Phototransfection of mammalian cells using femtosecond laser pulses: optimization and applicability to stem cell differentiation

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Abstract. Recently, femtosecond laser pulses have been utilized for the targeted introduction of genetic matter into mammalian cells. This rapidly expanding and developing novel optical technique using a tightly focused laser light beam is called phototransfection. Extending previous studies [Stevenson et al., *Opt. Express* **14**, 7125–7133 (2006)], we show that femtosecond lasers can be used to phototransfect a range of different cell lines, and specifically that this novel technology can also transfect mouse embryonic stem cell colonies with ~25% efficiency. Notably, we show the ability of differentiating these cells into the extraembryonic endoderm using phototransfection. Furthermore, we present two new findings aimed at optimizing the phototransfection method and improving applicability: first, the influence of the cell passage number on the transfection efficiency is explored and, second, the ability to enhance the transfection efficiency via whole culture treatments. Our results should encourage wider uptake of this methodology. [DOI: 10.1117/1.3430733]

Keywords: phototransfection; femtosecond; laser pulses; cell arresting; neuroblastomas; pluripotent stem cells; embryonic stem cell differentiation.

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1 Introduction

Cytoplasmic expression of genetic materials and other macromolecules has a crucial role in medicine and biology.[1] Essential applications involve delivery of a wide selection of potentially therapeutic agents [e.g., proteins, oligonucleotides, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA)].[2] Various methods including chemical (cationic polymers and lipids), viral, or physical approaches have been developed to promote uptake of foreign genes and other macromolecules into mammalian cells.[3] However, each of these delivery systems harbors limitations. For both *in vitro* and *in vivo* procedures, a gene, drug, and/or vaccine delivery scheme possessing a minimum cytotoxicity and immunogenic response, which can be applied under sterile tissue culture protocols and can also offer targeted treatment of individual cells, organelles, and organs, is highly desirable.

Optical cell transfection satisfies these criteria. Lasers of different wavelengths and type, ranging from continuous wave visible sources[4–9] to pulsed infrared[5,10–12] sources have been widely utilized for successful introduction and expression of genetic materials into mammalian cells with the use of femtosecond lasers being the most prominent. Additional benefits are that optical transfection setups can be easily integrated with other optical techniques, such as confocal laser scanning microscopy and optical tweezer systems.[13,14] For example, we have recently shown that gold nanoparticles can be optically tweezed to a desired location and subsequently introduced into mammalian cells by such phototransfection.[14] We have also demonstrated the possibility of optically transfecting mammalian cells via an axicon tipped optical fiber, thus opening the future prospect of coupling this phototransfection methodology with endoscopes for *in vivo* applications.[15]

The mechanisms that enables this femtosecond laser phototransfection have been previously determined by Vogel et al.[16] Briefly, the process is described as the accumulation of numerous free electrons resulting from irradiating the plasma membrane with ultrashort laser pulses. These generated electrons then react photochemically with the cell membrane, leading to the creation of transient pores that allow entry of extracellular media.

Previously, using a titanium sapphire femtosecond pulsed laser, we explored the successful laser parameters and conditions to establish the transfection efficiency of Chinese hamster ovary (CHO) cells.[5] To elucidate the versatility and adaptability of this optical transfection setup, in this paper we report on improvements in transient phototransfection of different types of eukaryotic cells derived from different tissue and/or organs. Specifically, we initially show that following successful phototransfection of various different cell lines,

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mouse embryonic stem (mES) cell colonies were also phototransfected. Notably, because stem cells are thought to be capable of advancing current therapies for tissue regeneration and/or engineering, these mES cell colonies were not only phototransfected to express a gene-encoding red fluorescent protein but also induced to differentiate into a specific tissue type via phototransfection with the Gata-6 transcription factor.

Second, our investigations provide a comparative analysis of the influence of the cell passage number on the phototransfection efficiency and found that as cells increase in age, the efficiency decreases. Third, we show that it is possible to improve the phototransfection efficiency by simply aligning the cells to distinctive points in the cell division cycle specifically, the pro-metaphase (M-phase) or the DNA synthesis (S-phase) stages of the cell cycle. Overall, these studies will pave the way for increased uptake and applicability of this methodology.

