

Cellular tolerance to pulsed heating

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ABSTRACT

Many laser therapies involve significant heating of tissue with pulses varying from picoseconds to minutes in duration. In some of the applications heating is a primary goal, while in others it is an undesirable side effect. In both cases, if a hyperthermia is involved, the knowledge about the threshold temperature leading to irreversible cellular damage is critically important. We study the dependence of the threshold temperature on duration of the heat exposure in the range of 0.3 ms to 5 seconds. Thin layer of cells cultured in a Petri dish was exposed to a pulsed CO₂ laser radiation. Laser beam was focused onto sample providing Gaussian intensity distribution in the focal plane with a beam diameter ($2w$) 2-10 mm. Surface temperature in the central part of the focal spot (1mm in diameter) was measured by thermal infrared (IR) emission from the sample, recorded with a fast IR detector. For pulses shorter than 1 s the temperature profile across the focal spot was found to closely correspond to the radial distribution of the laser beam intensity, thus allowing for accurate determination of temperature at any given distance from the center of the spot. Immediate cellular damage was assessed using vital staining with the live/dead fluorescent assay. Threshold temperatures were found to vary from 65 °C at 5 s of heating to 160 °C at pulses of 0.3 ms in duration. The shorter end of this range was limited by vaporization, which occurs during the laser pulse and results in mechanical damage to cells. Dependence of the maximal temperature on pulse duration could be approximated by Arrhenius law with activation energy being about 1 eV.

Keywords: thermal shock, protein denaturation, cell thermal damage.

1. INTRODUCTION

Many medical procedures involve significant hyperthermia, i.e. exposure of tissue to elevated temperatures. A large variety of laser and radiofrequency therapies induce thermal stress in tissue with exposure time varying in a very broad range – from nanoseconds to minutes. In some of the therapies (such as thermal therapy of tumors, or rejuvenation of skin) heating is a primary goal, while in others it is undesirable side effect. For optimization of these therapies the knowledge of the threshold temperatures leading to irreversible cellular damage is very important. Living organisms can survive at temperatures 42-47 °C for prolonged periods of time, while higher temperatures can only be tolerated for a limited duration. It has been reported that mammalian cells can survive temperatures of about 65 °C with exposure time not exceeding 5 s [1]. There is a very few data about cell survival after shorter thermal exposures – in a millisecond or microsecond domains, which are very common during the laser- or electro-surgery [2-4]

Duration of the heat shock is determined not only by the time of energy deposition (e.g. laser pulse duration), but also by the heat dissipation time, which can greatly exceed the energy deposition time. Heat transfer rate out of the exposed sample sets a limit to biologically relevant duration of thermal exposure. Taking a typical value of heat conductivity for biological tissue of $0.6 \text{ W m}^{-1} \cdot \text{K}$ and cells size of 10 μm one can find that the heat dissipation time from a single cell surrounded by a cold tissue is on the order of 100 μs . This is the shortest heat pulse which can be applied to a single cell as a whole. Situation, where a single cell layer is exposed to a short pulse of heat is not uncommon. It takes place, for example, during selective therapy of the retinal pigment epithelium with microsecond pulses of visible laser [5], or when tissue surface is treated by a mid-infrared laser having penetration depth of a few microns. Such conditions can also occur in the vicinity of microplasma generated by a femtosecond laser-induced dielectric breakdown [6].

In this article we study cellular viability under conditions of pulsed thermal stress with exposures extending from 1 second down to 300 μs . In this study we also aim at verifying whether dependence of the cellular thermal

damage threshold on pulse duration can be described by the Arrhenius law - an idea proposed long time ago [7], but never verified at short pulse durations.

2. EXPERIMENTAL DETAILS AND PROCEDURES

We performed measurements of cells viability under pulsed heating conditions, using a CO₂ laser as a heat source and a single layer cell culture as a sample. Penetration depth for a CO₂ laser radiation with wavelength 10.6 μm into the organic tissue, comprised mainly of water, is about 12 μm. This penetration depth, which is practically equal to cell diameter, matched perfectly to experimental requirements. It provided absorption of the major part of laser energy ($E_{\text{abs}} > 60\%$) within a monolayer of cells and at the same time allowed for rapid cooling due to thermal diffusion into the sample substrate.

