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BIOPHYSICS LETTER

# Single cell viability and impact of heating by laser absorption

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**Abstract** Optical traps such as tweezers and stretchers are widely used to probe the mechanical properties of cells. Beyond their large range of applications, the use of infrared laser light in optical traps causes significant heating effects in the cell. This study investigated the effect of laser-induced heating on cell viability. Common viability assays are not very sensitive to damages caused in short periods of time or are not practicable for single cell analysis. We used cell spreading, a vital ability of cells, as a new sensitive viability marker. The optical stretcher, a two beam laser trap, was used to simulate heat shocks that cells typically experience during measurements in optical traps. The results show that about 60% of the cells survived heat shocks without vital damage at temperatures of up to 58 ± 2°C for 0.5 s. By varying the duration of the heat shocks, it was shown that 60% of the cells stayed viable when exposed to 48 ± 2°C for 5 s.

**Keywords** Cell viability · Heat shock · Optical stretcher · Cell adhesion

Non-contact cell trapping and micromanipulation techniques using electrical, optical, or magnetic fields to induce forces on cells have been increasingly used in biophysics during the last two decades (Guck et al. 2001; Huang et al. 1993; Molloy and Padgett 2002; Winkleman et al. 2004). Many of these techniques cause a temperature increase in the cells as a side effect (Ebert et al. 2007; Jaeger et al. 2007; Peterman et al. 2003). Optical tweezers show a relatively low temperature increase of about 1.4–1.7 K/100 mW (when using the usual 1,064 nm laser) due to the low applied laser power (Kuo 1998; Liu et al. 1995). Dielectrophoresis techniques cause temperature increases up to 25 K depending on the applied voltage and the conductivity of the medium (Jaeger et al. 2007). In the microfluidic optical stretcher (MOS), a dual beam laser trap is coupled to a microfluidic device for single cell analysis. In the MOS the laminar flow is stopped, and subsequently cells are trapped and then stretched by increasing the laser power from 100 mW to stretching power of 0.5–1.5 W. For 1.0 W this results in a temperature increase of 23 ± 2 K (Fig. 1) after approximately 1 s, and the temperature after 0.5 s was measured to be 92% (21 K) of this maximum temperature. Heating is in a range of 2.3 ± 2 K for trapping and up to 34 ± 2 K for stretching experiments with a power of 1.5 W per fiber. A temperature close to equilibrium is reached within tens of milliseconds after switching on the laser. The temperature drop after the stretch also largely occurs within tens of milliseconds followed by a slower decay with the starting temperature reached after approximately 1 s. After stretching, the flow is established again and takes the cell away from the heated region.

The cell's viability can be affected by temperature changes that cause damage to the cytoskeleton (Morley et al. 1995; Rao and Cohen 1991; Straface et al. 2001) as well as changes in gene expression (Pirkkala et al. 2001). The impact of heat shocks on single cell viability has been barely investigated up to now. Most research done in this field has applied a temperature increase of 5–10 K at time scales of minutes and hours (Huang et al. 1999). In optical traps using infrared laser light, cells experience higher temperature increases on shorter time scales (Ebert et al.

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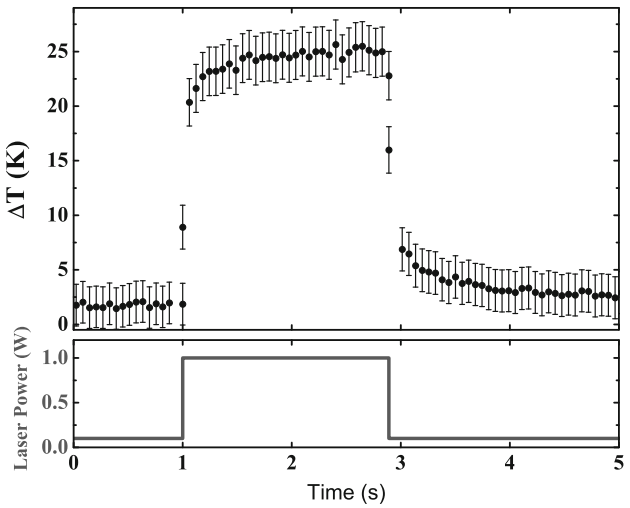