2 Materials and Methods

2.1 *Cell Culturing*

All cell lines were purchased from the European collection of cell cultures (ECACC, distributor Sigma-Aldrich Poole, UK). E14g2a cells were donated. Cells were cultured in a 37 °C, 5% carbon dioxide (CO_2), and 85% humid incubator. Cells were routinely grown in T25 vented-top culture flasks (Nunc™), subcultured twice weekly at a ratio 1:4 Chinese hamster ovary (CHO-K1), human neuroblastoma (SK-N-SH) and human embryonic kidney (HEK-293) cells were grown in minimum essential medium (MEM) (Invitrogen, United Kingdom) with 1% penicillin-streptomycin (PEST) (Sigma, United Kingdom) supplemented with 10% foetal bovine serum (FBS). The mouse/rat neuroblastoma x glioma hybrid (NG108-15) cells were grown in Dulbecco's modified eagle's medium (DMEM) (Invitrogen, United Kingdom) with 1% PEST and 10% FBS. Mouse embryonic stem cells (E14g2a) were grown in Knockout DMEM (KDMEM) (Invitrogen, United Kingdom) modified with 1% nonessential amino acids (Invitrogen, United Kingdom), 1% L-glutamine (Invitrogen, United Kingdom), 1% sodium pyruvate (Invitrogen, United Kingdom), 0.1% β-mercaptoethanol (Sigma, United Kingdom), supplemented with the cytokine leukemia inhibitory factor (LIF) (Millipore, United Kingdom) and 10% FBS (Biosera, United Kingdom). They were grown in 0.2% gelatin (Sigma, United Kingdom) coated on the surface of T25 culture flasks.

To prevent detachment of the monolayer when working with NG108-15 and HEK-293 cell lines, the glass surface of the petri dishes was coated with a thin layer of 2 $\mu g/cm^2$ laminin (Sigma, United Kingdom).

2.2 *DNA Preparation*

Preparation of all DNA plasmids and a description of the plasmid pDsRed2-Mito has been previously reported.[5,6] A plasmid encoding the transcription factor Gata-6 gene (pCAGSIH-Gata-6) was donated.

2.3 *Optical Setup*

The optical setup is shown in Fig. 1. Femtosecond laser beam pulses emitted by a titanium sapphire laser (790 nm, 80 MHz, 200 fs, average power at the beam focus=60 mW) were magnified by a simple two-lens telescope to match the back aperture of the 60× Nikon objective lens with a numerical aperture (NA) of 0.8. This created a diffraction limited spot of ~1.1 μm diam. A mechanical shutter (Newport, United Kingdom, model no. 845HP-02) placed in the beam path regulated the time of beam exposure at the cell sample plane. The sample was placed on an xyz stage (Newport, United Kingdom) and illuminated using a Koehler arrangement. The cell sample was imaged by a long working distance objective onto a Watec color camera (WAT-250D) placed below the sample stage.

2.4 *Sample Preparation and Phototransfection*

For phototransfection experiments, $\sim 10^4$ cells in 2 mL of complete medium were seeded in 35-mm-diam type-zero glass-bottomed petri dishes (23 mm diam=glass working area (World Precision Instruments, Stevenage, United Kingdom). These were incubated to subconfluence over 24 h in optimum growth conditions. Then, the monolayer was washed twice with 2 mL of OptiMEM (Invitrogen, United Kingdom) each time to remove the serum. Thereafter, the cells were covered in 60 μL of serum-free medium containing 10 $\mu g/mL$ of pDsRed2-Mito plasmid DNA (pDNA). The sample chamber was then covered with a 22-mm-diam type-1 coverslip (BDH, Poole, United Kingdom). Targeted phototransfection of individual cells was then performed via, first, imaging the beam at the bottom coverslip. Then, by carefully adjusting the translation stage in the axial direction while both the sample and the beam were imaged through a bright field, the beam was focused onto the plasma membrane of the cell. Laser irradiation per cell was thereafter administered by three shots of ultrashort duration while avoiding visual cellular response. Because the Rayleigh range of the beam was ~5 μm, we estimate the cell membrane to be within one Rayleigh range for the creation of the transient holes necessary for successful phototransfection.

