Photothermal responses of individual cells

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Abstract. Photothermal (PT) responses of individual intact cells are studied with a thermal lens dual-laser scheme. A multiparameter model for analysis of PT responses as a function of cell size, structure, and optical properties is suggested and verified experimentally for living cells, red blood cells, lymphocytes, tumor cells (K 562), hepatocytes, and miocytes, by applying pulsed laser radiation at 532 nm for 10-ns duration. PT responses for noninvasive and damaging modes of laser-cell interaction are investigated. It is shown theoretically and experimentally that specific optical and structural features of cells influence the polarity, shape, front, and tail lengths of their PT responses. Common for different cells, features of PT responses are evaluated. It is found that in cells with a highly heterogeneous light-absorbing structure, the PT response of a whole cell differs from that of the local absorbing area. The model suggested allows us to interpret PT responses from single cells and to compare cells in terms of their diameter, degree of spatial heterogeneity of light absorbance, and laser-induced damage thresholds. © *2005 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.1854685]

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1 Introduction

Calorimetric methods (mainly photoacoustic and photothermal) are intensively used in different biomedical applications.[1–5] These methods are based on measuring thermal effects through acoustical, optical, and mechanical phenomena.[6–13] However, little progress was made in applications of calorimetric methods for studying individual living cells. This situation is caused mainly by the lack of techniques and equipment for sensing photoinduced thermal effects in individual cells. The difference of the cell as an optical object from solutions and other homogeneous samples may be described as follows.

- Cell size (micrometer range), geometry of sample chambers [closed volume with solid (glass as a rule) boarders], and duration of most available laser sources (nanosecond to microsecond range) make preferable optical registration of laser-induced thermal phenomena.

- Heterogeneity of the cell structure may lead to extremely nonuniform light absorption and, therefore, nonuniform initial laser-induced heating with consequent redistribution of thermal field, with temporal kinetics of such a field being different from that in homogeneous media.

- Local overheating may cause damage to the cell through vaporization and bubble formation,[14–17] even when cell-averaged thermal effects are well below the damage level.

Realistic analysis of laser-induced thermal phenomena in individual cells is limited due to absence of experimental methods for direct probing temperature with high temporal and spatial resolution. Macrolevel samples are usually investigated during the studies of the processes that occur in individual cells.[18,19] In a theoretical approach, the main problem appears to be the lack of quantitative data on optical and thermal parameters of cellular structural elements that absorb light and on their distribution within specific cells. Up to now, such parameters were determined mainly as averaged values for whole organs at the macrolevel.[20] Current models of photothermal (PT) phenomena consider, as a rule, homogeneous samples,[21,22] and even when assuming some heterogeneity[23] they do not count cell-specific optical and structural features.

Among laser-induced thermal phenomena, damage is the most crucial. Cell damage phenomena were studied better,[12,13] probably due to the significance of the subject and also due to the fact that damage is easier to detect in comparison with noninvasive heating. Damage is associated mainly with bubble formation due to overheating of local light-absorbing targets above critical temperatures[16,17,24] or by rarefaction waves[25,26] that may occur after absorption of a laser pulse. All laser-induced phenomena can be detected by photoacoustic and photothermal methods capable of registering such signals and interpreting them in terms of specific phenomena. Applied to the studies of individual cells, this task may be referred to as cytometric. Recently, several attempts were undertaken to apply calorimetric methods to the investigation of an individual cell.[27–29] As a result, the concept of PT cytometry was introduced.[27,30] This concept suggested an analysis of

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Table 1 Cell and laser parameters of the model for laser-cell interaction for three types of samples.

Model parameter	Symbol and unit	Hb solution	RBC (red blood cell)	K562 (tumor cell)
Radius of cell	R_c, μm	r_{pump}	3.5 to 4	5 to 10
Radius of absorbing area	r_a, μm	r_{pump}	3.5 to 4	0.5
Number of absorbing areas	N	1	1	9
Light absorption coefficient at pump laser wavelength (532 nm)	α, cm^{-1}	3	135	0.8 to 6.6
Characteristic radius of displacement of absorbing areas from cell center	c_a, μm	0	0	0 to R_c
Initial laser-induced temperature rise	ΔT, K	0 to 10	0 to 300	0 to 300
Difference between thermal coefficients for refractive indexes of solution and cell	$\Delta\beta_c$, 1/°C	0	0 to $2.5 \cdot 10^{-4}$	0 to $2.5 \cdot 10^{-4}$
Difference between refractive indexes of solution and cell	Δn_c	0	0 to 0.4	0 to 0.4
Radius of probe beam	r_{pr}, μm	4 to 8	4 to 8	5 to 12

living nonstained cells through optical monitoring of cell reaction (in the form of PT images or PT response) to a laser pulse. All the works mentioned were mainly experimental and their results showed a great difference in signals from cells and those obtained from homogeneous samples.

Also, a correlation between PT effects and cell physiological properties was found, such as respiratory chain activity, ion balance, oxygen content, cell death processes, and drug influence.[28-32] In the nearest future, we expect the emerging of a new type of microscopy and cytometry that will be based on photothermal analysis of intact cells. Therefore, we have tried to analyze principal features of photothermal responses of cells and to suggest the model that would allow estimating such PT responses as a function of cell and laser beam properties and of thermal phenomena type such as heating-cooling or bubble generation.

