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FAST TRACK COMMUNICATION

Selective inactivation of micro-organisms with near-infrared femtosecond laser pulses

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Abstract

We demonstrate an unconventional and revolutionary method for selective inactivation of micro-organisms by using near-infrared femtosecond laser pulses. We show that if the wavelength and pulse width of the excitation femtosecond laser are appropriately selected, there exists a window in power density that enables us to achieve selective inactivation of target viruses and bacteria without causing cytotoxicity in mammalian cells. This strategy targets the mechanical (vibrational) properties of micro-organisms, and thus its antimicrobial efficacy is likely unaffected by genetic mutation in the micro-organisms. Such a method may be effective against a wide variety of drug resistant micro-organisms and has broad implications in disinfection as well as in the development of novel treatments for viral and bacterial pathogens.

1. Introduction

Selective inactivation of pathogenic micro-organisms such as viruses or bacteria remains a significant challenge. Current antimicrobial strategies, including the use of ultraviolet (UV) irradiation or other modern biochemical and pharmaceutical methods, evoke problems of drug resistance and have limited long-term efficacy. Furthermore, these methods frequently damage essential sensitive materials such as mammalian cells, leading to clinical side effects. It is, therefore, important and necessary to develop an innovative experimental technique that not only provides efficient inactivation of target micro-organisms but also avoids undesirable side effects. In this letter, we demonstrate a non-traditional and revolutionary method for accomplishing this goal through proper manipulation of a near-infrared (IR) femtosecond laser via impulsive stimulated Raman scattering (ISRS) to produce damage (e.g. on the protein coat of a virus) by forced resonance. We measured the near-IR femtosecond laser-induced inactivation of simple viruses and bacteria, and determined the effects of the laser irradiation on

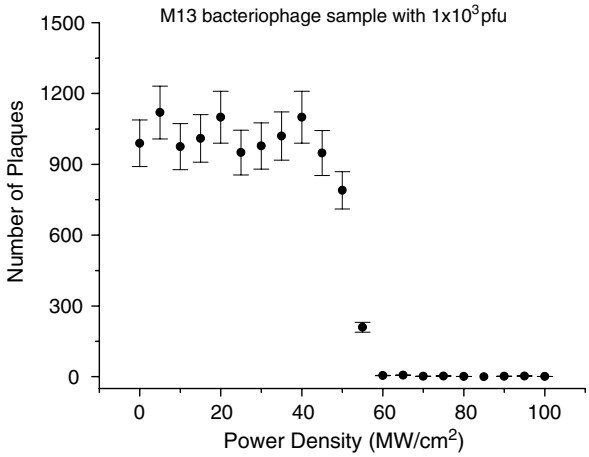

Figure 1. Number of plaque counts as a function of the excitation power density for M13 bacteriophage samples containing about 1×10^3 pfu in water solution after laser irradiation for about 1 h. Each data point is represented by its mean over three replicas and the standard deviation.

the viability of mammalian cells. Specifically, we show that if the wavelength and pulse width of the excitation femtosecond laser are appropriately selected, there exists a window in power density that enables us to achieve selective inactivation of target viruses and bacteria without causing cytotoxicity in mammalian cells. This strategy targets the mechanical (vibrational) properties of micro-organisms, and thus its antimicrobial efficacy is likely unaffected by genetic mutation in the micro-organisms. Such a method may be effective against a wide variety of drug resistant micro-organisms and has broad implications in disinfection as well as in the development of novel treatments for viral and bacterial pathogens. Some of the immediate plausible applications of this technique in biomedical research will be the efficient and safe treatments of blood-borne diseases such as AIDS and hepatitis, disinfection of blood supply and disinfection of biomaterials in hospitals.

