

## Cellular tolerance to pulsed hyperthermia

D. M. Simanovskii,<sup>1</sup> M. A. Mackanos,<sup>2</sup> A. R. Irani,<sup>2</sup> C. E. O'Connell-Rodwell,<sup>2</sup> C. H. Contag,<sup>2,3</sup>  
H. A. Schwettman,<sup>1</sup> and D. V. Palanker<sup>1,4</sup>

<sup>1</sup>*Hansen Experimental Physics Laboratory, Stanford University, Stanford, California 94305, USA*

<sup>2</sup>*Department of Pediatrics, Stanford University, Stanford, California 94305, USA*

<sup>3</sup>*Department of Radiology, Stanford University, Stanford, California 94305, USA*

<sup>4</sup>*Department of Ophthalmology, Stanford University, Stanford, California 94305, USA*

(Received 3 March 2006; revised manuscript received 30 May 2006; published 24 July 2006)

Transient heating of tissues leading to cellular stress or death is very common in medicine and biology. In procedures involving a mild (below 70 °C) and prolonged (minutes) heating, such as hyperthermal tumor therapy, the cellular response to thermal stress is relatively well studied. However, there is practically no data on cell viability at higher temperatures and shorter exposures, while the demand for this knowledge is growing. Two main reasons motivate this research: (i) a growing number of laser therapies and surgical procedures involving pulsed heating, and (ii) cellular viability data at short exposures to high temperatures provide a unique insight into the understanding of processes leading to thermally induced cellular death. We designed a technique and performed a study of cell viability under pulses of heat from 0.3 to 100 ms in duration with peak temperatures as high as 130 °C. We found that the threshold of cellular death in this range can be accurately approximated by the Arrhenius law with the activation energy of 1 eV, a significantly lower value than was reported in studies based on multisecond exposures.

DOI: [10.1103/PhysRevE.74.011915](https://doi.org/10.1103/PhysRevE.74.011915)

PACS number(s): 87.17.-d, 87.54.Br

### I. INTRODUCTION

Many medical procedures involve significant hyperthermia, i.e., exposure of tissue to elevated temperatures. A variety of laser and radio frequency therapies induce thermal stress in tissue with exposure times varying in a very broad range—from nanoseconds to minutes. In some therapies (such as thermal therapy of tumors or rejuvenation of skin) heating is a primary goal, while in others it is an undesirable side effect. For optimization of these therapies knowledge of the threshold temperatures leading to irreversible cellular damage is critically important. Mammalian cells can survive at temperatures of 42–47 °C for prolonged periods of time [1], while higher temperatures can only be tolerated for a limited duration [2]. It has been reported that cells die at temperatures of about 70 °C in a few seconds [3]. There is a lack of data, however, about cellular survival following shorter exposures to higher temperatures, i.e., in millisecond and microsecond domains, which are very common during pulsed laser—or electro-surgery [4].

It is generally assumed that denaturation of proteins which are critical for cellular metabolism is the underlying cause of cellular damage [1]. While there is some data on protein denaturation at high temperatures [5–7], cellular viability at these temperatures remains unknown. It was our expectation that cellular viability data at short exposures might contribute to an understanding of processes leading to thermally induced cellular death. One might anticipate that as the exposure time decreases, the threshold temperature will increase monotonically. The rate of this increase, or possibly even the structure within it, however, might elucidate mechanisms and dynamics. The study was limited to short pulses specifically to eliminate the possibility of cellular repair during the hyperthermal exposure.

Most of the experimental techniques used to quantify cell viability at elevated temperatures are based on keeping the

sample at a constant temperature for a fixed period of time. For short exposures, this approach becomes impractical, because temperature ramp-up and ramp-down times become comparable or even exceed the isothermal portions of thermal pulses. In fact, the heat transfer rate out of the exposed sample sets a limit to the biologically relevant duration of thermal exposure. By taking a typical value of heat conductivity for soft tissue of 0.6 W m<sup>-1</sup> K and a cell size of 10 μm, one can find that the heat dissipation time from a single cell or cell layer into the surrounding tissue or liquid medium is on the order of 100 μs. This is the shortest heat pulse to which a single cell or a cellular monolayer can be exposed without strong thermal gradients across the cell body. Situations in which a single cell layer is exposed to a short pulse of heat are not uncommon. It takes place, for example, during selective therapy of the retinal pigment epithelium with microsecond pulses of a visible light laser [8] or when a tissue surface is irradiated by a short-pulse mid-infrared laser having a penetration depth of a few micrometers [9].

In this article we describe a technique that enables the study of cellular viability under conditions of short pulsed thermal stress with exposures extending from 100 ms down to a few hundred microseconds. We used a CO<sub>2</sub> laser as a heat source, which provided homogeneous heating of a thin sample at any desirable rate. Fast cooling was achieved by using a sample of minimal possible thickness—a monolayer of cells. Another key point of this technique was the precise temperature monitoring throughout heating and cooling phases, which allowed for the analysis of cellular damage produced at different temperatures during a single heat pulse.

