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Abstract. We report a virus-free optical approach to human cell reprogramming into induced pluripotent stem cells with low-power nanoporation using ultrashort Bessel-shaped laser pulses. Picojoule near-infrared sub-20 fs laser pulses at a high 85 MHz repetition frequency are employed to generate transient nanopores in the membrane of dermal fibroblasts for the introduction of four transcription factors to induce the reprogramming process. In contrast to conventional approaches which utilize retro- or lentiviruses to deliver genes or transcription factors into the host genome, the laser method is virus-free; hence, the risk of virus-induced cancer generation limiting clinical application is avoided. © 2015 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: [10.1117/1.JBO.20.11.115008](https://doi.org/10.1117/1.JBO.20.11.115008)]

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1 Introduction

Induced pluripotent stem (iPS) cells closely resemble embryonic stem cells in many aspects such as differentiation potency, protein content and gene expression, proliferation, morphology, and embryoid body formation.¹ Typically, iPS cells are generated through the reprogramming of somatic cells by forced expression of a combination of appropriate transcription factors.^{1–3} Conventional approaches utilize retro- or lentiviruses for gene delivery.¹

iPS cells derived from patients' cells have tremendous potential for the investigation of disease pathology and the development of pharmaceuticals,⁴ in particular, since they open the possibility of using the patient's own cells for transplantation therapy and circumvent problems associated with immune compatibility.⁵

iPS cells were first derived from mouse fibroblasts¹ by Yamanaka's group in Japan in 2006 (Nobel Prize in 2012). It was shown that the viral introduction of the transcription factors Oct4, Klf4, Sox2, and c-Myc transferred fibroblasts into iPS cells. Although only one decade has passed since 2006, iPS cells have been generated from a range of tissue-specific stem cells and fully differentiated somatic cells such as peripheral blood cells, hepatocytes, keratinocytes, oral mucosa, and umbilical cord blood, as well as dental pulp cells.⁶

Alternatively, functional somatic cells have been generated from other fully differentiated cells as well as from committed, but not yet fully differentiated, cells (so-called progenitor cells) through "direct reprogramming" (also known as trans-differentiation) without reversion to an intermediate pluripotent state.^{7–9} This approach provides a faster method for the generation of functional cells and excludes the risk of generating potentially tumorigenic iPS cells⁷ due to their pluripotency.

Direct reprogramming was realized by the forced expression of the gene *MyoD* in dermal fibroblasts, retinal cells, and immature chondrocytes that converted them into muscle cells.¹⁰ The combination of transcription factors such as Ascl1, Brn2, and Myt1l converted fibroblasts into neurons,¹¹ whereas Gata4, Mef2c, and Tbx5 could reprogram mouse cardiac and dermal fibroblasts directly into functional cardiomyocytes.^{12,13}

The delivery of transcription factors into the genome of a target cell is typically achieved through the use of retro- or lentiviruses.¹ Clinical translation of iPS cells generated with these viral techniques is challenging due to insertional mutations caused by the random integration of genes carried by viruses.⁶ Moreover, the integration of viral genes into the cell genome may result in residual transgene sequences, thereby increasing the risk of cancer formation.¹⁴

Alternative strategies of reprogramming through nonviral gene vectors such as small interfering ribonucleic acid and plasmid deoxyribose nucleic acid (DNA) have also been employed.⁶ A variety of delivery approaches such as electroporation, magnet-assisted transfection, DNA guns, ultrasound, microinjection, and liposome-mediated transfection have been applied to assist penetration of the genes into the cytoplasm and further into the nucleus. However, the issues of cytotoxicity, contamination with chemicals, low posttransfection viability due to irreversible damage, and low transfection efficiency are still unsolved problems.

