Combined multiphoton imaging and automated functional enucleation of porcine oocytes using femtosecond laser pulses

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Abstract. Since the birth of "Dolly" as the first mammal cloned from a differentiated cell, somatic cell cloning has been successful in several mammalian species, albeit at low success rates. The highly invasive mechanical enucleation step of a cloning protocol requires sophisticated, expensive equipment and considerable micromanipulation skill. We present a novel noninvasive method for combined oocyte imaging and automated functional enucleation using femtosecond (fs) laser pulses. After three-dimensional imaging of Hoechst-labeled porcine oocytes by multiphoton microscopy, our self-developed software automatically identified the metaphase plate. Subsequent irradiation of the metaphase chromosomes with the very same laser at higher pulse energies in the low-density-plasma regime was used for metaphase plate ablation (functional enucleation). We show that fs laser-based functional enucleation of porcine oocytes completely inhibited the parthenogenetic development without affecting the oocyte morphology. In contrast, nonirradiated oocytes were able to develop parthenogenetically to the blastocyst stage without significant differences to controls. Our results indicate that fs laser systems have great potential for oocyte imaging and functional enucleation and may improve the efficiency of somatic cell cloning.
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Keywords: femtosecond laser; multiphoton microscopy; cell surgery; somatic cell nuclear transfer; SCNT; oocyte enucleation.

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1 Introduction

Since the birth of "Dolly" in 1996 as the first clone of an adult mammal by somatic cell nuclear transfer (SCNT),[1] this technique has been successfully applied to a variety of mammalian species, including cattle,[2] mice,[3] goats,[4] pigs,[5] rabbits,[6] and horses.[7] When combined with genetically modified donor cells, mammalian cloning offers the possibility to produce transgenic offspring for applications in agriculture and biomedicine.[8] To overcome the shortage of human organ donors, transgenic pigs are considered to be best suitable as donors of xenografts.[9,10]

The production of cloned animals by SCNT involves multiple steps.[11,12] At first, the maternal deoxyribonucleic acid (DNA) is usually removed from the recipient oocyte by aspiration of the metaphase II (MII)-spindle and the adjacent first polar body. This procedure is called enucleation. Because of the high amount of lipids in oocytes of farm animals, the metaphase plate must be visualized by staining with Hoechst dye under UV illumination. In less opaque mouse and rhesus monkey oocytes, the spindle can be visualized using polarization microscopy without previous Hoechst staining.[13,14] In a second step, a single donor cell is inserted under the zona pellucida as close as possible to the oocyte membrane. Both cells are then electrically fused and the inserted donor cell nucleus is epigenetically reprogrammed to a pluripotent state. Finally, the reconstructed embryo is artificially activated to initiate the embryonic development.

Despite intense research over the past decade, the number of viable offspring compared to the number of reconstructed embryos transferred into surrogate mothers has remained low. Species-dependent success rates between 0.1 and 15% have been reported.[8] The influencing factors can be divided into biological and technical aspects. Biological factors include the characteristics of oocytes and somatic cells and their epigenetic reprogramming after electrofusion.[15] The removal of maternal DNA during enucleation belongs to the technical aspects. The highly invasive mechanical enucleation requires sophisticated, expensive equipment and considerable micromanipulation skill.[16] To penetrate the oocyte plasma membrane without lysis, oocytes must be pretreated with the microfilament inhibitor cytochalasin B,[15] which is associated with dramatic changes of the cytoskeleton.[17,18] DNA staining is usually done with the DNA-specific fluorochrome Hoechst 33342. For localization of the metaphase plate before its as-

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piration, the oocytes are exposed to UV light for a short time. Although viable offspring have been produced by this technique, it has been suggested that UV irradiation causes damage to the oocyte cytoplasm, especially the mitochondrial DNA.[19] Therefore, possible long-term damaging effects cannot be ruled out.[20] During aspiration of the metaphase plate, small amounts of cytoplasm next to the DNA, containing matrix proteins, are inevitably removed, which has detrimental effects on the embryonic development.[21] To avoid these problems, novel noninvasive methods for oocyte imaging and manipulation must be developed and evaluated.

Over the past four decades, laser pulses have been used in developmental biology for several applications.[22–26] Successful laser inactivation (functional enucleation) of metaphase chromosomes was first demonstrated by McKinnell et al. in leopard frog eggs using microsecond pulses.[27] Recently, Karmenyan et al. used picosecond pulses for functional enucleation of mouse oocytes by targeted irradiation of the metaphase plate.[28] However, these protocols had several major drawbacks. The interaction of microsecond laser pulses with biological tissue is dominated by thermal effects. Depending on the thermal relaxation time, significant thermal damage may occur to the adjacent cytoplasm.[29] Karmenyan et al. used conventional UV illumination in combination with Hoechst staining to visualize the metaphase plate in mouse oocytes, which had significant detrimental effects on their developmental potential.[28] The metaphase plate was also manually moved into the laser focus prior to manipulation. This time-consuming step significantly slowed down the whole process. Because of targeted irradiation of the metaphase plate, several chromosomes or large chromosome fragments most likely remained in the cytoplasm. The resulting aneuploid embryos after SCNT would still contain residual maternal DNA, which may impede the development to term.[4] As a consequence of these drawbacks, current methods do not allow for automation of the enucleation procedure.

Femtosecond (fs) laser are an excellent minimally invasive tool for imaging and precise manipulation of single cells. Compared to continuous UV illumination, the interaction with biological tissue is based on nonlinear absorption. This enables higher penetration depths and impedes out-of-focus absorption and photodamage.[30] Because of these advantages, multiphoton microscopy allows long-term imaging of whole embryos without compromising cell viability.[31] Above a certain pulse intensity threshold, a so-called low-density plasma is produced in the focal volume of the laser beam. It is assumed that these low-density plasmas mediate intracellular dissection by cumulative free-electron-mediated chemical effects.[32] In this regime, no significant heat or mechanical energy transfer to surrounding regions occurs.[32] On this basis, the ablation of single-cell organelles, such as microtubules or mitochondria, without damaging surrounding structures and other organelles has been successfully accomplished.[33–36] Moreover, using the very same fs laser system for both three-dimensional imaging and manipulation of single cells facilitates the automation of the ablation procedure.