2. Experimental technique

The excitation source employed in this work is a diode-pumped cw mode-locked Ti–sapphire laser. The excitation laser, which has an average power of about 500 mW is chosen to operate at a wavelength of $\lambda = 850$ nm. It provides a nearly transform-limited pulse train having a pulse width of FWHM $\cong$ 80 fs, spectral width of FWHM $\cong$ 80 cm^{-1} and at a repetition rate of 80 MHz. The typical average power used in the experiments is about 100 mW. The various excitation power densities ranging from 1 MW cm^{-2} to 60 GW cm^{-2} can be achieved by varying the laser spot size with an appropriate lens. Here, the laser power density is defined as that at the most tightly focused region formed by the focusing lens. The interaction of photons with micro-organisms or mammalian cells suspended in liquid solution inside a quartz cuvette of about 2 ml was facilitated by using a magnetic stirrer.

3. Experimental results and analysis

The inactivation of laser-irradiated M13 bacteriophages was measured by plaque assay on *E. coli* bacterial cells [1]. Figure 1 shows the number of plaque counts as a function of the excitation power density for a M13 bacteriophage sample containing about 1×10^3 pfu

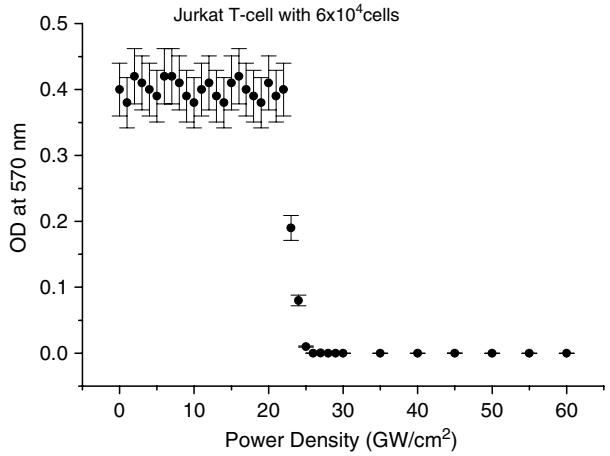

Figure 2. Optical density (OD) measured at 570 nm of the samples containing about 6×10^4 Jurkat T-cells in serum solution as a function of the excitation power density after laser irradiation for 1 h. Each data point is represented by its mean over three replicas and the standard deviation.

(plaque forming units) in water solution after laser irradiation for 1 h (data for zero power density represent the control). Our experiments find that M13 bacteriophages are inactivated at a threshold excitation power density of about 60 MW cm^{-2}. Similar experiments on tobacco mosaic virus (TMV) (data not shown) indicate that TMV becomes inactivated at a threshold power density of about 250 MW cm^{-2}. Therefore, our data provide strong support that viruses have an inactivation threshold of the order of a few hundred MW cm^{-2} or lower when excited with a near-IR 80 fs laser.

The survival of laser-treated mammalian cells was measured by MTT assay [2]. Figure 2 shows the optical density (OD) of a human Jurkat T-cell sample in serum solution as a function of the excitation power density after laser irradiation for 1 h (data for power density $= 0$ represent the control). The OD was measured at 570 nm and the sample contained 6×10^4 cells. These data show that human Jurkat T-cells fail to survive at a threshold power density of about 22 GW cm^{-2}, an intensity threshold *far* higher than the threshold for viruses. Previous investigations have shown behavior consistent with our experimental results. Chinese hamster ovarian (CHO) cells have been demonstrated to have a damage threshold of about 60 GW cm^{-2} when subject to irradiation by a 200 fs, 780 nm laser [3]. Recently, a 1 TW cm^{-2} power density derived from a femtosecond laser system has been used to open up a transient perforation in the membrane of CHO as well as rat-kangaroo kidney epithelial (PtK2) cells without causing signs of apoptosis or cell death [4]. In summary, previous data and our current data strongly suggest that mammalian cells (which are significantly larger in size than viruses) have a relatively high damage threshold when excited with femtosecond lasers—of the order of 10 GW cm^{-2} or higher.