The scheme of the irradiation setup is shown in Fig.1. The pulsed CO₂ laser beam with Gaussian spatial profile irradiated a monolayer of cells grown in a Petri dish. Thermal emission from the sample was collected by a spherical mirror, focused on a small aperture, which selected radiation emitted from the central part of the laser spot, and detected by a Mercury-Cadmium-Telluride (MCT) detector. Thermal emission signal was used for the time-resolved temperature measurements.

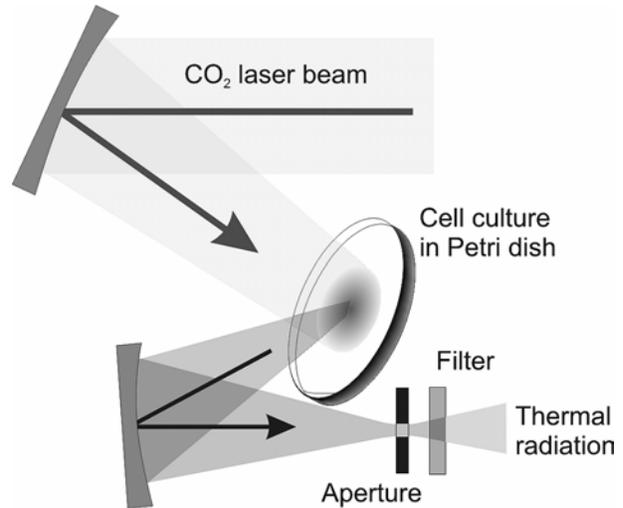


Fig. 1 Experimental arrangement for pulsed laser irradiation of living cells and temperature measurements.

2.1 Laser setup

We used a PLX-100 CO₂ laser (Paralax Technology Inc.), which can be operated in both, pulsed and CW regimes, producing up to 100 W of power in a single TEM₀₀ mode. Sample was positioned in a focus of a spherical mirror, where laser beam has Gaussian spatial intensity

distribution: $I = I_{\text{max}} \exp\left(-\frac{2x^2}{w^2}\right)$. Depending on the

focal length of the mirror, beam diameter at the focal plane was either 2 mm, or 10 mm. Beam profile was measured using knife-edge method and found to be of a Gaussian shape with accuracy of about 5%. Larger beam was used for pulses from 10 ms to 1 s, while smaller diameter was used for pulses from 300 μs to 10 ms, when higher laser intensities were required.

Temperature rise in the irradiated sample in the absence of thermal diffusion is proportional to the amount of the absorbed laser energy. At 10.6 μm wavelength water is a dominant absorber in a biological tissue. Proteins and lipids don't have pronounced absorption bands in this spectral region. This allowed us to consider cell culture in a water-based media as a homogeneous absorber. Since focal spot size was by 2 to 3 orders of magnitude larger than the radiation penetration depth, thermal diffusion in the sample was mainly one dimensional - with a heat flow from the surface to the

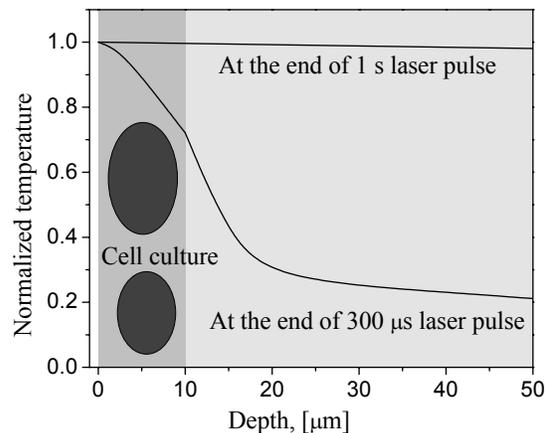


Fig. 2 a) Calculated temperature distribution in a sample and underlying polymethylmetacrilate substrate at the end of 300 μs and 1 s laser pulses.

sample substrate. Thus the lateral profile of heat distribution remained practically unchanged during the laser pulse and well after it. To substantiate these statements we performed numerical simulation of the heat transfer process in the model object comprised of 10 μm water layer on a 1 mm thick polymethylmethacrylate (Petri dish material) substrate. Results of these calculations are presented in Fig. 2a and 2b.