In this study we also aimed to verify whether dependence of the cellular thermal damage threshold on pulse duration could be described by the Arrhenius law. The Arrhenius law, which predicts the rate of chemical reaction as a function of temperature, was also found to be applicable for describing

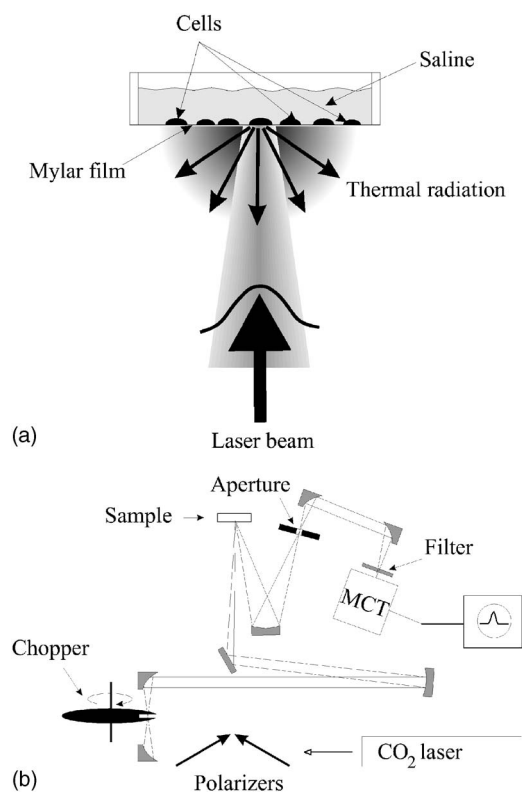


FIG. 1. (a) Pulsed laser heating of a single layer cell culture. (b) Laser heating pulses of variable intensity and duration were produced by transmitting the  $\text{CO}_2$  laser beam through a pair of polarizers that served as an attenuator and a rotating disk chopper. Thermal radiation emitted from the central region of the irradiated sample area was used to determine sample temperature. An aperture in the collection optics selected radiation emitted from the central part of the sample and a filter rejected scattered  $\text{CO}_2$  laser radiation.

cell killing by thermal shock [10–12]. We demonstrate that the Arrhenius law is applicable to quantification of thermal damage leading to immediate cellular death under short pulses of heat, and that kinetic parameters characterizing this process differ significantly from those reported for longer heat pulses when repair processes can play an important role [1–3,13].

## II. EXPERIMENTAL SETUP AND PROCEDURES

We have measured cell viability under pulsed heating conditions using a  $\text{CO}_2$  laser as a heat source and a single layer cell culture as a sample. The experimental arrangement is shown in Fig. 1(a). The pulsed  $\text{CO}_2$  laser beam was focused on a monolayer of cells, generating a temperature profile across the sample surface that replicates the Gaussian shape of the laser beam. Thermal emission from the central region of the irradiated area was used to measure cell temperature, and shortly after irradiation, a two-color fluorescence cell viability assay was performed.

### A. Sample preparation and viability assay

To provide a sample of uniform thickness, NIH 3T3 cells were grown nearly confluent on a  $3\ \mu\text{m}$  thick Mylar film in

a specially designed container under a few millimeters thick layer of cell culture medium. Cells were irradiated by a  $\text{CO}_2$  laser through the Mylar film, which is relatively transparent to the  $10.6\ \mu\text{m}$  radiation of the  $\text{CO}_2$  laser (3% absorption losses in a  $3\ \mu\text{m}$  film). This configuration, with cells attached to the film, allowed for highly uniform irradiation conditions and also prevented cell dehydration, which would be a problem if cells were exposed to the air.

Cell containers were made from 3 cm diameter Petri dishes with perforated bottoms and a thin Mylar film (Goodfellow, Inc.) attached to the open side of the dish using biocompatible UV glue (Norland Products, Inc.). Cells were grown to a surface density of about  $10^5\ \text{cm}^{-2}$  to ensure that they form a nearly confluent monolayer. Before irradiation the medium was replaced with a saline solution. Afterward the laser pulse cells were stained with a standard LIVE/DEAD® Viability/Cytotoxicity Assay Kit (L-3224, Invitrogen, Inc.). This kit provides a two-color fluorescence cell viability assay that is based on simultaneous determination of live and dead cells with probes that measure two recognized parameters of cell viability: the intracellular esterase activity and the plasma membrane integrity. After staining, the live cells fluoresced green due to enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. Dead cells fluoresce red because the red dye EthD-1 penetrates damaged membranes, binds to nucleic acids, and undergoes a 40-fold enhancement of fluorescence.

