Journal of Innovative Optical Health Sciences
Vol. 4, No. 2 (2011) 113−125

DOI: 10.1142/S179354581100123X

TRANSFECTION AND CELL FUSION BY FEMTOSECOND LASERS

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Biophotonics is an exciting and fast-expanding frontier which involves the fusion of advanced photonics and biology. It has not only created many novel methodologies for biomedical research, but also achieved many significant results as an independent field. Thanks to femtosecond (fs) laser technologies, important progresses have been made regarding the manipulation, imaging, and engineering of biological samples ranging from single molecules to tissues in the last 20 years. The ultrashort pulses at near-infrared band provide many advantages: high nonlinear efficiency, low absorption by biological samples, high spatial and temporal resolution and confinement, and low phototoxicity. They are noninvasive and easy to control. Although the mechanism of how fs laser pulses interact with cells remains unclear, experimental results have shown that they could open up the cell membrane and hence made optical transfection and optical cell fusion possible. In this review, some of the seminal works on transfection and cell fusion by fs lasers are presented. The ideas behind and the experimental details will be described together with a highlight on their significances. Specifically, the thermal effect is analyzed based on multiphoton excitation and plasma formation in an aqueous environment to explain the nontoxic characteristic of fs laser irradiation. Last, some applications of fs laser induced transfection and cell−cell fusion with potential major impact in biomedical sciences are proposed.

Keywords: Biophotonics; transfection; cell fusion; femtosecond laser.

1. Introduction

Biophotonics has advanced rapidly, thanks to the development and applications of femtosecond (fs) lasers in the early 1990s.[1] Significant progresses have been made on multiphoton imaging,[2,3] optical coherence tomography,[4] and nanosurgery of cells.[5,6] The fs beam at the near-infrared (NIR) range, around 700−1100 nm, has shown a lot of important advantages when irradiating on biological samples, such as less scattering in cells, little absorption by water, and possessing low photon energy. Hence, both cells and water exhibit good transparency in this range, which is termed the "optical window." Besides, the ultrafast pulse can significantly protect the cell from optical damage when compared with traditional continuous-wave (CW) and long-pulsed lasers. In most practical cases, the laser beam would be focused by an objective of high numerical aperture (NA) onto the sample. The spatial resolution can be as high as submicron level, which is a little

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less than the diffraction limit of the NIR beam, because of the nonlinear effect of photons. Inside the small focused spot, the photon density at the peak of the pulse can reach a very high level, resulting in nonlinear absorption and excitation in this region.[7] This means the fs pulses can be focused onto any single subcellular structure,[8,9] such as a mitochondrion,[10] a chromosome,[11] and even tissue structures,[12] to disrupt or image those targeted organelles without creating any harm to other parts of the cell within a very short interval.[13] This is exemplified in nonlinear imaging methods by fs beams, such as coherent anti-Stokes Raman spectroscopy (CARS) developed by the group of Sunney Xie.[14−17] The disruption caused by fs pulses can be very precise and controllable while guaranteeing the safety of the cells.[18] Regeneration of neurons has been observed after fs laser irradiation,[19] which will help the rapid identification of genes and molecules that affect nerve regeneration and development and is an important step toward developing treatments for human neurological diseases. Such kinds of surgeries can even produce vascular disruptions within the rat brain parenchyma that targets single microvessels and achieved three forms of vascular insult.[20] The resolution of cell surgery has also been progressing swiftly. Nanoprocessing by fs beams at nanometer scales has been reported.[21] Aided with some nanoparticles, the manipulation of single molecules, like manipulating genes and DNA, can be achieved by fs beams.[22,23] This advancement is very promising in gene engineering. Clinical applications of fs beams for nano-/micro- surgery are expected as evidenced by the demonstration of ablation of subepithelial voids in porcine vocal fold tissue within 100 μm below the surface to enable the ablation of a larger void in the active area of vocal fold mucosa in about 2 min.[24] Corneal surgery by laser-assisted *in situ* keratomileusisusing (LASIK) has also been improved by using fs pulses.[25,26]

When the focus of the fs laser is localized on a cell membrane, its permeability can be changed if the photon density in that volume exceeds a certain level.[27] Besides, holes may be opened on it, although there is still no direct experimental proof. This allows the materials inside the cell to exchange with the environment outside. Molecules which originally are not permitted to pass through the cell membrane can now diffuse into cells. Based on this idea, researchers have used fs laser in cell transfection and cell−cell fusion. In this review, we first give a brief introduction to fs beams focused by a microscope system for biological samples. The development of transfection then follows, with a discussion on the thermal effect of fs pulses and a protocol for transfection. After that, cell−cell fusion by fs laser is introduced and its mechanism is proposed. Finally, prospective future applications of these optical methods are given.

2. Properties of Femtosecond Pulses Focused by Objective Lenses

In practice, the fs beam is usually focused by objectives to irradiate one specific spot of the biological sample. If the NA of the objective is large enough (usually > 1), the photon density at the peak of the pulse can reach a level of more than $10^{12}\,\mathrm{W\,cm^{-2}}$, causing nonlinear absorption to dominate in the main part inside the focus.[28] Hence, fs pulses exhibit a much weaker dependence on the absorption coefficient of the target material. However, the photon density decays rapidly outside of the focus region and will therefore not induce any nonlinearity there. Hence, fs laser effects are finely confined in the focal volume, whose spatial extent is below the optical diffraction limit as shown in Fig. 1. Consequently, all parts of the sample outside the laser focus are protected from photon damage.

Plasma formation is now considered as the most probable mechanism for changing the cellular

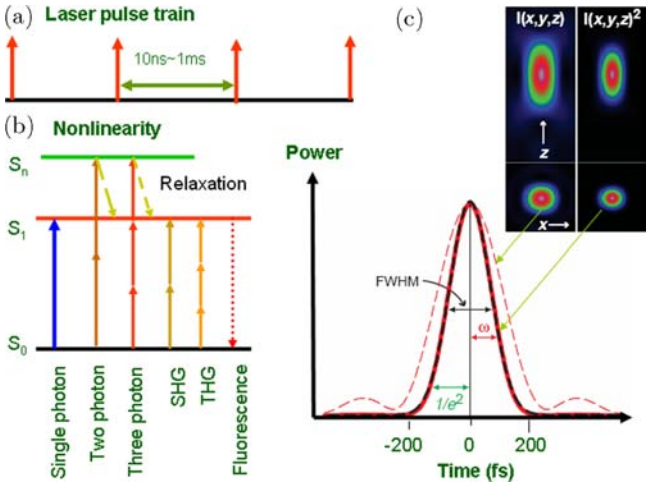


Fig. 1. Properties of fs laser pulses. (a) Typical fs pulse train. (b) Nonlinearities induced by the fs pulse. (c) Spatial optical intensity distribution of fs pulses after focusing. Dashed red line: original intensity distribution of a pulse. Solid red line: intensity distribution squared.

membrane permeability by fs lasers.[29] Generally, valence band electrons in lipids or other materials within the cell can absorb a sufficient number of photons from the fs laser pulse to become free electrons, which in turn can also absorb photons to excite more free electrons. A low-density plasma can thus be formed by this kind of avalanche ionization inside the focus volume to damage the membrane structure placed at the focus. As the plasma is confined in the focal volume, the fs laser below a certain power level generates only a slight thermal effect, thereby avoiding damage to the irradiated cells. This will be discussed in more detail later. Ultrashort laser pulses thus provide a lot of advantages: high nonlinear efficiency, low absorption by biological samples, high spatial and temporal resolution, and low phototoxicity, clean, noninvasive, and controllable.