Therefore, the top surface of the cell's plasma membrane was directly exposed to the pDNA and fs beam focus, the area where the multiphoton effect is confined[17] (see Fig. 2). Alignment of the beam focus to the cell's plasma membrane therefore promotes the generation of free electrons (e^-), which photochemically react with the membrane resulting in the induction of transient holes[16] through which pDNA diffuses into the cytosol. Following irradiation, the DNA-containing medium was aspirated, the monolayer washed once with OptiMEM, covered in 2 mL condition medium, and incubated under optimum growth conditions for 48 h before live cell fluorescence analysis.

All experiments were performed in triplicate, and each experiment was repeated five times. In all cases, the transfection efficiency (measured in percent) was calculated according to Tsukakoshi et al.[12] and Stevenson et al.[13] using the expression: $N_{cor}=[(E/D).100]/X_D$, where N_{cor} is the population corrected transfection efficiency, E is number of cells transiently expressing the pDNA after a suitable amount of time has passed, D number of cells dosed on a given experiment, and X_D is the ratio of proliferation that has occurred by the dosed cells between dosing and the measurement of expression.

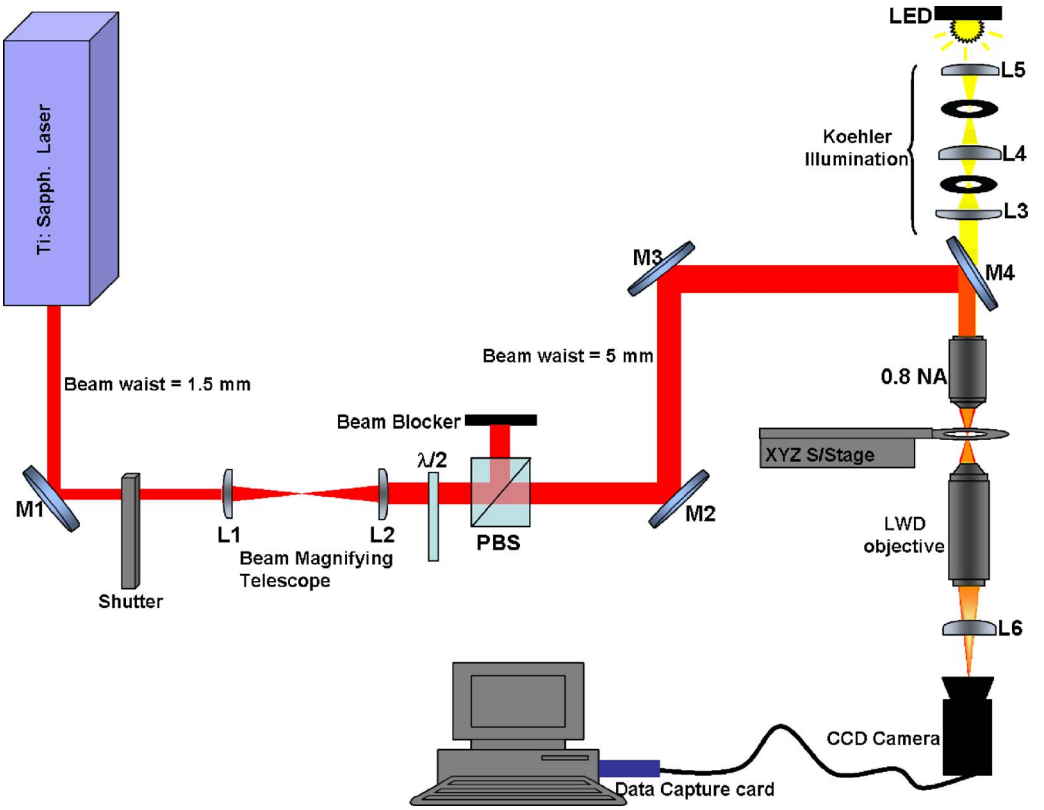

Fig. 1 Phototransfection setup for our studies. An infrared Gaussian beam (beam diameter=1.5 mm) is emitted by a titanium sapphire laser bounced off mirror (M1) and expanded via lenses L1 (f=50 mm) and L2 (f=175 mm). A half-wave plate ($\lambda/2$) and polarizing beamsplitter cube (PBS) were used to attenuate the power output, while mirrors (M2 and M3) served as a periscope. Mirror M4 reflected the beam onto the back aperture of a 60× objective lens (NA=0.8). A light-emitting diode (LED) provided sample lighting when arranged into Koehler illumination via passing through lenses (L5, L4, and L3) and two apertures. The sample imaging system consisted of a long working distance (LWD), f=200 mm, 50× objective lens (NA=0.55), a tube lens (L6), and a charge-coupled device (CCD) camera. These were connected to the output computer by a data-capture card.