As one can see in Figure 2A, even for the shortest laser pulse of 300 μs, temperature variation across a cell layer doesn't exceed 20%, while for longer pulses temperature distribution becomes practically flat. As shown in Figure 2B, lateral distribution of temperature retains a shape very close to Gaussian even after 1 second-long laser pulse.

The fact that lateral temperature profile remained practically unchanged during heating means that cells located at different distances from the center experience heat pulse of the same temporal profile, but with different amplitude. Temperatures at any given radial position x

can be calculated as:
$$T(t) = T_{\max}(t) \exp\left(-\frac{2x^2}{w^2}\right),$$

where $T_{\max}(t)$ is a current temperature at the center of laser spot, which was measured for each pulse, and w is a half of laser beam diameter. Thus, in a single laser shot we were able to collect information on cells exposed to thermal pulses from the strongest ones, which can kill the cells, to weakest, that didn't produce any visible damage.

2.2 Temperature measurements

Temperature of a sample during and after irradiation was measured by detection of a thermal radiation from the sample (Fig.1). Thermal emission from a 1 mm diameter spot in the center of the irradiated area was collected with an $f/2$ focusing mirror and focused onto an MCT detector - MCT20-010 (Electro Optical Systems) which is sensitive to IR radiation from 2 μm to 20 μm. To suppress a scattered laser radiation a narrowband filter blocking 10.6 μm was positioned in front of the detector. Time resolution of the detector was 1 μs, allowing for the accurate measurement of the temporal profile for all used pulse durations.

Detector was calibrated using a hot plate as a thermal radiation source in place of a sample. Hot plate was covered with glass - since absorption coefficient of glass in mid-IR spectral region is close to that of water, it provided emissivity similar to that of the biological sample. Mechanical shutter, kept at room temperature, was positioned close to the detector and periodically opened allowing thermal radiation to reach the detector. Electrical signal from the MCT detector was amplified by a low-noise amplifier (SR 560 - Stanford Research Systems) and recorded by a Tektronix oscilloscope TDS 620B. Temperature traces exemplified in Figure 3 were calculated from the MCT signals using the calibration curve.

Fig. 4 demonstrates a different type of temperature evolution during the laser pulse. It was observed with short pulses of high intensity. Abrupt temperature fall during the laser pulse can be explained by explosive vaporization of the surface layer, which was also visually observed during such shots. This process happened sporadically at temperatures

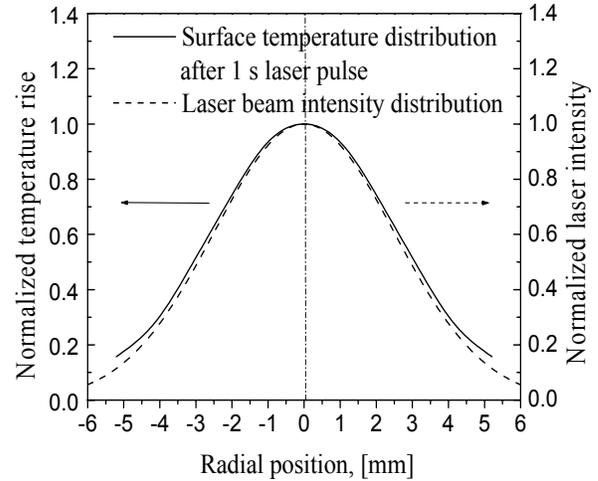


Fig. 2 b) Calculated surface temperature distribution at the end of 1 s laser pulse and intensity profile of the incident laser beam.

