Supplementary Material for:

SCATTERING OF EXCITING LIGHT BY LIVE CELLS IN FLUORESCENCE CONFOCAL IMAGING – PHOTOTOXIC EFFECTS AND RELEVANCE FOR FRAP STUDIES

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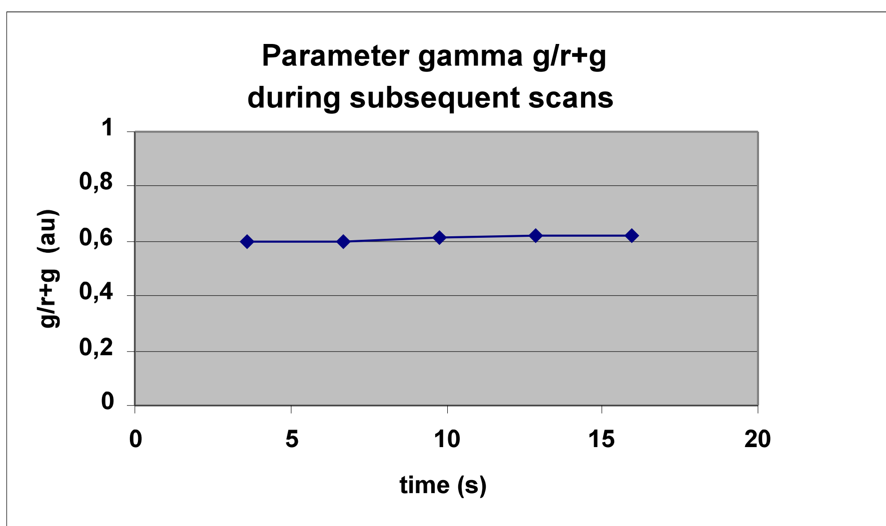
1. Stability of a parameter γ during collection of subsequent images.

Differential bleaching rates of red and green emissions of AO [Bernas et al., 2005], redistribution of the dye in the cell, and potential loss through the damaged plasma membrane are the factors that may influence measurements of γ. This experiment was performed to investigate if any changes of parameter γ occur during necessary data collection.

5 subsequent images of sublethally damaged cells (γ = 0.60) were collected (0.3scan/s; 512x512 pixels; 0.15 mW laser beam, AO present in culture medium, 37°C). Parameter γ was measured in subsequent scans and plotted against time. The value of γ increased from 0.60 to 0.62 (3%), i.e. 0.7%/scan. This increase was caused by a higher bleaching rate of AO stacks (red) than monomers (green). Since collecting 1-2 frames is sufficient to measure γ, the differential bleaching rates of the green and red emissions of AO appear to have negligible influence on assessment of photodamage based on this parameter.