A virus-free delivery method is the targeted femtosecond laser transfection of adherent cells.¹⁵ Due to a cell's innate self-repair mechanism, transient pores in the cell membrane can be created by highly focused femtosecond laser beams in the near-infrared (NIR) range to allow genes or vectors to enter the cell without any adverse effect on cellular viability. Femtosecond laser transfection is sterile and nontoxic, and it achieves high posttransfection viability. This method has been tested on a variety of mammalian

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cells including stem cells.¹⁶ Thus, femtosecond laser transfection has been employed for the insertion of the green fluorescence protein (GFP) gene in stem cells¹⁷ as well as to activate mouse stem cell differentiation through GATA6.¹⁸ NIR femtosecond lasers can create nanoholes in biological objects with a diameter of less than 80 nm.¹⁹

In this paper, we present a proof-of-principle study for virus-free optical reprogramming of human somatic cells by the introduction of a cocktail of four transcription factors as well as the GFP gene with femtosecond laser nanoporation.

2 Materials and Methods

2.1 Laser Scanning Microscope for Cell Detection and Nanoporation

Nanoporation was performed with a 10 fs multiphoton microscope (JenLab GmbH, Jena, Germany).¹⁷ The mode-locked Ti:sapphire laser Integral Pro 400 oscillator with a broad M-shaped emission spectrum centered around 800 nm, a repetition rate of 85 MHz, and a mean output power of 420 mW was employed. For cell nanoporation, the scanning mirrors of the microscope were kept fixed in the center position. A 40× NA1.3 objective (Zeiss EC Plan-Neofluar) was used. A motorized stage (Märzhäuser, Wetzlar, Germany) was employed to move the sample in $x-y$ directions, and a piezoelectric z -translator (nanoMipos400, Piezosystems Jena, Germany) was used to shift the objective along the z -direction. The laser illumination time was controlled by a mechanical shutter (Thorlabs Inc., New Jersey).

An axicon lens (apex angle of 0.5 deg, Thorlabs Inc., New Jersey) was used to elongate the laser focal region along the axial direction by creating a quasi-Bessel beam [Fig. 1(a)]. The lateral laser intensity pattern is described by a Bessel function of the first kind, that is, it consists of an intense central part

with several concentric rings. Experimentally, however, only an approximation to a Bessel beam with the properties of the mathematical entity over a finite distance can be realized.²⁰ This intensity pattern is here referred to as a quasi-Bessel beam. The quasi-Bessel beam extends along the optical axis leading to an effectively elongated focal region compared to a Gaussian beam shape. The elongated focal region relaxes the need for precise fine-focus positioning onto the cell membrane, which is necessary with Gaussian beam focal shape. Due to the enhanced focal length by a factor of 2 in our setup in the case of the quasi-Bessel beam, the exact positioning of the laser focal volume is less critical for the realization of efficient nanoporation.

Chirped mirrors were employed to precompensate for the group velocity dispersion (GVD) of all optics (Fig. 1). The *in situ* pulse duration behind the objective, that is, at the sample position, was determined by interferometric autocorrelation measurements. Figures 1(b) and 1(c) show an autocorrelation trace measured behind the objective as well as the pulse spectrum. Although the autocorrelation trace still indicates the presence of some uncompensated components of GVD, a pulse duration of ~ 19 fs (27/1.41) can be derived assuming pulses with Gaussian temporal shapes.

Typically, we used a mean power of 40 mW *in situ* for the nanoporation. Nanoporation was observed over an axial range of 4 μm , that is, the acquired focusing precision for the focal volume on the cell membrane was about 4 μm . This range was enough to potentially optoporate all adherent cells inside a culture dish and to balance different x - y -position-dependent vertical offsets due to slight horizontal misalignments of the cell dish in the microscope stage. While the axial focus region was extended, the lateral focal was not significantly widened compared to a Gaussian focal beam shape. Axial and lateral extensions of the focal region of the quasi-Bessel beam as