In this paper, we show that fs laser pulses offer great potential for combined multiphoton imaging and automated functional enucleation of porcine oocytes. Efficient functional enucleation was achieved by three-dimensional ablation of the metaphase plate in the low-density-plasma regime. Subsequent artificial activation of enucleated oocytes was done to determine their developmental potential in comparison to several control groups.

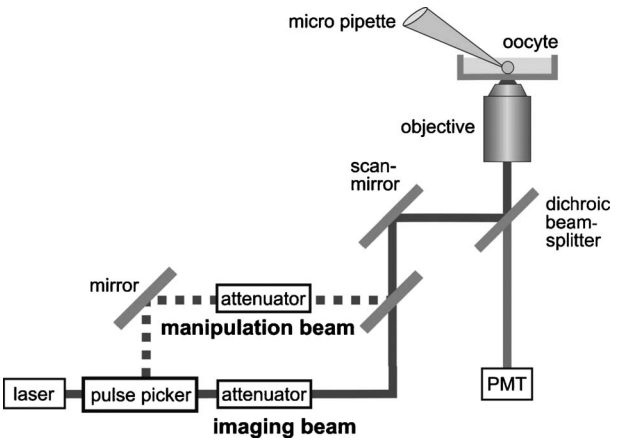

Fig. 1 Schematic setup for multiphoton imaging and manipulation of single oocytes.

2 Materials and Methods

2.1 *Laser System and Microscope*

The laser system used in this study was a tunable Ti:sapphire laser (Chameleon Ultra II, Coherent, Santa Clara, California), which generates ultrashort pulses of 140 fs at a repetition rate of 80 MHz with a $M^2 < 1.1$ beam quality. The accessible wavelength ranges from 680 to 1080 nm. For imaging and manipulation of oocytes, the central wavelength was set to 720 nm corresponding to the two-photon absorption maximum of the Hoechst 33342 dye in this range.[37] At this wavelength, the maximum pulse energy at the laser output is 30 nJ. An acousto-optical pulse picker (Pulse Select, APE GmbH, Berlin, Germany) was applied to regulate the pulse frequency for the manipulation of oocytes by selecting pulses of the laser beam with a division ratio between 1:20 and 1:5000 (corresponding to 4 MHz and 16 kHz). These pulses are diffracted into the first order with a diffraction angle of ~3.5 deg. The diffracted and initial laser beam were used for oocyte manipulation and multiphoton microscopy, respectively (see manipulation and imaging beam in Fig. 1).

Both laser beams were guided through a mechanical shutter and an attenuator, consisting of a half-wave plate and a polarizing beamsplitter cube, before being superimposed. After entering the tubus of an inverted microscope (Axiovert 100, Carl Zeiss AG, Oberkochen, Germany) via a dichroic mirror, they were focused into the sample by a 0.8-NA water-immersion objective (C-Achroplan NIR, Carl Zeiss AG). At a central wavelength of 720 nm, this results in a theoretical diffraction-limited spot diameter of ~1080 nm. Because of dispersion in the optics, the pulse duration in the sample was ~275 fs determined with the autocorrelator "CARPE" (APE GmbH). Laser beam scanning in the x-y plane was achieved by a pair of high-speed galvanometer mirrors (Cambridge Technology, Lexington, Massachusetts). A piezoelectric objective-lens positioning system (nanoMIPOS 400, Piezosystem Jena GmbH, Jena, Germany) was used to move the

laser focus along the z-axis. The fluorescence induced by multiphoton excitation at low pulse energies around 0.1 nJ passed the objective and the dichroic mirror in the backward direction and was detected by a photomultiplier tube (R6357, Hamamatsu Photonics, Hamamatsu, Japan). A micropipette with an inner diameter of ~50 μm was attached to the microscope to hold single oocytes in position by applying a slight low pressure.

2.2 *Fs Laser-Based Functional Enucleation of Porcine Oocytes*

The procedures and media used to obtain matured porcine metaphase II (MII)-oocytes have recently been described.[38] MII-oocytes were incubated in groups of five in TL-Hepes 296 medium supplemented with 5 μg/ml Hoechst 33342 (Invitrogen, Carlsbad, California) for 10 min at 37 °C. They were washed once and placed in a drop of TL-Hepes 296 medium on a glass bottom dish with a thickness of 170 μm (MatTek Corporation, Ashland, Massachusetts) at room temperature. Individual oocytes were rotated by alternately applying low and high pressure until the first polar body and the metaphase plate could be identified in the equatorial plane by multiphoton microscopy. Oocytes were divided into three groups: laser enucleation, sham enucleation, and nonirradiation.

Fs laser-based functional enucleation of porcine oocytes comprised three steps. At first, a three-dimensional stack of multiphoton microscopy images containing the metaphase plate was generated. Subsequent image postprocessing by our self-developed LabView-based software automatically determined the exact position and shape of the metaphase plate in each plane, based on the contrast between bright Hoechst fluorescence and background. Thereafter, the manipulation beam was solely scanned over the marked areas with higher pulse energy and lower scan speed. The distance between two scan lines in each plane and the distance between two planes were set to 0.5 and 1.5 μm, respectively, corresponding to the diffraction-limited focal volume of the laser beam. Finally, the same three-dimensional stack as before was generated to verify the success of the metaphase plate ablation. The whole procedure lasted ~30 s.