3. Optical Transfection

3.1. *Femtosecond optical transfection*

The delivery of foreign molecules such as DNA, proteins, and other macromolecules into cells through the plasma membrane, known as transfection, is a key technique in cell and molecular biology with many important biochemical applications. It provides an efficient way to study the temporal and spatial regulation of protein systems that underlie basic cellular functions, and is expected to be used in innovative therapy by the introduction of healthy copies of mutated or absent genes into target cells so as to promote the expression of normal protein and to restore correct cellular function.[30] Traditional methods like electroporation and liposomal transfection can transfect a large population of cells but without any selectivity, and the efficiency is not very high.[31] Microinjection can be operated at single cell level but it is an invasive procedure.[32]

Lasers, especially fs lasers working in the near-infrared region, have shown distinct advantages in biological fields. In 2002, König *et al.* first reported the use of Ti:sapphire fs laser at 800 nm with a repetition frequency of 80 MHz and a mean power of 50−100 mW to transfect Chinese hamster ovarian (CHO) and rat−kangaroo kidney epithelial (PtK2) cells.[33] In their study, the photon density at the focus was around $10^{12}\,\mathrm{Wcm}^{-2}$ so that the laser beam produced a single, site-specific, transient perforation in the cell membrane through which DNA could enter. To reduce the damage to cells, they suggested that the exposure time should not be longer than 16 ms and the laser beam should be focused on the edge of the membrane of target cells. Subsequently, a report by the group of K. Dholakia in 2006 indicated that the efficiency of opto-transfection by the Ti:sapphire laser was around 50% ± 10% by testing photoporation on 4000 CHO cells with a different average laser power,[34] which was also focused by a high *N.A.* objective on the cell membrane. They found that many cells died even when the exposure time was strictly controlled within 250 ms. This problem arises probably because fs lasers can generate reactive oxygen species, perturb the cell plasma membrane integrity, deform the nuclei, and introduce DNA strand breaks if the exposure time is too long.[35]

In 2008, we coupled a fiber fs laser at 1554 nm (Calmar FPL-04) into an inverted microscope (Nikon, TE2000U) to examine its effect on transfection and photoporation of human liver cancer HepG2 cells.[36] The optical design is shown in Fig. 2. In our setup, the fs laser was coupled to a 40× objective lens ($N.A. = 1.0$). The fs fiber laser had a central wavelength at 1554 nm and a repetition frequency of 20 MHz with a mean power around 100 mW and the pulse width around 170 fs. The system coupling efficiency was around 60%. The diameter of the laser beam focus was about 2 μm, and thus the peak power was $10^{12}\,\mathrm{Wcm}^{-2}$ at the focus, which was high enough to perforate the cell membrane. We tested 50 HepG2 cells adhering on a cover glass in 0.5 ml of culture medium containing

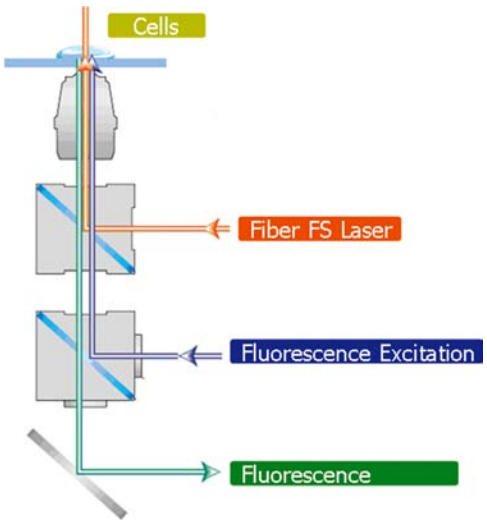


Fig. 2. Optical design of the transfection system by the fs laser at 1554 nm.

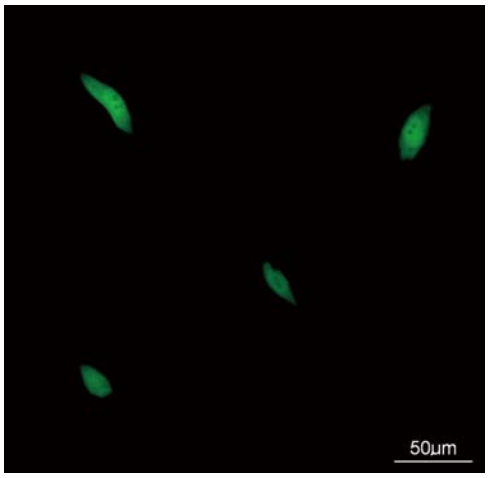
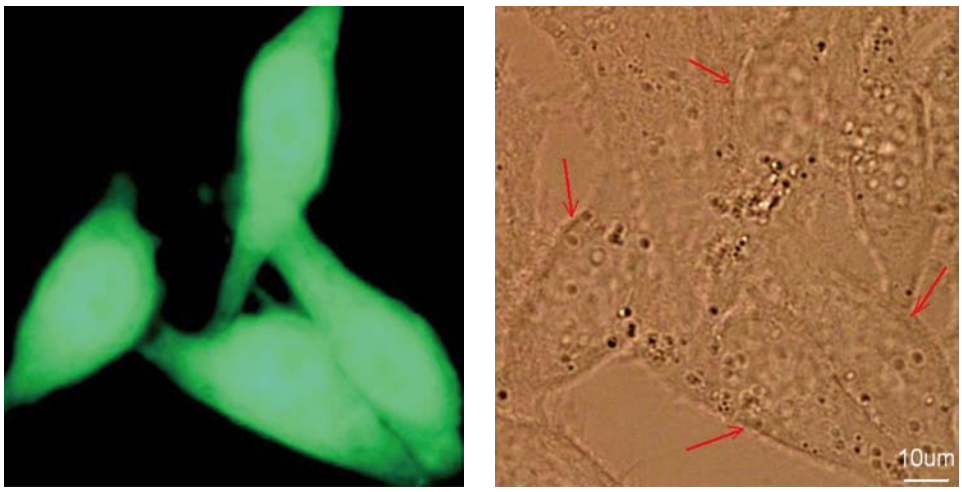


Fig. 3. GFP fluorescence in HepG2 cells with targeted transfection. HepG2 cells were cultured at 37°C and 5% CO_2 for 24 h. Adherent cells were then exposed to the 1554 nm fs beam for 7 s in the medium with a plasmid encoding enhanced GFP (final concentration 40 μg/ml).

20 μg of plasmid DNA [GFP gene pEGFP-C1 (4.7 kb)] by individually exposing them to the laser beam for 7 s. After illumination, the cells were cultured at 37°C and 5% CO_2 for GFP expression for 24 h. Thirty-eight healthy cells in an elongated form emitted the green fluorescence as shown in Fig. 3, indicating that GFP was expressed. To eliminate the error introduced by cell proliferation (the doubling time of HepG2 is $\sim$48 h), adjacent cells were intentionally not exposed to achieve a minimum distance of around 50 μm between any two exposed cells. Consequently, if one single cell with green fluorescence was observed in an area of 50 μm by 50 μm, it should be the mother cell. In the case of two or more neighboring cells emitting green fluorescence, we only counted them as one transfected cell. This experiment was repeated two more times and 36 and 42 healthy cells with the GFP expressed were observed separately out of 50 exposed cells. Thus, the transfection efficiency was around 77.3% with a p-value of 2.82e-52 according to *Fisher exact test*. A control experiment was also performed in which cells were cultured with the same amount of DNA plasmids for 24 h but without laser exposure and no fluorescence was found from all those cells. The proliferation of the transfected cells is shown in Fig. 4 after 48 h since the transfection event. It shows that the cells still survived after a 7 s irradiation of fs laser.