2.5 *Stem Cell Differentiation and Selection*

E14g2a cells were seeded at concentration 10^6 cells/mL in gelatin-coated glass-bottomed petri dishes and incubated overnight. To stimulate cell differentiation, these were phototransfected with 15 μg/mL of Gata-6 pDNA at 50-mW average power levels at the beam focus and 40-ms pulse duration. After laser treatment, the cells were rinsed with serum-free KDMEM and incubated in optimum growth conditions in complete medium and selecting with 200 μg/ml of hygromycin (Invitrogen, United Kingdom). The medium was changed every two days.

2.6 *Reverse Transcriptase Polymerase Chain Reaction (rtPCR)*

Total RNA was isolated from differentiated embryonic stem (ES) cells by trizol reagent (Invitrogen, United Kingdom). For rtPCR analysis, cDNA was synthesized from 5 μg of total RNA, with an oligo-dT primer and transcriptor reverse transcriptase (rt) (Roche, United Kingdom). One-twentieth of the single-strand cDNA products were utilized for each polymerase chain reaction (PCR) amplification experiment. PCR was performed according to the manufacturer's instructions using the ready-mix taq PCR reaction kit (Sigma, United Kingdom). Primer (Eurofins, United Kingdom) sets (Gata-4, Oct-4, and Nanog) described by Fujikura et al.[18] were used at 10 pmol and 40 cycles per reaction. Amplified cDNAs were run on a 1.5% agarose gel.

2.7 *Cell Cycle Arrest by Thymidine and Colcemid*

CHO-K1 and HEK-293 cells were reversibly synchronized at the M and S phases of the cell-division cycle. Cells were plated in complete medium in T25 tissue culture flasks at concentration 10^6 cells/mL and incubated in optimum growth conditions over 48 h prior to arresting. Next, a nonlethal metaphase arrest was induced by treating the cells with 10 μg/mL of colcemid (Invitrogen, United Kingdom), followed by a further incubation of 6 h. Thereafter, the cells were harvested using 0.25% trypsin (Sigma, United Kingdom), plated for phototransfection and left to recover for one cell cycle ($\sim$18 h).[19–21] Addition of colcemid to the cells prevents the centrioles from organizing the microtubules, which are necessary for chromosome migration to the poles during cell division.[22] Cells were synchronized at the S phase via a thymidine-1 (TdR) (Sigma, United Kingdom) double block. Briefly, they were treated with 2 mM of TdR for 18 h, released into complete medium for 9 h, and then incubated in medium containing 2 mM TdR for another 18 h. Afterward, the cells were again harvested with 0.25% trypsin, plated for