To examine the mitochondrial distribution and morphology before and after metaphase plate ablation, oocytes were incubated in TL-Hepes 296 medium supplemented with 0.5 μM MitoTracker Orange (Invitrogen) for 20 min prior to Hoechst staining. Two-photon excitation of MitoTracker Orange was done at a wavelength of 900 nm. The manipulated oocytes were restained with Hoechst 33342 and the double-stranded DNA-specific dye SYBR Green I (1:1000 of the original 10,000x stock solution, Invitrogen) to discriminate photobleaching and ablation. For sham enucleation, an arbitrarily selected area in the cytoplasm close to the metaphase plate with a similar volume was irradiated using the same parameters. Nonirradiated oocytes underwent the same procedure as laser-enucleated oocytes except for the metaphase plate ablation. One control group remained in the incubator during the enucleation procedure to determine possible negative side effects due to multiphoton microscopy and other work steps. To have a direct comparison to conventional methods, mechanical enucleation was done as described in Hoelker et al.[38]

2.3 *Embryo Culture and Quality Assessment*

To verify the success of the fs laser-based functional enucleation of porcine oocytes, all five experimental groups (laser enucleation, sham enucleation, nonirradiation, mechanical enucleation, and control) were parthenogenetically activated. Parthenogenesis is defined as an embryo development without fertilization by a male gamete and can be artificially induced in mammalian oocytes by different methods.[11,39] In nuclear transfer protocols, the development of parthenogenetic embryos generally serves as an indicator of oocyte quality and culture conditions.[12] Successful metaphase plate ablation (functional enucleation) should inactivate the DNA and arrest the parthenogenetic development directly at the MII-stage with minimal damage to the cytoplasm and other organelles. In our study, we used electrostimulation for parthenogenetic activation as described in Hoelker et al.[38] Parthenogenetic embryos were cultivated in NCSU 23 medium supplemented with 0.4 mg/ml BSA for seven days at 38.5 °C in 5% CO_2 in humidified air.

The *in vitro* development of the parthenogenetically activated embryos was evaluated 19 or 168 h after activation. To assess successful pronucleus formation after 19 h, the embryos were fixed for 24 h in a solution of ethanol and acetic acid (3:1 ratio), stained with lacmoid, and analyzed using phase contrast microscopy. Rates of pronucleus formation were defined as the number of embryos with at least one pronucleus divided by the total number of activated oocytes. Morphological criteria, including cell shape and blastocoele formation, were used to determine the successful development up to the blastocyst stage after 168 h. Quality of the embryos was assessed by 5 μg/ml Hoechst 33342 staining for 10 min and subsequent counting of the total number of cell nuclei in each embryo under a conventional fluorescence microscope. Embryos with more than one nucleus were classified as cleaved. Blastocyst and cleavage rates were defined as the number of blastocysts and cleaved embryos divided by the total number of activated oocytes, respectively.

The statistical significance of the rates of pronuclear and blastocyst formation, intact morphology, and cleavage was evaluated by the χ-squared test. Differences between experimental groups were considered significant at $P<0.05$.

3 Results

To demonstrate the suitability of multiphoton microscopy (MPM) for imaging of porcine MII-oocytes, a three-dimensional reconstruction from a stack of multiphoton images is shown in Fig. 2. In this representation, the autofluorescence of the cytoplasm (levels of gray) and the Hoechst fluorescence of the polar body (orange) are visible, whereas the adjacent metaphase plate is hidden in the cytoplasm. A sharp decrease of the detected fluorescence signal occurred in the deepest layers of these oocytes, which had a diameter of ~150 μm (see Fig. 2, at the bottom of the images). Therefore, the metaphase plate and polar body were placed in the equatorial plane before metaphase plate ablation in further experiments.

The first set of experiments was made to identify optimal laser parameters for efficient metaphase plate ablation with minimal collateral damage to the cytoplasm. To this end, the repetition rate, scan speed, and pulse energy of the manipula-

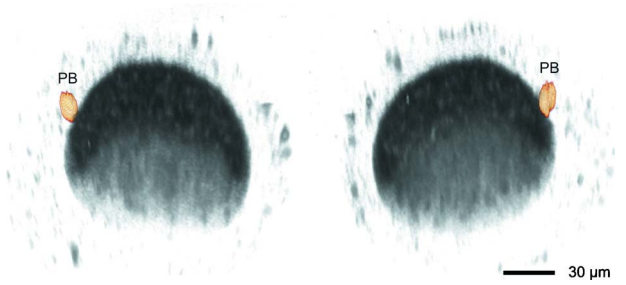

Fig. 2 Three-dimensional reconstruction of a porcine MII oocyte from a stack of multiphoton microscopy images shown at two different angles. The autofluorescence of the cytoplasm and the fluorescence of the polar body (PB) stained with Hoechst 33342 are marked in levels of gray and orange, respectively. The metaphase plate is hidden in the cytoplasm. (Color online only.)

tion beam were varied. At a constant spatial pulse overlap, the ablation efficiency was independent of the laser repetition rate. Therefore, a high repetition rate of 1 MHz was selected to minimize the duration for the whole procedure and to exclude possible temperature accumulation effects at the same time.[32] Figure 3 shows the metaphase plate and polar body both before and after manipulation at 1 MHz repetition rate, 2.5 nJ pulse energy, and 100 μm/s scan speed. Following metaphase plate irradiation, the Hoechst fluorescence completely vanished in this area [see Fig. 3(c)]. In contrast, the nearby fluorescence of the polar body remained unaffected. Restaining of the oocyte with Hoechst 33342 and SYBR Green I did not result in any detectable fluorescence recovery of the metaphase plate (data not shown). Therefore, the fluorescence decrease after irradiation could be attributed to ablation and not to photobleaching. Numerous repetitions of this experiment at the same parameters resulted in a high ablation efficiency and intact oocyte morphology over at least 3 h. In addition, no changes in the mitochondrial distribution and morphology were observed within this period (see Fig. 4). Varying the laser pulse energy at 100 μm/s scan speed and 1-MHz repetition rate exhibited two other regimes. Whereas residual Hoechst fluorescence was detected below 2.5 nJ, gas bubble formation in the irradiated area occurred above this value indicating severe cell damage (data not shown).[32] Consequently, a pulse energy of 2.5 nJ was used in further experiments.

The next step involved the evaluation of the early development after parthenogenetic activation of all five experimental groups: laser enucleation, sham enucleation, nonirradiation, mechanical enucleation, and control. All experimental groups, including laser-enucleated oocytes, maintained intact morphology (>95%) over 19 h. The efficiency of functional oocyte enucleation was 96%, pronucleus formation occurred in only 2 of 50 oocytes (see Table 1), compared to 100% efficiency after mechanical enucleation. Small chromosome fragments were found in ~10% of laser-enucleated oocytes [see Fig. 5(a)], whereas none of them continued the parthenogenetic development and underwent cleavage. After seven days of *in vitro* culture, cytoplasmic fragmentation without detectable DNA in these fragments was observed in ~35% of laser-enucleated oocytes [see Fig. 5(b)]. The same observation was made after conventional mechanical enucleation (data not shown). Sham-enucleated oocytes, whose cytoplasm was irradiated at the same parameters, cleaved and developed to the blastocyst stage with no significant differences in comparison with nonirradiated and control oocytes.