In this experiment, it is very important that the focus of the laser must be localized on the cell membrane. However, the effective focal length in the axial direction of a Gaussian beam is very short (around 4 μm), which necessitates an adjustment of focus for different cells. It is worth to note that Dholakia's group used a Bessel beam for transfection.[37] The Bessel beam offers transfection at axial distances 20 times greater than that of its Gaussian equivalent. This means the need for exact focusing of the laser beam upon the membrane surface has been alleviated, thereby enabling the automation of this technique. Besides, the self-healing properties of the beam have been shown to permit transfection through a distorting medium. Thus, one can envisage much higher throughput for laser transfection



(a) (b)

Fig. 4. Proliferation of transfected cells. (a) Green fluorescence image of several HepG2 cells transfected with GFP plasmid. (b) Image of the same cells with GFP expression under white light. Red arrows: cells transfected.

when multiple cell planes are combined with a laser beam multiplexing method in the lateral direction.

Using shorter fs pulses (~ 20 fs), transfection on stem cells has been also reported in 2008.[38] It was found that mean power of less than 7 mW (< 93 pJ) and low millisecond exposure times were found to be sufficient to transfect human pancreatic and salivary gland stem cells in these preliminary studies.

It needs to be stressed that the long axial focal length of the Bessel beam will also generate effects in the other parts of cells besides the membrane. Organelles inside cells and even localized ion store can respond to the laser exposure.[39,40] However, biological safety is a very complex problem. It has already been observed that fs lasers could harm cells when they were first put into experiments. The group of König has done a lot of research since 1990s.[41,42] They investigated the cellular damage and responses to the fs beam in nonlinear imaging and presented some safe threshold of scanning or exposing time. It is clear that the cellular response has a dependence on the pulse length of the fs beam[43] since the peak power and exposure time are both changed accordingly. Besides, during the scanning by fs beam for two photon imaging, the group also noticed the mitochondrial response and presented its safe scanning laser power.[44] Mitochondria are very important organelles in cells that are closely related with the viability and death of cells. König's group later found that the fs beam could generate reactive oxygen species (ROS) inside cells which led to apoptosis-like death.[35] More recently, our group discovered that the ROS generated by the fs laser could cause mitochondria depolarization or dysfunction.[45] The mechanism of ROS generation was also proposed. Thus, the mitochondrial transmembrane potential (MTMP) is a good indicator of cellular viability. By measuring the MTMP before and after laser exposure, the safe exposure time of the fs beam at 1554 nm was reported.[36] In short, even though fs laser irradiation can harm organelles like mitochondria or trigger cellular free Ca^{2+} release,[46] cells can still maintain sustainable viability if the exposure is controlled within a safe limit. Hence, a Bessel fs laser beam can also work as a safe tool to manipulate cells, although its special beam shape may induce more side effects.

In all common setups, a high NA objective is needed to focus the fs beam to reach a high photon density. In 2008, Dholakia's group reported the success of using fiber delivery for the transfection.[47] They engineered a standard optical fiber to generate an axicon tip with an enhanced intensity of the remote output field that delivers 800 fs pulses without requiring the fiber to be placed in very close proximity to the cell sample. The efficiency went up to 57%, which is comparable with free space transfection. This invention paves the way for optical transfection of tissue samples and endoscopic embodiments of this technique.

It is interesting to study the cellular response during the laser irradiation. Baumgart *et al.* measured membrane potential changes during optical transfection in 2008.[48] They calculated and experimentally verified that the relative volume exchanged was 0.4 times the total cell volume, which represents a quantitative estimate of the amount of uptaken molecules. Hence, quantification of the transfection process becomes possible.

Apart from GFP plasmid transfection, researchers also tried macromolecules for fundamental biological research and obtained dramatic exciting results. The opto-injection technique for macromolecules by fs pulses has been reported in 2005.[49] In 2006, a fs laser was used to introduce E-26−like protein 1 (Elk-1) mRNA into specific regions of live, intact primary rat neurons.[50] Introduction and translation of Elk-1 mRNA in dendrites produced cell death, whereas introduction and translation of Elk-1 mRNA in cell bodies did not produce cell death. Elk-1 translated in dendrites was transported to the nucleus, and the occurrence of cell death depended upon transcription, supporting the dendritic imprinting hypothesis, and highlighting the importance of the dendritic environment on protein function. Subsequently, transfection for miRNA has also been demonstrated in 2009.[51] This has enabled the measurement of target binding in the cytoplasm by two-photon fluorescence imaging.

Optical transfection can even produce conversion to different cell lines of differentiated cells. The group of James Eberwine used a fs laser to transfer the transcriptome from differentiated rat astrocytes into a nondividing differentiated rat neuron,[52] which resulted in the conversion of the neuron into a functional astrocyte-like cell in a time-dependent manner. The RNA population from astrocytes contains RNAs in appropriate relative abundances that give rise to regulatory RNAs and translated proteins that enable astrocyte identity. When transferred into the postmitotic neuron, the astrocyte RNA

population converts 44% of the neuronal host cells into the destination astrocyte-like phenotype. This technique is called transcriptome-induced phenotype remodeling and may potentially be capable of controlling the differentiation of differentiated cells and, even more importantly, the conversion of differentiated cells to stem cells.

3.2. *Thermal effect of the femtosecond pulses*

The thermal effect of the fs laser is very important to biological samples since they are very sensitive to temperature. However, as the fs pulses are too short and the focus is too tiny, there is no experimental report of any direct measurement of the temperature rise caused by the fs laser. Here we would like to use the model developed by Vogel *et al.*[29] to analyze at least the theoretical temperature rise. This rise is mainly dependent on the free electron density induced and not much related with the linear absorption of the cell.

To estimate the temperature rise, two parameters need to be considered, the characteristic time for electron cooling (the transfer of kinetic electron energy during collisions, around a few picoseconds[53]) and the electron recombination time, which is around only 300 fs.[54] Hence these two time constants are both longer than our fs pulses (170 fs). Therefore, the energy density deposited into the interaction volume is simply given by the total number density of the free electrons produced during the pulse period multiplied by the mean energy gain of each electron. The mean energy gain of an electron is given by the sum of the ionization energy, $\tilde{\Delta}$, and the average kinetic energy, 5/4 $\tilde{\Delta}$, for free electrons produced by cascade ionization. Thus, the plasma energy density is given by:

$$\varepsilon = \rho^* 9/4^* \tilde{\Delta}, \tag{1}$$

where ρ is the electron density. The temperature rise is then

$$\Delta T = \varepsilon / \rho_m C_m, \tag{2}$$

where ρ_m is the mass density and C_m is the heat capacity of the medium. Equation (2) can be used to estimate the temperature rise during each pulse period.