Fig. 1-SM. Changes of parameter γ during collection of subsequent images

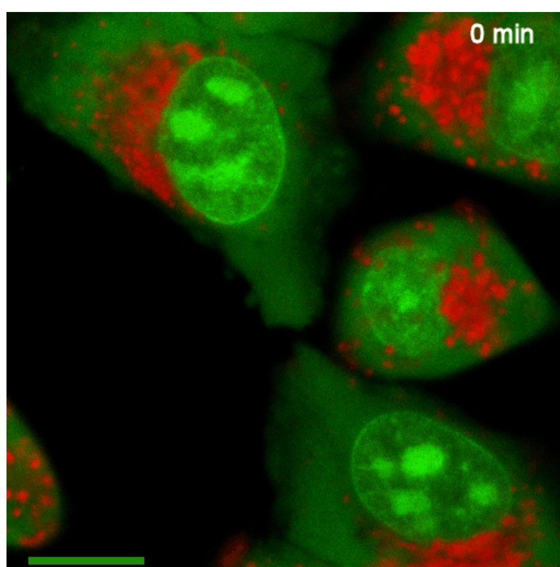


2. Timing of the test for photodamage, and spreading of photodamage throughout the cell

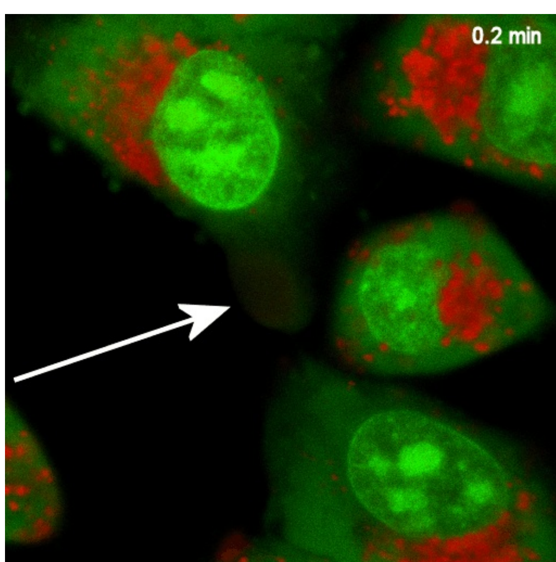
TIMING OF THE TEST

This series (Fig. 2-SM) demonstrates a typical time course of events leading to stabilization of the red signal in a photodamaged area of the cell. Changes of AO red and green emission following local photodamage are shown in a sequence of images below. Optimal time for measurements of AO luminescence following cell damage is 15-20 min.

Fig. 2-SM. Stabilization of the red signal after photodamage.

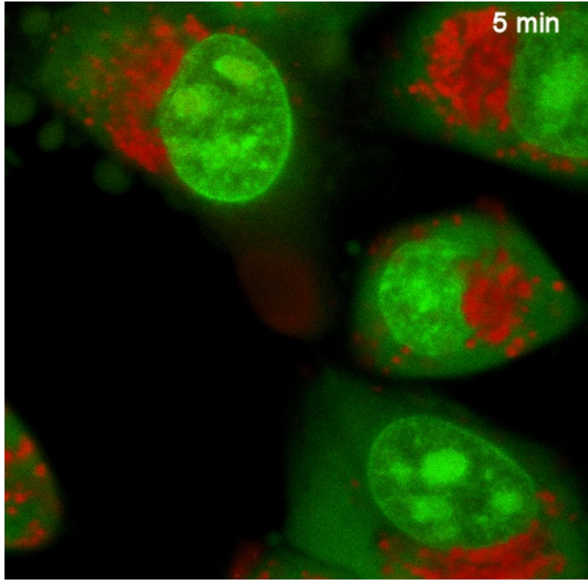


prior to photodamage
(bar - 10 μm)

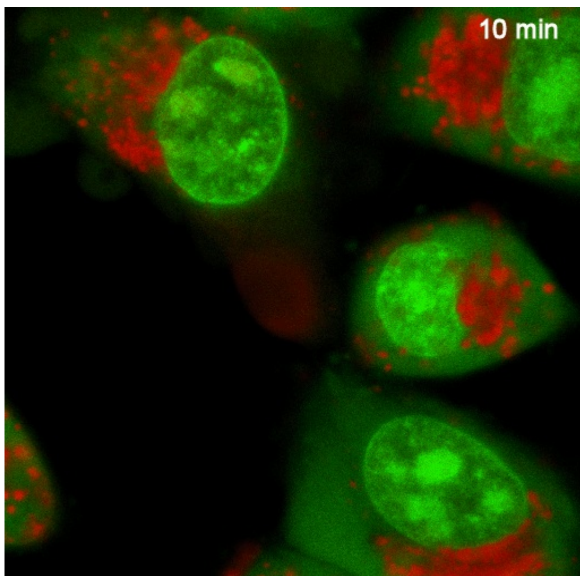


immediately after inflicting photodamage in the region marked with an arrow

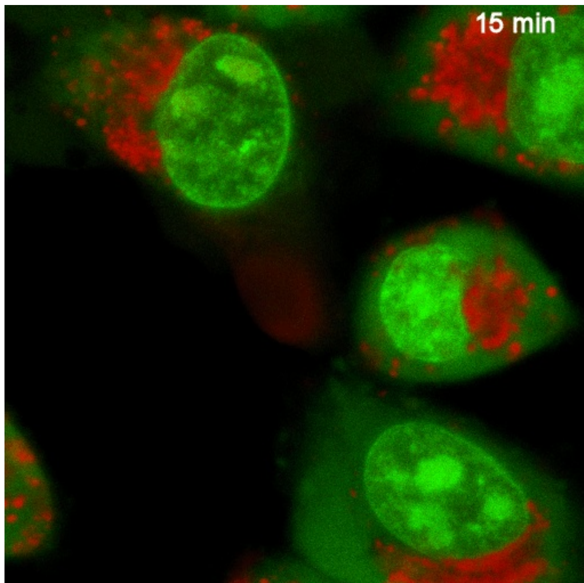
(The conditions of direct illumination were: 458 nm, 150 μW at the lens exit, 10 scans, 0.3 scan/s, area 5x5 μm).