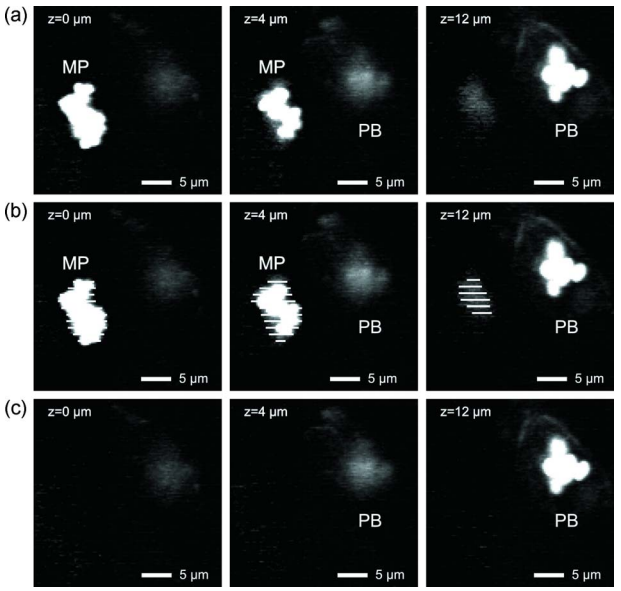

Fig. 3 Multiphoton microscopy images from various depths (a) before and (c) directly after metaphase plate ablation of a porcine MII oocyte stained with Hoechst 33342 at 1 MHz laser repetition rate, 2.5 nJ pulse energy, and 100 μm/s scan speed (MP: metaphase plate, PB: polar body). The manipulation beam was solely scanned over the metaphase plate along the white lines in (b) whose shape was automatically determined by our software.

4 Discussion and Conclusion

The presented results demonstrated the successful combination of three-dimensional imaging and automated functional oocyte enucleation using fs laser pulses. Compared to previous laser-based functional enucleation protocols, this method had several major advantages.

The generated three-dimensional reconstruction of a Hoechst-stained porcine MII-oocyte showed the feasibility of MPM for three-dimensional oocyte imaging. However, a sharp decrease of the detected fluorescence intensity occurred in the deepest layers of these oocytes (see Fig. 2). Although the use of near-infrared (NIR) wavelengths generally improves the imaging depth of fluorescence microscopy,[30] a slightly different behavior is observed in porcine oocytes probably attributed to its high cytoplasmic lipid content. Transmission drops ~30% from 300 to 500 nm followed by a constant increase up to the initial value at 1000 nm.[40] Therefore, longer NIR wavelengths combined with other DNA stains such as Sybr14 are promising to further optimize the imaging depth.[19]

Using MPM and Hoechst staining for metaphase plate visualization prior to manipulation did not affect the viability and developmental potential of oocytes after parthenogenetic activation with no significant difference to controls (see Table 1). These findings are consistent with the work done by Squirrell et al., indicating the great potential of multiphoton microscopy for noninvasive long-term imaging of whole

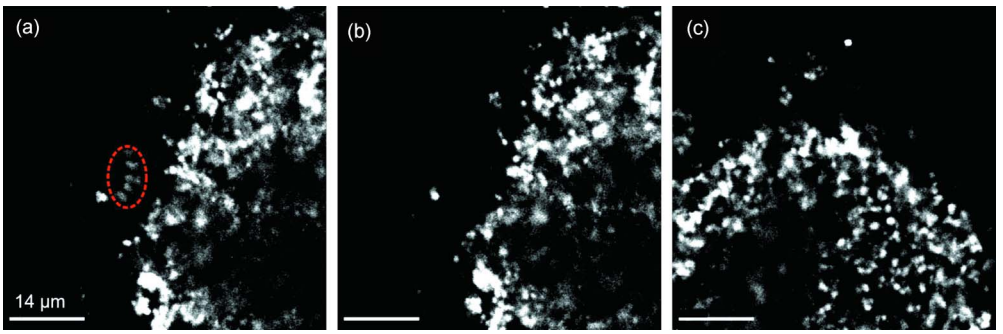

Fig. 4 Mitochondrial distribution and morphology of porcine MII oocytes (a) before, (b) directly after and (c) 3 h after metaphase plate ablation. The metaphase plate position in (a) is indicated by the red dashed ellipse. No changes in mitochondrial distribution and morphology were observed within this period. (Color online only.)

embryos.[31] The great advantage of using fs laser pulses is the negligible multiphoton absorption outside the focal volume and hence reduced damage to adjacent structures including membranes.[30] Moreover, no incubation with the microfilament inhibitor cytochalasin B was required prior to metaphase plate ablation (functional enucleation) in contrast to conventional mechanical enucleation. This may positively influence the embryonic developmental potential after nuclear transfer.[41]

To the best of our knowledge, this is the first time that the localization, shape acquisition, and subsequent three-dimensional ablation of a cell organelle were fully automated by means of MPM images. This automation of the whole procedure with our self-developed software significantly increased the throughput. Further optimization of the entire experimental protocol allowed for processing 20–25 oocytes per hour. Compared to conventional enucleation methods, this was about two times slower. However, the presented noncontact and noninvasive method using fs laser pulses offers the potential for automation of the enucleation procedure since every step can be done by a microfluidic technique.[42]

