Fs-laser-induced Ca^{2+} concentration change during membrane perforation for cell transfection

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Abstract: Fs-laser based opto-perforation is a gentle method for gene transfer into sensitive cells such as stem cells or primary cells. The high selectivity and the low damage to the cell lead to a high efficiency of transfection. However, there are side effects which induce stress to the cell due to the exchange of intra- and extracellular media as well as the disintegration of the structure of biomolecules resulting from the laser exposure. Moreover, the mechanisms of the optical transfection are still unclear. In this paper, we present our study on calcium (Ca^{2+}) homeostasis during cell surgery, especially during laser induced membrane perforation. We show that the manipulation of cells can induce an increase in the cytosolic Ca^{2+} concentration. This increase was not observed if the manipulation of the cells was performed in absence of the extracellular calcium indicating the importance of the Ca^{2+} uptake. We found, that the uptake of extracellular Ca^{2+} strongly depends on the repetition rate and the irradiation time of the laser pulses. The exposure for several seconds to kHz pulses even induces Ca^{2+} induced Ca^{2+} release. Dependent on the location of perforation, probably in the vicinity of an intracellular Ca^{2+} stock, an instantaneous intracellular Ca^{2+} release can be induced. Since Ca^{2+} could be involved in negative side effect by cell surgery, we propose an application of the optoperforation technique in nominal Ca^{2+}-free external solution.

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1. Introduction

Transfection or introduction of particles or molecules into a living cell is an important tool in cell biology to analyse dynamic pathways and the function of cell organelles. In the last decades the technology has gained impulse by the introduction of the GFP and GFP derivative labeled fusion protein which allows life imaging of cellular structures using the fluorescence microscopy.

A gentle method for the delivery of particles or molecules into cells is the perforation of the membrane by fs laser pulses [1–3]. Whereas the whole membrane is perforated by high electric fields during electroporation, the fs laser pulses are focused on a small region of the membrane of the order of one micrometer. There is almost no heating of the irradiated volume as the pulse duration is shorter than the thermal conduction time. The manipulation induced by the pulses is reduced to the focal volume because there is no linear absorption but non-linear multiphoton absorption [4,5]. The multiphoton absorption can solely occur in the focal volume where a high

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photon density is achieved. Therefore, the fs laser based perforation of the cell membrane by the fs laser pulses only harms a small volume in the cell of the order of some femto liters [2]. Compared to the electroporation for example, the number of the manipulated cells is very small but the selectivity on the other hand is very high.

The opto-perforation is usually performed at pulse energies below the threshold of optical breakdown, a so called low density plasma is produced in the very focus of the laser beam which results in cumulative free electron mediated chemical effects [6]. It is assumed that in the direct vicinity of the focal volume, chemical bonds are broken liberating free electrons which dissociate and ionize water and biomolecules. This disintegration of the structure of molecules leads to a dissection of the material. Furthermore reactive oxygen species (ROS) are yielded by the multiphoton ionization and dissociation of the material in the laser focus [6–11]. Additionally to the ROS formation during opto-perforation, extracellular solution diffuses into the cells, which disturbs the intracellular ionic equilibrium. Changes in cytosolic Ca^{2+} concentration are second messengers, which can affect various cellular function such as proliferation, differentiation, cell motility or cell death [12]. In general, the extracellular Ca^{2+} concentration is a factor of about 10^4 above the intracellular concentration. Beside the extracellular reservoir, intracellular Ca^{2+}-stores are Ca^{2+} binding proteins, mitochondria and the endoplasmic reticulum (ER). As Ca^{2+} store, the ER is of a particular interest because it allows a rapid release of Ca^{2+} into the cytosol. Laser exposure can open the cell membrane yielding to an increase of cytosolic Ca^{2+} due to Ca^{2+} influx from the extracellular space. In some cells such as muscle cells or endothelial cells the increase of the cytosolic Ca^{2+}-concentration can be generated or reinforced by the intracellular Ca^{2+}-induced Ca^{2+} release of the ER [13, 14].