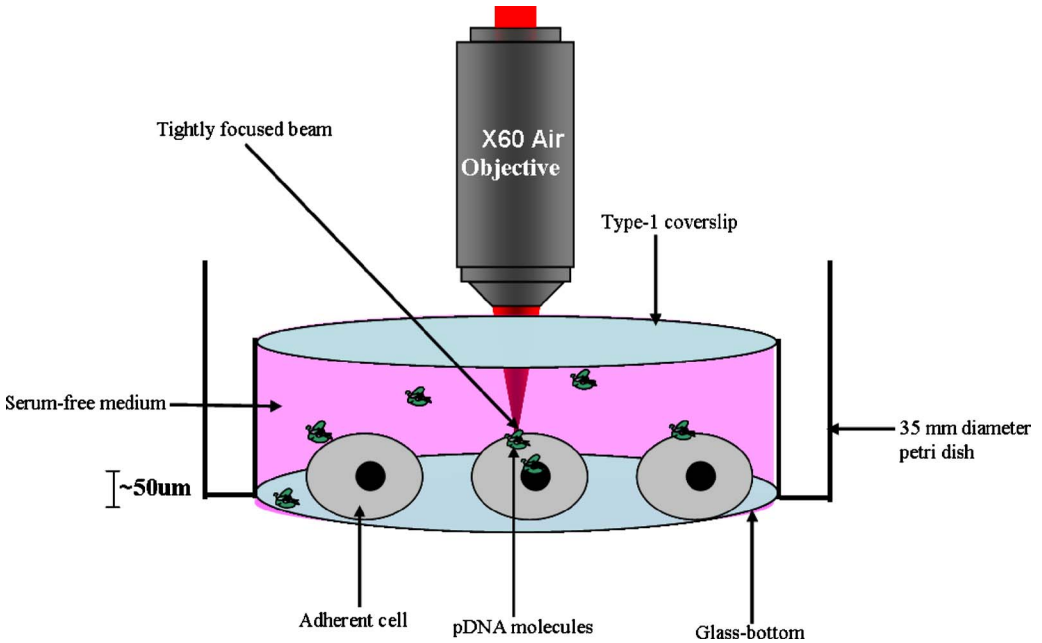

Fig. 2 Sample chamber illustrated. Irradiation of the top surface of adherent cells facilitated by targeted delivery of the infrared fs laser beam permitted transient opening of the plasma membrane and subsequent diffusion of surrounding plasmid DNA.

phototransfection, and allowed to recover for one cell cycle (~18 h). Essentially, TdR is uptaken by cells and subsequently phosphorylated to thymidine-monophosphate and thymidine-triphosphate (TIP). The accumulation of TIP results in the feedback inhibition of other nucleoside triphosphates, particularly deoxycytidine-triphosphate (dCTP), which is critical for DNA synthesis.[23]

3 Results

3.1 *Phototransfection and Differing Cell Types Including Stem Cells*

Our previous studies have focused on the commonly used cell line, the CHO-K1.[5] Therefore, using the same optical parameters and optical setup, we established the transient transfection efficiency (see Table 1) of a range of differing mammalian cell types, including pluripotent stem cells (E14g2a) and neuroblastomas (SK-N-SH and NG108-15), that are difficult to transfect by conventional transfection technologies.[24]

Table 1 Summary of the cell lines phototransfected with a gene-encoding red fluorescent protein (pDsRed2-Mito). Experiments were performed in triplicate with a minimum of 150 individual cells treated per experiment. The transfection efficiency values reported were corrected as previously described.

Cell type	Tissue of origin	Transfection efficiency (%)
CHO-K1	Ovary (hamster)	63 (2500 cells treated)
HEK-293	Kidney (human)	52 (2000 cells treated)
NG108-15	Brain (mouse/rat)	40 (1500 cells treated)
SK-N-SH	Brain (human)	45 (1500 cells treated)
E14g2a	Embryo (mouse)	25 (1000 cells treated)

All cell types were successfully transfected, but of particular note, was the possibility of phototransfecting embryonic stem cell colonies. Indeed, the effect of the exposure of stem cells to transfection reagents and/or chemicals is important because they may alter their metabolic balance and influence their biochemistry. Figure 3 depicts successful phototransfection of nondifferentiated pluripotent E14g2a stem cells and also indicates that our system allows the selection of specific cells within a mass of cells.

The successful laser transfection of stem cells has been previously reported,[25] but crucially we explored the possibility of taking this technology an important step further. Previously, we and others have commonly transfected a fluorescent reporter gene into mammalian cells, but within the field of stem cell biology, it would be a significant step forward to use physiologically relevant genes. Indeed, Uchugonova and Konig[26] reported on an urgent requirement in stem cell research on technologies for noninvasive, marker-free observation of growth, proliferation, and stability of living stem cells under physiological conditions. Therefore, here we report the induced differentiation of mouse embryonic stem cells into a defined tissue by introducing a specific marker-free plasmid DNA via femtosecond laser pulses.

