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Plasma membrane temperature gradients and multiple cell permeabilization induced by low peak power density femtosecond lasers

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ABSTRACT

Calculations indicate that selectively heating the extracellular media induces membrane temperature gradients that combine with electric fields and a temperature-induced reduction in the electropermeabilization threshold to potentially facilitate exogenous molecular delivery. Experiments by a wide-field, pulsed femtosecond laser with peak power density far below typical single cell optical delivery systems confirmed this hypothesis. Operating this laser in continuous wave mode at the same average power permeabilized many fewer cells, suggesting that bulk heating alone is insufficient and temperature gradients are crucial for permeabilization. This work suggests promising opportunities for a high throughput, low cost, contactless method for laser mediated exogenous molecule delivery without the complex optics of typical single cell optoinjection, for potential integration into microscope imaging and microfluidic systems.

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1. Introduction

By introducing exogenous DNA into living cells while maintaining viability, gene therapy may potentially treat numerous diseases and conditions [1–4]. An ideal delivery method would be inexpensive and efficient (high delivery efficiency with high viability) with high throughput. Early gene therapy frequently used viral vectors for delivery [5]; however, side effects, such as inflammation and leukemia, prompted nonviral delivery method development [6]. Alternatives include chemical approaches, such as lipofection [7], or physical approaches, such as electric fields [8], ultrasound [9], nanoparticles [10], or lasers [11].

Electromagnetic techniques are the most common physical approach. Pulsed electric fields (PEFs) of appropriate duration and intensity electropermeabilize the membrane in a process called electroporation [12] with the membrane either resealing, as in gene therapy [13], or failing to reseal, as in cancer treatment [14] and sterilization [15]. Electroporation pulses are typically microseconds to milliseconds in duration with field strengths of approximately hundreds of volts per centimeter. PEFs of similar energy, but shorter duration (10–300 ns) and higher field strength (30–300 kV/cm) [16], may induce intracellular effects, such as apoptosis [17], changes in calcium dynamics [18], and mitochondria permeabilization [19], because the pulse duration is shorter than the charging time of the cell and on the order of the charging time of the smaller organelles. These nanosecond PEFs (nsPEFs) also create membrane pores smaller than those induced by traditional electroporation [20]. Low (~kHz–MHz) and high (~MHz) frequency AC fields similarly target external and internal membranes, respectively [21]. Lasers enable non-contact treatment and seamless integration with microscopic imaging and microfluidic systems [22]. Optical transfection has successfully delivered multiple substances, including ions, small interfering RNAs (siRNAs), and plasmids, using an automated, high-throughput process [23].

1.1. Laser mediated exogenous molecule delivery – overview

Laser-based gene delivery typically uses tightly focused beams, making it mostly a single cell permeabilization technique (optoinjection). While successful, the mechanism remains

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Table 1
Absorption coefficients for water and lipids at relevant laser wavelengths and the ratio (Water/Lipid) between them.

	Laser wavelength (nm)			
	532	800	1550	2080
Absorption Coefficient (cm^{-1})				
Water	0.000447	0.02	10.5	32
Lipid	0.01002	0.004	0.1996	1.625
Water/ Lipid	0.045	5	52.6	19.7

incompletely understood. Analogous to PEFs, laser pulse duration impacts the light-cell interaction. Continuous wave (CW) lasers generally favor delivery by plasma membrane heating [24]. Nanosecond lasers create shockwaves spanning multiple cell widths [24–26] while additionally inducing heat and thermoelastic stress [27]. Femtosecond lasers create free electrons at the cell surface to trigger a low density plasma that permeabilizes a single cell [23].

Laser wavelength also influences the permeabilization mechanism. The energy required for optical breakdown at femtosecond duration increases with wavelength [28]. Also, the volume of laser absorption depends upon the laser spot size (or illumination area) and absorption coefficient, which varies with wavelength [29–34] and medium temperature [35]. Table 1 summarizes absorption coefficients for water [29] and lipids [34, 36–37]. At 532 nm, the absorption coefficient of lipids ($\sim$0.01 cm^{-1}) is much higher than for water ($\sim 4.47 \times 10^{-4}$ cm^{-1}), making water much more transparent to the beam than lipids. Thus, the resulting laser exposure predominantly interacts with the plasma membrane at the membrane/buffer interface, making heating and thermoelastic stress dominant mechanisms [24]. At longer wavelengths, laser radiation increasingly interacts with the surrounding medium. At 800 nm, the most successful wavelength for single cell optoporation [24], the absorption coefficient for water (0.02 cm^{-1}) is five times larger than for lipids (0.004 cm^{-1}), indicating that the laser preferentially heats the surrounding buffer to induce plasma cloud formation at the plasma membrane [23] and a membrane temperature gradient (∇T) analogous to that calculated for electric fields [38]. The ∇T may additionally induce membrane voltages due to the thermoelectric effect that could contribute to permeabilization [38]. At 1550 nm, the absorption coefficient of water (10.5 cm^{-1}) exceeds that of lipids (0.2 cm^{-1}) by approximately a factor of fifty, further increasing ∇T. Similarly, the absorption coefficient of water (32 cm^{-1}) exceeds that of lipids (1.625 cm^{-1}) at 2080 nm by approximately a factor of twenty, suggesting that ∇T may contribute to membrane permeabilization, although the mechanism remains unknown [24]. The first experimental effects of ∇T in biological samples were observed previously during microwave exposures. Greater absorption of microwave energy in the extracellular fluid [39] creates ∇T that may induce various physiological responses, without bulk heating [40–41], such as membrane permeabilization [40], or that are irreproducible with convection heating alone [42].