The increase of Ca^{2+} concentration due to fs-laser pulses focussed into a single cell has been described in literature before for different applications as for example multiphoton microscopy or intracellular surgery [13–18]. The case of membrane perforation we discuss in the present paper is an example of cell manipulation, for which not only intracellular Ca^{2+} release is an important issue but also extracellular Ca^{2+} uptake.

The optimum parameters for opto-perforation in terms of cell viability after the transfection and the efficiency are not yet well known. In this paper we investigate the side effect of a disturbance of the Ca^{2+} homeostasis accompanying the application of the opto-perforation method. With the better knowledge of negative influences, they can be minimized to increase the viability of the treated cells and therefore the efficiency of transfection, which are the most important parameters for comparison with common methods and to establish this method in the broad field of transfection applications. As a result of our experiments we showed differences between Ca^{2+} uptake and Ca^{2+} release from intracellular stores depending on the laser parameters and the location of exposure. Additionally we reduced the disturbance of the Ca^{2+} homeostasis after perforation by manipulating the cells in Ca^{2+}-free culture medium which possibly increases the viability of the perforated cells.

2. Materials and methods

The described experiments were realized with a setup consisting of a fs-laser system and a microscope including fluorescence equipment. In the following, these elements are described in detail.

2.1. Laser system and pulse picker

The laser system used in this study was a tunable Ti:sapphire laser (*Coherent, Chameleon*) which generates ultrashort pulses of 140 fs at a repetition rate of 80 MHz. The accessible wavelength ranges between 690 nm and 1050 nm and the maximum pulse energy at 800 nm is 44 nJ at the laser output. An acousto optical modulator (AOM, *APE, Pulse Select*) was used to

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regulate the pulse frequency for the repetition rate dependent experiments by deviding the beam into two parts, one at the initial repetition rate (imaging beam) and one at a lower repetition rate (manipulation beam, see Fig. 1). It consists of a Bragg cell which selects pulses of the laser beam. These pulses are diffracted into the first order. The pulse picker was configured to change the repetition rate from 80 MHz to either 4 MHz or 40 kHz.

2.2. Microscope

The laser beams were guided through a mechanical shutter (*Thorlabs, SC10*) and an attenuator before being superimposed (see Fig. 1). Afterwards, both were deflected in x- and y-direction by galvanometer scan-mirrors (*Cambridge Technology*) into the microscope (*Carl Zeiss AG, Axiovert* 100). They entered the tube via a dichroitic beam splitter and were further guided onto the sample. The 1.3 NA oil immersion objective focused the beams into the sample with a theoretical spot size of approximately 370 nm at a central wavelength of 800 nm. The fluorescence induced by multiphoton excitation at very low laser pulse energies of about 0.2 nJ for the Fluo-4 and FM4-64 labeled samples passed a dye specific bandpass filter (525 nm for Fluo-4, 655 nm for FM4-64, *Semrock*) and was detected by a photomultiplier tube (*Hamamatsu, R6357*). The pixel dwell time was about 11 µs and the size of the scanned area is 67×67 µm.

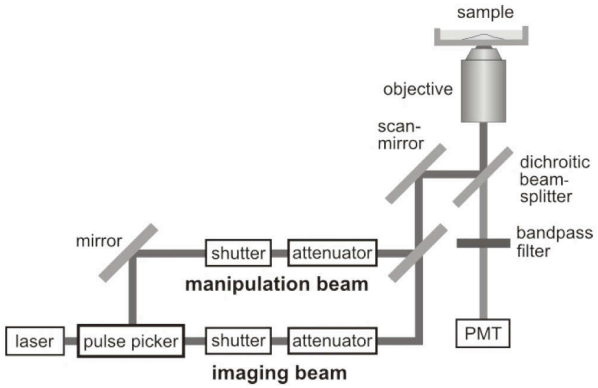

Fig. 1. Schematic setup for the manipulation and the multiphoton imaging of single cells.

2.3. Cells and labeling

Bovine endothelial cells were cultivated in glass bottom dishes with a thickness of the cover slip of 170 µm (*MatTek Corporation*) using RPMI 1640 medium (*Roswell Park Memorial Institute*) supplemented with 10% FCS (fetal calf serum) and the antibiotics penicillin, streptomycin, and partricin at 37^{o}C and 5% CO_2 humidified atmosphere.