Mouse stem cells (E14g2a) can be differentiated into extraembryonic endoderm by the activation of the transcriptional factor Gata-6, which is considered to be one of the master regulator genes.[18] Using our established parameters, we phototransfected the endoderm-associated transcription factor Gata-6 gene into E14g2a cells. These cells were further cultured as outlined in the methods, and as a result of the Gata-6–induced differentiation events, a morphological change in E14g2a cells 96 h post-treatment was observed. Figure 4 displays images of this result, showing an altered

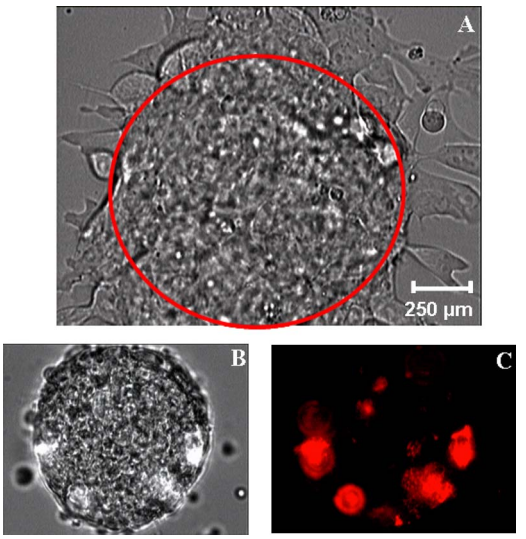

Fig. 3 A 50× Mitutoyo long working distance objective lens (NA =0.55) was used to capture image A displaying a brightfield view of the E14g2a cells. Surrounded within the red circle is the sample of interest (i.e., the nondifferentiated cell colony). 10 μg/mL of DsRed2-Mito pDNA was phototransfected into these cells at 50 mW (at the beam focus) and 40 ms. Then, 48 h later a 10× Nikon objective lens (NA=0.25) was employed to capture images B (brightfield image) and C (fluorescent image) in the presence of a TRITC filter cube. (Color online only.)

morphology of E14g2a colonies producing identifiable individual cells with spindle- and stellate-shaped morphology, which is characteristic of extraembryonic endoderm tissue.[18]

To biochemically prove this morphological change, the differentiated cells were tested by rtPCR for the expression of key transcriptional factors. In Fig. 5, the upregulation and/or downregulation of three different transcriptional factors, including Gata-4, Oct-4, and Nanog, is shown. Our results confirm that the stem cells have been differentiated as we can see: first, the amplification of Gata-4 (lane 2) in the transfected cells as compared to the undifferentiated cells (lane 5) and, second, downregulation of both Oct-4 and Nanog (lanes 3 and 4) in the differentiated cells as compared to the undifferentiated cells (lanes 6 and 7). This is in agreement with what has been previously observed for these transcription factors.[18]

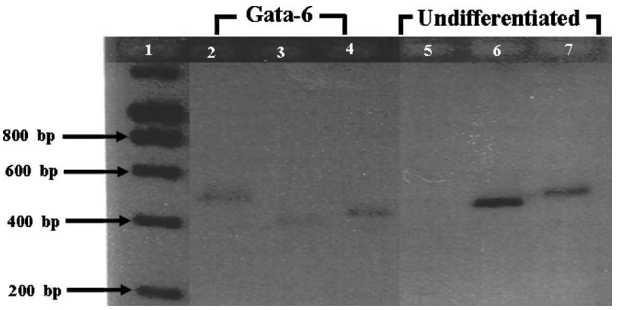

Fig. 5 rt-PCR analysis of germ layer markers. Lane 1: Molecular weight marker: Hyperladder 1 molecular weight marker (Bioline), Lane 2: rtPCR product for Gata-4, Lane 3: rtPCR product for Oct-4, Lane 4: rtPCR product for Nanog gene transcripts in differentiated cells (Gata-6). Lane 5: rtPCR product for Gata-4, Lane 6: rtPCR product for Oct-4, Lane 7: rtPCR product for Nanog gene transcripts in undifferentiated cells.