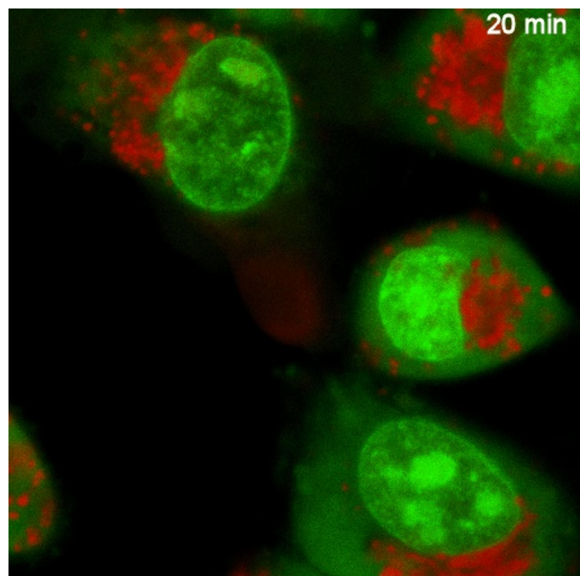
5 min after photodamage



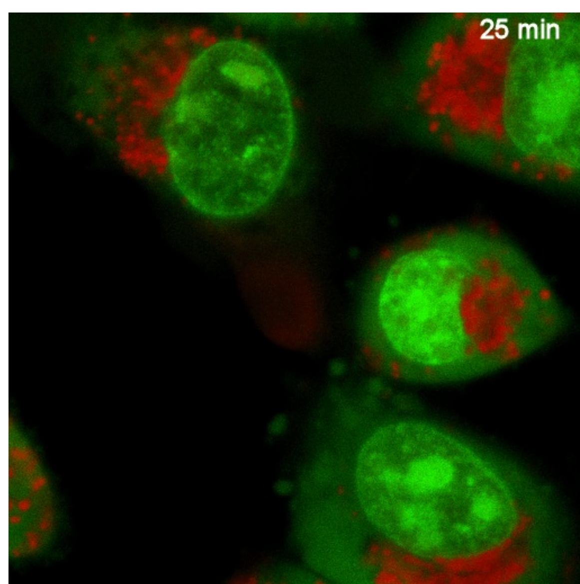
10 min



15 min



20 min



25 min

SPREADING OF PHOTODAMAGE

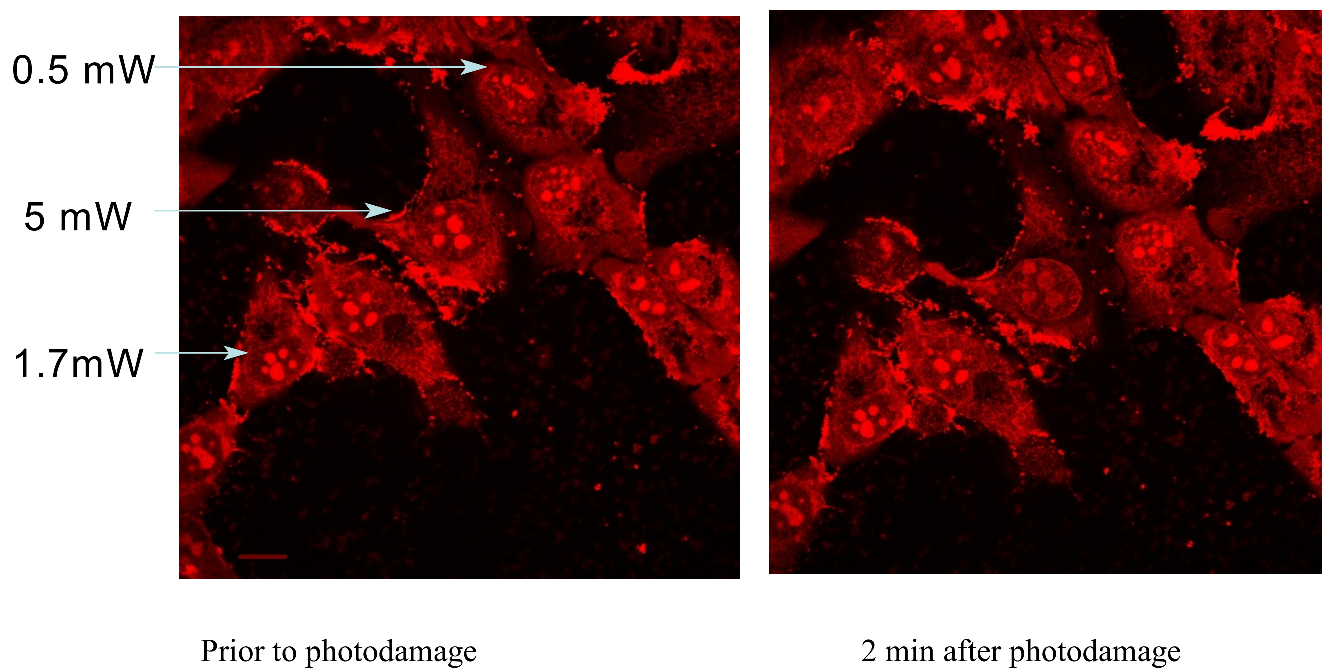
In the experiment shown above the red color did not spread throughout the cell. It expanded no more than 2 μm beyond the edges of the illuminated rectangle. This indicates that phototoxic compounds that may be generated by interaction between light and AO diffused out of the damaged region by no more than 2 μm. Scattering of exciting light may also contribute to this limited spreading of red luminescence.

3. Detection of photodamage exerted by ethidium (EB) and green light.