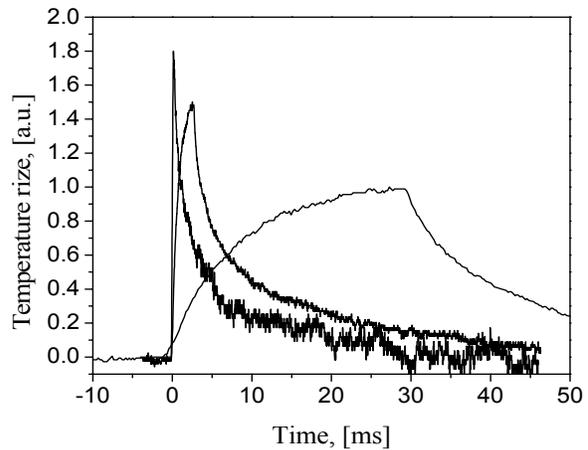


Fig.3 Typical temperature evolution of the samples surface irradiated by 0.3 ms, 3 ms, and 30 ms laser pulses.

higher than 180° C and becomes dominant above 220 ° C. These values correspond well to temperatures, which can be reached in overheated water for a short period time. Besides additional cross-checking of our temperature measurement technique, observation of the explosive vaporization is important for the following analysis of the mechanisms of cellular damage.

2.3 Sample preparation and staining

NIH 3T3 cells were grown confluent in 5cm diameter Petri dishes. Cells surface density was kept at 10⁵ cm⁻² to ensure that they form a uniform monolayer on the Petri dish surface. Before irradiation the medium from the Petri dish was removed by setting it at 45° angle to the horizon and allowing the liquid to drain from the upper part of the Petri dish for about 10 seconds. This procedure left a cell culture exposed to the laser beam with a layer of residual liquid not thicker than a few microns. After irradiation the samples were returned to horizontal position and refilled with liquid.

To distinguish between the live and dead cells after laser treatment cell cultures were stained with a standard LIVE/DEAD® Viability/Cytotoxicity Assay Kit (L-3224). This two-color fluorescence cell viability assay is based on simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cells viability - intracellular esterase activity and plasma membrane integrity. After staining the live cells are fluorescing green due to enzymatic conversion of the virtually nonfluorescent cell-permiant calcein AM to the intensely fluorescent calcein. Dead cells are fluorescing red because red dye EthD-1 penetrates the damaged membranes, binds to nucleic acids and undergoes a 40-fold enhancement of fluorescence.

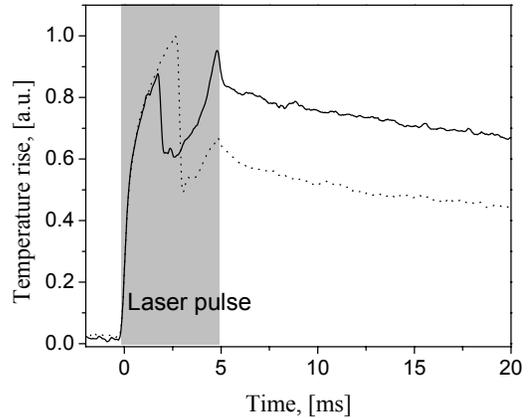


Fig.4 Temperature evolution of the sample surface irradiated by laser pulses of high intensity. Rapid temperature fall observed during laser pulses is associated with explosive boiling of the overheated liquid, which happens at temperatures well above 100°C. Temperatures corresponding to explosive boiling are determined by the rate of energy deposition and sample surface microstructure.