However, it should be noted that this estimation is only for the average thermal effect in the focal volume and within only one pulse duration. In reality, the temperature rise is related to the pulse width, pulse repetition rate, and the NA of the objective. It is obvious that the pulse width and repetition rate determine the accumulation of heat. If the pulse duration, and hence the heating time, is short, the excess thermal energy can diffuse away more rapidly. When the interval between two pulses is long enough for the heat produced by the leading pulse to decay away, there will be no accumulation of heat during the trailing pulse duration. The critical repetition rate is probably around 1 MHz, and lasers with repetition rate less than 1 MHz can raise the temperature only within a single pulse.[29]

The NA of the objective determines the focal volume. If the beam is tightly focused, the resulting tiny focal volume facilitates the heat diffusion to the surroundings. Usually, if the NA is larger than 1.0, there will be little accumulation of heat. If the NA is 0.6 or smaller, the heat accumulation will significantly increase the cell temperature.

The temperature rise caused by our fs laser at 1554 nm can be calculated in the following manner. Considering the laser power reaching the focus spot of diameter 2 μm is 60 mW, the power density is around 10^{12} W/cm^2 and the free electron density is calculated to be 10^{14}/cm^2 in the focal volume. The mean energy gain of an electron is estimated to be ~14.6 eV from the discussion above. The average temperature rise in the focal volume calculated from Eq. (2) above is ~10 K. The temperature rise in the central part of the focal volume will naturally be much larger than this value, but it is confined within a small part of the focal volume and will decay very quickly in less than one pulse duration. Hence the heating effect generated by the fs laser pulses should not result in any major harm to the cells if the exposure time is limited to a few seconds.

3.3. *Protocol of optical transfection*

In our experiments, we used the DNA plasmid of GFP for the optical transfection in HeLa and HepG2 cells. The protocols for the transfection are as follows.

3.3.1. *Preparation of cells*

Human HepG2 hepatocellular carcinoma cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco) or phenol-red free RPMI 1640 medium (Invitrogen) at 37°C and 5% CO_2 for more than

12 h and settled on the dish bottom. Cells (3×10^5/ml) were seeded on a 35 mm culture dish with a glass slide (0.17 mm thick) at the bottom (MatTek).

3.3.2. *DNA plasmid incubation*

The cell medium was at first removed and only 0.5 mL new medium with 20 μg GFP plasmid DNA was added to get a final concentration of 40 μg/mL. The cells were then incubated for another 3 h for the DNA molecules to attach to the membrane of cells.

3.3.3. *Laser treatment*

The cell dish was placed on the microscope and only adherent cells on the bottom were randomly selected to be illuminated by the fs laser for 7 s. The focus of the laser should be at the upper membrane of the exposed cell, whose position could be adjusted by the lens pair and the objective. The exposure time was controlled by an electrical or mechanical shutter.

3.3.4. *Cell incubation*

After the laser treatment, 2 mL of cell medium was added and the cells were incubated for 24 h at 37°C and 5% CO_2 for the GFP expression.

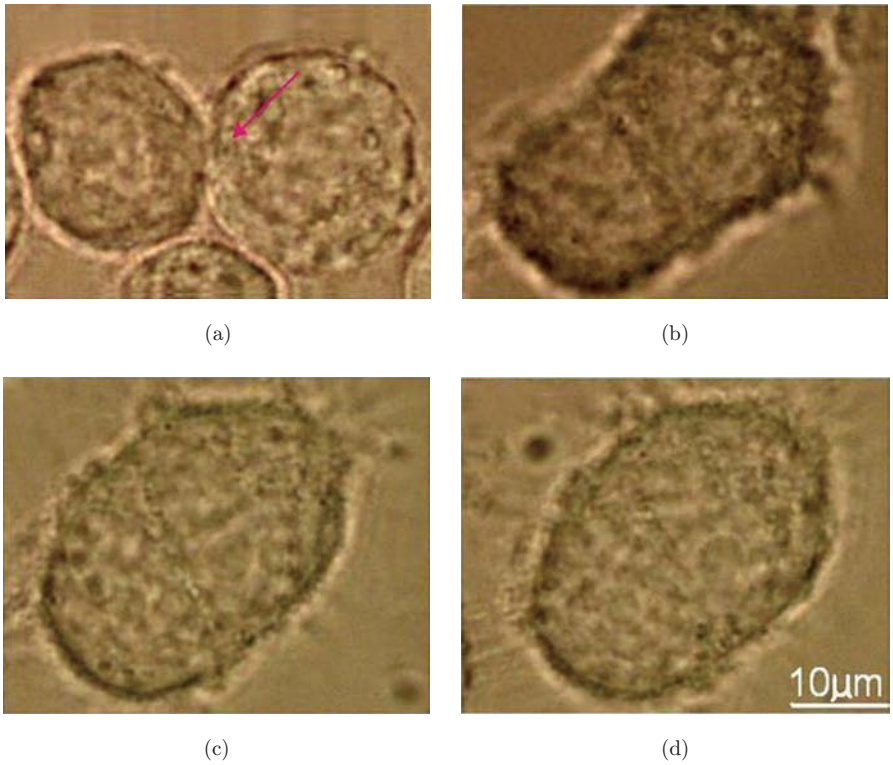
4. Optical Cell–Cell Fusion

4.1. *Femtosecond laser fusion*

Cell−cell fusion is a powerful tool for the analysis of gene expression, chromosomal mapping, monoclonal antibody production, and cancer immunotherapy.[55] One of the challenges of *in vitro* cell fusion is to improve the fusion efficiency without the need of adding extra chemicals while maintaining the cells alive and healthy. In 1991, Steubing *et al.* described the use of a nanosecond-pulsed UV laser to fuse two single cells with an efficiency less than 10%.[56] In their study, the scattered high-energy UV photons of the laser damaged the cells, and the thermal effect caused by the laser could not be confined within the focal volume. To overcome these disadvantages, Gong *et al.* employed polyethylene glycol (PEG) and a Ti:sapphire fs laser at 800 nm to fuse yeast cells with a very high efficiency (80%).[57] Such a high fusion efficiency was probably due to the better confinement of the fs beam and the ultrahigh pulse peak power. However, as PEG is used conventionally as a fusogen of cells to generate hybridomas for monoclonal antibody production,[58] it is not clear whether the high cell fusion efficiency in the yeast system was due more to the effect of fs laser or PEG. In fact, PEG is toxic and it imposes undesirable side effects to cells.[59] Besides, the cells were paired up randomly in their system by PEG and it would be difficult to find the proper targets for cell fusion, especially if they were from different cell lines. In this connection, we sought to develop a better method to fuse human cells solely optically that would not involve PEG or any other chemicals.

The same fs laser at 1554 nm was used for cell fusion and another CW laser at 980 nm was employed as an optical tweezer to manipulate the targeted cells.[60] As the fs laser power was maintained at 100 mW, it could photoionize the lipid and water at the interface between two contacting cells. The free electrons generated would destroy the membrane in the focal volume of the beam. After the laser irradiation, the broken lipid membranes of the two cells would connect together and create a stable connecting channel as a result of the membrane fluid mosaic property. The channel gradually became broadened and finally a hybrid cell was formed after cytoplasm mixing.[61] As shown in Fig. 5(a), a HepG2 cell was moved to contact with another, and this cell pair was then illuminated by the fs beam at 1554 nm for 10 s at the contact interface. Each pulse had a power at 1.6×10^4 W. After illumination, the two HepG2 cells were incubated at 37°C and 5% CO_2. Ninety minutes later, fusion was observed and a hybrid cell was formed 3 h later, as shown in Figs. 5(b)−5(d).