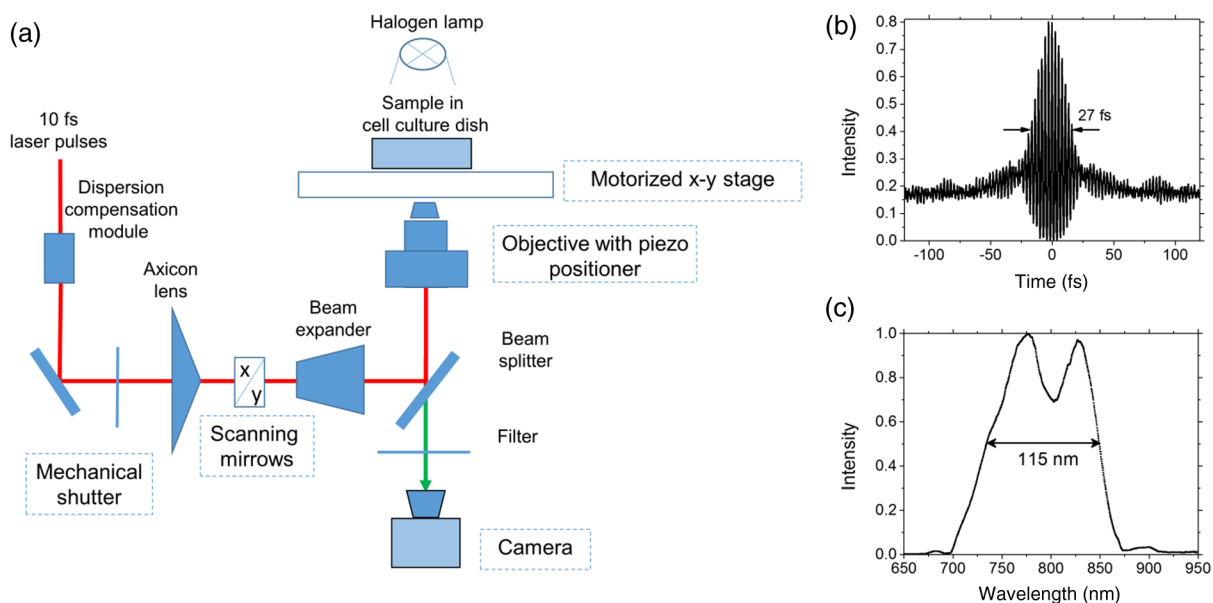


Fig. 1 (a) Scheme of the femtosecond laser nanosurgery microscope with quasi-Bessel beam focal geometry. The dashed rectangles indicate the objects controlled by the automation software. (b) Interferometric autocorrelation trace of the femtosecond laser pulses in the focal plane of the NA1.3 objective. The width of about 27 fs of the autocorrelation trace indicates a sub-20 fs pulse width assuming Gaussian temporal shape. (c) The laser spectrum exhibits an FWHM bandwidth of 115 nm with two peaks at 770 and 830 nm.

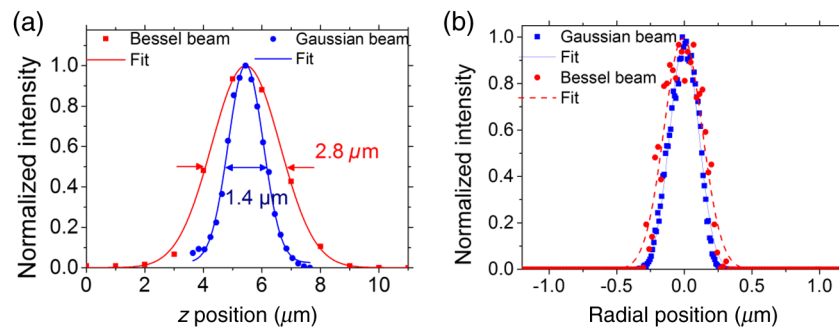


Fig. 2 Gaussian and axicon-generated quasi-Bessel beam focus dimensions along (a) axial (z) and (b) radial axes.

determined from imaging a subresolution fluorescent sphere with 5 mW are shown in Figs. 2(a) and 2(b), respectively, together with the measurement data for the Gaussian-shaped focal volume (obtained with the axicon removed).