For the Ca^{2+} labeling, the cells were incubated in NaCl-bath solution (NaCl-BS) media containing 121 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 6 mM $NaHCO_3$, 5.5 mM glucose, 25 mM HEPES, pH 7.4 and an osmolarity 295 ± 5 mOsmol/l and 8 µM Fluo-4 (*Invitrogen*) and 4 µM FM4-64 (*Invitrogen*) for 60 minutes and 10 minutes respectively. Then the cells were washed once with NaCl-BS. All experiments were performed in NaCl-BS or in Ca^{2+}-free NaCl-BS at room temperature (22^{o}C). The Ca^{2+}-free NaCl-BS additionally contains ethylene glycol tetra-acetic acid (EGTA), which is a Ca^{2+} specific chelator (Kd $\cong$ 175 nM).

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2.4. *Manipulation and imaging of the cells*

The Ca^{2+} concentration change during fs laser cell surgery was studied by measuring the fluorescence intensity of Fluo-4 (Invitrogen), which is a Ca^{2+} indicator, while a fs laser beam was targeted into the fluorescently labeled membrane (FM4-64, Invitrogen) of the cells using an 1.3-NA objective. The fluorescence intensity was measured and averaged in a region of interest of approximately 225 μm^2 at the nucleus of the perforated cells as this was the brightest region due to the thickness of the cell. The manipulation was performed in NaCl-BS or NaCl-BS containing EGTA at a pulse energy of 1.0 nJ to 1.5 nJ, typical energies usually used for transfection at these high NA [1–3], which both are below the theoretical optical breakdown threshold [6], so that the manipulation effect was due to low density plasma generation. The membrane was targeted three times at a slightly different position (at a distance of less than 1 µm between two exposures), which was shown to be most effective for transfection [2, 3].

Two different repetition rate regimes were used at 4 MHz and 40 kHz respectively. In the kHz regime, an irradiation time of 4 s was performed, which is equivalent to an exposure of 160,000 laser pulses corresponding to the threshold for Ca^{2+} release in the kHz regime [14]. For the 4 MHz repetition rate, an irradiation time of 40 ms was performed, so that the same number of pulses was introduced into the cells as in the kHz regime (see also [11]). Iwanaga et al. showed, that at repetition rates between 10 and 400 kHz, at 1.0 nJ pulse energy and an exposure of 160,000 pulses of a duration of 80 fs, the probability for Ca^{2+} wave formation is at 50%. At 1 MHz repetition rate, only 40,000 pulses lead to a 50% probability of Ca^{2+} wave formation [14]. Additionally, those parameters are within an order of magnitude similar to those typically used for cell transfection.

The imaging of the reaction of the cell by fluorescence after the manipulation was performed by multiphoton microscopy for 18 minutes, scanning the field of view once every 30 s to take an image. For the instantaneous Ca^{2+} increase, one image every 5 s was taken during the first two minutes after laser manipulation (first image was taken 40 ms after exposure), which is sufficient to visualize slow Ca^{2+} reactions [12, 16], that are not reduced by cell mechanisms and therefore damage the cell. Some images were taken before the manipulation to measure the initial fluorescence level. At least one control cell, which was not manipulated, was observed additionally. The change in fluorescence in the manipulated cells was compared to that in non manipulated cells.

3. Results

The influence of laser induced manipulation to the Ca^{2+} homeostasis was observed during membrane perforation. The behavior of the Ca^{2+} concentration can be divided into 6 groups. The behavior of each group did not differ significantly at different pulse energies and the different repetition rates but not all of the described behaviors were observed at all parameters. The principal behavior of each group is specified and described in the following. Only in group 3, the behavior after perforation at MHz repetition rate was different compared to manipulation in the kHz regime. The higher pulse energy did not induce a significantly different behavior in all groups.