**Fig. 1** Temperature characteristics in MOS. When the laser is switched on, the temperature rises within tens of milliseconds. After approximately 1 s of laser application at constant power, a maximum value is reached. When the laser is switched off the temperature falls very quickly and reaches the ground state within 1 s. For 1 W of applied laser power per fiber, the temperature increase was determined to be 21 ± 2 K after 0.5 s, and the equilibrium value was 23 ± 2 K. *Error bars* indicate the uncertainty of imaging of the fluorescence signal and its temperature calibration. The *lower panel* shows the applied laser power [see Ebert et al. (2007) for method]

2007). Reliable assays determining the physical condition of cells after such treatments have to be fast yet highly sensitive to functional damage.

Proliferation and epigenetic analysis are the most accurate assays to investigate changes in viability. However, these methods are often not applicable since they are time-consuming (i.e., the fate of the cells has to be tracked in cell culture for several days) or require larger numbers of cells. Our aim was to find a highly sensitive test that can be applied to rare cells in a reasonable period of time. The existing gold standard, which insufficiently fulfills these requirements, is the vital stain, the most common of which, calcein acetoxymethylester (AM), served as a comparison to our newly developed viability assay using cell spreading as a viability marker. Cell spreading is a vital feature of nonmalignant cells since adherent cells become apoptotic in suspension. It requires an intact cytoskeleton as well as working complex signalling pathways. When a cell spreads, actin filaments are reassembled at the cell's edge. This mechanically spreads the plasma membrane against the membrane tension (Dubin-Thaler et al. 2004). The cell has to be in a highly vital state to perform such an intricate action, which suggests that cell spreading is a marker of viability, as it is highly sensitive to immediate damages leading to necrosis as well as functional damage inducing apoptosis (Chen et al. 1997). In our experiments the ability of a cell to spread in a certain time frame was therefore correlated to the cell's viability.

NIH/3T3, a Swiss mouse embryonic fibroblast cell line purchased from the American Type Culture Collection (ATCC) and cultured according to the provided protocol was used for the experiments. In preparation for each measurement, cells were detached from the culture flask using 0.05% Trypsin-EDTA (PAA) and resuspended in medium after centrifugation. For fluorescence stain, cells were incubated with 5 μM calcein AM (Invitrogen) for 20 min at 37°C. Calcein AM is membrane-permeable and hydrolyzed by endogenous esterases into the green fluorescent calcein ($\lambda_{ex}$ = 485 nm, $\lambda_{em}$ = 530 nm) that in turn is retained in the cytoplasm of living cells (Wang et al. 1993). After incubation, cells were washed with PBS and prepared for the measurements as described above.

The suspended cells were injected into the fluidic system of the MOS, a further development to the optical stretcher, which uses a glass capillary chip combined with a microfluidic device for better throughput and reproducible measurement conditions (Lincoln et al. 2007). Two opposing laser fibers originating from a 10 W single mode yttrium fiber laser (YLD-10-1064, IPG Photonics Germany) are aligned perpendicular to a square glass capillary. They emit divergent infrared laser beams forming an optical trap in the center of the channel. Single cells are transported through the channel by laminar flow into the trap. After the flow is stopped the cell is trapped and subsequently stretched by increasing the laser power. Ambient temperature was set to 37°C to keep cells as close to culture conditions as possible.

After injecting the cells into the microfluidic system, single cells were trapped and exposed to laser-induced heating with laser power ranging from 0.5 to 1.5 W. For an exposure time of 0.5 s, this results in temperatures of 47 ± 2 to 68 ± 2°C, which are almost the final temperatures of 48 ± 2 to 71 ± 2°C for 1–10 s. Subsequently cell viability was measured by an established viability assay—the vital fluorescence stain calcein AM—and a newly developed method based on cell spreading. Fluorescence images were taken with a silicon intensified target (SIT) camera (VE-1000-SIT, DAGE-MTI, USA) right after the heat shock. For cell spreading experiments, the laser was entirely switched off. The cell sank down to the ground of the glass capillary and was observed for 15 min. Phase contrast images were taken every minute. Control cells were treated the same way without trapping and subsequent heat shock.