The optical reprogramming procedure was controlled with automated software that was developed in our laboratory.²¹ A CCD camera was used for online monitoring of the fibroblasts during the laser treatment. The software recognizes the cell positions by fast gray-level evaluation and steers the hardware components to center the periphery of the target cell on the laser beam position, and it controls the number and duration of laser shots per cell. Mean powers of 10 and 40 mW were required for the laser treatment in the case of the Gaussian and the quasi-Bessel beams, respectively.

2.2 Cell Lines

Human dermal fibroblasts (passage 2) isolated from a healthy donor were purchased from LONZA (Adult Human Normal Dermal Fibroblasts #CC-2511, Lonza) and cultured in fibroblast growth medium containing high-glycose Dulbecco's modified Eagle's medium (# 41965-039, Life Technologies), 15% fetal bovine serum (# 10270-106, Life Technologies), and 1% Gentamicin (# 1570, Gibco). Cells were passaged in a 1:4 ratio and split every three to four days upon reaching the cell density of about 80%.

Adherent cells in a 170- μ m glass bottom dish with 500- μ m grids and removal inserts were prepared one day before the transfection. The growth medium was aspirated from the flask, cells were washed once with Dulbecco's phosphate-buffered saline medium free of Ca^{2+} and Mg^{2+} (# 10010, Life Technologies), and 1 ml of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (# 25200, Gibco) was added. The cells in the flask were incubated for about 3 to 4 min at 37°C (5% CO_2) until all cells were completely detached. Subsequently, the trypsin-EDTA was inactivated by adding 3 to 5 ml fibroblast growth medium. Dislodged cells were transferred into a 15-ml conical tube and centrifuged at 200 rpm for 5 min. A defined number of cells were transferred into 500- μ m-grid cell culture dishes (#81168 Ibidi) and incubated overnight in a growth medium. Cell inserts (# 80209 Ibidi) were applied to minimize the amount of plasmids. Before the laser treatment, the cell samples were washed with a serum-free medium and incubated in an 80 ml Opti-Mem medium (#11058-021, Life Technologies). Three microliters of genes were added into the medium before transfection. The inserts were removed after laser transfection to avoid overgrowth. After transfection, cells were incubated in

fresh growth medium at 37°C (5% CO_2) and monitored for GFP expression and colony formation for several days after laser exposure using a standard fluorescence microscope.

2.3 Nonviral Minicircle Deoxyribose Nucleic Acid

Nonviral minicircle DNA, as described by Narsinh et al.,²² was used. The minicircle vector contained the reprogramming factors OCT4, SOX2, NANOG, and LIN28 as well as the GFP reporter gene.²⁰

2.4 Optoporation with Accelerated Control Software

In order to be able to treat a large number of cells, we implemented means to speed up the process of laser targeting, which is time-demanding if done solely by hand. Of particular importance, in this context, is the automatic identification of the target positions on the cells for laser illumination. The exact locations on the cell, namely, the cell membrane, cytoplasm, or nucleus, as well as the number of illuminated positions per cell, greatly influence the optoporation and the cell survival rate.²¹ Briefly, the identification is based on the contrast evaluation in bright-field microscope images of the sample. To determine a position for laser targeting, the camera images are subdivided into parallel lines in subsquare regions with edge lengths corresponding to the typical length of the targeted cells, for instance, 10 to 20 μ m. The cells are identified by determining image contrast changes along the horizontal square edge lines with the built-in LabView function IMAQ Rake 3 (National Instruments, LabView Vision Development Module), which evaluates changes in the image contrast to determine the so-called edge positions along a set of parallel search lines. Figure 3 shows a microscope bright-field image with overlaid search lines and identified "contrast edges," that is, positions on the cell membranes. The positions are marked by filled circles (blue and yellow). The image contrast changes are only evaluated from left to right along the horizontal lines such that at most one edge position is identified per square, specifically, between neighboring vertical lines. The filled circles mark the automatically determined positions for later laser illumination, which correspond to the periphery of a cell. This results in laser poration of the cell's membrane away from the nucleus, which is typically located at the center of the cell.