### B. Optical system

Pulsed heating of the monolayer cell culture was achieved by irradiating it with a  $\text{CO}_2$  laser (PLX-100, Parallax Technology, Inc.), which produced up to 100 W of power in a single  $\text{TEM}_{00}$  mode. Selection of a  $\text{CO}_2$  laser for this experiment was motivated by two considerations. First, the penetration depth of  $\text{CO}_2$  laser radiation ( $10.6\ \mu\text{m}$ ) in water is about  $12\ \mu\text{m}$ , a value comparable to the thickness of our single layer cell culture, which was about  $3\text{--}4\ \mu\text{m}$  in our case. Thus, a significant fraction of the laser energy is deposited within the monolayer of cells without forming a strong temperature gradient across the cell. Second, at the  $\text{CO}_2$  laser wavelength, water is the dominant absorber in biological tissue, while proteins and lipids do not have pronounced absorption bands in this spectral region. This allows one to consider the cell culture in the water-based medium as a homogeneous radiation absorber, a situation that can be analyzed easily. Although the laser energy is absorbed by water, thermalization of proteins and other molecules occurs on a subnanosecond timescale [14], so their temperature can be considered practically equal to water temperature in our experiments. The  $\text{CO}_2$  laser was operated in a pulsed regime, with pulse length controlled electronically in the 10–100 ms range. Shorter pulses, from 0.3 to 3 ms, were achieved by mechanical chopping of longer pulses.

The layout of the experimental setup is shown in Fig. 1(b). The sample was positioned at the focus of a spherical mirror where the laser beam has a Gaussian spatial intensity distribution,

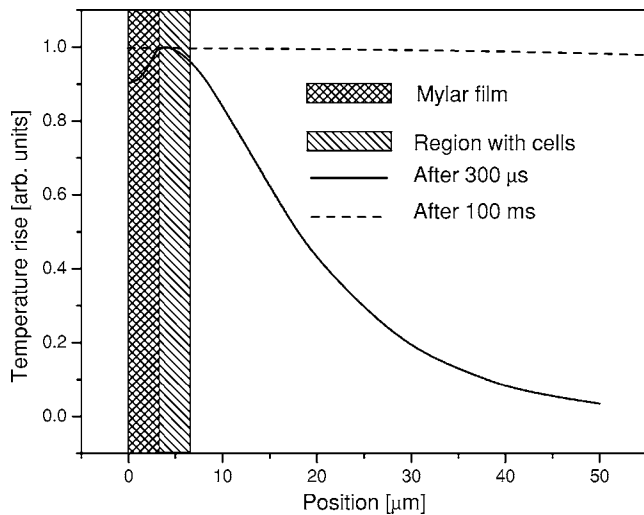


FIG. 2. Calculated axial temperature distribution in a sample at the end of 300  $\mu\text{s}$  and 100 ms laser pulses.

$$I = I_{\text{max}} \exp\left(-\frac{2r^2}{w^2}\right),$$

where  $r$  is the radial coordinate in the sample plane. The focusing system was designed to produce a focal spot size, defined as  $2w$ , equal to 2 mm. In the absence of thermal diffusion the incremental temperature rise generated by the  $\text{CO}_2$  laser pulse in the monolayer of cells is

$$\Delta T(r, z) = \frac{\mu F}{C_v} \exp\left(-\frac{2r^2}{w^2}\right) \exp(-\mu z),$$

where  $\mu$  is the absorption coefficient of the sample at 10.6  $\mu\text{m}$ ,  $F$  is the laser fluence at the surface of the sample,  $C_v$  is the specific heat capacity of the sample, and  $z$  is the axial coordinate (perpendicular to the sample plane). Thermal diffusion changes the temperature distribution in the sample, although for the conditions of our experiment its effect is moderate in the axial direction and very limited in the lateral one.

Since the focal spot in our experiment (2 mm) was more than two orders of magnitude larger than the radiation penetration depth ( $\mu^{-1} = 12 \mu\text{m}$ ), and since the laser pulse duration did not exceed 100 ms, thermal diffusion in the sample was nearly one-dimensional, with the heat flow proceeding predominantly axially from the front surface into the medium. To quantify this phenomenon, we performed a numerical simulation of the heat transfer in a model system comprised of a 3  $\mu\text{m}$  Mylar film and a 3 mm water layer.

In the absence of thermal diffusion, the axial temperature gradient caused by laser beam attenuation across the 3  $\mu\text{m}$  thick cell layer was  $\sim 20\%$ , which makes cell temperature not very well defined. However, thermal diffusion significantly improves the situation. Results of the numerical simulation, shown in Fig. 2, demonstrate that at the end of 300  $\mu\text{s}$  pulses thermal diffusion reduces the temperature gradient to 5%. For longer pulses, the temperature gradient across the cell layer decreases further, and with 3 ms pulses it becomes negligible.

With respect to the radial heat transfer, simulations have shown that even for the longest pulse (100 ms) used in our experiment, the radial distribution of temperature did not deviate from the initial Gaussian profile of the laser beam by more than 3%. To avoid more significant deviations, we limited pulse durations in this study to 100 ms. Since the radial temperature distribution was practically preserved throughout the heating and subsequent cooling phases, cells located at different distances from the center of the laser beam experienced a heat pulse of the same temporal profile but of different amplitudes. The temperature rise at any given radial position  $r$  can be calculated as  $T(t) = T_{\text{max}}(t) \exp[-(2r^2/w^2)]$ , where  $T_{\text{max}}(t)$  is the time dependent temperature rise at the center of the laser spot, a quantity that was measured for each pulse.