2 Methods and Materials

2.1 Photothermal Model for Laser-Cell Interaction

We consider three main processes: 1. irradiation of the cell with a pump laser beam and its absorption by cellular chromophores; 2. nonradiative heat release in absorbing areas and related thermal processes; 3. thermally induced changes of cell optical properties and of a second (probe) light beam that is used for sensing a thermal field in a cell.

A cell is a multicomponent system. The composition and structure of the cells may differ significantly even if they belong to the same culture. In ultraviolet and visible regions, light is most efficiently absorbed by hem-proteins (hemoglobin, cytochromes, catalasa, peroxidasa).[33,34] Intracellular concentration and spatial distribution of these absorbers may be very heterogeneous for most of the cells. The light absorption coefficient of local absorbers can be 2 to 3 orders of magnitude higher than the spatially averaged absorption coefficient for a whole cell (Table 1). Such heterogeneity of optical scattering and absorption properties of cells was verified during several experiments that involved PT imaging of individual lymphocytes,[30] lymphoblasts,[35] and neutrophils:[32] numerous heated microareas with size close to 1 μm, which is about the diffraction limit for the microscope used, were discovered in cells. Those structures may have an actual size in the range of 10 to 1000 nm. The cell may be modeled as an object with several absorbing elements being located in a nonabsorbing sphere of radius R_c (Fig. 1). The size, amount, and location of these absorbers are expressed through several parameters (Table 1): r_a is the absorber radius, N is the number of absorbers, and c_a is the characteristic radius of absorbers offset from the cell center. Of course, real cells are not only spheres (for example, red cells have a disk shape and miocytes are rod shaped), but a sphere still seems to be a good approximation for general analysis. Laser-pulse parameters are fluence Φ and duration τ_{pump}. It is assumed that all absorbed optical energy is converted into heat.

2.1.1 Photoinduced thermal phenomena

After absorption of a laser pulse, nonradiative relaxation of optical energy occurs within 10^{-11} s.[36] This is approximately

Fig. 1 Model for laser-cell interaction: uniform laser beam is larger in diameter than spherical cell with local absorbing areas.

the time of initial heating of absorbing areas that act as heat sources as long as a laser pulse lasts. The next process is heat diffusion into surrounding media (cooling), with a characteristic time τ_d that depends mainly on the size r_a of a spherically symmetrical absorbing area:

$$\tau_d = 0.9011 \frac{r_a^2}{a}, \tag{1}$$

where a is the heat diffusion coefficient, and τ_d is defined as the time of temperature decrease by 10 times.

We have made several assumptions:

- $\alpha^{-1} \gg r_a$, $\alpha^{-1} > R_c$,
- $r_a \ll r_{pump}$, $r_a \ll r_{pr}$,
- some physical properties of light-absorbing zones are equal to those for water.

Comparing laser-pulse duration τ_{pump} to cooling time τ_d, we consider two modes: a short pulse mode ($\tau_{pump} \ll \tau_d$) when all thermal energy is immediately released within the absorbing area, and a quasistationary mode (long-pulse mode) when the laser-pulse width is much longer than cooling time τ_d ($\tau_{pump} \gg \tau_d$). These two modes have different dynamics of the photoinduced thermal field. Depending on the initially induced temperature, we also consider the linear mode of laser-tissue interaction when only heating and cooling processes occur (temperature is proportional to deposited laser energy), and the nonlinear mode, which assumes phase transitions when temperatures exceed the vaporization threshold (temperature is no longer proportional to deposited energy). In this mode, various photodamage mechanisms may develop, and they depend not only on energy of the pulse but also on its length. Due to the small size (10 to 1000 nm) of initial heat sources in the cell and the range of laser pulse duration (1 to 1000 ns), the build up of a significant pressure wave is not likely, because the pressure relaxation time for such local absorbers is much shorter than the laser pulse length. Therefore, further analysis includes evaluation of the thermal field only as the acoustic field relaxes within the cell during the time of about 1 ns.

Linear regime of light-cell interaction. This mode is analyzed with the heat-transfer equation for a laser-generated heat source:[37]

$$\frac{\partial T}{\partial t} = a \Delta T + \frac{I(t, \vec{x})}{\rho c}, \tag{2}$$

where $I(t,x)$ is the heat source intensity, ρ is the density of the absorbing zone, and c is the heat capacity of the absorbing zone.

For the short-pulse mode, we may describe the heat source intensity as:

$$I = \frac{\alpha(\vec{x}) \Phi \delta(t)}{\tau_{pump}}, \tag{3}$$

where $\delta(t)$ is the delta function, α is the light absorption spatial distribution in the absorbing zone, and Φ is the laser fluence.

For the long-pulse mode under the previous assumptions within the time of relaxation, the beam is taken to be constant in time, then the heat source intensity is described as:

$$I = \Phi \frac{\alpha(\vec{x})}{\tau_{pump}}. \tag{4}$$

Linear regime of light-cell interaction: the long-pulse mode. We assume also that inside the absorbing zone, light absorption is uniform and is equal to zero outside this zone:

$$\alpha(r) = \begin{cases} \alpha_0 & \text{for } r \leq r_a \\ 0 & \text{for } r > r_a \end{cases}, \tag{5}$$

where r is the distance between the center of the absorbing zone with the coordinates (x_i, y_i, z_i) and the point where temperature is analyzed (x, y, z):

$$r = [(x - x_i)^2 + (y - y_i)^2 + (z - z_i)^2]^{1/2}.$$

In this case the stationary heat-transfer equation may be expressed as:

$$\frac{1}{r^2} \cdot \frac{\partial}{\partial r} \cdot \left(r^2 \cdot \frac{\partial T}{\partial r} \right) + \frac{\alpha(r) \Phi}{k \tau_{pump}} = 0, \tag{6}$$

where k is the thermal conductivity. Spatial distribution of the resulting thermal field may be obtained by Eqs. (6) and (5) with the boundary conditions being $T(\infty) = T_\infty$, $T(0) < \infty$, and:

$$T(r) - T_\infty = \begin{cases} \dfrac{1}{2} \dfrac{\alpha_0 \Phi r_a^2}{k \tau_{pump}} \left(1 - \dfrac{r^2}{3 r_a^2} \right) & \text{for } r \leq r_a \\[4mm] \dfrac{1}{3} \dfrac{\alpha_0 \Phi}{k \tau_{pump}} \dfrac{r_a^3}{r} & \text{for } r > r_a \end{cases}. \tag{7}$$

Maximal temperature at the center of the absorbing zone may then be expressed as:

$$\Delta T_{max} = T(0) - T_\infty = \frac{1}{2} \frac{\alpha \Phi r_a^2}{k \tau_{pump}}. \tag{8}$$

Linear regime of light-cell interaction: the short-pulse mode. For this case we assume a spherically symmetric heat source with the light absorption coefficient distribution inside the absorbing zone being:

$$\alpha(r) = \alpha_0 \exp\left[-\left(\frac{r}{r_a}\right)^2\right], \tag{9}$$

where α_0 is the constant. In this case, the heat-transfer equation may be expressed as:

$$\frac{\partial T}{\partial t} = a \frac{1}{r^2} \cdot \frac{\partial}{\partial r} \cdot \left(r^2 \cdot \frac{\partial T}{\partial r}\right) + \frac{\alpha(r)\Phi\,\delta(t)}{\rho c\,\tau_{\text{pump}}}. \tag{10}$$

With solution for the infinite area:

$$T(r) = \frac{\alpha(r)\Phi}{\rho c\,\tau_{\text{pump}}\left(1 + \frac{4at}{r_a^2}\right)^{3/2}} \exp\left(-\frac{r^2}{4at + r_a^2}\right). \tag{11}$$

For several absorbing zones with total number N and arbitrary spatial positions, the resulting thermal field may be considered as superposition of the fields described by Eqs. (5), (10), and (11), because of the linearity of Eq. (10):

$$T(x,y,z) = \sum_{i=1}^{N} \frac{\alpha(r)\Phi}{\rho c\,\tau_{\text{pump}}\left(1 + \frac{4at}{r_a^2}\right)^{3/2}}$$

$$\times \exp\left[-\frac{(x-x_i)^2 + (y-y_i)^2 + (z-z_i)^2}{4at + r_a^2}\right]. \tag{12}$$

Nonlinear regime of light-cell interaction. The most universal thermal mechanism of cell damage relates to local vaporization and bubble formation. The bubbles may emerge when the photoinduced temperature reaches the critical value (this mechanism is referred to as local overheating). Also, the bubbles may be induced by rarefaction waves. We assume that light intensities are below the optical breakdown threshold, thus the two mechanisms are assumed. The probability of bubble generation and its dynamics greatly depend on the properties of initial heterogeneity around which a bubble emerges. They are difficult to be described for such an object as a cell; there can be numerous sources for heterogeneities—gas, membrane-cytoplasm border, etc. For that reason we do not consider the mechanisms of bubble generation but analyze its kinetics after emergence of the bubble under the assumption that the initial radius of a bubble has the size of heterogeneity R_0. During laser-cell interaction, any bubble, regardless of the mechanism of its generation, can be a source for cell damage. The bigger the bubble, the higher the probability of cell damage. We analyze the dynamics of a bubble radius $R(t)$; this parameter characterizes damage of the cell. The basic equation that describes the bubble behavior at the stage when pressure stress has already relaxed (i.e., at stages after first nanoseconds) is the Rayleigh-Plesset equation:[38–40]

$$R\frac{d^2R}{dt^2} + \frac{3}{2}\left(\frac{dR}{dt}\right)^2 = \frac{1}{\rho}\left(P_v(t) - P_\infty - \frac{2\sigma}{R} - \frac{4\eta}{R}\frac{dR}{dt}\right), \tag{13}$$

where σ is the surface tension coefficient, η is the viscosity, P_∞ is the outer pressure, and P_v is the inner pressure.

For short laser pulses, the most universal mechanism of bubble formation is vaporization of liquid with the initial vapor pressure being equal to critical pressure P_{v_0}.[41] For initial conditions such as:

$$\frac{dR}{dt} = 0, \quad R = R_0 \quad \text{at } t = 0, \tag{14}$$

Eq. (13) was solved numerically to obtain $R(t)$ under the assumption of adiabatic approximation of bubble expansion:

$$P_v(t) = P_{v0} \cdot \left[\frac{R_0^3}{R(t)^3}\right]^\gamma, \tag{15}$$

where γ is the adiabatic coefficient, and P_{v0} is initial pressure in the bubble.

In the nonlinear mode, we use two basic input parameters: R_0 as the radius of heterogeneity that gives birth to the bubble, and P_{v0} as initial pressure that depends on absorbed optical energy. The output parameters that can be obtained numerically are bubble duration (lifetime) and maximal radius. Like temperatures, they cannot be measured directly and are analyzed later with the thermal lens approach through the parameters of a probe laser beam.