Our experiments determined for the first time the optimal parameters for efficient metaphase plate ablation of porcine MII-oocytes. At a pulse energy of 2.5 nJ, the bright fluorescence of the metaphase plate completely vanished (see Fig. 3). Even after restaining with different nucleic acid stains, no fluorescence recovery was observed above the background noise. Because our parameters are >20% above the photobleaching threshold,[43] we can be sure of metaphase plate ablation.[35] However, it is still possible that very small DNA fragments remain that are likely not able to recruit sufficient dye molecules. To disprove this assumption, we scanned a weakly focused amplified Ti:Sa fs laser beam over a small droplet with 100 ng/μl linearized plasmid DNA (pEGFP-C1, Clontech, Mountain View, California) at the same laser fluences. The plasmid encodes a wild-type GFP and has a length of 4.7 kbp, which roughly corresponds to the average gene length.[44] Gel electrophoresis revealed that the intensity of the 4.7 kbp band decreased with increasing pulse energy until it completely disappeared. However, no fragments with detectable fluorescence intensity were observed in the range of 4.7 kbp to 20 bp by both agarose and polyacrylamid gels.[45] Therefore, we assume that possible DNA fragments produced by metaphase plate ablation were smaller than genes or transposons whose lengths are >100 bp.[44] In addition, they were much smaller than the residual chromosomes or large chromosome fragments after previous laser-based functional

Table 1 Early development in parthenogenetically activated control and nonirradiated as well as sham-, laser- and mechanically enucleated porcine MII oocytes. Pronucleus formation and oocyte morphology were evaluated 19 h after activation, whereas cleavage and blastocyst rates were examined after 168 h.

	After 19 h		After 168 h	
Experimental group	Intact morphology	Pronucleus formation	Cleavage rate	Blastocyst rate
Control	64/64 (100%)*	61/64 (95%)*	102/119 (86%)*	36/119 (30%)*
Nonirradiation	51/51 (100%)*	50/51 (98%)*	55/66 (83%)*	17/66 (26%)*
Sham enucleation	Not measured	Not measured	62/85 (73%)*	17/85 (20%)*
Laser enucleation	48/50 (96%)*	2/50 (4%)†	0/60 (0%)†	0/60 (0%)†
Mechanical enucleation	42/42 (100%)*	0/42 (0%)†	0/80 (0%)†	0/80 (0%)†

Within columns, different symbols indicate that values are significantly different ($P<0.05$).

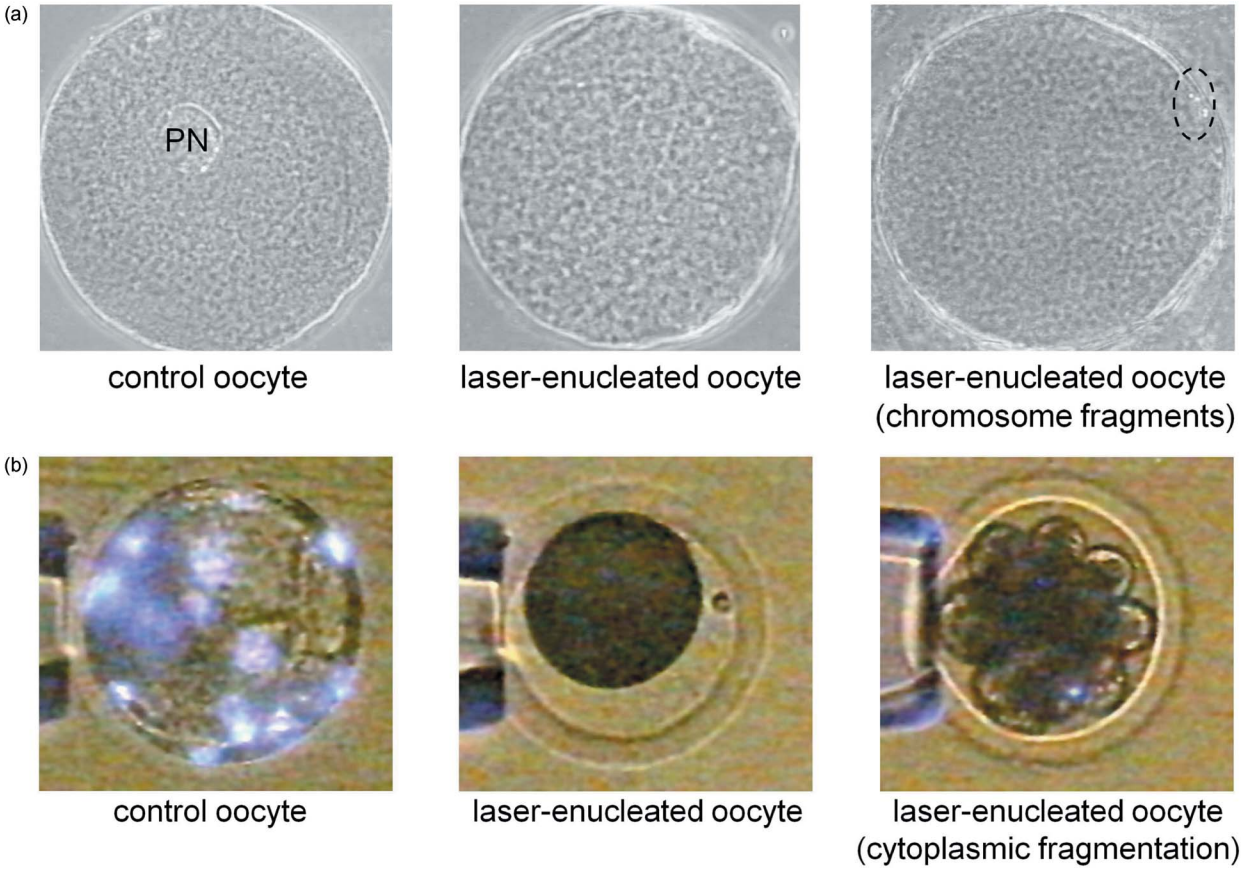

Fig. 5 Phase contrast and light-microscopy images of porcine embryos (a) 19 h and (b) 7 days after parthenogenetic activation, respectively. Chromosome fragments, indicated by the black dashed ellipse in (a), and cytoplasmic fragmentation (b) were observed in some laser-enucleated oocytes. Control oocytes predominantly (a) formed a pronucleus (PN) and (b) developed to the blastocyst stage. Embryos in (b) are stained with Hoechst 33342 (blue). (Color online only.)

enucleation protocols. As the cytotoxicity increases with the molecular weight of macromolecules,[46] our method should be less cytotoxic and may thus improve the embryonic development.