The paper embraced selected data demonstrating photodamage caused by 458 light exciting acridine orange (AO). AO served as a photosensitizer and damage reporter. Here we demonstrate an example of detecting photodamage to drug efflux caused by ethidium (DNA photodenaturation occurs as well [Bernaś et al. 2005]). The same method of detecting damage is used.

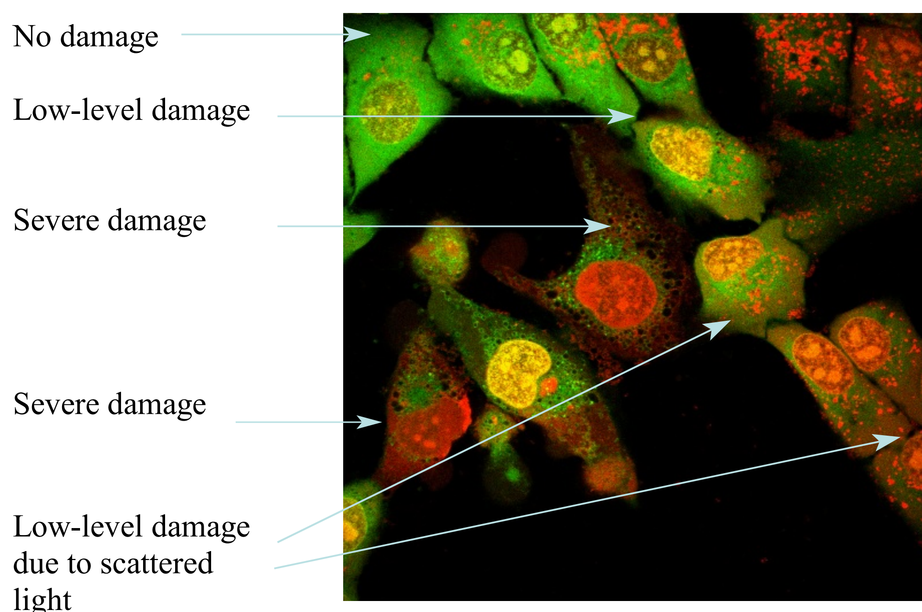
HeLa cells were incubated with ethidium bromide. Ethidium slowly penetrates into cells and stains nuclear and mitochondrial DNA, as well as RNA. Selected areas in three cells were exposed to green light (area 25μm^2, 514 nm, 10 scans, beam 5 mW, 1.7 mW or 0.5 mW) (Fig. 3-SM). Subsequently AO was added and red and green emissions of AO were imaged (excitation 458 nm; ethidium is excited very poorly at this wavelength, thus a contribution of EB to the red signal of AO is negligible).