Cells mainly consist of water (70–85% of mass) and proteins (10–20% of mass) (Loeffler and Petrides 1998). Proteins do not absorb radiation at near infrared, but water does. Since an infrared laser beam ($\lambda$ = 1,064 nm) is used in the MOS, cells experience an increase in temperature depending on the absorbed laser power. These thermal effects can be characterized experimentally by using a combination of temperature sensitive and insensitive

fluophores (Ebert et al. 2007), but they can also be verified theoretically. The maximum temperature reached at equilibrium in the MOS can be described by the Poisson equation $\Delta^2 T = -\frac{1}{k} S$ (Ebert et al. 2007), where T is the temperature distribution, k is the thermal conductivity, and $S$ the thermal source distribution given by $S = \alpha I$, with I being the optical irradiance distribution and angle $\alpha$. Heating occurs basically in a narrow region along the laser profile inside the capillary. Applying a laser power of 1 W per fiber, a temperature rise of 23 ± 2 K in the center of the dual beam trap was obtained (Fig. 1) measured by the method proposed in Ebert et al. This study concentrated on the laser absorption of water. There might be other absorption effects of significance inside the cell that Ebert et al. did not investigate.

Cells were trapped in the MOS with 1.5 W per fiber for 0.5 s, which results in a temperature increase of 31 ± 2 K to a total temperature of 68 ± 2°C. The fluorescence intensity of the vital dye, before and after heating, was evaluated as the integral of gray scale value over the cell area. Comparing the images of the cell before and after the heat shock yields the value for the loss of fluorescence intensity. Figure 2 illustrates the intensity distribution in one dimension.

In order to identify the loss of fluorescence intensity most accurately, the overall loss of intensity was analyzed. After exposing the cell to 68 ± 2°C (1.5 W laser power per fiber) for 0.5 s, an average loss of fluorescence intensity of about 15% was obtained.

Cells were exposed to the HBO light only during snapshots. Nevertheless, the fluorescence dye showed bleaching of 10–20% after imaging. Thus, the loss of fluorescence intensity measured after the heat shock is insignificant being in the range of bleaching. It appears to be unlikely that cells can be exposed to 68 ± 2°C without any loss of viability. The vital dye is not sensitive enough to determine changes in viability on time scales of seconds. Thus, cell spreading was evaluated as a new cell viability test.

Figure 3a shows an example of cell spreading. Very characteristic are the fimbriate membrane extensions and the ribs of actin within the lamellipodium (see also Dubin-Thaler et al. 2004). In contrast to cell spreading, non-spread cells show a sharp edge and membrane blebbing (see Fig. 3b). These cells are only weakly attached to the surface and can be detached easily by introducing a small flow.

Seventy-five percent of all measured cells spread within 9 min (see Fig. 4). Only a very few cells treated with severe conditions (i.e., cells exposed to more than 68 ± 2°C for 0.5 s or exposed to 48 ± 2°C for 10 s) were observed to spread between 11 and 15 min. We conclude that highly vital cells have spreading times below 10 min. For the measurements, cells were only counted as viable if they adhered within 10 min (checked by introducing a small flow of medium) and showed clear spreading after a maximum of 15 min. Only cells with no obvious damage (poor contrast, membrane blebbing) prior to trapping were chosen for experiments. One hundred percent of the controls, measured at 37°C, showed a clear spread during the preset time frame.

Two kinds of measurements were performed: one varying the temperature increase by changing the applied laser power and the other varying the duration of the applied heat shock.

First, cells were exposed to a heat shock for 0.5 s. The temperature increase during the heat shock was varied from 10 ± 2 to 31 ± 2 K (0.5–1.5 W per fiber), which corresponds to absolute temperatures of 47–68°C. The critical temperature, i.e., the threshold at which about 60% of the cells stayed viable during heat shocks was found to be about 58 ± 2°C (see Fig. 5a). When exposed to 68°C for