2.1.2 Optical detection of thermal phenomena in a cell

The phenomena considered before for linear and nonlinear modes of laser-cell interaction can be detected and measured by the optical method (thermal lens) as they influence another cell parameter, refractive index, which generally depends on temperature as:

$$\Delta n(x,y,z,t) = \beta \Delta T(x,y,z,t), \tag{16}$$

where $\beta = (dn/dT)_p$ is the thermal coefficient for the refractive index of solution and ΔT is the initial temperature rise.

Therefore, optical sensing of thermal processes in a cell can be done with an additional light beam by probing refractive index alterations in an individual cell. If a cell does not absorb the probe beam, heating of the cellular structures causes a phase shift in a probe-beam wavefront in the cell plane (Fig. 2):

$$\Delta\varphi(x,y,t) = \frac{2\pi}{\lambda_0}\int_{-\infty}^{\infty} \Delta n(x,y,z,t)\,dz$$

$$= \frac{2\pi}{\lambda_0}\int_{-\infty}^{\infty} \beta(x,y,z)\Delta T(x,y,z,t)\,dz, \tag{17}$$

where λ_0 is the probe-light wavelength, ΔT is described by Eq. (12), and

Fig. 2 Schemes for optical detection of photoinduced thermal phenomena in single cell: heated cell (left) and bubble (right) cases.

$$\beta(r)=\begin{cases}\beta_0 & \text{for } r\geq R_c \\ \beta_0+\Delta\beta_c & \text{for } r<R_c\end{cases},$$

where r is the radial coordinate with the origin at the center of the cell and another optical parameter for the cell $\Delta\beta_c$ is the difference between the thermal coefficients for the refraction indices of the cell and solution due to the differences in their composition (Table 1).

In our model we consider the difference between the refraction indices of the solution and cell Δn_c as the model parameter (Table 1), which provides additional phase shift into the probe beam:

$$\Delta\varphi_c(x,y)=\frac{4\pi\Delta n_c}{\lambda_0}[R_c^2-(x^2+y^2)]^{1/2}. \quad (18)$$

In the case of a bubble, we may consider a spherical area with a low index of refraction, and Eq. (17) may be presented as:

$$\Delta\varphi(x,y,t)=\frac{4\pi\Delta n_0}{\lambda_0}[R(t)^2-(x^2+y^2)]^{1/2}, \quad (19)$$

where Δn_0 is the difference between the refraction indices for vapor and cell media.

The wave function $f(x,y,t)$ for the probe beam in the cell plane and after passing through it can be described in the linear mode as:

$$f(x,y,t)=\exp\left(-\frac{x^2+y^2}{r_{pr}^2}\right)$$
$$\times\exp\{i[\Delta\varphi_c(x,y)+\Delta\varphi(x,y,t)]\}, \quad (20)$$

and in the nonlinear (bubble) mode as:

$$f_b(x,y,t)=\exp\left(-\frac{x^2+y^2}{r_{pr}^2}\right)\exp\{i[\Delta\varphi_b(x-x_b,y-y_b)]\},$$
$$(21)$$

where x_b and y_b are bubble displacements off the probe beam axis.

Probe-beam detection is realized through the thermal lens scheme (Fig. 2): the probe beam passes through the object and the collecting lens, then through the axial pinhole with a dimensionless radius R_a (this is equal approximately to one third of the probe beam radius in the detector plane) and is registered with a photodetector. Axial power of probe beam $I(t)$ can be described through Fraunhofer diffraction:[42]

$$I(t)=C\int_{-R_a}^{R_a}\int_{-\sqrt{R_a^2-Y^2}}^{\sqrt{R_a^2-Y^2}}|F(X,Y,t)|^2dXdY, \quad (22)$$

where X and Y are the dimensionless coordinates in the detectors plane, x and y are the coordinates in the objects plane, C is the devise constant, and $F(X,Y,t)$ is the wave function of the probe beam in the detector plane:

$$F(X,Y,t)=\int_{-\infty}^{\infty}\int_{-\infty}^{\infty}f(x,y,t)$$
$$\times\exp\left[-\frac{2\pi i}{\lambda_0}(X\cdot x+Y\cdot y)\right]dxdy. \quad (23)$$

The optical signal that is derived from $I(t)$ and describes the photoinduced thermal field in the cell is defined as the photothermal response $S(t)$ of the cell to the pump laser pulse:

$$S(t)=\frac{I(t)-I(0)}{I(0)}, \quad (24)$$

where $I(0)$ is the baseline signal in the absence of the pump pulse.

The amplitude of PT response was obtained numerically as a function of time and other model parameters (total of nine) according to Eq. (24), where $I(t)$ was calculated by Eqs. (22) and (23). The wave function is calculated by Eq. (20) with account for Eqs. (18) and (17) (linear mode) and Eq. (21) (nonlinear mode). The 2-D integral Fourier transform in Eq. (23) was calculated by fast Fourier transform (MathCAD 2000, MathSoft Incorporated, Cambridge, MA) using a 512 $\times$512 grid. In the case of bubble, by virtue of the presence of a sharp boundary in the phase shift region on the bubble boundary, the response calculated by the discrete scheme has a fluctuation component due to digitization of the integral transform (discretization noise). This component was smoothed by multivariate polynomial regression.