3.2 *Culture Passage Number and Transfection Efficiency*

Successful phototransfection of stem cells into recognizable tissue is in itself significant, but next we explored methods to enhance this technology even further. Generally, in all transfection experiments, the passage number of cell lines can affect not only the transfection efficiency, but also protein expression.[27] We therefore phototransfected a plasmid encoding a mitochondrially targeted red fluorescent protein (pDsRed2-Mito) into CHO-K1 cells of different culture passage number to establish its effect on phototransfection. As can be seen in Fig. 6, from passage 19 to 30 then, the effi-

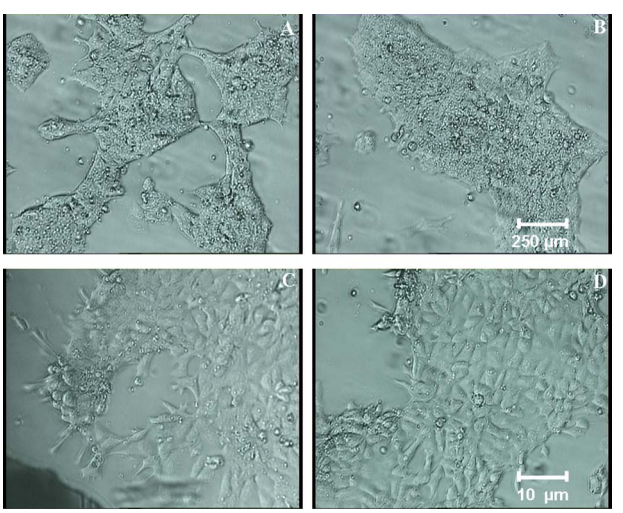

Fig. 4 (a) and (b) (negative controls) are brightfield images of the E14g2a cell colonies 48-h postroutine subculturing, growing in the presence of LIF. Obtained 96 h after phototransfection at 50 mW (at the beam focus), 40 ms with the Gata-6 vector. (c) and (d) are the morphologically altered E14g2a cells selected in the presence of LIF. All images were captured through a 10× Nikon microscope objective lens (NA=0.25).

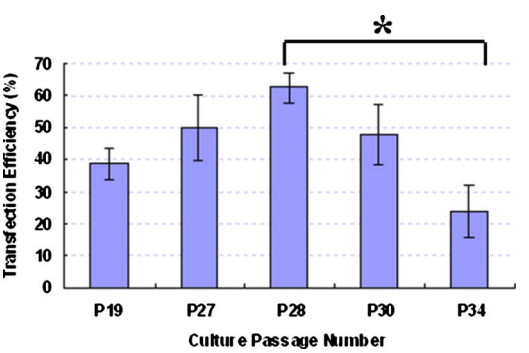

Fig. 6 CHO-K1 cells phototransfected (at an average power at the beam focus=60 mW and 40 ms time of exposure) illustrate the influence of culture passage number on the phototransfection efficiency. Error bars represent the standard error of the mean (n=5 experiments of 50 treated cells). Using analysis of variance (ANOVA) followed by Dunnett's and Fisher's tests: The asterisk means data sets are significantly different from each other.