In average, about 50% of all manipulated cells did not show any reaction in the Ca^{2+} concentration due to the laser irradiation. One third of the cells manipulated at 40 kHz in NaCl-BS containing Ca^{2+} and two thirds in calcium free NaCl-BS showed no disturbance in their Ca^{2+} homeostasis (see Table 1 group 6, Fig. 2A, and 3F), at 4 MHz, half of the cells did not change their Ca^{2+} concentration, and two thirds in Ca^{2+}-free medium. For the rest of the manipulated cells, the behavior of the Ca^{2+} concentration due to laser exposure can be divided into groups 1 to 5:

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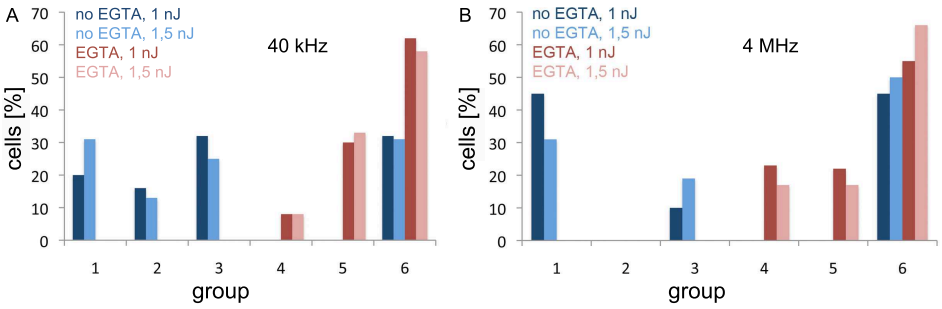

Fig. 2. (Color online) The percentage of cells that showed the Ca^{2+} concentration behavior of the different groups due to membrane perforation (see also Fig. 3) A) at 40 kHz and B) at 4 MHz repetition rate. The membrane was perforated three times at a pulse energy of 1.0 nJ or 1.5 nJ. The blue bars represent the percentage of cells, that were manipulated in NaCl-BS, the red bars represent those cells perforated in Ca^{2+} free NaCl-Bs containing EGTA. At 1.0 nJ pulse energy: n = 25 and 13 at 40 kHz repetition rate with and without Ca^{2+} in the NaCl-Bs respectively, n = 29 and 9 at 4 MHz with and without Ca^{2+} in the NaCl-BS respectively. At 1.5 nJ pulse energy: n = 16 and 12 at 40 kHz repetition rate with and without Ca^{2+} in the NaCl-Bs respectively, n = 16 and 12 at 4 MHz with and without Ca^{2+} in the NaCl-BS respectively.

Group 1 is characterized by a very fast increase to 0.3 units. The Ca^{2+} concentration stayed constantly on this level for about 4 minutes and decreased to the initial level afterwards [see Fig. 2 and 3(a)]. At 1.0 nJ pulse energy 16% of the cells perforated at 40 kHz showed the behavior described in group 2, which is characterized by a very fast increase of 0.6 units after manipulation. The Ca^{2+} concentration stayed constant on this higher level for 70 s, then another fast increase was initiated. This second instantaneous jump was followed by a rapid decrease to the first level of 0.6 units until it dropped to the initial level, 4 minutes in total after perforation [see Fig. 3(b)]. In both groups, Ca^{2+} oscillations were observed after the decrease to the initial level (see Fig. 5). The same behavior was observed in 13% of the cells perforated at 1.5 nJ pulse energy.

In group 3 a very high jump of about 1.8 units was measured. The laser was focused into the membrane at a position near the cell nucleus. The increase was followed by a fast decrease to

Table 1. The different groups of Ca^{2+} concentration behavior due to the laser exposure and the source of Ca^{2+} for the concentration change (+ = takes place, - = does not take place): The influx of extracellular Ca^{2+} (Ca^{2+} influx), the laser induced intracellular Ca^{2+} release (LICR), the Ca^{2+} induced Ca^{2+} release (CICR), the efflux of intracellular Ca^{2+} (Ca^{2+} efflux), the location of the laser focus (+ = location near the nucleus, - = location far from the nucleus), and the presence of extracellular Ca^{2+} ($[Ca^{2+}]_o$).