The main purpose of the developed model is to analyze the influence of the cell and laser parameters on the experimentally detectable PT signal. The developed model is not intended for evaluation of absolute values of the temperature in space and time. Estimation of thermal fields in the cell requires the use of correct values of all constants. As these data are often unknown for specific cells, we have to use available data such as for water, for example. This approach does not allow calculating the thermal field correctly, but this is not required when the resulting value—PT signal—can be registered with proper temporal and spatial resolution. In this case, the cell parameters can be compared by analyzing their PT responses without preliminary determination of all thermal, optical, and physical properties of all possible cell components that contribute into the PT response. Therefore, a PT

model can be applied for quantitative analysis of individual cells even in cases when specific values of all physical constants are not available.

2.2 Experimental Setup

Next we describe the PT microscope that supports two modes of PT measurements for individual cells: integral PT response and time-resolved PT imaging. The experimental setup[43] is arranged on the basis of the upright optical microscope MBI-15 (LOMO, Russia). Pulsed pump pulse and cw probe beams were directed coaxially into a standard microscope condenser that was used for focusing laser beams on the sample plane. The parameters of the lasers were as follows:

- Pulsed pump beam (custom-made dye laser pumped with Nd-Yag pulsed laser, model 1340, Lotis TII, Belarus): wavelengths are 532 nm; pulse length is 10 ns; beam diameter at the level of 0.5 of maximum is 18 μm; and energy (fluence) is variable by the polarizing filter in the range 0.1 to 300 μJ (0.03 to 84 J/cm^2).
- cw probe beam (He-Ne laser, model LGN-224, Russia): 633 nm, 0.2 mW, and the beam diameter at sample plane at the level of 0.5 of maximum is 22 μm.

The output signal from the photodetector $I(t)$ was registered with a digital oscilloscope (computer board Bordo-210 from Diagnostic Systems, Belarus) with 10-ns temporal resolution and analyzed by a computer with a PT signal being calculated by Eq. (19).

2.3 Sample Preparation

Three types of samples were investigated: 1. homogeneous solution of cellular (red blood cell) content (hemoglobin), 2. red blood cells as cells with a nearly homogeneous content, and 3. several cell types that *a priori* are highly heterogeneous, these being tumor cells (blasts) K 562, lymphocytes, hepatocytes, and miocytes. These four types of cells strongly differ in size (from 9 μm for lymphocytes to 15 to 18 μm for hepatocytes), shape (spherical K 562, lymphocytes, hepatocytes, and rod-shaped miocytes), content of light absorbers (hepatocytes and miocytes are rich with cytochromes, while blasts and lymphocytes have not much light-absorbing components at 532 nm), and inner structure. Our aim was to compare integral PT responses from different cells.

Cell suspensions were prepared on the basis of native tissues by standard preparation techniques. The concentration of cells in a suspension was adjusted to be in the range of 1 to 10 mln cell/ml. Suspension in amount of 6 to 8 μl was placed into the chamber S-24737 (Molecular Probes, Oregon) and sealed with coverglass. Up to four of such chambers were fixed on the standard microscope slide. Due to natural sedimentation, the cells formed a monolayer of individual cells at the bottom of the slide with an average distance between them being at least several of their diameters. Each cell was positioned at the center of the laser beams using the microscope stage, and after that was irradiated by one pump pulse. All experiments were performed at room temperature.

3 Results and Discussion

3.1 Linear Mode

3.1.1 Theoretical estimation of laser-induced thermal effects

The model developed was applied to analysis of three different samples (Table 1): homogeneous solution of hemoglobin (Hb), red blood cell (RBC) (quasiuniform content of light absorber Hb inside the cell), and tumor cell K 562 (heterogeneous content of light absorber, cytochromes and other hemproteins, inside the cell).

RBC is different from a homogeneous solution of Hb because this substance in the cell is confined by a cell membrane. The disk-shaped cell has a diameter of about 7 to 8 μm. The tumor cell (K 562) is 12 to 20 μm in diameter and has various light-absorbing components of different size in the range 0.01 to 1 μm. For a tumor cell, the spatial location of absorbing areas was taken as spherically symmetric through displacement of six areas for ±30 deg off the coordinate (x,y) plane in sequential order. We have estimated initial temperatures for short- and long-pulse modes (Table 2). Cell-averaged and local temperatures induced by laser-pulse absorption were compared. The main feature of the thermal field in the short-pulse mode is great spatial and temporal heterogeneity (Table 2): initial local values of temperature may be rather high and differ from cell-averaged values. In comparison with the short-pulse mode, the long-pulse mode is characterized by a more spatially uniform thermal field (Table 2) with lower local and higher cell-averaged temperatures. The size of the initial photoinduced thermal field will also be larger than that for the short-pulse mode. The short-pulse mode is preferable when high temperatures localized within or near absorbing areas are desired. The long-pulse mode is safer for a cell because at the same laser energies the temperatures are lower and thus, risk for thermal damage is lower. As can be seen from Table 2, in the cell with a heterogeneous absorbing structure, local temperatures may exceed vaporization thresholds. Also, the difference between short- and long-pulse modes increases with cell heterogeneity. The highest contrast between cell-averaged and local temperatures was found for the short-pulse mode and the lowest for the long-pulse mode. Therefore, the shorter the laser pulse, the more the result of laser-cell interaction will depend on the properties of local absorbing components.