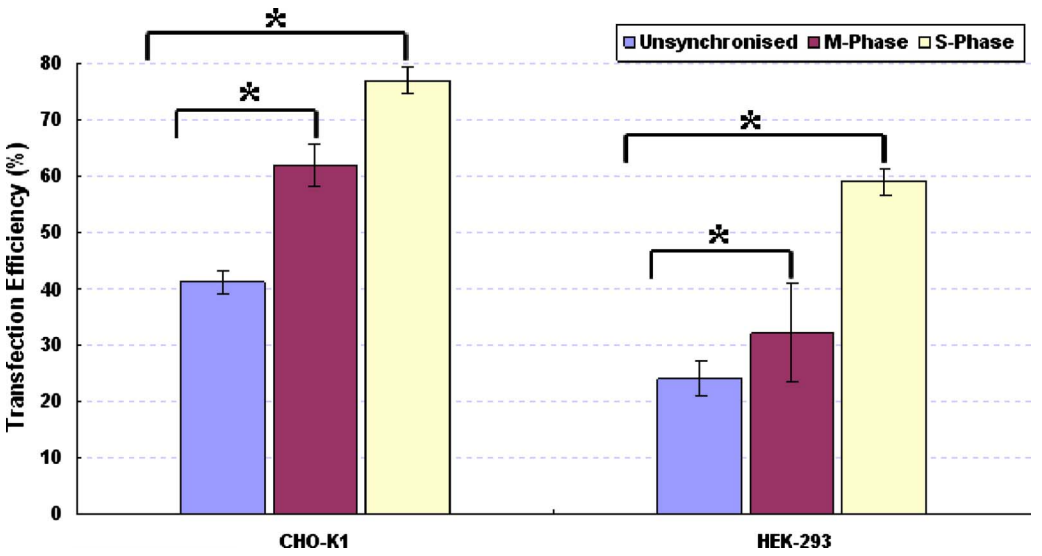

Fig. 7 Arresting CHO-K1 and HEK-293 at the M phase gave a higher phototransfection compared to cells treated while at random stages of the cycle. However, arresting at the S phase gave highly enhanced phototransfection efficiency; this was observed in both cell lines. The corrected values of transfection efficiency are presented. Error bars represent the standard error of the mean (n=5 experiments of 50 dosed cells). Using ANOVA followed by Dunnett's and Fisher's tests: The asterisk means data sets are significantly different from each other.

ciency is between 40 and 60%, peaking at approximately passage 28, but then drops significantly once the cells are at passage 34. This confirmed that, as with other transfection techniques, the passage number of a cell line plays a significant role in establishing the efficiency of phototransfection.[27]

3.3 *Cell Arresting and Phototransfection Efficiency*

We next explored the role of the cell cycle in the phototransfection of mammalian cells. In Fig. 7, is shown the effect of chemically inducing CHO-K1 and HEK-293 cells to be arrested at two different phases (M and S) of the cell cycle, thereby synchronizing the population of the cells. The results show that arresting cells at two different stages of the cell cycle can significantly enhance phototransfection efficiency, with those in the S-phase the most efficient. The reasons for this are dependent on the processes occurring in the different phases of the cell cycle. In the M phase, the nuclear membrane disappears and, as a result, gene transfection efficiency increases as plasmid DNA can easily access the nuclear machinery.[28,29] While in the S phase, this is the point in the cycle where DNA is synthesized; thus, plasmid DNA will be also copied and transcribed, increasing its overall copy number and thus subsequently allowing more transcription.

4 Discussion

Phototransfection is becoming an applicable transfection technology. This technique allows the delivery of both molecular and/or nonmolecular particles of various sizes into mammalian cells. Key characteristics to this all-optical methodology include its ability to keep cell viability intact, retain the quality of genetic materials being introduced, producing a minimum immunogenic response and its use under sterile tissue culture protocols. Notably, in contrast to other methodologies, phototransfection permits the delivery and expression of small molecules, including mRNA, into specific subcellular regions within cells,[30] and also the ability of targeting individual cells within a population of cells.

We have previously explored the transfection of mammalian cells with fs lasers and established its transfection efficiency in CHO-K1 cells.[5] Here we explored new parameters about this process. Initially, we show that this technology can be applied to a variety of different cell types and, specifically, it has the capability to phototransfect successfully mES both in a targeted manner but also with the ability of transforming these cells into a new cell type.

We also show that it is possible to improve phototransfection efficiency even further than our standard procedure. First, it appears that there is a strong dependency of the phototransfection efficiency to the cell passage number. Therefore, for future studies, it is imperative to establish the passage number under which a cell line is at to optimize transfection efficiency. The effects of passage number on cell lines are intricate and can be influenced by factors such as the type of cell line, the tissue and species of its origin, and also the culture conditions of the cells.[31]

Our results also demonstrated the influence of the metabolic state of the cells on transfection efficiency. In fact, in cells phototransfected while harboring S-phase DNA content, the efficiency of transfection was significantly higher than in cultures treated while accumulated in the M phase and even those in a nonarrested state. Notably, a phototransfection efficiency of ~80% for CHO-K1 rivals many chemically induced methodologies. This observation therefore demonstrates that the phototransfection efficiency reflects cell-cycle dependency. This effect was also reported for the improvement of both transient and stable cell transfection efficiencies using electroporation technology, where cells were synchronized in the G2/M phase and then treating them with butyrate.[32] This combination allowed a decrease of electropo-

ration voltage and DNA concentration while greatly enhancing the transfection efficiency.

In summary, we have shown that fs laser phototransfection has the ability of becoming a standard transfection method, because it now has the ability of transfecting cells at a high efficiency, a wide variety of cell types, but with the additional capability of being targeted when required.

Acknowledgments

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