	Ca^{2+} influx	LICR	CICR	Ca^{2+} efflux	focus location	$[Ca^{2+}]_o$
group 1	+	-	-	-	-	+
group 2	+	-	+	-	-	+
group 3	+	+	+	-	+	+
group 4	-	+	-	+	+	-
group 5	-	-	-	+	-	-
group 6	-	-	-	-	+/-	+/-

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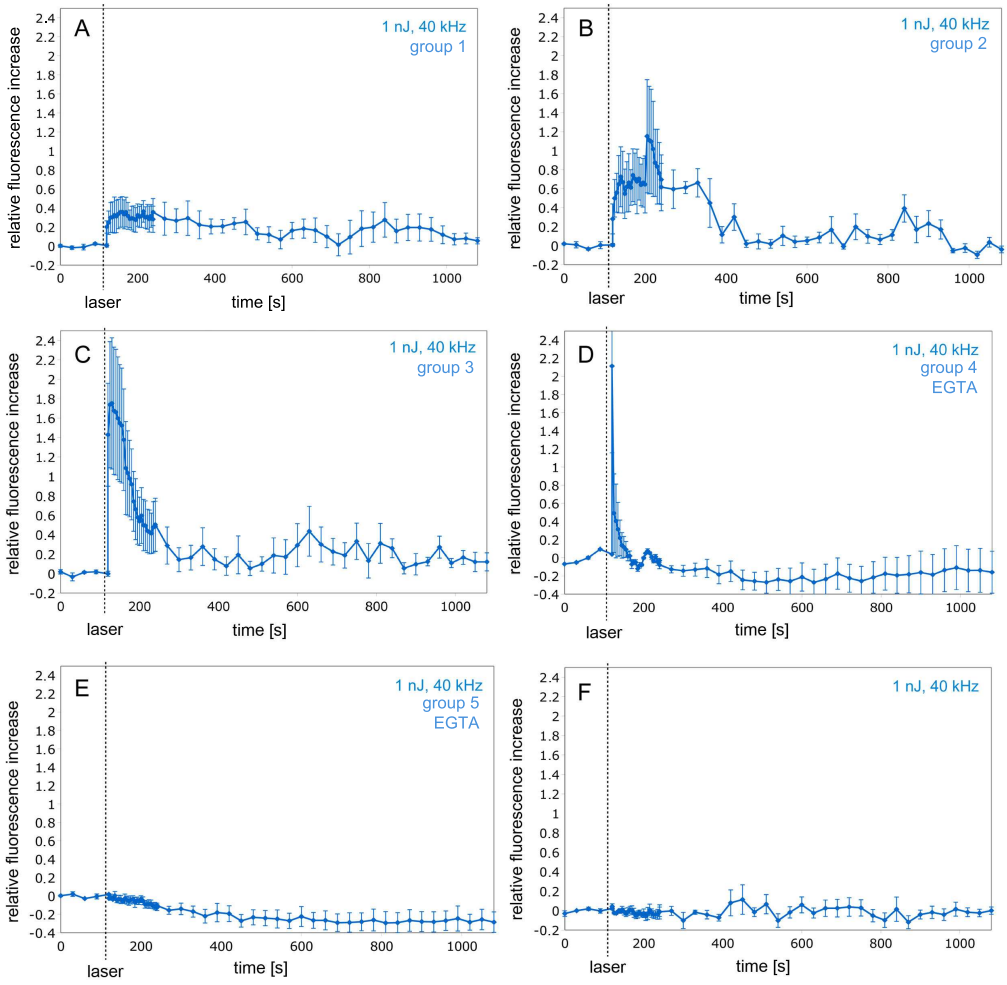