In this study, we had irradiated 56 HepG2 cell pairs by the fs laser, and 21 pairs were fused. The efficiency was therefore around 37.5%. The unsuccessful cases might be due to the fact that cells were in loose contact so that they could not stick together during fusion. It was difficult in our system to move two cells to contact each other tightly by the optical tweezer as this was a mechanical operation. It has been reported that a spacing larger than 50 Å between two cells poses a barrier to molecular exchange.[62] This is the main reason why our fusion efficiency was low. Besides, the fs beam also acted as an optical tweezer[63] and forced the cells under exposure to move or vibrate toward the central part of the beam. Hence the exchange channel would be cut off and thus the exchange process terminated. Furthermore, since lipid molecules could flow along the membrane, the cells might have repaired the holes in the membrane soon after the laser



(a) (b) (c) (d)

Fig. 5. A pair of HepG2 cells fused by a 1554 nm fs laser. (a) Two HepG2 cells were fused by a fs laser with a power of 100 mW for 10 s. Red arrow: the laser spot at the interface of the two cells. (b) 1.5 h after exposure, the membranes fused together. (c) 3 h after exposure, the hybrid cell became rounded. (d) 4 h after exposure, the two cells became one single hybrid cell. Bar: 10 μm. This is a typical example of 21 similar cases.

illumination. Apart from HepG2 cell pairs, we also fused HeLa−HeLa and HepG2−HeLa cell pairs and observed the exchange of cytoplasm, as shown in Figs. 6 and 7.

Our results represent the first all-optical homotypical and heterotypical cell fusion with high efficiency, and have been highlighted by Nature Photonics ["Femtosecond fusion," *Nature Photonics*, **2**, 709 (2008)]. This simple method without any chemical fusogen addition may become an important tool for cell−cell fusion in biotechnology and bioengineering and sets the stage for further research to understand the complex mechanisms of cell−cell fusion.

4.2. *Protocol of optical cell−cell fusion*

In the experiment, we used HeLa and HepG2 cell suspensions. The protocols for the cell−cell fusion are as follows.

4.2.1. *Preparation of cells*

Human HepG2 hepatocellular carcinoma cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum (FCS) (Gibco) or phenol-red free RPMI 1640 medium (Invitrogen). Cells (3×10^5/ml) were seeded on a 35 mm culture dish with a glass slide (0.17 mm thick) at the bottom (MatTek) and one dish of cells can be labeled by fluorophores like calcein/AM (Molecular Probes). The cells should be suspended in the medium during the experiment.

4.2.2. *Cell mixture*

The cells with calcein labeling were at first centrifuged and washed two times by PBS and then mixed with the other group without any labeling. The cell mixture could be observed by the fluorescence of calcein.

4.2.3. *Laser treatment*

The cell mixture was then put in the incubator (Nikon) on microscope stage (37°C and 5% CO_2). One cell with calcein labeling was randomly selected and trapped by the optical tweezer at 980 nm. It was moved to attach to another cell without any

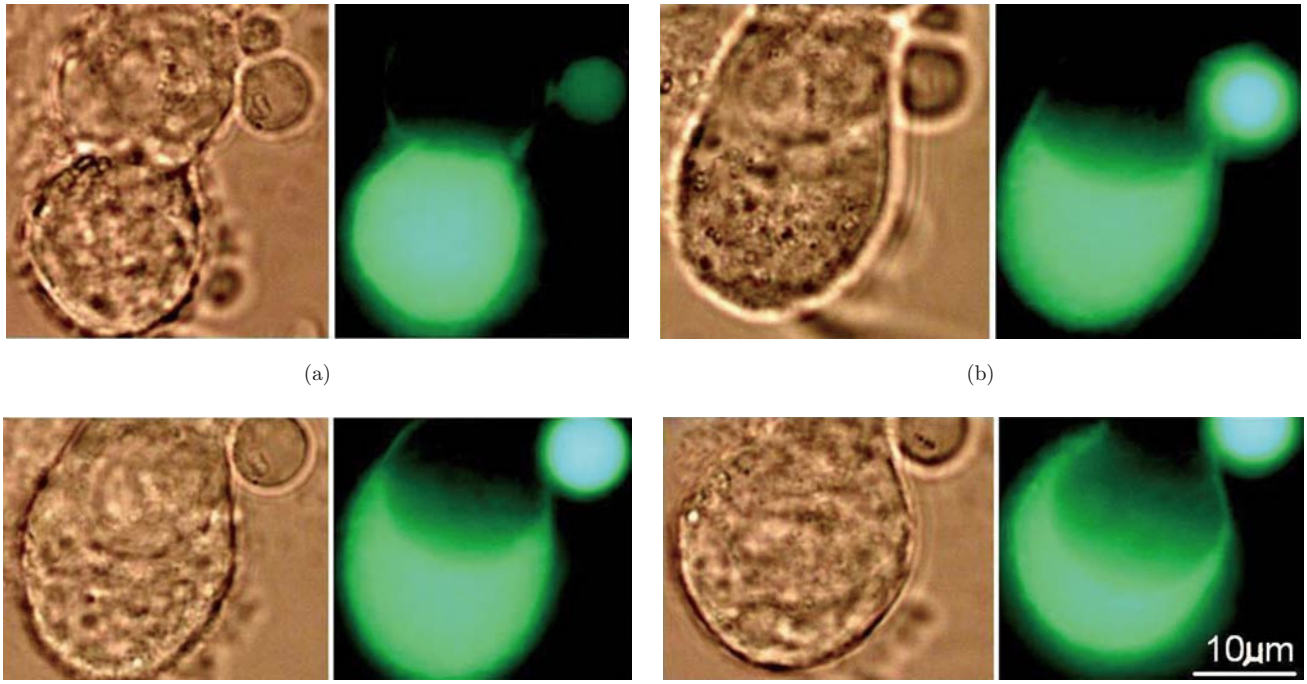


Fig. 6. Diffusion of cytoplasm within HeLa cells during optical fusion. (a) Two HeLa cells were fused together in which one was loaded with calcein and the other was not. The two cells were exposed to the fs laser for 10 s and cultured at 37°C and 5% CO_2. (b) 2 h later, the two membranes fused into one and calcein diffused into the upper cell. (c) 4 h later, more calcein diffusion was observed. (d) The mixing of cytoplasm continued while the photo was taken at 8 h after fusion. The bubble on the right was a fragment from other cells. Bar: 10 μm.