Thus, in a single shot we were able to collect information on cells exposed to a broad range of heat pulses of the same shape but different amplitudes. The laser intensity and consequently, the maximum temperature at the center of the focal spot was chosen to be high enough at each pulse duration to kill cells. Cells located at the periphery of the laser spot experienced a heat pulse of lower amplitude, and thus at a certain distance from the center, the hyperthermia became tolerable and did not produce any visible damage.

### C. Temperature measurement

Although model thermal calculations are straightforward, we felt it prudent to measure the course of temperature in the sample directly by measuring its thermal IR emission. For materials highly absorbing in the mid-IR, the emission spectrum is well approximated as a “black body” radiation spectrum. This makes temperature measurements material independent for a broad range of samples and experimental conditions, and allows for a precise calibration of the “transient radiation thermometer” using a static heat source. There is, however, a well known limitation of this technique. When the temperature within the sample changes significantly on a length scale comparable to the thermal radiation penetration depth, thermal emission is no longer a good measure of the surface temperature. In our experiments this situation was encountered only for laser pulses shorter than 1 ms, where a small correction factor had to be introduced into the calibration data, as described below.

As shown in Fig. 1(b), thermal emission was collected from a 0.3 mm diameter spot at the center of the 2 mm irradiated area with an  $f/4$  spherical mirror and focused onto a mercury cadmium telluride (MCT) detector (MCT20-010, Electro Optical Systems, Inc.), which is sensitive to IR radiation from 2 to 15  $\mu\text{m}$ . To suppress scattered  $\text{CO}_2$  laser radiation, a narrowband filter blocking 10.6  $\mu\text{m}$  wavelength was positioned in front of the detector. Temperature variation within the 0.3 mm collection area in the center of the 2 mm diameter Gaussian spot did not exceed 4% and thus allowed accurate measurement of the maximum temperature.

The MCT detector was calibrated using a hot plate with a glass surface as a thermal radiation source. A mechanical chopper, kept at room temperature, was positioned close to the aperture, providing alternating pulses of IR radiation to

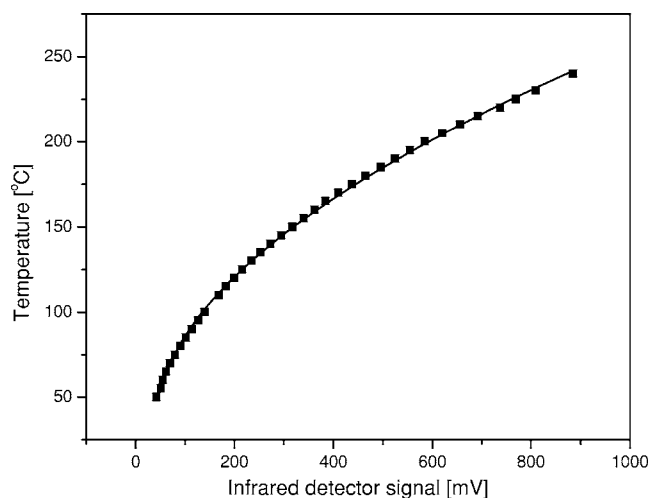


FIG. 3. Calibration curve for temperature measurements using infrared thermal emission from the sample.

the detector. The electrical signal from the MCT detector was amplified by a low-noise amplifier (SR 560, Stanford Research Systems, Inc.) and recorded by a Tektronix oscilloscope, TDS 620B. The experimental calibration curve of system sensitivity is presented in Fig. 3. This curve, which is a convolution of the detector spectral sensitivity, filter transmission, and surface emission spectrum, has been measured for all relevant temperature values, providing a basis for direct temperature measurements on the laser irradiated samples.

As we already mentioned, for the shortest laser pulses, the layer of material with elevated temperature is no longer optically thick for its thermal radiation and some correction factor to the measured temperature is required. Since it was quite difficult in our case to calculate this correction with sufficient precision, we did it experimentally. Correction of the radiation thermometer for short pulse durations was achieved by comparing its readings with a reliable reference temperature obtained by other means. For this purpose we used the threshold of explosive vaporization of water. It is well known that on a short time scale water can be overheated up to a spinodal limit, which has a well defined value [9]. This limit is set by the rate of homogeneous nucleation, either in bulk water, or in our case, at the Mylar-water interface. For bulk water the spinodal point is  $\sim 300^\circ\text{C}$  [15], while for water adjacent to an organic film it is lower, in the range of  $200\text{--}280^\circ\text{C}$ , depending on the surface hydration state [16].