Fig. 3. (Color online) Relative Ca^{2+} concentration increase after membrane perforation. Representative diagrams of the different groups of results obtained at 1.0 nJ pulse energy and 40 kHz repetition rate. A) Behavior of Fluo-4 labeled endothelial cells in group 1, the manipulation was followed by an increase of fluorescence intensity which slowly declined back to the control level. B) Ca^{2+} induced Ca^{2+} release due to previous Ca^{2+} uptake (group 2). C) Group 3 represents the superposition of intra- and extracellular Ca^{2+} on the concentration change which is characterized by an intensity peak that declines rapidly. The peak was so rapid that we could not discriminate it from the first increase shown in (B). D) Intracellular Ca^{2+} release due to exposure in Ca^{2+} free NaCl-BS containing EGTA (group 4). E) The first increase could be suppressed if the manipulation was performed in presence of EGTA in the bath solution in group 5. In this case a continuous slow decline of the fluorescence intensity was observed suggesting a Ca^{2+} efflux and a probable uptake of EGTA. In this group of cells, the manipulation did not affect the fluorescence of the fluo-4, indicating that the optoperforation could be achieved without change in the cytosolic Ca^{2+} concentration in the cells. F) No change of the fluorescence intensity due to the manipulation was observed. The laser exposure is indicated by the black dotted line. (n = 3 to 5, error bars represent standard error of the mean)

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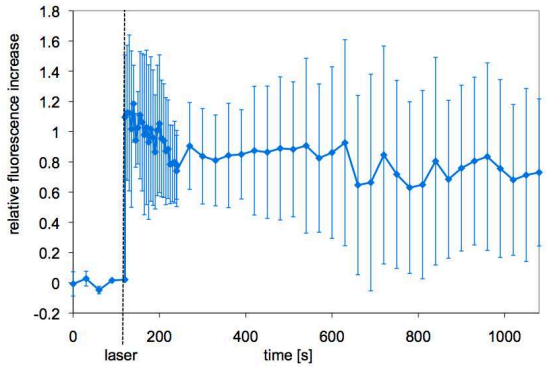

Fig. 4. (Color online) Relative Ca^{2+} concentration increase after membrane perforation at 1.5 nJ pulse energy and 4 MHz repetition rate, representing group 3. It shows the superposition of intra- and extracellular Ca^{2+} on the concentration change which is characterized by an intensity peak that declines rapidly but in 3 cells out of 5, it was not reduced to the initial level. As indicated by the high standard error of the mean, the level to where the Ca^{2+} concentration is reduced or to which it even increases is not constant. (n = 5, error bars represent standard error of the mean)

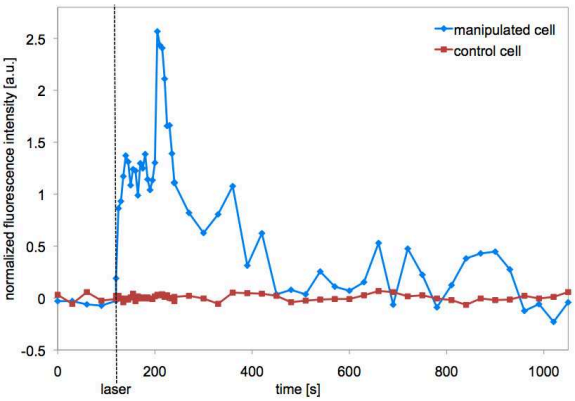

Fig. 5. (Color online) Representative example of Ca^{2+} oscillations after manipulation in group 2. The fluorescence intensity of one perforated (blue rhombus) and one control cell (red square) are shown. The initial fluorescence intensity before manipulation is normalized to zero. The Ca^{2+} concentration of the control cell stays constant whereas the concentration of the perforated cell oscillates even after the initial level was achieved at about 450 s. The dotted black line indicates the laser irradiation time.

the initial level during 100 s (see Fig. 3C). In the following, Ca^{2+} oscillations were observed as in group 1 and 2 (at 1.0 nJ pulse energy 25 cells were perforated in total in the kHz regime, 29 in the MHz regime, at 1.5 nJ pulse energy, 16 cells were perforated at both repetition rates, see Fig. 2). The increase due to the perforation can be induced by either uptake of extracellular Ca^{2+} or the release of intracellular Ca^{2+}. In the case of MHz exposure, in 40% of the cells the Ca^{2+} concentration was not reduced to the initial level after manipulation at 1.0 and 1.5 nJ but stayed on a higher level during the observation time, in average at 0.8 units (see Fig. 4).