Circles that lie very close to each other are in a further step removed from the set of illumination positions by the software to avoid stressing the cells by laser illumination (removed circles are indicated by the yellow color). The separation distance

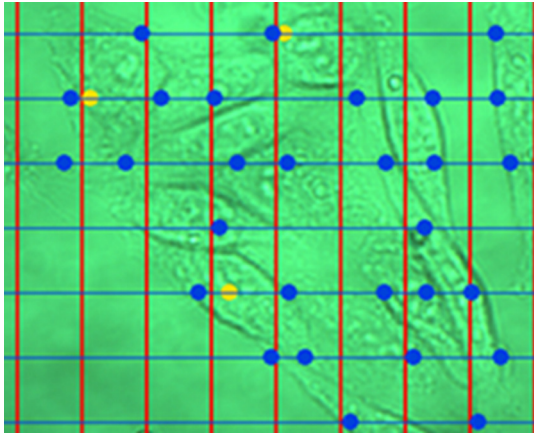


Fig. 3 Microscopic bright-field image of the cell sample with illustration of the cell position finding. Contrast changes in the image are evaluated along the horizontal lines. Between neighboring vertical lines, a maximum of positions is determined. The filled circles mark the found positions. Too close-lying circles (yellow) are removed from the position set.

between the horizontal blue lines can be fine-tuned by the user to accommodate different cell sizes and alter the average number of shots per cell. The software for the automated cell optoporation further controls several hardware components such as the microscope stage and optical shutter. After identifying the positions for laser illumination, these are centered in the focal volume of the microscope objective (i.e., “laser focus”), and the

mechanical shutter in the laser beam path is opened for a preset illumination duration. Afterward, the same procedure is repeated with all other identified positions inside the microscope field of view (FOV). After addressing all located cell positions inside a microscope FOV, the stage moves to the adjacent region in a meander shape such that a large sample area can be covered in a mosaic pattern.

2.5 Atomic Force Microscopy

To visualize and characterize holes in cells induced with the quasi-Bessel beam focus geometry, laser-processed cells were imaged with an atomic force microscope (AFM) DimensionTM 3100 (Digital Instruments, Veeco Metrology Group) in tapping mode. The cantilevers (Micro Cantilever, OMCL-AC160TS-C2, Olympus) with silicon tips with a tip diameter of about 5 nm, a spring constant of 40 N/m, and a resonance frequency of 300 kHz were used. WSxM 4.0 software was used to analyze the AFM (.stp) files.

2.6 Fluorescence Microscopy

An Olympus IX70 fluorescence microscope equipped with a high-pressure mercury lamp was used to evaluate GFP expression after the laser procedures. An excitation wavelength of 435 nm (Bandpass 434/17) was used to induce the GFP fluorescence with a maximum at 530 nm. Detection filters BP510/42 and BG 39 were used for GFP fluorescence and bright-field images, respectively.