Fig. 3-SM. Phototoxicity of ethidium bound to nucleic acids. Top panels – fluorescence of ethidium in cells, below - luminescence of AO added after inflicting photodamage on three selected cells. Bar - 10 μm.



Prior to photodamage

2 min after photodamage

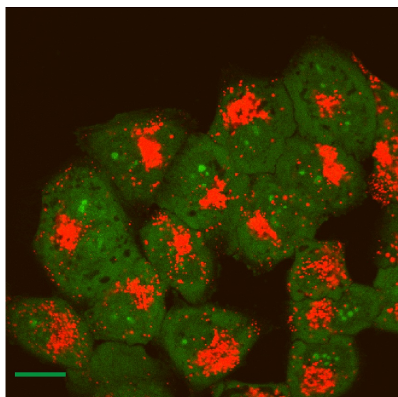


After staining cells with AO – cells that were photodamaged by direct illumination acquire red color in cytoplasm (note that this image is shifted downwards by approx. 15 μm in comparison with the image collected prior to photodamage).

Red color of cytoplasm of illuminated cells indicates photodamage, which resulted from interaction of green light with ethidium (photodenaturation of DNA in nuclei occurs as well [Bernas et al., 2005]. Cells affected by scattered light also exhibit various degrees of damage manifested by red luminescence of cytoplasm.

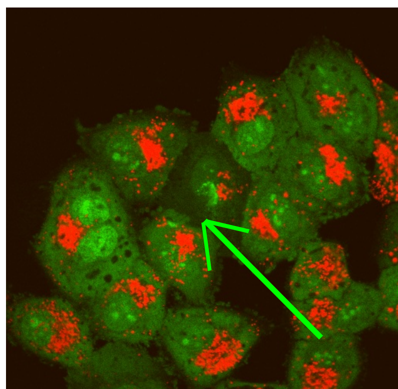
4. Effects of a single, short exposure to a high intensity laser beam

Fig. 4-SM. Damage caused by a single dose of high intensity light incident on a cell labeled with AO.



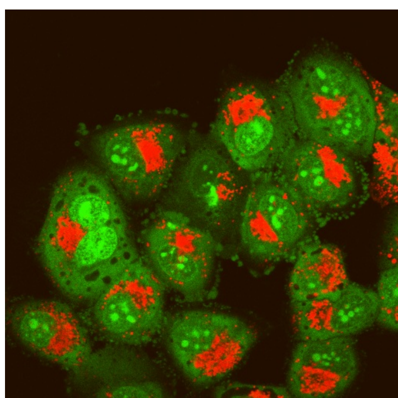
A single dose of high intensity excitation light (488 nm, 1scan (1s), 4 mW, illuminated area 17x17 μm) resulted in severe damage to the cell, a loss of integrity of plasma membrane, and a loss of low molecular components from the cell. This light dose exceeded the highest dose depicted in Fig 2 (graph).

Image prior to light insult. Bar - 20 μm.



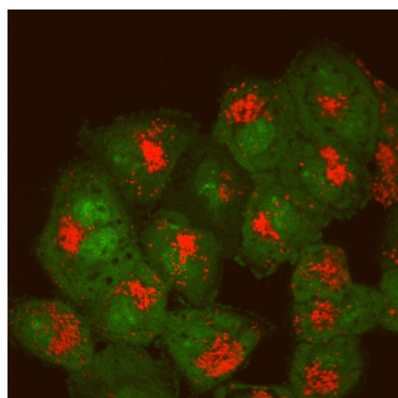
5 min after the light insult. The arrow points at the region, where exciting light was delivered.

Exciting light results in damage to endosomes and an immediate release of accumulated AO. Red luminescence from endosomes turns into green.



10 min after the insult

Neighboring cells (left of the damaged cell) are damaged by scattered light, and AO influx has grown – green fluorescence increases.



20 min

The damaged cell is losing RNA and low molecular weight compounds, including AO; green fluorescence diminishes. Endosomes are damaged and their red luminescence is decreasing, too.