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As a control measurement, the cells were perforated in Ca^{2+} free NaCl-BS containing EGTA so that no uptake of extracellular Ca^{2+} can take place and only intracellular Ca^{2+} release can contribute to a concentration change.

In most of the cells (55 to 66%) perforated in the kHz and the MHz regime respectively, the exposure in Ca^{2+} free NaCl-BS did not induce an increase of the Ca^{2+} concentration (see Fig. 2). In approximately one third of the cells, the Ca^{2+} level even decreased to -0.2 units after manipulation in the kHz and MHz regime, represented by group 5 [at 1.0 nJ pulse energy 13 cells were perforated in total in the kHz regime, 9 cells in the MHz regime, 12 cells in both regimes at 1.5 nJ pulse energy, see Fig. 2 and 3(d) and 3(e)]. Only 8% of the cells perforated at 40 kHz (at 1.0 and 1.5 nJ) showed a very high jump of the Ca^{2+} concentration in response to the exposure, which is due to intracellular Ca^{2+} release, followed by a very fast decrease even below the initial concentration level (group 4). At 4 MHz repetition rate, 23% of the cells showed this behavior at 1.0 nJ, 17% at 1.5 nJ [see Fig. 2 and 3(e)].

4. Discussion and outlook

The results of the Ca^{2+} measurements showed a very complex behavior. However, it is shown that the external Ca^{2+} is a suitable parameter at which it can be act to reduce the disturbance of the cell during the perforation procedure. The change of the Ca^{2+} concentration can be classified in 6 groups. The differences of the observed Ca^{2+} signals can be explained by the uptake of extracellular Ca^{2+} or an intracellular Ca^{2+} release. The differentiation of the signal due to intracellular Ca^{2+} release was determined by performing the membrane perforation in Ca^{2+} free NaCl-BS.

Approximately 30 to 66% of the cells manipulated at kHz or MHz repetition rate in presence or in absence of extracellular Ca^{2+} did not show any reaction, which is in good agreement to the perforation rates of about 70% usually obtained [2, 3]. No change in Ca^{2+} concentration was observed most likely due to non-adequate positioning of the laser focus. The inaccuracy of the focussing is due to the small extent of the membrane of only several nanometers. Additionally, the focus position was defined before the manipulation which was performed 2 minutes after starting the measurement, movements of the cell and especially of the membrane relative to the laser focus may occur.

The Ca^{2+} concentration in the extracellular medium is about a factor of 10^4 higher compared to the intracellular concentration. Therefore, Ca^{2+} diffuses into the cell, when perforating the membrane. Depending on the size and the opening time of the created pore, the Ca^{2+} concentration reaches a certain threshold at a certain time, so that Ca^{2+} induced Ca^{2+} release (CICR) from intracellular Ca^{2+} stocks is activated [19–21]. This threshold was not reached in those cells related to group 1. In the case described in group 2, it was reached marginally whereas the cells of group 3 exceed the threshold very fast. This indicates that perforation at 40 kHz induces higher uptake of Ca^{2+} compared to 4 MHz (see Fig. 2). This is due to the longer exposure time needed to apply the same number of pulses. The laser irradiation induces a longer disturbance of the integrity of the membrane molecules, so that Ca^{2+} ions can diffuse into the cell in a higher concentration than due to MHz exposure. At the same time, the location of the laser irradiation is more important in the kHz regime, as there is no gas bubble formation (in contrast to the MHz regime [2, 3, 6]), which means that there is no influence in the vicinity of the focal spot and therefore, the membrane has to be localized very precisely in this volume. Additionally, the Ca^{2+} concentration change does not depend on the pulse energy as well as the probability of inducing a Ca^{2+} wave above the threshold of an energy density of 0.07 J/cm^2 is constant [15]. The inaccuracy of the position of the focal volume relative to the membrane therefore explains the increase of the number of cells in group 1 at 40 kHz at higher pulse energy. Almost the same number of cells was observed to be classified to group 2 at both pulse

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energies.