For this calibration we used a container filled with pure water. The onset of explosive vaporization could be easily monitored by an abrupt change of the surface thermal emissivity and the appearance of laser radiation scattered from a transient vapor bubble formed at the water-film interface. A typical infrared signal corresponding to explosive vaporization of water is shown in Fig. 4(a). Applying laser pulses of increasing power, the moment of explosive vaporization and the corresponding temperatures have been measured. The temperature of explosive vaporization as a function of heat pulse duration (delay after the beginning of the laser pulse) is shown in Fig. 4(b). For heat pulses shorter than 20 ms the

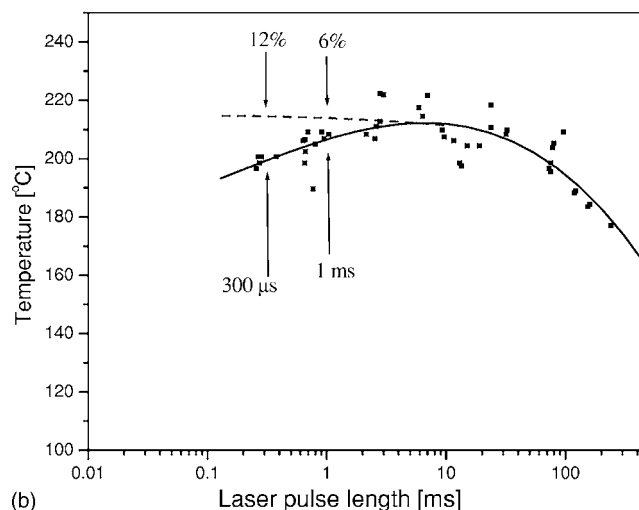
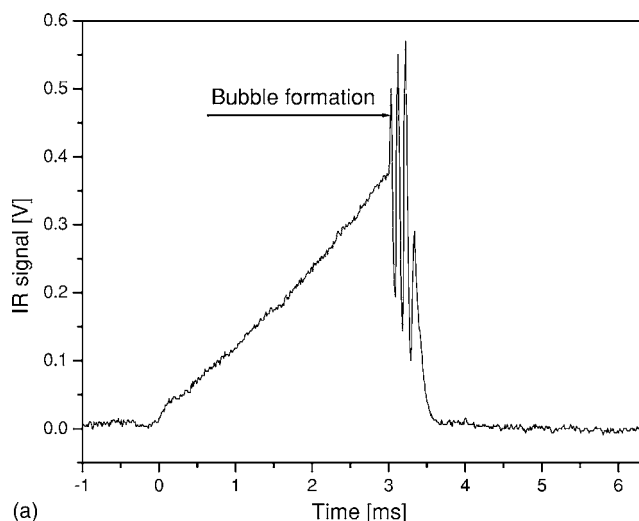


FIG. 4. (a) Infrared emission signal from the target. Moment of explosive vaporization is marked by an abrupt change in surface emissivity and appearance of a signal from the  $\text{CO}_2$  laser radiation scattered on an oscillating bubble. (b) Threshold of explosive vaporization of water adjacent to Mylar film measured by IR detection system as a function of laser pulse duration. For short laser pulses thermal emission measurements give underrated values of temperature due to optical thinness of the hot surface layer.

temperature of explosive vaporization measured by thermal emission reached a maximum at about  $220^\circ\text{C}$  and remained constant down to approximately 2 ms. For pulses shorter than 2 ms thermal emission at vaporization threshold started to decrease, providing an apparent temperature that is less than the limiting  $220^\circ\text{C}$  value, which should be constant in the microsecond to nanosecond domain. This additional calibration provided us with a correction factor for short pulse temperature measurements, which was found to be about 6% for 1 ms laser pulses and 12% for  $300\ \mu\text{s}$  pulses.

### III. EXPERIMENTAL RESULTS AND ANALYSIS

We measured survival of the NIH 3T3 cells under transient heating using laser pulses ranging from



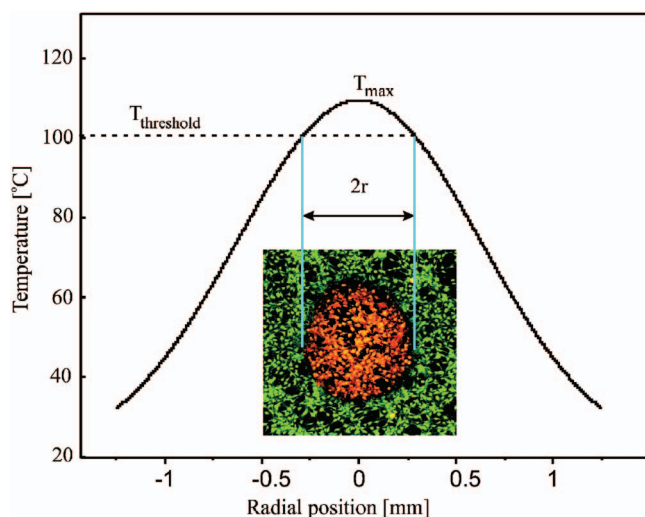


FIG. 5. (Color) Microscope image of the stained cells after irradiation showing live (green) and dead (red) zones within the laser-irradiated spot. Temperature corresponding to the boundary between live and dead cells was defined as a threshold temperature.