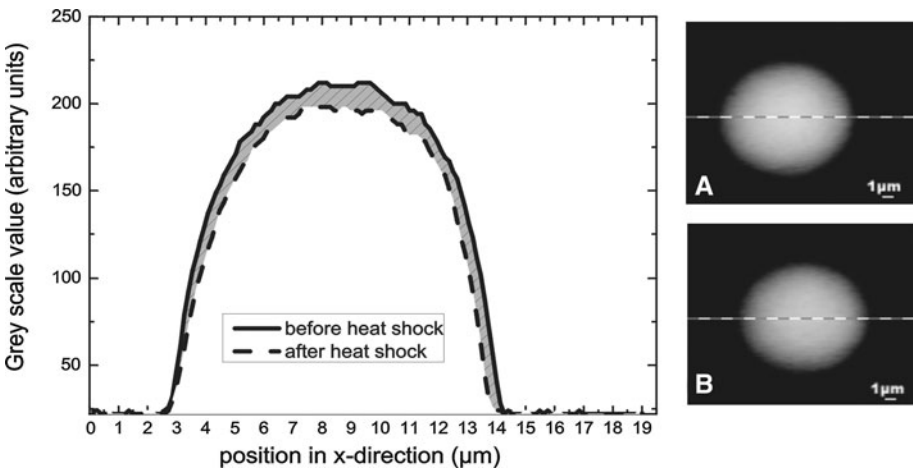


**Fig. 2** Calcein AM fluorescence intensity as standard viability marker. The graph illustrates the intensity distribution along the laser axis. *Gray area* marks loss of fluorescence intensity (analysis in one dimension) after heat shock. Background was set to zero. The pictures show the fluorescence image of the cell before (**a**) and after (**b**) heat shock exposure. A loss of intensity is visible. Photo bleaching was not taken into account