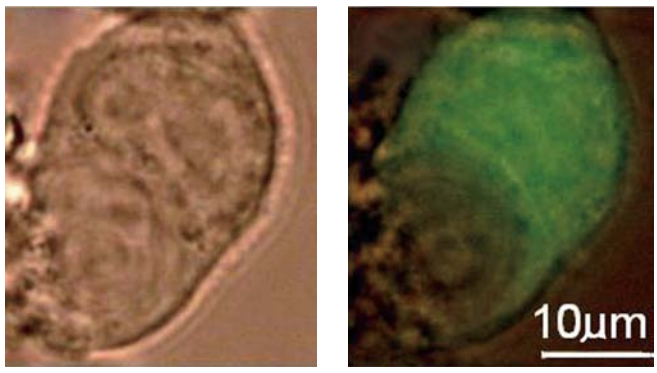


Fig. 7. A labeled HeLa cell was fused with a HepG2 cell without calcein. (a) A white light image of the hybrid cell taken at 3 h after laser illumination. (b) Corresponding fluorescence image of the hybrid cell. The cytoplasm of HeLa cell with calcein was shown in green. Fusion condition: exposure for 10 s with 100 mW laser power. Bar: 10 μm.

labeling. After the attachment, the optical tweezer was turned off and the common part of the membrane of the two cells was illuminated by the fs beam for 1−10 s. It should be noted that it would be better if the two cells had similar size and weight, because the optical tweezer effect due to the fs beam would induce significantly different forces to the two cells if they are too different to cause the cell pair to rotate in the vertical direction.

4.2.4. *Cell incubation*

After the laser treatment, the cells were incubated in the incubator for around 4 h and some would

fuse together successfully. One could observe the diffusion of the cytoplasm of the fused cell by the fluorescence of calcein.

5. Discussion

Here we would like to propose the mechanism of plasma generation in the culture medium by the fs pulses to explain how the fs pulses can open up the cell membrane. However, this is only a hypothesis without any direct proof. Again the model developed by Vogel *et al.*[29] is employed here. The low-density plasma induced by fs laser is a versatile tool for manipulation of transparent biological samples with little damage and yet very precise excitation. For simplicity, we only discuss the plasma formation in water here since the optical breakdown threshold in water is very similar to that in ocular and other biological media.[64] Besides, the refractive index of water (1.34) is very similar to those of most cells (1.45−1.55).

The band gap energy of electron in H_2O molecule is 6.5 eV. In some breakdown model, it is often assumed that a free electron can be produced as soon as the pump energy exceeds the band gap either by the sum of the simultaneously absorbed photons or by the kinetic energy of an impinging free electron. However, if the electron is ionized by a large volume of photons, one must add in the oscillation energy of the electron due to the electrical laser field. Then, the effective band gap is

$$\tilde{\Delta} = \Delta + \frac{e^2E^2}{4m\omega^2}, \tag{3}$$

where Δ is the original band gap, e, m, E and ω represent the electrical charge and effective mass of the electron, the electric field intensity and angular frequency of the light, respectively. The second term can be neglected if the laser pulse is long, but must be included for fs pulses since the electric field there is very large.

The ionization of electrons by photons can be explained by the following mechanism. If an electron in the valence band absorbs multiple photons simultaneously and the sum of whose energy is larger than the band gap energy, the electron will be excited by such a multiphoton absorption and become free. The photon energy is 1.2 eV at 1040 nm and 0.8 eV at 1554 nm. It means that at least six photons at 1040 nm or nine photons at 1554 nm are needed to excite one electron. The chance that one electron absorbs so many photons is usually very small. Only when the photon density is high enough will this nonlinear absorption be able to occur readily. As a macro-criterion, an electron in water can be ionized only when the electric field intensity of the light is larger than 100 MV/cm, which means that the photon density should be larger than $1.5 \times 10^{13}\,\mathrm{W/cm^2}$ at 1040 nm or $2.5 \times 10^{13}\,\mathrm{W/cm^2}$ at 1554 nm.

Once a free electron is produced in the medium, it can absorb photons in a nonresonant process, inverse Bremsstrahlung, during its course of collisions with heavy charged particles. The energy gain through inverse Bremsstrahlung should be more rapid than the energy loss by collisions with heavy particles occurring without simultaneous absorption of a photon. The energy of the photons can be converted to the kinetic energy of the free electron. After a sequence of inverse Bremsstrahlung absorptions, the electron can gain a sufficiently high kinetic energy to produce another free electron through impact ionization. Thus, there are two mechanisms in the ionization process by fs pulses in media, viz., direct multiphoton ionization and impact ionization. According to the assumption stipulated in other reports,[65,66] the critical energy for impact ionization in water can be estimated as around $1.5\tilde{\Delta}$. Since all free electrons can gain energy through inverse Bremsstrahlung absorption and then produce more electrons by impact ionization, an avalanche growth of free electrons will result if the irradiance is high enough to overcome the loss of free electrons through diffusion out of the focal volume and recombination. The whole process is called "avalanche ionization" or "cascade ionization."

6. Conclusion

In this study, the development of optical transfection and cell−cell fusion are discussed. Femtosecond lasers have played the key role in these processes. The optical method has a lot of advantages over traditional chemical/biological and physical methods, and is attracting more and more worldwide attention. The Ti:sapphire fs lasers have been widely used. But they are bulky, expensive, and more difficult to maintain and use. We have developed the protocols for transfection and cell−cell fusion using a commercial fiber fs laser at 1554 nm, which is compact, less expensive, and convenient to

operate. The protocols are also flexible regarding the control of exposure time. It is expected that researchers in this area may prefer to use fiber fs lasers as their tools of choice, not to mention that other wavelengths are also readily available in these lasers to test for better protocols. However, it should be noted that fs lasers in the longer wavelength range (longer than 1100 nm), such as 1554 nm, are not suitable for performing two-photon imaging since most fluorescence can be only excited by photons whose wavelength is shorter than 550 nm.

Optical transfection and cell−cell fusion have demonstrated important applications. Optical methods are not cell-type specific. They can be used on very special cell lines that are difficult to transfect by traditional methods, like the virus vector. Recent reports have shown that optical transfection can easily transfer mRNA and transcriptome into cells. It would be no surprise to witness optical transfection being used to control cell differentiation successfully in the near future. The mechanism of optical cell−cell fusion still remains unclear. Nevertheless, that should not hinder its great potential in such important applications as monoclonal antibody production and cancer immunotherapy because of its purely optical process that obviates any need for additional chemicals. It can be expected that both optical transfection and optical cell−cell fusion will contribute significantly to future biomedical research.

Acknowledgment

This work was supported in part by The Research Grants Council of HKSAR Government under GRF Grant Nos. CUHK410708 and 410809.