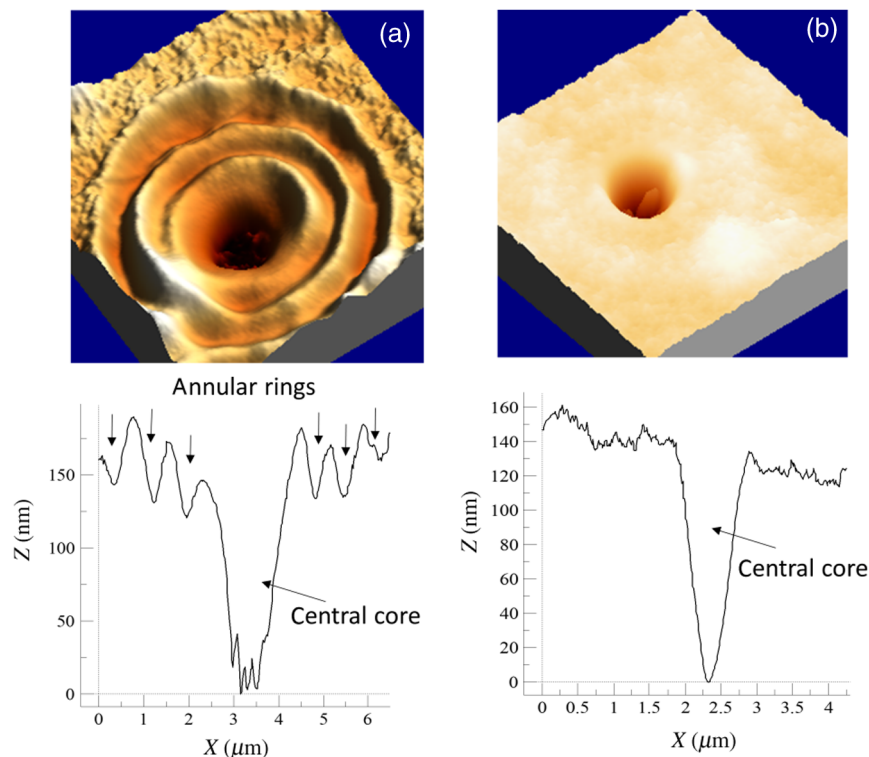


Fig. 4 Atomic force microscope (AFM) images of a cell nucleus, taken after illumination with pulses with a quasi-Bessel beam focal geometry at different mean powers. (a) The AFM image shows the destructive effects of a high-power quasi-Bessel beam consisting of an intense central core surrounded with several concentric rings. (b) Destructive effects occur in the central part of the beam only when using lower mean powers.

3 Results

3.1 Fast Cell Recognition and Low Power Nanoporation

The laser scanning microscope with automated cell recognition software and an elongated focus was employed to perform nanoporation on a high number of cells. At first, the destructive effects of the quasi-Bessel beam were studied using AFM. If the power and beam dwell times are too high, destructive effects occur not only in the central part of the beam but also in the microenvironment due to the symmetric pattern of a quasi-Bessel beam that consists of an intense central core surrounded with several concentric rings [Fig. 4(a)]. However, only the highly localized destructive effects of the central core are desired. The destructive effects of the less-intense “ring structure pattern” should be avoided [Fig. 4(b)]. When using a beam dwell time of 100 ms and a mean power of 40 mW, precise sub-micron holes in the cell’s membrane could be achieved. This corresponds to about 8 million laser pulses with a 12-ns pulse interval.

Cells were recognized by fast gray-level evaluation from images obtained by white light source illumination. The simple edge detection algorithm needs about 0.2 ms to identify a cell position. In fact, in our setup, the duration of moving the stage

and the laser illumination time itself overwhelmingly determine the speed of the optoporation procedure. The microscope stage needs about 15 ms for a position change of 10 μm . Therefore, with an illumination time of 100 ms, about 115 ms is required to identify, position, and laser illuminate a cell position. With an FOV of about $400 \times 400 \mu\text{m}^2$, up to 500 cells can be theoretically nanoporated within 1 min. However, we employed typically up to three positions per cell for laser exposure (Fig. 3). Not all cells were found to be perfectly recognized by the software. Therefore, a realistic value is about 100 nanoporated cells per minute.