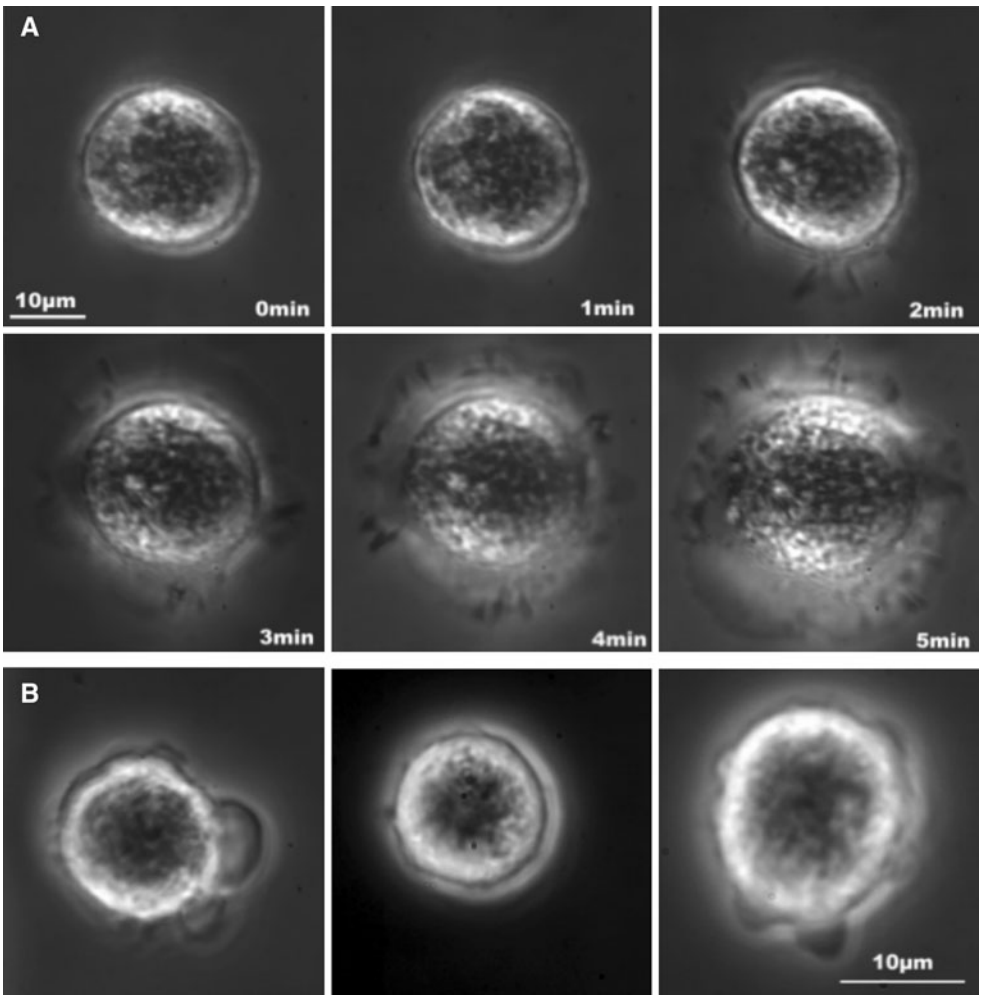


**Fig. 3a, b** Cell spreading. **a** Within 5 min the cell shows a clear random spread. Fimbriate membrane extensions and ribs of actin within the lamellipodium are prominent features of spread cells. **b** Three examples of non-spread cells. Characteristic are the sharp edge and membrane blebbing

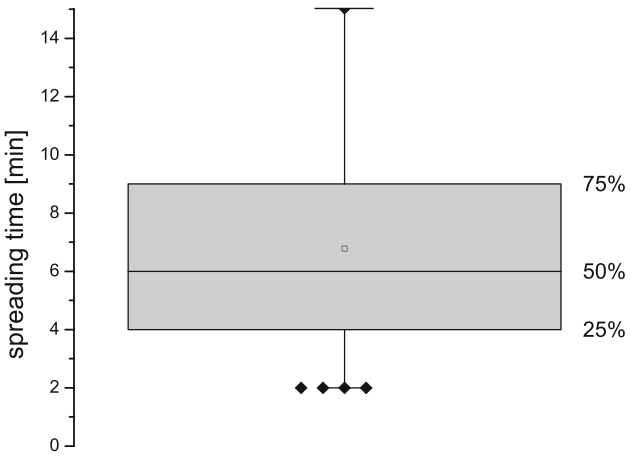


**Fig. 4** Spreading time of treated and untreated cells. Seventy-five percent of all measured cells spread within 9 min. *Black diamonds* mark outliers

0.5 s, only 27% of the cells stayed viable. This is in contradiction to the results from the fluorescence measurements, where an insignificant loss of fluorescence intensity in the range of bleaching suggested that all cells stayed viable.

The impact of the duration of a heat shock around 48 ± 2°C was investigated, varying the exposure time from 0.5 to 10 s. There was almost no loss in viability at heat shocks up to 1.5 s. After a 5 s heat shock still about 60% of the cells stayed viable (see Fig. 5b).

The influence of optical trapping was determined by holding cells at trap power of 100 mW for 60 s, which causes a temperature increase of 2.3 ± 2 K to a total of 39°C. Fourteen of the measured 15 cells stayed viable according to the spread test. Usual trapping times of MOS elasticity measurements are in the range of 3–10 s, therefore the influence of trapping on cell viability is minimal.