Most lasers used for transfection have peak power levels of $\sim$10 kW, pulse durations of 17–150 fs, and wavelengths of 800–1000 nm to permeabilize a single cell [43] through low density plasma formation. Reduced cell viability [44] motivated research into femtosecond pulses of longer wavelength [45]. A 170 fs, 120 mW, 1554 nm laser transiently induced propidium iodide (PI) uptake with reduced temperature rise, shock waves, and cavitation bubbles compared to 800 nm lasers [45]. However, this study used relatively narrow focus ($\sim$2 μm) with high peak power densities at the optical breakdown level to perforate a single cell at the time [45].

This paper uses a simple analytic model to quantify and assess the potential impact of ∇T on experimental results showing that a 1550 nm, 100 fs laser with wide-field illumination in both pulse and CW modes will permeabilize cells with peak power densities three orders of magnitude below the typical optoporation threshold [45]. Comparing permeabilization levels and ∇T between pulse and CW modes will further show that ∇T may drive permeabilization and that bulk temperature rise alone is insufficient. The approach outlined here promises to be inexpensive with a higher throughput than conventional optoporation.

2. Materials and methods

Fig. 1 shows the experimental setup. The laser wavelength was $\sim$1550 nm, average power W_{avg}=120 mW, pulse duration τ_p $\sim$100 fs, repetition rate ν_{rep}=50 MHz (or 20 ns between pulses), peak power $W_{peak}\sim$24 kW ($W_{peak}=W_{avg}/[\tau_p \nu_{rep}]$), and spot diameter of $\sim$50 μm. The peak energy of the pulse ($E_{peak}=\tau_p W_{peak}$) is 2.4×10^{-9} J. Despite similar W_{peak} ($\sim$10 kW) and τ_p (17–150 fs) to standard optoinjection [43], the power density here is three orders of magnitude lower due to the wider illumination area ($\sim$2500 μm^2 compared to 4 μm^2). The resulting peak energy and average power densities are 9.6×10^{-4} J/cm^3 and 4.8×10^4 W/cm^3, respectively, assuming an exposure volume equal to the product of the illumination area and the absorption coefficient. In terms of area, peak energy and average power densities are 9.6×10^{-5} J/cm^2 and 4.8×10^3 W/cm^2, respectively. The typical laser spot measures approximately 50 μm, illuminating approximately 10–20 cells simultaneously with the exact number of cells a function of initial confluence density (approximately 80%) and the geometrical distribution of cells in the treated area.

We followed the cell preparation method presented in more detail elsewhere [46]. We cultured adherent Chinese Hamster Ovarian cells (CHO, American Type Culture Collection (ATCC)) in F12K media supplemented with 10% FBS according to the ATCC protocol. The cells were used at early passages, typically between passage four and ten. We used a Countess® Automated Cell Counter (Invitrogen) for cell counts. We seeded the cells at approximately 50% confluency with between 7×10^4 and 1×10^5 cells/well in 24 well-plate dishes. We verified cell morphology and viability twenty-four hours after seeding and observed that the cell confluency exceeded 80%. Fresh media was added to the cells to obtain a final volume of 1 ml for cells seeded in 24 well plates. The cells were incubated with propidium iodide (PI, Sigma) at 1 μg/ml for 5 min prior to laser exposure. We generated a negative

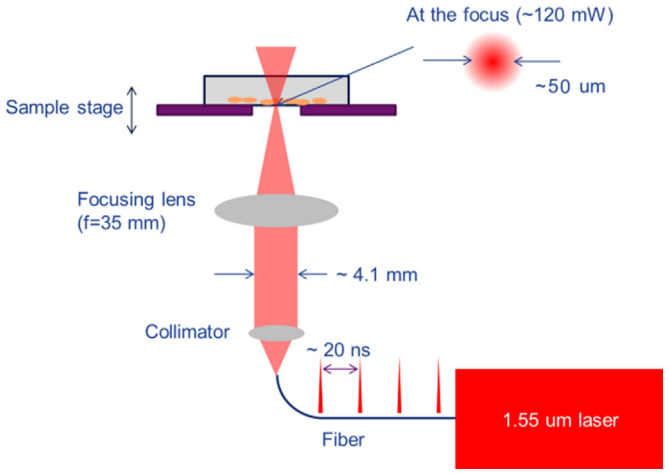

Fig. 1. Experimental setup for laser treatment of cells. The mechanical shutter controlling exposure time is not shown for clarity. The cells adhere to the bottom of the dish.

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