300  $\mu$ s to 100 ms in duration. For each pulse duration the measurement was repeated ten times using two sample containers with five irradiation spots in each. All samples were stained with the live-dead fluorescent assay within 5 min after the irradiation and examined under the microscope 20 min later, when fluorescence reaches its maximum level. Red and green fluorescent images (corresponding to dead and live cells, respectively) of the same irradiated area were recorded with a digital camera and then combined using image editing software. A typical composite image, showing zones of live (green) and dead (red) cells, is shown in Fig. 5.

The staining procedure employed in these experiments provides information about the distribution of live and dead cells at the moment of staining. To determine whether cells that survive immediately after irradiation might die later, we made a series of measurements in which cells were irradiated with a laser pulse of 30 ms duration and stained at 2, 10, 30, and 60 min after the pulse. No significant difference in the threshold temperature was found in these measurements.

As explained above, the radial temperature profile in the monolayer of cells was Gaussian, providing heat pulses of different amplitude at different radial locations within the spot. To maximize the precision of the temperature measurement at the live-dead boundary we adjusted the laser intensity for each pulse length so that the dead zone was significantly smaller than the laser spot size. Only shots creating a dead zone smaller than half the laser spot diameter were selected for further analysis. The threshold temperatures (i.e., temperatures at the red-green boundary) are plotted in Fig. 6 as a function of laser pulse duration.

Our cellular viability data extending from 0.3 to 100 ms shows no structure; within experimental error the temperature rises smoothly as the laser pulse duration decreases. This behavior prompted us to attempt a self-consistent analysis of this data based on the Arrhenius law. For such a description one might assume (i) the existence of a critical component of cellular metabolism with lowest heat tolerance, (ii) a descrip-

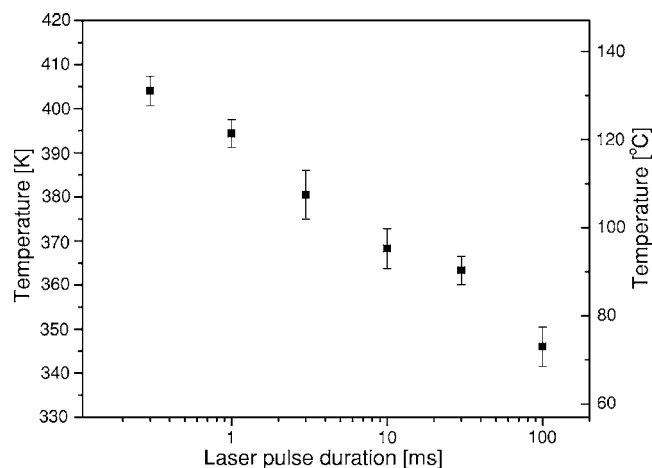


FIG. 6. Temperatures at the end of a laser pulse corresponding to the live-dead transition region.

tion of its denaturation in terms of chemical reaction theory, and (iii) providing the time scale is short enough, cellular repair during the exposure can be ignored. In this approximation one expects that cells will tolerate the same total amount of damage independent of heat pulse length, where damage is defined as a decrease of the critical component concentration. This total damage can be calculated by integrating the temperature-dependent damage rate during the heat pulse. In the Arrhenius approximation a decrease in concentration of a chemical component  $D$  can be expressed as

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

where  $D(t)$  is the current concentration,  $A$  is the reaction rate constant,  $E^*$  the reaction activation energy,  $R$  the universal gas constant, and  $T(t)$  the current value of absolute temperature. The criterion for cell viability can then be determined as a maximum tolerable decrease in concentration of the critical chemical component ( $D_{thresh}/D_0$ ), which can then be written as follows:

$$\int_0^\tau A \exp\left(-\frac{E^*}{RT(t)}\right) dt = -\ln\left(\frac{D_{threshold}}{D_0}\right), \quad (2)$$

where  $\tau$  is the duration of the thermal pulse. While the exact value of the damage integral  $\ln(D_{threshold}/D_0)$  corresponding to cell death is not known, it is usually assumed to be equal to  $-1$  [13], which means that the concentration of the critical component is reduced by a factor of  $e$ . There have been other reports claiming that cells will not survive if even 5-10% of a critical protein is denatured [1]. Selection of the value for the damage integral will affect the value of the rate constant  $A$  deduced from the experimental data, but it will not affect the value of  $E^*$ . Since this value is our primary interest in this study, and for the sake of simplicity, we will assume the damage integral to be equal to  $-1$ .