In the experiments, the successfully nanoporated cells took up the minicircle DNA including the GFP reporter gene. The integration of the foreign DNA into the cell’s genome resulted in the formation of a green fluorescence as well as significant changes of the cell morphology. Human dermal fibroblasts have a spindle-shaped morphology and grow as monolayer adherent cells *in vitro* (Fig. 5). Only some of the laser-exposed cells started to change their morphology as well as their fluorescence behavior. In particular, iPS cell-like green-fluorescent dome-shaped colonies appeared as early as four to six days after a single optical surgery procedure (Fig. 5). The number of cell colonies varied from experiment to experiment. In a successful experiment, up to 20% to 30% of colonies per experiment were generated, which shows about 2% to 3% colony-forming

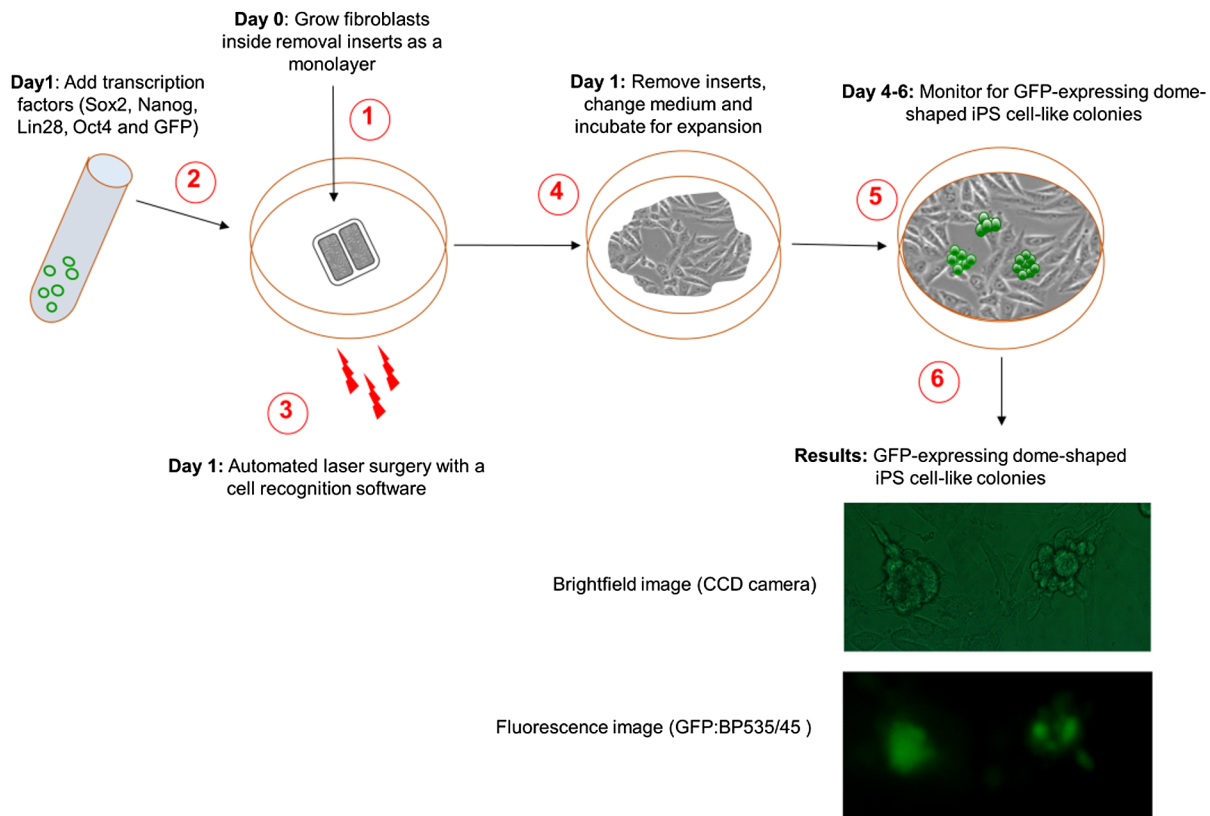


Fig. 5 Optical reprogramming procedure and detection of induced pluripotent stem (iPS) cell-like colonies. Cell monolayers were prepared in removal inserts 24 h prior to the experiment. Plasmids with transcription factors were given to the medium shortly before the laser nanoporation. Laser holes were created with quasi-Bessel beams at 40 mW mean power at 85 MHz at sub-20 fs. Inserts were removed; cells were washed and then incubated for expansion. Green fluorescence protein (GFP)-expressing dome-shaped iPS cell-like colonies were expected on days 4 to 6. Transfected cells were monitored with a fluorescence microscope for GFP fluorescence. iPS cell-like colonies generated from human dermal fibroblasts as a result of optical reprogramming were detected, where strong GFP expression indicated successful integration of minicircle DNA comprising GFP and four reprogramming factors.

efficiency. These colonies are typical for iPS cells and were found in all previous nonoptical iPS generation techniques such as lipofectamine and nucleofector transfection. Positive green fluorescence shows the successful synthesis of the reporter gene GFP and ensured coexpression of the whole set of reprogramming genes OCT4, SOX2, NANOG, and LIN28 in the genome. Figure 5 shows the procedure of virus-free optical reprogramming as well as images of GFP-expressing iPS cell-like colonies. Typically, 10 to 20 cells are estimated per colony.

4 Discussion

Reprogramming of somatic cells was achieved without the use of viruses. Approximately, 8 million femtosecond laser pulses were applied to the cell's membrane to create transient holes. This allowed diffusion of the DNA minicircle containing a cocktail of four genes required to induce cell reprogramming as well as reporter gene GFP. Normally, the exact focusing of the laser beam to the cell's membrane periphery takes a relatively long alignment time. When using quasi-Bessel beams with an elongated focus as demonstrated in this paper, this alignment procedure is no longer required. The fast software-assisted transfection system with cell recognition can realize a high-throughput transfection.