The activity of laser-irradiated bacteria was measured by a Petrifilm plating method [5]. Figure 3 shows the number of cfu (colony forming units) as a function of the excitation power density for the green-fluorescence *E. coli* bacteria in beef-broth solution with about 300 cfu after laser irradiation for about 1 h (data for power density $= 0$ represent the control). The green-fluorescence *E. coli* bacteria have been found to inactivate at a threshold excitation power density of about 900 MW cm^{-2}. These results suggest that bacteria, which generally have sizes between those of viruses and mammalian cells, have an inactivation threshold of 1 GW cm^{-2} or smaller.

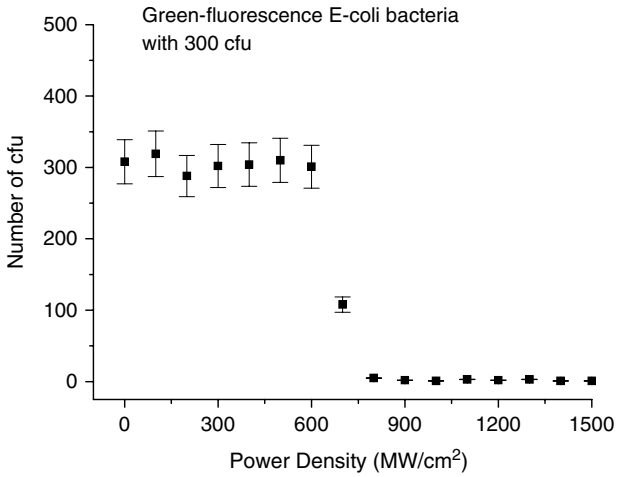

Figure 3. Number of cfu as a function of the excitation power density for green-fluorescence *E. coli* bacteria samples with about 300 cfu in beef-broth solution after laser irradiation for about 1 h. Each data point is represented by its mean over three replicas and the standard deviation.

Table 1. Threshold power density for inactivation of micro-organisms.

	Micro-organisms			
	M13	TMV	Green-fluorescence bacteria	Jurkat T-cell
Threshold power density for inactivation (MW cm^{-2})	60	250	900	22 000

All of our experimental results are summarized in table 1. The results indicate that, for excitation with a near-IR femtosecond laser having $\lambda = 850$ nm and pulse width of 80 fs, a window in excitation power density exists (bounded approximately by $\widetilde{=}1$ GW cm^{-2} and $\widetilde{=}10$ GW cm^{-2}) that inactivates both viruses and bacteria while leaving sensitive materials such as mammalian cells unharmed. In other words, selective inactivation of micro-organisms is feasible with proper manipulation of near-IR femtosecond laser pulses.

4. Discussion

ISRS has been shown to be a viable technique for producing large-amplitude vibrational modes in solid state systems and in molecules in liquid solution [6]. The ISRS process is one in which excitation of a coherent molecular (or lattice) vibration is driven by a sufficiently short laser pulse providing an impulsive force. The force is produced by the electric energy density of the laser as it couples to a Raman-active vibrational distortion of the molecule. The impulse occurs even with a single (ultrashort) laser pulse. There is no laser intensity threshold for producing an atomic displacement pattern within the molecule. However, an intensity threshold is necessary for producing large enough displacements to produce damage. Viewed in the context of light scattering, ISRS is a forward-scattering Raman process and is stimulated because the Stokes frequency is contained within the spectral width of the excitation pulse.

ISRS has recently been used to coherently excite the Raman-active axial mode (with $\omega_0 \widetilde{=} 8.5$ cm^{-1}) of the protein capsids of M13 bacteriophages with a 100 fs, 425 nm ultrafast

laser. Inactivation of M13 bacteriophages has been observed when the excitation laser peak intensity (which is proportional to the amplitude of the excited coherent vibrations) exceeds a certain threshold value [7, 8].