Using the obtained laser parameters for efficient metaphase plate ablation, functional enucleation was achieved in 96% of oocytes with simultaneous maintenance of intact morphology over a long period (see Table 1). This behavior was comparable to that observed after conventional mechanical enucleation. None of the laser-enucleated oocytes underwent cleavage and continued the parthenogenetic development. In striking contrast, UV-C irradiation of the metaphase plate does not block but only delays the first cleavage by <48 h.[47] The significantly higher peak intensity of fs laser pulses causes frequent simultaneous multiphoton absorption above the ionization threshold of DNA molecules.[48] We assume that subsequent free electron and free radical formation induce further severe DNA fragmentation and base modifications.[49] Minor damage by continuous UV-C irradiation is most likely, at least partially, repaired by DNA repair mechanisms.[50]

Following fs laser-based functional enucleation, no changes in the mitochondrial distribution and morphology were observed within 3 h (see Fig. 4). As mitochondrial DNA (mtDNA) damage and apoptosis are associated with rapid changes in mitochondrial morphology,[51] we assume that the laser treatment did not damage the mtDNA. Irradiation of the oocyte cytoplasm at the same parameters (sham enucleation) did not compromise the developmental potential compared to controls (see cleavage and blastocyst rates in Table 1). In previous studies using nanosecond, picosecond, and fs laser pulses, subcellular ablation without damaging surrounding organelles or cytoskeletal structures was demonstrated.[36,52,53] Therefore, our results suggest that the metaphase plate ablation did not affect the oocyte cytoplasm and organelles.

In $\sim$10% of fs laser-enucleated oocytes, small chromosome fragments were observed after 19 h [see Fig. 5(a)]. A detailed analysis of the MPM images after metaphase plate ablation and restaining revealed that about the same percentage was incompletely ablated, leaving small fluorescently labeled DNA fragments in the cytoplasm (data not shown). To avoid these fragments in every oocyte, all laser parameters for metaphase plate ablation have to be further optimized. After seven days of *in vitro* culture, $\sim$35% of laser-enucleated oocytes showed cytoplasmic fragmentation without detectable DNA in these fragments [see Fig. 5(b)]. Because this behavior was also observed in mechanically enucleated oocytes[54] cytoplasmic fragmentation is unlikely caused by fs laser irradiation of the metaphase plate.

Compared to the aspiration of the maternal DNA during mechanical enucleation, DNA fragments and free radical-induced DNA base modifications remained in the oocyte cytoplasm after fs laser-based functional enucleation. It has been shown that fs laser irradiation of cell nuclei causes accumulation of several DNA repair factors and proteins at the irradiation sites.[55,56] Furthermore, this accumulation results in cell-cycle checkpoint activation and significant delay of mitotic cleavage.[57] For this reason, further studies have to investigate potential negative consequences of DNA repair mechanisms on embryonic cleavage after fs laser-based metaphase plate ablation and subsequent nuclear transfer.

In conclusion, we demonstrated the suitability of fs laser as a novel tool for oocyte enucleation. The use of ultrashort pulses with NIR wavelengths instead of UV illumination for imaging and metaphase plate localization improved the oocyte viability and developmental potential. The high functional enucleation efficiency of >95% combined with a high throughput (20–25 oocytes per hour) is promising for the application of fs laser systems as a fast, easy-to-use, and reliable enucleation tool. Compared to conventional mechanical enucleation, it may improve the efficiency of somatic cell clone production. Further advancement of the presented method combined with microfluidic technique offers the possibility to automate oocyte enucleation and to significantly increase the speed of this step of the cloning protocol.