Nevertheless, Iwanaga et al. found a higher threshold for intracellular Ca^{2+} waves at low repetition rates on the order of kHz compared to MHz [14]. The same effect was observed in this work at intracellular Ca^{2+} release (group 4). There is almost no intracellular Ca^{2+} release in the kHz regime, when no uptake of extracellular Ca^{2+} takes place. Additionally, at MHz repetition rate, the instantaneous increase of Ca^{2+} was not completely reduced to the initial level (see Fig. 4) whereas at kHz exposure the Ca^{2+} concentration was always reduced to the initial value. A similar result was shown for reactive oxygen species in previous work done in our group, where manipulation at kHz repetition rate showed less damage to the cell compared to manipulation at MHz repetition rate [11]. This indicates that the damage due to the MHz exposure was more severe and the membrane integrity, i.e. Ca^{2+} impermeability, was not completely restored in these cases within the observation time. With respect to an application in optical transfection using MHz repetition rates, a shorter irradiation time and by this a lower total number of pulses seems therefore to be advantageous.

Group 3 is characterized by a fast and strong Ca^{2+} concentration increase directly after exposure. As this case occurred only after focusing the laser into the membrane near the nucleus, it supposably describes the combination of extracellular Ca^{2+} influx by diffusion through the perforated membrane and the intracellular Ca^{2+} release by the ER. The ER is located at the nucleus membrane and is an important intracellular Ca^{2+} reservoir. The group 3 also covers cells of group 2, where the laser pulses induced instantaneously an intracellular Ca^{2+} release without a time delay. The behavior of group 2 did not occur at the MHz regime (see Fig. 2).

Group 3 and 4 are therefore probably random effects because the intracellular Ca^{2+} stores, i.e. the ER, were not labeled and it is independent of the repetition rate. When focusing the laser into the membrane at a position near the nucleus, the cells reacted as described for these groups. This effect has to be proven by ER targeting to induce the intracellular Ca^{2+} release. As no extracellular Ca^{2+} exists in group 4, the description of this behavior seems to correlate well to an intracellular release. The characteristics of the increase in group 3 are very similar to those in group 4.

The cells that showed the behavior of group 1 to 3 after exposure, tend to Ca^{2+} oscillations even when they achieved the initial level, which are not well shown in Figs. 3(a) to 3(c) as they are averaged. Figure 5 shows these oscillations representatively. Additionally, those oscillations are faster than 30 s, the time between each image at the end of the observation time. Compared to this, in absence of extracellular Ca^{2+}, none of the manipulated cells showed Ca^{2+} oscillations. Therefore, a longer observation of the cells would show the influence of Ca^{2+} disturbance on the cell viability.

The manipulation in Ca^{2+}-free medium containing EGTA led in some cases first to an increase of the Ca^{2+} concentration that was reduced and then followed by a continuous slow decline of the fluorescence intensity even below the initial concentration level (groups 4 and 5). The first rapid decline of the fluorescence was probably related to Ca^{2+} reuptake in intracellular stores and to a possible sequestration by the EGTA which diffused into the cell during opto-perforation [15], which suppresses any CICR.

The results on the influx of Ca^{2+} or intracellular release lead to new insights to the side effects during and after fs laser based opto-perforation for cell transfection. At the usual parameters for this method, the cells are highly influenced at the molecular level by the laser exposure. Even the reduction of Ca^{2+} was no prove of the viability of the cell which especially is shown by the oscillating behavior of the cells. We showed that extracellular Ca^{2+} uptake can be avoided by manipulating the cells in Ca^{2+}-free medium. In previous works, it was shown, that the efficiency of transfection is dependent on the formation of a gas bubble, which leads to an adequate exchange of intra- and extracellular media [2,3]. As there was no gas bubble formation

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observed in the kHz regime, the exchange might not be sufficient for transfection at a reasonable irradiation time. Nevertheless, the application of kHz pulses should be more thoroughly studied, as the side effects at the molecular level seem to be reduced when compared to MHz pulses. On the contrary, it should be possible to increase the viability and therefore the efficiency of fs-laser based transfection even at MHz repetition rate by using a Ca^{2+}-free medium.

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