3. EXPERIMENTAL RESULTS AND DISCUSSION

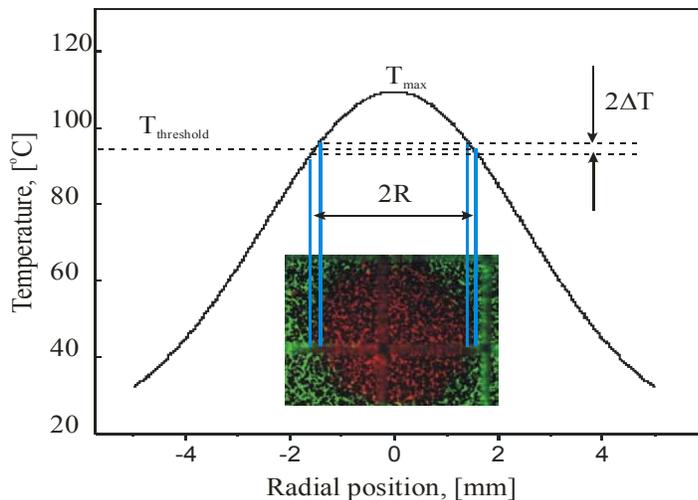


Fig. 5 Microscope image of the stained cells after irradiation showing live and dead zones within laser irradiated spot. Threshold temperature $T_{\text{threshold}} \pm \Delta T$ corresponds to the border between live and dead cells.

Cells survival was measured with laser pulse durations ranging from 300 μ s to 1 s. Each time point was measured on 8 samples, namely using two Petri dishes and applying 4 spots per dish. All samples were stained with the live-dead fluorescent assay within 5 minutes after the irradiation and examined under the microscope 20 minutes later, when fluorescence reached its maximum value. Red and green fluorescent images (corresponding to dead and live cells) of the same area on the sample were recorded with a digital camera and then combined using an image editing software. Typical composed image, demonstrating zones of the live - green (light gray) and dead - red (dark gray) cells is shown in Fig.5. This staining procedure provides information about distribution of the live and dead cells at the moment of staining. To verify whether the cells that appeared live right after the pulse could be dying later we

made a series of measurements with staining delayed by 2, 10, 30, and 60 minutes after the pulse. No significant difference in the damage thresholds was found in these measurements, though the width of the transition region (i.e. a mixture of the live and dead cells) seems to slightly increase at a 1 hour delay. Temperature variation across the transition zone was within a statistical error of our measurements.

Maximum temperatures, corresponding to the center of the transition zone between the live and dead cells are plotted in Fig.6 as a function of laser pulse duration. While laser pulses in our experiment had a nearly rectangular shape, temperature pulses associated with them have much slower rising and falling edges, with their shapes being dependent on duration of the laser pulse. Strong variation of temperature with time makes the analysis of cellular reaction to thermal stress much more complex.

In a steady-state situation a cell remains viable if the rate of damage does not exceed the rate of repair. In the pulsed regime this balance becomes dynamic and does not require instantaneous equality of rates, i.e. a cell might be able to survive after accumulating certain amount of damage if it can repair it after the pulse. It is reasonable to assume that when damage is produced during the time much shorter than the metabolic repair time constant, cells viability is determined by the integrated amount of damage, no matter how fast it was induced.

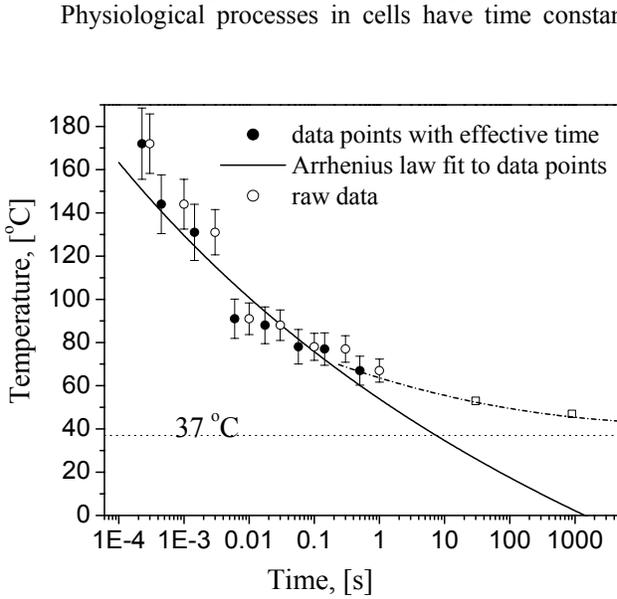


Fig. 6 Threshold temperatures as a function of the laser pulse length - open circles (raw data) and as a function of the effective time of the thermal pulse - solid circles. Solid line represents a Arrhenius law fit to the threshold temperatures as a function of the effective time. Dashed line demonstrates deviation from Arrhenius law at times longer than several hundred milliseconds.