Coherent excitation of Raman-active vibrational modes by ISRS can be understood in the following way. The vibrational mode excited by the laser is represented by normal coordinate Q. If we ignore dispersion in the index of refraction and assume that the incident electric field from the excitation laser is not depleted by the stimulated scattering, the equation of motion for Q can be written as [9, 10]

$$\frac{\partial^2 Q}{\partial t^2} + 2\gamma \frac{\partial Q}{\partial t} + \omega_0^2 Q = f(t) \tag{1}$$

where ω_0 is the angular frequency of vibration, γ is a damping constant and $f(t)$ is the impulsive driving force produced by the excitation laser and is described next.

The laser electric field $\vec{E}_{\mathrm{L}}$ induces a dipole on the molecule due to its polarizability α as $\vec{P} = \alpha \vec{E}_{\mathrm{L}}$, where for simplicity we neglect the tensor properties of α. The polarizability has a static part that produces elastic Rayleigh scattering, and a part that is modulated by the oscillating displacement Q. It is this modulated contribution that produces the Raman effect and is of interest here. The polarizability is expanded in a Taylor series and to the lowest order in Q is $\alpha(Q) = \alpha_0 + \alpha_0' Q$, where $\alpha_0' = (\frac{\partial \alpha}{\partial Q})_0$ is the polarizability derivative at zero displacement. This quantity is proportional to the amplitude of the Raman scattering cross section. The potential energy stored in an induced polarization is $U(Q,t) = -\frac{1}{2}\vec{P}(Q,t) \cdot \vec{E}_{\mathrm{L}}(t)$. The generalized force driving equation (1) is $f(t) = -\frac{\partial U(Q,t)}{\partial Q}$ which leads to

$$f(t) = \tfrac{1}{2}\alpha_0' E_{\mathrm{L}}^2. \tag{2}$$

In our experiments we use an ultrashort laser pulse so that the force is impulsive. Although the oscillation of the electric field is quite rapid compared to that of the molecule, the forcing function $f(t)$ has a DC component due to the squaring of the electric field and is always of the same sign.

Equation (1) with $f(t)$ given by equation (2) can be solved to determine the normal coordinate $Q(t)$. In particular, for excitation by a single-beam ultrashort laser having a pulse width of τ_{L}, and intensity $I(t) = I_0 \mathrm{e}^{-(t^2/\tau_{\mathrm{L}}^2)}$, assuming small damping, the displacement is $Q(t) = Q_0 \mathrm{e}^{-\gamma t} \sin(\omega_0 t)$. Of greatest importance is the amplitude Q_0 of the displacement away from the equilibrium produced by ISRS and this is given by [6]

$$Q_0 = \frac{\sqrt{\pi}}{2} \frac{n}{cK\varepsilon_0} \alpha_0' \frac{\tau_{\mathrm{L}}}{\omega_0} I_0 \mathrm{e}^{-(\omega_0^2 \tau_{\mathrm{L}}^2/4)}. \tag{3}$$

Here I_0 is the peak intensity of the excitation laser, α_0' is the polarizability derivative proportional to the amplitude of the Raman scattering cross section, n is the index of refraction, c the speed of light, and $K\varepsilon_0$ the permittivity of the dielectric medium.

It is clear from equation (3) that larger Raman cross sections (α_0'), higher laser power densities (I_0), and lower vibrational frequencies (ω_0) produce larger excited vibrational amplitudes. As a matter of fact, in small molecules with a moderate Raman scattering cross section and sufficiently low vibrational frequency, and using a reasonable excitation power density, amplitudes of atomic motion during the vibrational excitation of 0.01–1 Å can be achieved through ISRS.