3.1.2 Analysis of PT responses from cells

PT responses were calculated for three samples with parameters as described in Table 1, and were obtained experimentally for the Hb solution and the following cells: RBC, K 562, lymphocytes, hepatocytes, and miocytes. The response calculated for a tumor cell (K 562) is shown in Fig. 3(a). The experimentally obtained PT response for the same cell is shown in Fig. 3(b). Both responses have positive polarities, rather long front (about 0.5 μs), and tail (about 20 μs). The PT response obtained for a red blood cell is given in Fig. 3(c). Its higher maximal amplitude reflects higher light absorption as compared to a blast cell. The shorter front (0.2 μs) is the result of a more homogeneous light-absorbing content of that cell (all cell volume is heated simultaneously), and a shorter tail length (8 μs) indicates both a smaller cell size (7 μm

Table 2 Laser-induced maximal temperatures and cooling times for cells and hemoglobin for linear mode of laser-cell interaction.

Sample		Average values		Local values	
		Short pulse $(\tau_{pump}=10^{-8}$ s$)$	Long pulse $(\tau_{pump}=10^{-3}$ s$)$	Short pulse	Long pulse
Hb solution, $\Phi=3$ J/cm^2	τ_d, s	10^{-4}		10^{-4}	
	ΔT, K	5 to 7	12 to 15	5 to 7	12 to 15
RBC, $\Phi=3$ J/cm^2	τ_d, s	10^{-5}		10^{-5}	
	ΔT, K	80 to 160	30 to 60	80 to 160	30 to 60
K 562, $\Phi=54$ J/cm^2	τ_d, s	10^{-5} to 10^{-4}		10^{-7}	
	ΔT, K	12 to 100	21 to 45	175 to 1461	294 to 630

compared to 12 μm for a tumor cell) and the fact that the RBC shape in not spherical but rather disk like. The latter factor shortens cooling time. PT responses were also obtained for other types of cells: hepatocytes, lymphocytes, and mio-cytes (not shown). Their shapes were very close to those presented in Figs. 3(b) and 3(c). PT response obtained for a homogeneous solution is presented in Fig. 3(d). Theoretical and experimental PT responses obtained for all considered cells

Fig. 3 PT responses of type 1 (linear mode) obtained numerically for the model of (a) K-562 cell and experimentally at 532 nm for: (b) K 562 cell, (c) red blood cell, and (d) hemoglobin solution. Y axis for experimental responses is given as a photodetector output signal in mV.

Table 3 Parameters of photothermal responses from intact cells.

Cell type	Maximal amplitude S, recalculated for pump energy 1 μJ at 532 nm	Front length, μs	Tail length, μs
Red blood cell (RBC)	0.8	0.1	6
Lymphocyte	0.0041	0.2 to 0.4	10
Hepatocyte	0.048	0.1 to 0.3	25
Miocyte	0.11	0.1 to 0.3	12
K 562 (blast cell)	0.0014	0.3 to 0.5	20
K 562, local area	0.1	0.02 to 0.04	0.6

revealed cell-specific feature: polarity of their PT response changes from negative [case of a homogenous sample, Fig. 3(d)] to positive. This means that any unexcited cell acts like a converging lens, it focuses the probe beam, thus creating an additional focal point between the collecting lens and the detector. The lengths of the tails of experimentally obtained PT responses (Table 3) are in agreement with the object size and with Eq. (1): the bigger the cell, the longer the tail of the response. Experimentally obtained cooling times are given in Table 3. Fronts of PT responses from cells are longer than those for homogeneous solutions (see Fig. 3).

To explore the nature of this effect, additional experiment with PT imaging of K 562 cells was undertaken. As a result, the temporal behavior of PT signals in local absorbing areas of those cells (Fig. 4) was registered experimentally. The amplitude of the PT image at the points inside the cells with a maximal signal was analyzed as a function of time after laser pulse. Temporal resolution of this measurement was 10 ns. According to PT images of the cells (not shown), the radius of the areas with a high PT signal was 0.3 to 0.6 μm. Based on the assumption that the radius of the absorbing area has an order of 0.5 μm, we calculated PT response from the local single absorbing area in the cell (Fig. 4). Both theoretical and experimental responses are similar and have a short front,

about 20 ns (Table 3). The more interesting result is that the length of a tail of local response (600 ns) is very close to the front length of an integral PT response from the whole cell [Table 3 and Figs. 3(a) and 3(b)]. Therefore, we may assume that for the cell with a local absorbing structure, an initial thermal field (with maximal temperatures) does not provide maximal amplitude of integral PT response of the cell. This response reaches its maximum after some time delay that corresponds to the time of cooling of local initial laser-induced thermal fields. As a result of thermal diffusion and interference of local thermal fields, the "secondary" thermal field emerges. It has a cell size, much lower temperature, and its influence on the probe beam is strong enough to create a maximal thermal lens effect. At that moment, the PT response from the whole cell reaches its maximum. Therefore, the front of the PT response from an individual cell contains information on both its averaged light absorbance and the absorbing structure. The longer the front of the PT response, the smaller the cellular light-absorbing zones. This assumption was also verified using our model by numerical analysis of the PT response front as a function of the absorbing area size (not shown).

