are generated with lifetimes up to 100 ns that mediate the cell poration process. In turn this is somewhat less “gentle” than the high repetition mechanism using the fs beams as too high an energy can cause bubbles that cause cell lysis rather than transfection. Experiments have looked and validated this approach where the size of the bubble generated is monitored in situ (online) with the aid of a probe laser beam [79].

It is intriguing to explore if one could replicate the “gentle” transfection at the single cell level that is seen with femtosecond beams with nanosecond pulses – the latter offers far more compact and
FIGURE 3.5: An overview of the physical breakdown induced by fs pulsed lasers, along with threshold values for the damage, transfection, or dissection of cells. The different effects are depicted together with the corresponding values of free-electron density and irradiance. The irradiance values are normalized to the optical breakdown threshold $I_{th}$ defined by a critical electron density of $n_e = 10^{21} \text{ cm}^{-3}$. All data refer to plasma formation in water with femtosecond pulses of about 100-fs duration and 800-nm wavelength [76]. With kind permission from Springer Science+Business Media: Applied Physics B, Lasers and Optics, Mechanisms of femtosecond laser nanosurgery of cells and tissues, Vol. 81, 2005, p. 1038, A. Vogel, J. Noak, G. Hütten, & G. Paltauf, Figure 21, © Springer-Verlag 2005.

Inexpensive laser systems make them more practicable. For the case of bulk dielectrics nanosecond optical breakdown is typically associated with significant plasma luminescence and dominant thermomechanical effects — this is not particularly suitable for gentle cell transfection. Very recent studies have explored ultraviolet-visible ns pulses with temporally smooth shapes [77]. Importantly the authors show the existence of a well-defined low-density regime at irradiances below the threshold for luminescent plasma formation. Experimental studies used seeded single-longitudinal-mode pulses with 7–11 ns duration. These are contrasted to more typical (nonseeded) ns pulses that always exhibit picosecond spikes due to longitudinal mode beating (making them less suitable). Using the single longitudinal mode ns laser pulses the study showed that optical breakdown occurred in two distinct steps. First, a nonluminescent low-density plasma is formed the expansion of which creates minute bubbles (typical radii $= 500 \text{ nm} – 10 \mu \text{m}$). Electron-hole recombination limits the free-electron density. The conversion efficiency of laser energy into bubble energy is typically very low ($\ll 1\%$). For energies one order of magnitude or more above the bubble formation threshold, the generated plasma suddenly assumes a much larger size. One observes a bright luminescence and large bubbles are produced with radii exceeding 0.1 mm. Accompanying this we have a conversion...
efficiency into bubble energy of 10%. Thermal ionization overcomes recombination processes. A runaway process leads to the generation of full-density plasmas. For infrared breakdown, luminescent plasmas are produced in one step already at the bubble formation threshold. For regular (nonseeded) laser pulses in the ns regime, no stable low-density plasma regime was observed at any laser wavelength. Most notably microchip lasers in the nanosecond regime may have smooth output pulses: this generates minute bubbles and thus delivers the low density plasma we desire [77].

3.6 Conclusion

Transfection by optical injection is now an established technology. It can provide a performance comparable to or better than existing nonviral transfection techniques, with one of its key advantages being the ability to treat individual cells in a targeted fashion under aseptic conditions. Moreover, its compatibility with other technologies such as confocal laser scanning microscopy or optical tweezers may pave the way towards the development of integrated systems where all modalities are on one microscope base: a bioworkstation. An armory of such exquisite cellular control and visualization will allow biomedical scientists to unravel some of nature’s secrets.

Acknowledgments

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References


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