References

1. D. E. Spence, P. N. Kean, W. Sibbett, "60-fsec pulse generation from a self-mode-locked Ti: sapphire laser," *Opt. Lett.* **16**, 42−44 (1991).
2. W. Denk, J. H. Strickler, W. W. Webb, "Two-photon laser scanning fluorescence microscopy," *Science* **248**, 73−76 (1990).
3. W. R. Zipfel, R. M. Williams, W. W. Webb, "Nonlinear magic: Multiphoton microscopy in the biosciences," *Nature Biotechnol.* **21**, 1369−1372 (2003).
4. W. Drexler, U. Morgner, F. X. Kartner, C. Pitris, S. A. Boppart, X. D. Li, E. P. Ippen, J. G. Fujimoto, "*In vivo* ultrahigh-resolution optical coherence tomography," *Opt. Lett.* **24**, 1221−1223 (1999).
5. K. König, "Multiphoton microscopy in life sciences," *J. Microsc.* **200**, 83−104 (2000).
6. I. Maxwell, S. Chung, E. Mazur, "Nanoprocessing of subcellular targets using femtosecond laser pulses," *Med. Laser Appl.* **20**, 193−200 (2005).
7. K. König, "Femtosecond laser microscopy in biomedicine," *Laser Optoelectron.* **2**, 40−45 (2000).
8. K. König, I. Riemann, P. Fischer, K. J. Halbhuber, "Intracellular nanosurgery with near infrared femtosecond laser pulses," *Cell. Mol. Biol.* **45**, 195−201 (1999).
9. K. König, O. Krauss, I. Riemann, "Intratissue surgery with 80 MHz nanojoule femtosecond laser pulses in the near infrared," *Opt. Exp.* **10**, 171−176 (2002).
10. W. Watanabe, N. Arakawa, S. Matsunaga, T. Higashi, K. Fukui, K. Isobe, K. Itoh, "Femtosecond laser disruption of subcellular organelles in a living cell," *Opt. Exp.* **12**, 4203−4213 (2004).
11. K. König, I. Riemann, W. Fritzsche, "Nanodissection of human chromosomes with near infrared femtosecond laser pulses," *Opt. Lett.* **26**, 819−821 (2001).
12. U. K. Tirlapur, K. König, "Femtosecond near-infrared laser pulses as a versatile noninvasive tool for intratissue nanoprocessing in plants without compromising viability," *Plant J.* **31**, 365−374 (2002).
13. U. Tirlapur, K. König, "Near-infrared femtosecond laser pulses as a novel non-invasive means for dye permeation and 3D imaging of localised dye-coupling in the Arabidopsis root meristem," *Plant J.* **20**, 363−370 (1999).
14. A. Zumbusch, G. R. Holtom, X. S. Xie, "Vibrational mircoscopy using coherent anti-Stokes Raman scattering," *Phys. Rev. Lett.* **82**, 4014 (1999).
15. A. Volkmer, J.-X. Cheng, X. S. Xie, "Vibrational imaging with high sensitivity via epidetected coherent anti Stokes Raman scattering microscopy," *Phys. Rev. Lett.* **87**, 23901 (2001).
16. C. L. Evans, E. O. Potma, M. Puoris'haag, D. Côté, C. P. Lin, X. S. Xie, "Chemical imaging of tissue *in vivo* with video-rate coherent anti-Stokes Raman scattering microscopy," *Proc. Natl. Aca. Sci.* **102**, 16807−16811 (2005).
17. C. L. Evans, X. S. Xie, "Coherent anti-Stokes Raman scattering microscopy: Chemically selective imaging for biology and medicine," *Annu. Rev. Anal. Chem.* **1**, 883−909 (2008).
18. A. Uchugonova, A. Isemann, E. Gorjup, G. Tempea, R. Bückle, W. Watanabe, K. König, "Optical knock out of stem cells extremely ultrashort femtosecond laser pulses," *J. Biophotonics* **1**, 463−469 (2008).
19. M. F. Yanik, H. Cinar, H. N. Cinar, A. D. Chisholm, Y. Jin, A. Ben-Yakar, "Neurosurgery Functional

regeneration after laser axotomy," *Nature* **432**, 822−822 (2004).
20. N. Nishimura, C. B. Schaffer, B. Friedman, P. S. Tsai, P. D. Lyden, D. Kleinfeld, "Targeted insult to subsurface cortical blood vessels using ultrashort laser pulses: Three models of stroke," *Nat. Methods* **3**, 99−108 (2006).
21. K. König, I. Riemann, F. Stracke, R. Le Harzic, "Nanoprocessing with nanojoule near infrared femtosecond laser pulses," *Med. Laser Appl.* **20**, 169−184 (2005).
22. F. Garwe, A. Czaki, G. Maubach, A. Steinbrück, A. Weise, K. König, W. Fritzsche, "Laser pulse energy conversion on sequence-specifically bound metal nanoparticles and its application for DNA manipulation," *Med. Laser Appl.* **20**, 201−206 (2005).
23. A. Czaki, F. Garwe, A. Steinbrück, G. Maubach, G. Festag, A. Weise, I. Riemann, K. König, W. Fritzsche, "A parallel approach for sub-wavelength molecular surgery using gene-specific positioned metal nanoparticles as laser light antennas," *Nanoletters* **2**, 247−253 (2007).
24. C. L. Hoy, W. N. Everett, J. Kobler, A. Ben-Yakar, "Towards endoscopic ultrafast laser microsurgery of vocal folds," *Proc. SPIE* **7548**, 754831−754840 (2010).
25. B. G. Wang, I. Riemann, H. Schubert, D. Schweitzer, K. König, K. J. Halbhuber, "Multiphoton microscopy for monitoring intratissue femtosecond laser surgery effects," *Lasers Surg. Med.* **39**, 527−533 (2007).
26. S. Toropygin, M. Krause, I. Riemann, M. Hild, P. Mestres, B. Seitz, E. Khurieva, K. W. Ruprecht, U. Low, Z. Gatzioufas, K. König, "*In vitro* noncontact intravascular femtosecond laser surgery in models of branch retinal vein occlusion," *Curr. Eye Res.* **33**, 277−283 (2008).
27. A. Vogel, J. Noack, K. Nahen, D. Theisen, S. Busch, U. Parlitz, D. X. Hammer, G. D. Noojin, B. A. Rockwell, R. Birngruber, "Energy balance of optical breakdown in water at nanosecond to femtosecond time scales," *Appl. Phys. B* **68**, 271−280 (1999).
28. J. Noack, A. Vogel, "Laser-induced plasma formation in water at nanosecond to femtosecond time scales: Calculation of thresholds, absorption coefficients, and energy density," *IEEE J. Quantum Electron.* **35**, 1156 (1999).
29. A. Vogel, J. Noack, G. Hüttman, G. Paltauf, "Mechanisms of femtosecond laser nanosurgery of cells and tissues," *Appl. Phys. B: Lasers and Optics* **81**, 1015−1047 (2005).
30. D. J. Wells, "Gene therapy progress and prospects: Electroporation and other physical methods," *Gene Therapy* **11**, 1363−1369 (2004).
31. S. Mehier-Humbert, R. H. Guy, Physical methods for gene transfer: Improving the kinetics of gene delivery into cells," *Adv. Drug Deliv. Rev.* **57**, 733−753 (2005).
32. D. J. Stephens, R. Pepperkok, "The many ways to cross the plasma membrane," *Proc. Natl. Acd. Sci. USA* **98**, 4295−4298 (2001).
33. U. K. Tirlapur, K. König, "Targeted transfection by femtosecond laser," *Nature* **418**, 290−291 (2002).
34. D. Stevenson, B. Agate, X. Tsampoula, P. Fischer, C. T. A. Brown, W. Sibbett, A. Riches, F. Gunn-Moore, K. Dholakia, "Femtosecond optical transfection of cells: Viability and efficiency," *Opt. Exp.* **14**, 7125−7133 (2006).
35. U. K. Tirlapur, K. König, C. Peuckert, R. Krieg, K.-J. Halbhuber, "Femtosecond near-infrared laser pulses elicit generation of reactive oxygen species in mammalian cells leading to apoptosis-like death," *Exp. Cell Res.* **263**, 88−97 (2001).
36. H. He, S. K. Kong, R. K. Y. Lee, Y. K. Suen, K. T. Chan, "Targeted photoporation and transfection in human HepG2 cells by a fiber femtosecond laser at 1554 nm," *Opt. Lett.* **33**, 2961−2963 (2008).
37. X. Tsampoula, V. Garcés-Chávez, M. Comrie, D. J. Stevenson, B. Agate, C. T. A. Brown, F. Gunn-Moore, K. Dholakia, "Femtosecond cellular transfection using a nondiffracting light beam," *Appl. Phys. Lett.* **91**, 053902−053904 (2007).
38. A. Uchugonova, K. König, R. Bueckle, A. Isemann, G. Tempea, "Targeted transfection of stem cells with sub-20 femtosecond laser pulses," *Opt. Exp.* **16**, 9357−9364 (2008).
39. N. I. Smith, K. Fujita, T. Kaneko, K. Katoh, O. Nakamura, S. Kawata, T. Takamatsu, "Generation of calcium waves in living cells by pulsed-laser-induced photodisruption," *Appl. Phys. Lett.* **79**, 1208−1210 (2001).
40. S. Iwanaga, T. Kaneko, K. Fujita, N. Smith, O. Nakamura, T. Takamatsu, S. Kawata, "Location-dependent photogeneration of calcium waves in HeLa cells," *Cell Biochem. Biophys.* **45**, 167−176 (2006).
41. K. König, H. Liang, M. W. Berns, B. Tromberg, "Cell damage by near-IR microbeams," *Nature* **377**, 20−21 (1995).
42. K. König, P. So, W. W. Mantulin, E. Gratton, "Cellular response to near-infrared femtosecond laser pulses in two-photon microscopes," *Opt. Lett.* **22**, 135−136 (1997).
43. K. König, T. W. Becker, I. Riemann, P. Fischer, K. J. Halbhuber, "Pulse-length dependence of cellular response to intense near-infrared laser pulses in multiphoton microscopes," *Opt. Lett.* **24**, 113−115 (1999).
44. H. Oehring, I. Riemann, P. Fischer, K.-J. Halbhuber, K. König, "Ultrastructure and reproduction behaviour of single CHO-K1 cells exposed to near