Selective excitation of modes of different frequency is an intriguing aspect of the ISRS technique. The τ_{L} dependence in equation (3) is $\tau_{\mathrm{L}} \mathrm{e}^{-(\omega_0^2 \tau_{\mathrm{L}}^2/4)}$ which is a maximum at $\tau_{\mathrm{L}} = 0.225 T_0$ where $T_0 (= 2\pi/\omega_0)$ is the period of the vibrational oscillation. This means that if the peak laser intensity I_0 is kept constant, the excited vibrational amplitude will be a maximum when the duration τ_{L} of the ultrashort pulse is near a quarter of a vibrational period. Thus

Table 2. Dependence of the status of the M13 bacteriophage on the laser pulse width. (The excitation laser intensity is kept at 5.6×10^{-6} J cm^{-2}. The numbers within the brackets indicate the spectral width in cm^{-1}.)

	Pulse width (fs)				
	80	250	500	800	1000
Status	(80)	(25)	(12)	(6.5)	(5)
Inactivation (Yes or No)	Yes	Yes	Yes	No	No

a window for laser intensity and pulse width exists—if τ_L is too long then little vibrational excitation occurs.

The presence of a window can also be viewed from the perspective of the frequency domain. For the one-beam excitation experiment employed in this work, the primary beam as well as the Stokes shifted beam, whose photon energies are $\hbar\omega_L$ and $\hbar\omega_s$, respectively, define the excited coherent vibrations with energy $\hbar\omega_0$ such that $\hbar\omega_0 = \hbar\omega_L - \hbar\omega_s$. As a result, the full width at half-maximum (FWHM) spectral width of the excitation laser due to time–frequency uncertainty has to be larger than the energy of the excited coherent vibrations. For the Gaussian distribution of the excitation laser in time (and space) the uncertainty principle gives rise to the factor $e^{-\omega_0^2\tau_L^2/4}$. This indicates that for a given ω_0, the excitation pulse width τ_L has to be chosen so that $\omega_0\tau_L \leqslant 1$ to significantly excite the mode. This explains why a longer excitation laser pulse, even with the same laser intensity, produces less inactivation. This behavior is evident in our experimental results of table 2 in which the dependence of the viability of M13 bacteriophage on the excitation laser pulse width is presented when the laser intensity remains constant.

Although it is not clear at the moment why there are large differences in inactivation power density threshold among the micro-organisms such as viruses and bacteria and mammalian cells produced through the ISRS method by near-IR femtosecond pulses, we believe that this difference most likely results from the nature of the structural compositions in the protein coats or membranes. Bacteria and cells have bi-layer membrane structure, whereas viruses like M13 phages are usually made up of capsids. Other possible factors include differences in the Raman scattering cross section and in the damping constant associated with the coherently excited vibrational modes of viral capsids, bacterial cell walls/membranes, and mammalian cell plasma membranes. The larger the size of the excited micro-organisms/cells, the greater the effect of damping by the surrounding water molecules; as a result, if the pulse width of the excitation femtosecond laser is appropriately chosen then the amplitude of the excited Raman-active vibrational modes can be larger for the smaller micro-organisms/cells.

Finally, since this method targets the intrinsic mechanical (vibrational) properties of protein capsids and membrane structures, it is relatively insensitive (perhaps completely immune) to genetic mutation in the target micro-organisms. This is because vibrational modes of low frequency characteristically have long wavelengths; as a result they are most influenced by global structure rather than specific details in local structure. Thus it is highly unlikely that target micro-organisms can avoid destruction through genetic mutations. Also, because a low power near-infrared femtosecond laser is employed, it is expected that the technique will have minimal effect on genetic materials within the micro-organisms.

5. Conclusion

In this work, we demonstrate a new and innovative method of selectively inactivating target micro-organisms with near-IR femtosecond laser pulses. We show that the basic

concept of coherently exciting a Raman-active mode (e.g. on the protein coat of a virus) with a femtosecond laser via ISRS is a novel and general method with the potential for inactivating undesired micro-organisms while leaving sensitive materials such as mammalian cells unharmed. This method should have numerous important applications in disinfection and may represent a novel strategy for the development of treatments against viral and bacterial pathogens.

Acknowledgments

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