All the results described were obtained under the assumption that no photodamage occurs. For all the experimentally studied cells, PT responses of this type were reproducible for at least 20 to 50 pump laser pulses. Thus, this mode of interaction may be considered noninvasive for living cells at least on a short time scale. Visual observation of irradiated cells showed neither any detectable signs of destruction, nor positive staining with Trepan Blue dye (the latter was added into the sample chamber as a standard indicator of cell damage). For these conditions we have also studied theoretically and experimentally the dependence of PT response on pump laser energy for each cell type. At low pump energies, the temperatures do not reach damage thresholds, and theoretical and experimental dependences of the amplitude S of the PT response on pulse energy were linear for all studied cells (not shown). Comparison of maximal amplitudes of PT responses obtained for different cell types (Table 3) showed that this parameter corresponds to light-absorbing properties of studied cells. Highest integral PT signals were detected for RBC due to high light absorption of hemoglobin. The lowest signals were detected for K 562 cells and for lymphocytes. Numerical

Fig. 4 PT responses from local absorbing areas in the K-562 cell: experimentally obtained at 532 nm using the PT imaging method with variable delays and numerically calculated.

analysis of the front width of the PT response revealed that for a homogeneous sample, this parameter is very short and does not depend on pump pulse energy, but for any cell front width, it increases with energy (not shown). The nature of this effect may be due to the local origin of heating in a cell: the smaller the absorbing areas, the longer it takes to form the thermal field in the whole cell and to reach the maximum of the PT response amplitude. Thus, the PT method in the linear mode provides at least three quantitative parameters that can be obtained experimentally for individual cells from their PT responses:

- the front length characterizes the cellular absorbing structure following the rule "the smaller the absorbing area, the longer the front"

- the maximal amplitude describes general light absorption and heat release in the whole cell

- the tail length depends on the cell size and shape.

All these parameters may be obtained experimentally for the individual intact cell without laser-induced damage to it. This mode makes possible the monitoring of individual cell properties during their interaction with the biological environment such as microbes, drugs, and other factors.

3.2 Nonlinear Mode

3.2.1 Numerical evaluation of the bubbles

Using the developed model, we have calculated the bubble dynamics for three samples. The short-pulse mode was used, as this mode assumes the highest local temperatures and the probability of bubble formation is much higher for the short-pulse mode rather than for the long-pulse mode. The mechanism of bubble generation was considered as overheating of a light-absorbing volume of radius R_0 and its transition into the vapor state at temperatures above critical. Initial conditions for three model samples were assumed as follows: R_0 for Hb solution was taken equal to the pump beam radius (6 μm); for RBC, R_0 was taken as a cell radius (3.2 μm); and for the K 562 cell, R_0 was taken as a radius of the absorbing zone (0.5 μm). The initial pressure was taken equal to critical for all three cases. This assumes approximately equal initial temperatures being about 300 K. The bubble radius dynamic is presented in Fig. 5 and the calculated curves show that the bigger the zone of initial vaporization, the longer the bubble lifetime and the larger its maximal radius. The smallest and shortest bubble (3 μm, 0.5 μs) is produced by the local absorbing zone in a K 562 cell, and the maximal bubble diameter is smaller than a cell diameter. For RBC, the bubble size (more than 20 μm and duration about of 5 μs) can exceed the cell diameter (7 μm) several times. This means that this bubble would rupture the cell membrane. For the case of overheating and vaporization of homogeneous media in the whole area of laser aperture, the biggest and longest bubble would be generated (up to 50 μm and 10 μs). The temporal behavior of the bubble radius is a quasisymmetrical process of growth and collapse. Also, the maximal radius R_{max} and duration of a bubble correlate to each other and linearly depend on its initial radius R_0 of inhomogeneity that gives birth to a bubble [see Fig. 6(a)]. The size and lifetime (duration) of the bubble

Fig. 5 Vapor bubble dynamics: numerical results calculated for various initial bubble radiuses.

may be considered as quantitative parameters of damage: when the bubble size is of the order of the cell size, the possibility of membrane rupture is very high. Also, through the experimental measurement of bubble lifetime, we can estimate R_0. This parameter according to our model estimation influences the bubble much stronger than, for example, initial laser-induced pressure [Fig. 6(a)], which may help to interpret the mechanisms of bubble generation.

3.2.2 Analysis of experimental photothermal responses from cells

PT responses obtained from the cells in the nonlinear mode greatly differ in shape from those obtained due to the heating-cooling process. We have followed (suggested earlier by us) the definitions of linear-mode responses as type 1 and nonlinear mode responses as type 2.[44] Type-2 responses are always negative and have a much higher maximal amplitude in comparison with type-1 responses. Experimental type-2 responses obtained for Hb solution and for cells (RBC, K 562) are given in Figs. 7(b), 7(c), and 7(d). Similarity of PT responses—theoretical that was predicted with the bubble model [Fig. 7(a)] and experimental [Fig. 7(b)]—means that the type-2 response is caused by the bubble. The front and tail of the type-2 response have almost the same lengths and can never be caused by the heating and cooling processes alone (the latter creates a long exponential tail). The amplitude of the type-2 response is too high to be caused only by temperature change; this amplitude indicates stronger gradients of the refractive index, which can be explained by the presence of an area with gas. The obtained responses of type 2 may superimpose on type-1 responses [Figs. 7(c) and 7(d)] because two processes—heating and bubble expansion—may coincide in time. Numerical estimation of the bubble maximal radius under the conditions of the experiment showed that it might be comparable or even exceed the cell radius (see Fig. 5). Thus, such a bubble would definitely damage the cell membrane. Independent measurements confirmed that the type-2 response is always accompanied by cell damage that results in either observable cell destruction or positive staining with an indicative dye such as Trypan Blue.[44] PT responses of type 2 were also obtained experimentally for lymphocytes, hepatocytes,

Fig. 6 Duration of a bubble as function of initial bubble radius as obtained numerically at (a) different initial pressure and obtained experimentally from (b) red blood cells at 532 nm at different laser fluence.