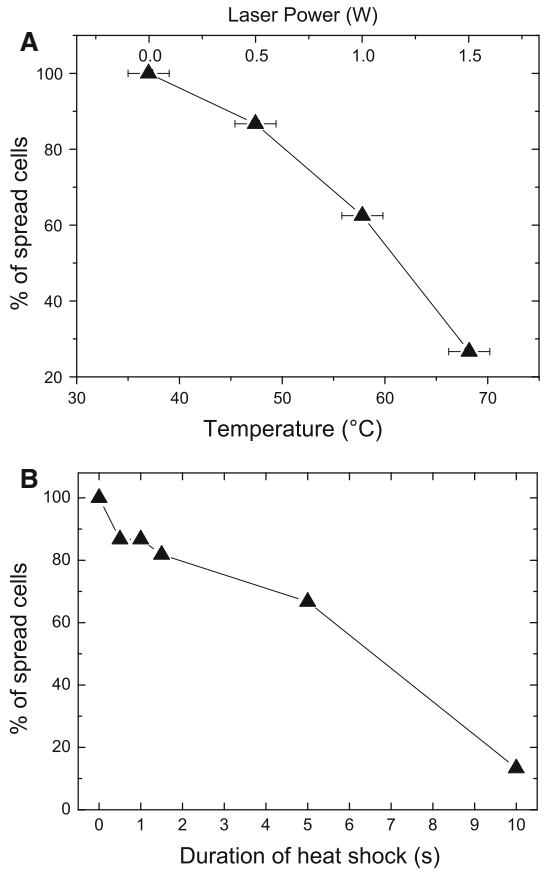


**Fig. 5a, b** Impact of heat shocks on cell viability. **a** Heat shocks were applied for 0.5 s. For each data point 15 cells were measured. For heat shocks of 58 ± 2°C, more than 60% of the cells stayed viable. The *upper x-axis* shows the applied laser power. Error bars result from uncertainty of temperature calibration. **b** Impact of heat shocks on cell viability. Cells were subjected to heat shocks of 48 ± 2°C for different durations. After 5 s of heat shock, 60% of the cells showed spreading

During the heat shocks, cells were also stretched along the laser axis. The resulting deformation of about 5–7% was obtained for the maximum applied laser power of 1.5 W. Such a deformation has no effect on the cell viability of mouse fibroblasts (Boccafoschi et al. 2007).

Neuman et al. (1999) observed vital damage on bacterial cells due to photochemical effects at laser intensities ($\cong 10^7$ W/cm$^2$) comparable to those used in our measurements ($\cong 3 \times 10^6$ W/cm$^2$). Significant loss in metabolic function of *Escherichia coli* was found at the earliest after 200 s of optical trapping (depending on power and wavelength). In our studies, cells experienced maximum exposure time of 10 s (besides trapping control, $3 \times 10^5$ W/cm$^2$ for 60 s). At this time scale, no effects of photo damage were observed in the studies from Neuman et al. (1999) or Liang et al. (1996). CHO cells after 60 s exposure to comparable intensities showed 90% cloning efficiency. Therefore heating is the predominant effect causing damage to the cells in our experiments.

Earlier studies investigated the impact of temperature changes on cells at time scales of minutes and hours for temperatures between 37 and 50°C (Huang et al. 1999). Proteins start to denature at 43°C. In cells the process of denaturation is reversible as long as DNA is not damaged and certain enzymes are still working (Lodish et al. 2000). In optical traps using infrared laser light, cells experience a higher temperature increase at shorter time scales (Ebert et al. 2007). In medicine, lasers are utilized for surgery and cosmetic corrections such as treatment of leg veins. Heating of the tissue occurs in some applications at comparable intensities and time frames to the parameter space in this study (Baumler et al. 2006). This study investigated the influence of heat shocks on cell viability using the heating effects of the MOS. First, the common fluorescence vital stain calcein AM was employed. Since the loss of fluorescence intensity in this viability assay corresponds to the loss of viability, the test is very fast and easy to handle. Cells stained with calcein AM and exposed to 68°C for 0.5 s showed an average loss of fluorescence intensity of 15%. Since the stain shows relatively strong bleaching with more than 10% loss of intensity per taken image, the data apparently indicated that the cells are nearly unaffected by this heat shock exposure. This seemed to be unlikely. Heat shocks not leading directly to necrosis might cause functional damage to the cells, which could induce apoptosis. During apoptosis, cytochrome-c release precedes the loss of membrane integrity, which could be identified by the loss of calcein AM signal intensity. Although the time line for the apoptotic process strongly differs with cells and triggering events, usually no release of cytochrome c before 3 h after treatment was observed (Goldstein et al. 2000). Calcein AM therefore only detected direct damage of the cell membrane in the observed time frame, but no functional damage inducing apoptosis. Cell spreading is a vital feature of cells requiring an intact cytoskeleton and working signalling pathways. Used as a viability marker it is a complex test probing structural integrity as well as functional capability. Therefore, experimental data of heat shock experiments using cell spreading as an indicator for viability are more trustworthy, though they might underestimate the number of viable cells. Measuring the proliferation rate would be a comparably complex viability assay, but it is not practicable for single cell analysis. Since it is known that NIH/3T3 cells adhere and spread within minutes, cells not showing such behavior after 15 min were counted as nonviable, but were not inevitably dead or apoptotic. It is possible that some of them could recover and successfully be recultured. The test can be seen as a worst case scenario for the impact of heat shocks. According to the data from our spreading experiments, cells survive short heat shocks up to 58 ± 2°C

with a survival rate over 60%. Heat shocks at 48 ± 2°C they can tolerate for up to 5 s.

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