infrared femtosecond laser pulses," *Scanning* **22**, 263−270 (2000).
45. H. He, K. T. Chan, S. K. Kong, R. K. Y. Lee, "Mechanism of oxidative stress generation in cells by localized near-infrared femtosecond laser excitation," *Appl. Phys. Lett.* **95**, 233702−233704 (2009).
46. H. He, S. K. Kong, K. T. Chan, "Identification of source of Ca^{2+} in HeLa cells by femtosecond laser excitation," *J. Biomed. Opt.* **15**, 057010-1-5 (2010).
47. X. Tsampoula, K. Taguchi, T. Čižmár, V. Garces-Chavez, N. Ma, S. Mohanty, K. Mohanty, F. Gunn-Moore, K. Dholakia, "Fibre based cellular transfection," *Opt. Exp.* **16**, 17007−17013 (2008).
48. J. Baumgart, W. Bintig, A. Ngezahayo, S. Willenbrock, H. Murua Escobar, W. Ertmer, H. Lubatschowski, A. Heisterkamp, "Quantified femtosecond laser based opto-perforation of living GFSHR-17 and MTH53a cells," *Opt. Exp.* **16**, 3021−3031 (2008).
49. F. Stracke, I. Riemann, K. König, "Optical nanoinjection of macromolecules into vital cells," *J. Photochem. Photobiol.* **81**, 136−142 (2005).
50. L. E. Barrett, J.-Y. Sul, H. Takano, E. J. Van Bockstaele, P. G. Haydon, J. H. Eberwine, "Region-directed phototransfection reveals the functional significance of a dendritically synthesized transcription factor," *Nature Methods* **3**, 455−460 (2006).
51. Z. Földes-Papp, K. König, H. Studier, R. Bückle, H. G. Breunig, A. Uchugonova, G. M. Kostner, "Trafficking of mature miRNA-122 into the nucleus of live liver cells," *Curr. Pharm. Biotechnol.* **10**, 569−578 (2009).
52. J.-Y. Sul, C.-W. K. Wu, F. Zenga, J. Jochems, M. T. Lee, T. K. Kim, T. Peritz, P. Buckley, D. J. Cappelleri, M. Maronski, M. Kim, V. Kumar, D. Meaney, J. Kim, J. Eberwine, "Transcriptome transfer produces a predictable cellular phenotype," *Proc. Natl. Acad. Sci. USA* **106**, 7624−7629 (2009).
53. S. Nolte, C. Momma, H. Jacobs, A. Tünnermann, B. N. Chichkov, B. Wellegehausen, H. Welling, "Ablation of metals by ultrashort laser pulses," *J. Opt. Soc. Am. B* **14**, 2716−2722 (1997).
54. D. N. Nikogosyan, A. A. Oraevsky, V. Rupasov, "Two-photon ionization and dissociation of liquid water by powerful laser UV radiation," *Chem. Phys.* **77**, 131−138 (1983).
55. U. Trefzer, G. Herberth, K. Wohlan, A. Milling, M. Thiemann, T. Sherev, K. Sparbier, W. Sterry, P. Walden, "Vaccination with hybrids of tumor and dendritic cells induces tumor-specific T-cell and clinical responses in melanoma stage III and IV patients," *Int. J. Cancer* **110**, 730−740 (2004).
56. R. W. Steubing, S. Cheng, W. H. Wright, Y. Numajiri, M. W. Berns, "Laser induced cell fusion in combination with optical tweezers: The laser cell fusion trap," *Cytometry* **12**, 505−510 (1991).
57. J. Gong, X. Zhao, Q. Xing, F. Li, H. Li, Y. Li, L. Chai, Q. Wang, "Femtosecond laser-induced cell fusion," *Appl. Phys. Lett.* **92**, 093901−093903 (2008).
58. R. Davidson, P. Gerald, "Improved techniques for the induction of mammalian cell hybridization by polyethylene glycol," *Somatic Cell Mol. Genetics* **2**, 165−176 (1976).
59. T. H. Norwood, C. J. Zeigler, G. M. Martin, "Dimethyl sulfoxide enhances polyethylene glycol-mediated somatic cell fusion," *Somatic Cell Mol. Genetics* **2**, 263−270 (1976).
60. H. He, K. T. Chan, S. K. Kong, R. K. Y. Lee, "All-optical human cell fusion by a fiber femtosecond laser," *Appl. Phys. Lett.* **93**, 163901−163903 (2008).
61. A. E. Wurmser, F. H. Gage, "Cell fusion causes confusions," *Nature* **416**, 485 (2002).
62. J. W. Wojcieszyn, R. A. Schlegel, K. Lumley-Sapanski, K. A. Jacobson, "Studies on the mechanism of polyethylene glycol-mediated cell fusion using fluorescent membrane and cytoplasmic probes," *J. Cell Biol.* **96**, 151 (1983).
63. Q. Xing, F. Mao, L. Chai, Q. Wang, "Numerical modeling and theoretical analysis of femtosecond laser tweezers," *Opt. Laser Technol.* **36**, 635 (2004).
64. F. Docchio, C. A. Sachhi, J. Marshall, "Experimental investigation of optical breakdown thresholds in ocular media under single pulse irradiation with different pulse durations," *Lasers Ophthalmol.* **1**, 83−93 (1986).
65. A. Kaiser, B. Rethfeld, M. Vicanek, G. Simon, "Microscopic processes in dielectrics under irradiation by subpicosecond laser pulses," *Phys. Rev. B* **61**, 11−437 (2000).
66. B. Rethfeld, "Unified model for the free-electron avalanche in laser-irradiated dielectrics," *Phys. Rev. Lett.* **92**, 187−401 (2004).