Acknowledgments

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References

1. I. Wilmut, A. E. Schnieke, J. McWhir, A. J. Kind, and K. H. S. Campbell, "Viable offspring derived from fetal and adult mammalian cells," *Nature* **385**, 810–813 (1997).
2. J. B. Cibelli, S. L. Stice, P. J. Golueke, J. J. Kane, J. Jerry, C. Blackwell, F. A. P. De Leon, and J. M. Robl, "Cloned transgenic calves produced from nonquiescent fetal fibroblasts," *Science* **280**, 1256–1258 (1998).
3. T. Wakayama, A. C. F. Perry, M. Zuccotti, K. R. Johnson, and R. Yanagimachi, "Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei," *Nature* **394**, 369–374 (1998).
4. A. Baguisi, E. Behboodi, D. T. Melican, J. S. Pollock, M. M. Destrempes, C. Cammuso, J. L. Williams, S. D. Nims, C. A. Porter, P. Midura, M. J. Palacios, S. L. Ayres, R. S. Denniston, M. L. Hayes, C. A. Ziomek, H. M. Meade, R. A. Godke, W. G. Gavin, E. W. Overstrom, and Y. Echelard, "Production of goats by somatic cell nuclear transfer," *Nat. Biotechnol.* **17**, 456–461 (1999).
5. I. A. Polejaeva, S. H. Chen, T. D. Vaught, R. L. Page, J. Mullins, S. Ball, Y. F. Dai, J. Boone, S. Walker, D. L. Ayares, A. Colman, and K. H. S. Campbell, "Cloned pigs produced by nuclear transfer from adult somatic cells," *Nature* **407**, 86–90 (2000).
6. P. Chesne, P. G. Adenot, C. Viglietta, M. Baratte, L. Boulanger, and J. P. Renard, "Cloned rabbits produced by nuclear transfer from adult somatic cells," *Nat. Biotechnol.* **20**, 366–369 (2002).
7. C. Galli, I. Lagutina, G. Crotti, S. Colleoni, P. Turini, N. Ponderato, R. Duchi, and G. Lazzari, "A cloned horse born to its dam twin—a birth announcement calls for a rethink on the immunological demands of pregnancy," *Nature* **424**, 635 (2003).
8. W. A. Kues and H. Niemann, "The contribution of farm animals to human health," *Trends Biotechnol.* **22**, 286–294 (2004).
9. M. Niekrasz, Y. Ye, L. L. Rolf, N. Zuhdi, and D. K. C. Cooper, "The pig as organ donor for man," *Transplantation* **24**, 625–626 (1992).
10. Y. Ye, M. Niekrasz, S. Kosanke, R. Welsh, H. E. Jordan, J. C. Fox, W. C. Edwards, C. Maxwell, and D. K. C. Cooper, "The pig as a potential organ donor for man—a study of potentially transferable disease from donor pig to recipient man," *Transplantation* **57**, 694–703 (1994).
11. K. H. S. Campbell, P. Fisher, W. C. Chen, I. Choi, R. D. W. Kelly, J. H. Lee, and J. Xhu, "Somatic cell nuclear transfer: past, present and future perspectives," *Theriogenology* **68**, S214–S231 (2007).
12. J. L. Edwards, F. N. Schrick, M. D. McCracken, S. R. Van Amstel, F. M. Hopkins, M. G. Welborn, and C. J. Davies, "Cloning adult farm animals: a review of the possibilities and problems associated with somatic cell nuclear transfer," *Am. J. Reprod. Immunol.* **50**, 113–123 (2003).
13. L. Liu, R. Oldenbourg, J. R. Trimarchi, and D. L. Keefe, "A reliable, noninvasive technique for spindle imaging and enucleation of mammalian oocytes," *Nature* **18**, 223–225 (2000).
14. J. A. Byrne, D. A. Pedersen, L. L. Clepper, M. Nelson, W. G. Sanger, S. Gokhale, D. P. Wolf, and S. M. Mitalipov, "Producing primate embryonic stem cells by somatic cell nuclear transfer," *Nature* **450**, 497–502 (2007).
15. C. Galli, I. Lagutina, and G. Lazzari, "Introduction to cloning by nuclear transplantation," *Cloning Stem Cells* **5**, 223–232 (2003).
16. J. Fulka, P. Loi, H. Fulka, G. Ptak, and T. Nagai, "Nucleus transfer in mammals: non-invasive approaches for the preparation of cytoplasts," *Trends Biotechnol.* **22**, 279–283 (2004).
17. S. MacLean-Fletcher and T. D. Pollard, "Mechanism of action of cytochalasin-B on actin," *Cell* **20**, 329–341 (1980).
18. H. Imahie, M. Takahashi, Y. Toyoda, and E. Sato, "Differential effects of cytochalasin B on cytokinesis in parthenogenetically activated mouse oocytes," *J. Reprod. Dev.* **48**, 31–40 (2002).
19. T. Dominko, A. Chan, C. Simerly, C. M. Luetjens, L. Hewitson, C. Martinovich, and G. Schatten, "Dynamic imaging of the metaphase II spindle and maternal chromosomes in bovine oocytes: Implications for enucleation efficiency verification, avoidance of parthenogenesis, and successful embryogenesis," *Biol. Reprod.* **62**, 150–154 (2000).
20. Y. Tsunoda, Y. Shioda, M. Onodera, K. Nakamura, and T. Uchida, "Differential sensitivity of mouse pronuclei and zygote cytoplasm to Hoechst staining and ultraviolet irradiation," *J. Reprod. Fertil.* **82**, 173–178 (1988).
21. C. Simerly, T. Dominko, C. Navara, C. Payne, S. Capuano, G. Gosman, K. Y. Chong, D. Takahashi, C. Chace, D. Compton, L. Hewitson, and G. Schatten, "Molecular correlates of primate nuclear transfer failures," *Science* **300**, 297 (2003).
22. J. C. Daniel, J. R. Takahashi, and K. Takahashi, "Selective laser destruction of rabbit blastomeres and continued cleavage of survivors *in vitro*," *Exp. Cell Res.* **39**, 475–482 (1965).
23. M. W. Berns, J. Aist, J. Edwards, K. Strahs, J. Girton, P. McNeill, J. B. Rattner, M. Kitzes, M. Hammerwilson, L. H. Liaw, A. Siemens, M. Koonce, S. Peterson, S. Brenner, J. Burt, R. Walter, P. J. Bryant, D. VanDyk, J. Coulombe, T. Cahill, and G. S. Berns, "Laser microsurgery in cell and developmental biology," *Science* **213**, 505–513 (1981).
24. S. Antinori, H. A. Selman, B. Caffa, C. Panci, G. L. Dani, and C. Versaci, "Zona opening of human embryos using a non-contact UV laser for assisted hatching in patients with poor prognosis of pregnancy," *Hum. Reprod.* **11**, 2488–2492 (1996).
25. M. Boada, M. Carrera, C. De la Iglesia, M. Sandalinas, P. N. Barri, and A. Veiga, "Successful use of a laser for human embryo biopsy in preimplantation genetic diagnosis: report of two cases," *J. Assist Reprod. Genet.* **15**, 302–307 (1998).
26. B. Schopper, M. Ludwig, J. Edenfeld, S. Al-Hasani, and K. Diedrich, "Possible applications of lasers in assisted reproductive technologies," *Hum. Reprod.* **14**, 186–193 (1999).
27. R. G. McKinnell, M. F. Mims, and L. A. Reed, "Laser ablation of maternal chromosomes in eggs of rana pipiens," *Z. Zellforsch.* **93**, 30–35 (1969).
28. A. V. Karmenyan, A. K. Shakhbazyan, T. A. Sviridova-Chailakhyan, A. S. Krivokharchenko, A. E. Chiou, and L. M. Chailakhyan, "Use of picosecond infrared laser for micromanipulation of early mammalian embryos," *Mol. Reprod. Dev.* **76**, 975–983 (2009).
29. J. L. Boulnois, "Photophysical processes in recent medical laser developments: A review," *Lasers Med. Sci.* **1**, 47–66 (1986).
30. K. Koenig, "Multiphoton microscopy in life sciences," *J. Microsc. Oxford* **200**, 83–104 (2000).