In conventional nonoptical methods, iPS generation requires from 3 to 27 repeated transfections of the same genes to achieve a sustained expression of introduced genes. Typically, the colony forms within 12 to 18 days after the transfection. Narsinh et al.²² have used minicircle DNA transfection of OCT4, SOX2, NANOG, and LIN 28 genes in human stem cells to induce iPS cell-like colonies with dome-like structures at approximately day 12 to 18 following nucleofection and repeated 2× lipofectamine transfection.

Interestingly, in this proof-of-principle study using femtosecond laser pulses, the bright GFP-expressing dome-shaped iPS cell-like colonies occurred even after one single optical nanosurgery procedure. Even more interestingly, the colonies were formed as early as four to six days after laser treatment. It remains challenging to know if and how ultrashort femtosecond laser NIR pulses accelerate the intricate iPS cell generation process. Nonetheless, most recent studies demonstrated that terahertz and low-level laser radiation increases stem cell proliferation and differentiation by the activation of transcription genes.^{23–25} The full iPS cell reprogramming procedure is very complex and is still being studied. Additionally, understanding the role of the laser in colony formation is crucial in future studies, since the complex reprogramming mechanism is dependent not only on the transfection procedure but also on many other chemical (medium, bioactive molecules, factors of reprogramming, precise gene balance, and culture conditions), physical (oxygen pressure, pH, and mechanical forces), and biological (type of cells, age, and origin of cells) factors. Furthermore, a variety of gene cocktails should also be investigated.

5 Conclusion

Optically generated iPS cells, such as immature pluripotent cells that are capable of developing into all types of cells in the body, are of great interest due to the avoidance of viruses or chemicals to realize gene transfer into the cell and would advance the clinical translation of iPS cells one step further. Our experimental finding demonstrates that the internalization of four

reprogramming factors and the reporter gene GFP can be optically realized. In contrast to other transfection and reprogramming methods, the optical reprogramming method provides a contamination-free transfection environment, and physical contact is not required. Virus-free minicircle DNA has been delivered without using further chemicals. The application of ultrashort NIR femtosecond laser nanosurgery enhanced colony-forming efficacy. However, further studies are required to optimize the optical procedure in particular to enhance the iPS cell generation efficiency.

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