and miocytes, and their shapes and parameters were similar (not shown) to those presented in Fig. 7. This means that a type-2 PT response has a universal origin. According to our model, the generation of the bubble is possible around any heterogeneity that is heated above the critical temperature. Duration of the type-2 response corresponds to bubble life-time. According to Fig. 7, the following lifetimes were registered experimentally: K 562 was 1 μs, RBC was 0.5 μs, and the Hb homogeneous solution was 0.5 μs. For a tumor cell, the experimentally obtained bubble lifetime is in agreement with that predicted theoretically (see Fig. 5, the curve for K 562) and, therefore, the bubble can be caused by overheating

Fig. 7 PT responses of type 2 obtained numerically for (a) the bubble and experimentally at 532 nm for: (b) K 562 cell, (c) red blood cell, and (d) hemoglobin solution. Y axis for experimental responses is given as a photodetector output signal in mV.

of the local light-absorbing area with a size (1 μm) much smaller than the cell size (12 μm). But for RBC and Hb solution, the experimentally obtained bubble lifetimes were much shorter (about one order of magnitude) than those predicted theoretically and presented in Fig. 5. Short bubble lifetimes in these objects mean that: 1. the maximal radius of the bubble is also much smaller than theoretically predicted, about several micrometers, though it is sufficient for cell damage, as was observed for RBC; 2. a bubble emerges around the heterogeneity of the size that is much smaller than the cell size (for RBC) or than the size of the heated volume (for Hb solution) [see also Fig. 6(a) for the numerically calculated dependence of bubble lifetime on the initial size R_0].

If for the cell there can be various heterogeneities due to complex cell structure, for the case of homogeneous Hb solution small bubbles may indicate that several mechanisms of bubble generation are involved. To explore the nature of these phenomena, we have measured the bubble lifetime as a function of pump pulse fluence for RBC [Fig. 6(b)]. With an increase of pump pulse energy, the lifetime of the bubble increases. Increased energy leads to increase of initial temperatures in the light-absorbing zones. If the size of such a zone is 1 μm and smaller, the laser-pulse width (10 ns) becomes comparable to or even longer than the thermal relaxation time [Eq. (1)] for this zone. Therefore, the actual size of the heated area may exceed the size of the absorbing zone and depends on temperature: the higher the initial temperature, the larger the heated area at the moment of laser pulse termination. The size of the heated area must be considered as R_0 (bubble nuclei radius) if the whole area turns into vapor during laser pulse action. Such explanation of the experimental result is in agreement with the theoretical dependence of bubble lifetime on R_0 [Fig. 6(a)]. Another feature of experimentally obtained dependence [Fig. 6(b)] is the threshold: at pump laser energies below this threshold, no bubbles emerge. Cell-averaged temperatures that correspond to applied pulse energies were calculated using Eq. (8). These temperatures were found to be in the range from 60 to 160 K [Fig. 6(b)]. Such temperatures are cell averaged, but assuming uniform light absorption in RBC, they are much below the critical temperature for vaporization of water (300 K). The results obtained mean that the mechanism of bubble generation in the case of RBC and Hb solution is not only overheating and vaporization of whole irradiated volume. A possible additional explanation of bubble formation under the previously discussed conditions (low temperatures and small size of the bubble) may include action of rarefaction waves and cavitation around small heterogeneities. Such phenomena were studied for macrosamples[26,45] but never were reported for microsamples that are confined by solid borders (in our case, all samples are studied inside chambers with solid glass bottom and top). Up to now, the mechanisms of bubble generation in individual cells have not been studied adequately and are beyond the scope of this report. Regardless of the mechanisms of bubble generation, they can be detected in individual cells with our PT technique and can be analyzed with the suggested model. In the nonlinear mode, the PT response obtained from an individual cell provides the following information that relates to cell damage: PT response shape allows one to detect the bubble in an individual cell; and PT response duration characterizes bubble lifetime and hence its maximal size.

4 Conclusions

The obtained experimental PT responses are in agreement with those predicted by the developed model in linear and nonlinear modes. The only disagreement of the model and experiment relates to durations of type-2 responses that correspond to bubble lifetimes. Also, we show that type-1 (linear) PT responses depend on the cell integral light absorbance, structure, and size of the light-absorbing zones, but do not depend significantly on the cell shape (PT responses from rod-shaped miocytes were very similar to those obtained from a spherical cell). The photothermal model of laser-cell interaction, together with the developed PT microscope, provides new data on individual cells. In the linear mode, the front length of PT response characterizes the size of the cellular absorbing structure; maximal amplitude describes general absorption and heat release in the whole cell; and the tail length depends on the cell size. All cells studied change polarity of PT responses (type 1) from negative to positive due to their content and structure. If a cell consists of small light-absorbing zones, its PT response is formed by the secondary thermal field, which results from heat diffusion and interference of initial thermal fields. In the nonlinear mode, laser-induced bubbles produce distinctive PT responses of type 2, regardless of the features or type of cell where they emerge, with the length of the response indicating the bubble lifetime and size. When some exogenous absorbers are used (highly absorbing particles inside the cell—gold particles, liposomes, etc.), the effect of those absorbing agents can also be estimated with our model. Photothermal measurements were done by a standard optical microscope equipped with pump and probe lasers and laser beam detectors, which are technically easier compared to, for example, laser scanning microscopes, because no scanning is used and all measurements may be a one-step procedure without any cell pretreatment. The time scale of measured PT processes is from 1 ns to 10 μs, and therefore the described PT method can be realized also with current flow cytometers, allowing analysis rates of about 100,000 cells per second.

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