31. J. M. Squirrell, D. L. Wokosin, J. G. White, and B. D. Bavister, "Long-term two-photon fluorescence imaging of mammalian embryos without compromising viability," *Nat. Biotechnol.* **17**, 763–767 (1999).
32. A. Vogel, J. Noack, G. Huettman, and G. Paltauf, "Mechanisms of femtosecond laser nanosurgery of cells and tissues," *Appl. Phys. B* **81**, 1015–1047 (2005).
33. F. Bourgeois and A. Ben-Yakar, "Femtosecond laser nanoaxotomy properties and their effect on axonal recovery in C. elegans," *Opt. Express* **15**, 8521–8531 (2007).
34. W. Watanabe, N. Arakawa, T. Higashi, K. Fukui, K. Isobe, K. Itoh, and S. Matsunaga, "Femtosecond laser disruption of subcellular organelles in a living cell," *Opt. Express* **12**, 4203–4213 (2004).
35. A. Heisterkamp, I. Z. Maxwell, J. M. Underwood, J. A. Nickerson, S. Kumar, D. E. Ingber, and E. Mazur, "Pulse energy dependence of subcellular dissection by femtosecond laser pulses," *Opt. Express* **13**, 3690–3696 (2005).
36. H. Niioka, N. I. Smith, K. Fujita, Y. Inouye, and S. Kawata, "Femtosecond laser nanoablation in fixed and non-fixed cultured cells," *Opt. Express* **16**, 14476–14495 (2008).
37. F. Bestvater, E. Spiess, G. Stobrawa, M. Hacker, T. Feurer, T. Porwol, U. Berchner-Pfannschmidt, C. Wotzlaw, and H. Acker, "Two-photon fluorescence absorption and emission spectra of dyes relevant for cell imaging," *J. Microsc. Oxford* **208**, 108–115 (2002).
38. M. Hoelker, B. Petersen, P. Hassel, W. Kues, E. Lemme, A. Lucas-Hahn, and H. Niemann, "Duration of *in vitro* maturation of recipient cocytes affects blastocyst development of cloned porcine embryos," *Cloning Stem Cells* **7**, 34–43 (2005).
39. E. Suomalainen, "Parthenogenesis in animals," *Adv. Genet.* **3**, 193–253 (1950).
40. R. Zeggari, B. Wacogne, C. Pieralli, C. Roux, and T. Gharbi, "A full micro-fluidic system for single oocyte manipulation including an optical sensor for cell maturity estimation and fertilisation indication," *Sens. Actuators B* **125**, 664–671 (2007).
41. H. Schatten, R. S. Prather, and Q. Y. Sun, "The significance of mitochondria for embryo development in cloned farm animals," *Mitochondrion* **5**, 303–321 (2005).
42. B. C. Heng, M. Stojkovic, G. Vajta, and T. Cao, "Mammalian oocyte polarity can be exploited for the automation of somatic cell nuclear transfer—in the development of a cloning biochip," *Med. Hypotheses* **67**, 420–421 (2006).
43. K. Kuetemeyer and A. Heisterkamp, "Influence of laser parameters on the intracellular ablation threshold" (unpublished data).
44. E. S. Lander et al., "Initial sequencing and analysis of the human genome," *Nature* **409**, 860–921 (2001).
45. K. Kuetemeyer, M. Schomaker, S. Willenbrock, and A. Heisterkamp, "Fragment size distribution after femtosecond laser ablation of plasmid DNA" (unpublished data).
46. S. Choksakulnimitr, S. Masuda, H. Tokuda, Y. Takakura, and M. Hashida, "*In vitro* cytotoxicity of macromolecules in different cell culture systems," *J. Controlled Release* **34**, 233–241 (1995).
47. C. L. V. Leal, C. Lee, and R. M. Moor, "UV irradiation of pig metaphase chromosomes: maturation-promoting factor degradation, nuclear cytology and cell cycle progression," *J. Reprod. Fertil.* **116**, 363–371 (1999).
48. D. N. Nikogosyan, "Two-quantum UV photochemistry of nucleic acids: comparison with conventional low-intensity UV photochemistry and radiation chemistry," *Int. J. Radiat. Biol.* **57**, 233–299 (1990).
49. M. Dizdarouglu, "Chemical determination of free radical-induced damage to DNA," *Free Radic Biol. Med.* **10**, 225–242 (1991).
50. C. M. Gedik, S. W. B. Ewen, and A. R. Collins, "Single-cell gel-electrophoresis applied to the analysis of UV-C damage and its repair in human-cells," *Int. J. Radiat. Biol.* **62**, 313–320 (1992).
51. M. Jendrach, S. Mai, S. Pohl, M. Voeth, and J. Bereiter-Hahn, "Short- and long-term alterations of mitochondrial morphology, dynamics and mtDNA after transient oxidative stress," *Mitochondrion* **8**, 293–304 (2008).
52. A. Kohdjakov, R. W. Cole, and C. L. Rieder, "A synergy of technologies: combining laser microsurgery with green fluorescent protein tagging," *Cell Motil. Cytoskeleton* **38**, 311–317 (1997).
53. E. L. Botvinick, V. Venugopalan, J. V. Shah, L. H. Liaw, and M. W. Berns, "Controlled ablation of microtubules using a picosecond laser," *Biophys. J.* **87**, 4203–4212 (2004).
54. M. Alikani, T. Schimmel, and S. M. Willadsen, "Cytoplasmic fragmentation in activated eggs occurs in the cytokinetic phase of the cell cycle, in lieu of normal cytokinesis, and in response to cytoskeletal disorder," *Mol. Hum. Reprod.* **11**, 335–344 (2005).
55. V. Gomez-Godinez, N. M. Wakida, A. S. Dvornikov, K. Yokomori, and M. W. Berns, "Recruitment of DNA damage recognition and repair pathway proteins following near-IR femtosecond laser irradiation of cells," *J. Biomed. Opt.* **12**, 020505 (2007).
56. X. Kong, S. K. Mohanty, J. Stephens, J. T. Heale, V. Gomez-Godinez, L. Z. Shi, J. S. Kim, K. Yokomori, and M. W. Berns, "Comparative analysis of different laser systems to study cellular responses to DNA damage in mammalian cells," *Nucleic Acids Res.* **37**, e68 (2009).
57. J. S. Kim, T. B. Krasieva, H. Kurumizaka, D. J. Chen, A. M. R. Taylor, and K. Yokomori, "Independent and sequential recruitment of NHEJ and HR factors to DNA damage sites in mammalian cells," *J. Cell Biol.* **170**, 341–347 (2005).