While laser pulses in our experiment had a nearly rectangular shape, the sample temperature increases monotonically throughout the laser pulse length and then falls at a rate determined by thermal diffusion from the heated layer. Tem-

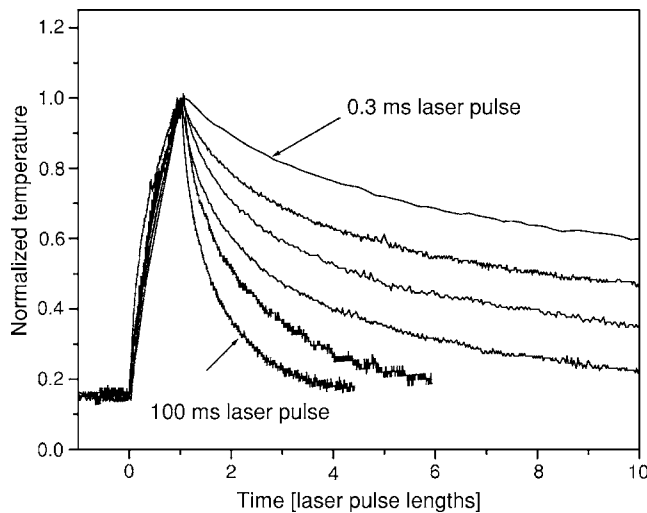


FIG. 7. Temporal profiles of the sample surface temperature corresponding to laser pulses of 0.3, 1, 3, 10, 30, and 100 ms. Time scale is normalized to laser pulse length. Shortest normalized temperature relaxation time corresponds to 100 ms laser pulse and longest one to 0.3 ms pulse.

poral profiles of the sample temperature  $T(t)$  were recorded with a calibrated IR detector for each laser pulse duration. Temperature profiles normalized to laser pulse duration are shown in Fig. 7. It is clearly seen that thermal diffusion rather than laser pulse length sets a limit in generating short thermal pulses.

Using the experimentally measured time-dependent values of temperature,  $T(t)$ , one can calculate damage integrals (2) for each given laser pulse length, treating  $E^*$  as a time independent constant. The value of  $E^*$  is then determined by assuming that the damage integrals are equal for all pulse lengths. By varying  $E^*$  and using the least squares method, we obtained a value of  $E^* = 96 \pm 7$  kJ/mol, or in terms of energy per single molecule expressed in electronvolts  $\varepsilon^* = E^*/N_A (1.6 \times 10^{-19}) = 1 \pm 0.08$  eV, where  $N_A$  is Avogadro's number. The rate constant  $A$  was found to be  $(1.5 \pm 3.6) \times 10^{16} \text{ s}^{-1}$ . As already mentioned, this parameter is rather arbitrary because it is affected by selection of the value of the damage integral in Eq. (2).

To compare our results with the data on viability of cells exposed to long isothermal pulses, we introduce a new term that we call the “effective time of thermal exposure,”  $t_{eff}$ . It is defined as the length of an imaginary rectangular heat pulse of a constant temperature  $T_{max}$ , which produces the same amount of damage as the actual heat pulse with peak temperature  $T_{max}$ .

$$t_{eff} = \frac{\int_0^\tau \exp\left(-\frac{E^*}{RT(t)}\right) dt}{\exp\left(-\frac{E^*}{RT_{max}}\right)}.$$

Using the already determined values of  $E^*$  we calculated effective times for all laser pulse lengths and plotted them in

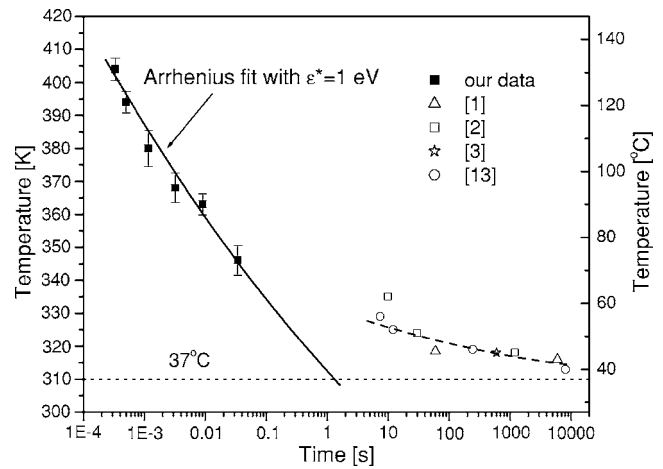


FIG. 8. Cell viability threshold as a function of “effective time” (solid black squares) fitted with the Arrhenius law (solid line). Open symbols—experimental data of cell viability thresholds from various sources with dashed line showing general trend.

Fig. 8. The Arrhenius graph, calculated as  $T = E^*/R \ln(A\tau)$ , fits the experimental points remarkably well.

#### IV. DISCUSSION

It is not clear whether the Arrhenius representation of our cellular viability data is simply a convenient fitting function or whether it has some deeper significance. In either case the data parameterization it provides is very useful. It is surprising, even for short pulse durations, that a complex biological process such as cellular death could be described as a chemical reaction proceeding at a rate expressed by the Arrhenius law. Dynamics of protein denaturation at short pulse durations and high temperatures, similar to the ones used in our experiment, was studied recently using a light scattering technique [6]. The authors established that kinetics of protein denaturation in a porcine cornea can be described by the Arrhenius equation with an activation energy of 106 kJ/mol (=1.1 eV). This value agrees remarkably well with our 1.0 eV activation energy for cell viability.