$$\int_0^{\tau} A \exp\left(-\frac{E^*}{kT(t)}\right) dt = D_{threshold} \quad (2)$$

where τ is a duration of the temperature pulse and $D_{threshold}$ characterizes the maximum amount of instantaneous damage which cell can tolerate. Using the experimentally measured values of $T(t)$ one can calculate such integrals for each experiment, and find the value of E^* which satisfies condition (2) for all experimental points (pulse durations) the best. Using the least square method we obtained a value of $E^* = 1 \pm 0.2$ eV.

Once we know the value of the activation energy E^* we can predict the maximum temperature that cell can tolerate under a “square” pulse of heat, i.e. heating to a constant temperature T during a given time t . This temperature can be calculated according to Arrhenius law as following:

Physiological processes in cells have time constants ranging from tens of seconds to several hours. Thus, damage caused by the heat pulses shorter than one second can be considered instantaneous. Under such conditions cells can tolerate exactly same amount of damage (which we will denote as D) induced by the heat pulses ranging from microseconds to one second. We will assume that cellular damage produced by heating below the vaporization threshold is due to denaturation of proteins. The rate of this process can be approximated as a rate of chemical reaction described by the Arrhenius law:

$$D(t) = A \exp\left(-\frac{E^*}{kT(t)}\right), \quad (1)$$

where A is a rate constant, E^* - an activation energy corresponding to a particular chemical reaction, k - Boltzmann constant, and $T(t)$ current value of the absolute temperature. While protein denaturation is a complicated process, it is not unnatural to ascribe it a certain effective value of E^* [Birngruber et al].

Criterion of cells viability can then be written as following:

$$T = \frac{E^*}{k \ln \left(\frac{At}{D_{threshold}} \right)},$$

Solid line in Fig. 6 represents this function. In order to re-plot experimental data in the same scale, i.e. for “rectangular” pulses of heat, we calculate τ_{eff} – the “effective time”, which is the duration of the imaginary rectangular temperature pulse of temperature T_{max} producing same amount of damage as a real pulse having the same maximal temperature T_{max} . τ_{eff} can be written as:

$$\tau_{eff} = \frac{\int_0^{\tau} A \exp\left(-\frac{E^*}{kT(t)}\right) dt}{A \cdot \exp\left(-\frac{E^*}{kT_{max}}\right)}$$

Experimental values of T_{max} and their effective times are plotted in Fig. 6 as solid circles. As can be seen in this Figure experimental data fits Arrhenius temperature dependence reasonably well for pulses shorter than 1 second. As we already discussed, Arrhenius approximation becomes inapplicable at longer times when slow thermal damage is competing with processes of cellular repair. Experimental data from other authors obtained by different techniques fills the gap between our measurements, and the steady state regime where cells can survive for a long time at temperatures around 47 °C. These data points are also shown in Fig.7 as open squares.

At pulses shorter than 300 μ s cellular death was induced by explosive vaporization which we observed at temperatures slightly above 200 °C. This effect sets an absolute limit on temperatures that biological cells can withstand at any pulse durations.

4. CONCLUSIONS

We have studied viability of mammalian fibroblast cells NIH 3T3 exposed to heat shocks with durations in the range of 300 μ s to 1 s. We found that temperatures leading to cellular death are steadily rising with decreasing pulse duration. For shortest heat pulses of 300 μ s cells were found to survive at temperatures as high as 180 °C (much higher than water boiling point at normal conditions). At shorter pulses the damage was produced by explosive vaporization of water. Analysis of the results have shown that the dependence of the maximal temperature on pulse duration can be described by Arrhenius law, assuming a constant value of the maximal amount of accumulated damage that cell can tolerate .

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