Despite this interesting agreement there are serious issues raised by a strict interpretation of the data in terms of the Arrhenius law. First, the two experiments also determine absolute rates of the corresponding processes—cell death and protein denaturation. In comparing these rates one must conclude that denaturation of only 1% of the cellular proteins leads to cell death. We find this value surprisingly low, since there are indications that cells can tolerate significantly more severe damage [1]. Second, if our cell viability data is extrapolated to longer exposure times (and thus to lower viability temperatures), the curve intersects 37 °C at an exposure time of less than 1 s. The Arrhenius law would tell us that in this time the cell has accumulated enough damage that cell death should occur. That clearly is not the case. Cells tolerate hyperthermia for exposure times that become arbitrarily long as the temperature is decreased toward physiological levels (37–40 °C).

Cell death may not require extensive irreversible denaturation. Exposure to high temperatures, even for short times, may disable normal protein function, inducing rapid changes in cells. For example, cross-membrane ion pumping can have a time constant as short as several milliseconds, and even a transient loss of control over the ion concentration in the cytoplasm may lead to lethal outcome.

Measurements of cell viability at long exposure times, from tens of seconds to minutes [1–3,13], are shown in Fig. 8, together with our short exposure data. Use of the Arrhenius expression as a fitting function in this range leads to activation energies in the range of 5–8 eV. Dramatic change in the slope of threshold temperature vs duration of exposure can be related to either cellular repair mechanisms taking place at multisecond durations, or to a higher activation energy of protein denaturation at lower temperatures.

Measurements of cellular viability at short pulse durations are more likely to provide an insight into the primary processes of thermal damage while avoiding the complexity of secondary mechanisms associated with cellular repair that might play a significant role at longer exposure times. We hope that this new data will help elucidate these processes.

## V. CONCLUSIONS

We have studied the viability of mammalian fibroblast cells, NIH 3T3, exposed to heat shocks with durations in the range from 0.3 to 100 ms. We found that the threshold temperature leading to cellular death rises with decreasing pulse duration, and for pulses of 300  $\mu$ s it reaches 130 °C, thus exceeding the water boiling point at normal conditions. Dependence of the threshold temperature on duration of hyperthermia can be described by the Arrhenius law, assuming that cells can tolerate a constant cumulative amount of damage produced during short exposures. The activation energy was found to be  $\varepsilon^* = 1 \pm 0.08$  eV. At pulse durations longer than a second, cellular tolerance deviates from such an Arrhenius behavior.

## ACKNOWLEDGMENTS

The authors would like to thank Philip Huie for his help with cell cultures. This work was funded by the U.S. Air Force Office of Scientific Research (AFOSR) (contract FA9550-04-01-0075).

- 
- [1] J. R. Lepock, *Int. J. Hyperthermia* **19**, 252 (2003).
  - [2] C. E. O'Connell-Rodwell, D. Shriver, and D. M. Simanovskii *et al.*, *FASEB J.* **18**, 264 (2004).
  - [3] X. M. He and J. C. Bischof, *Ann. Biomed. Eng.* **33**, 502 (2005).
  - [4] A. J. Welch, *IEEE J. Quantum Electron.* **20**, 1471 (1984).
  - [5] G. Huettmann and R. Birngruber, *IEEE J. Quantum Electron.* **5**, 954 (1999).
  - [6] J. Kampmeier, B. Radt, and R. Birngruber *et al.*, *Cornea* **19**, 355 (2000).
  - [7] G. Huttmann, B. Radt, J. Serbin *et al.*, *Proc. SPIE* **5142**, 88 (2003).
  - [8] C. Framme, G. Schuele, and J. Roider *et al.*, *Ophthalmic Surg. Lasers* **33**, 400 (2002).
  - [9] A. Vogel and V. Venugopalan, *Chem. Rev. (Washington, D.C.)* **103**, 577 (2003).
  - [10] F. H. Johnson, H. Eyring, and B. J. Stover, *The Theory of Rate Processes in Biology and Medicine* (Wiley, New York, 1974).
  - [11] R. Birngruber, *Springer Ser. Opt. Sci.* **1980**, 22.
  - [12] N. T. Wright, *Trans. ASME, Ser. K* **125**, 300 (2003).
  - [13] S. Bhowmick, J. E. Coad, D. J. Swanlund *et al.*, *Int. J. Hyperthermia* **20**, 73 (2004).
  - [14] T. Q. Lian, B. Locke, Y. Kholodenko *et al.*, *J. Phys. Chem.* **98**, 11648 (1994).
  - [15] V. P. Skripov, *Metastable Liquids* (John Wiley, New York, 1973).
  - [16] O. C. Thomas, R. E. Cavicchi, and M. J. Tarlov, *Langmuir* **19**, 6168 (2003).