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Short and long-term phototoxicity in cells expressing genetic reporters under nanosecond laser exposure

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ABSTRACT

Nanosecond-duration laser pulses are exploited in a plethora of therapeutic and diagnostic applications, such as optoacoustic imaging. However, phototoxicity effects of pulsed radiation in living cells, in particular those expressing genetic reporters, are not well understood. We established a three-dimensional fluorescent protein expressing cellular model in order to reliably investigate the extent and major exposure parameters responsible for both photobleaching and phototoxicity under pulsed laser exposure, unveiling a variety of possible effects on living cells, from reversible photobleaching to cytotoxicity and cell death. Significant losses of fluorescence levels were identified when exposing the cells to illumination conditions considered safe under common standards for skin exposure in diagnostic imaging applications. Thus, the use of photolabile fluorescent proteins and their *in vivo* exposure parameters have to be designed carefully for all applications using pulsed nanosecond radiation. In particular, loss of signal due to bleaching may significantly alter signals in longitudinal measurements, making data quantification challenging.

1. Introduction

It has been long understood that the biological effects of pulsed laser radiation may considerably differ from those observed under continuous exposure [1]. Among a plethora of therapeutic and diagnostic methods using nanosecond-duration laser pulses [2], optoacoustics has recently gained significant momentum amid its highly compelling advantages as a bioimaging modality. These include the capacity for rapid volumetric imaging [3,4], intrinsic sensitivity to functional tissue parameters [5–7] and molecular optical reporters [8,9] as well as excellent spatial resolution in imaging optical contrast at scalable depths, from millimeters to centimeters in scattering living tissue [10–12]. The efficiency of optoacoustic signal conversion may be altered by photobleaching, which occurs when a chromophore (e.g. fluorophore or fluorescent protein) permanently loses its reporting ability [13,14]. Although effects related to photochemical destruction and melting of inorganic nanoparticles under exposure to nanosecond laser radiation have been reported recently [15], little is known about the extent of photobleaching of organic chromophores in various optoacoustic imaging scenarios.

Genetically encoded fluorescent proteins (FPs) are of particular interest for *in vitro* and *in vivo* imaging, as they can act as reporter molecules for specific cellular structures, whole cells or tissue in manifold bio-medical applications [16]. FPs are valuable tools in biological research to study the structures of cellular components or to conduct functional studies, where they act as biosensors [17].

For *in vivo* imaging, the recently developed FPs with excitation and emission wavelengths in the far-red and near-infrared spectral windows, so-called near-infrared fluorescent proteins (iRFPs) are of particular interest since tissue optical scattering and absorption are relatively low in this wavelength range, thus significantly deeper imaging depth is achieved [8,18,19]. iRFPs also possess low quantum yields, which makes them ideal candidates for absorption-based imaging with optoacoustics.

The application of FPs in optoacoustic imaging is not very common yet as compared to their ample use in fluorescence

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microscopy and spectroscopy. Nonetheless, FPs have shown great promise for *in vivo* optoacoustic imaging in zebrafish [9,20], glioma visualization [8], multi-contrast optoacoustic imaging [21] and optoacoustic flow cytometry [22].

Many fluorescent proteins lack photostability [16,18], which may impede quantitative measurements [23]. To this end, nanosecond-laser-induced photobleaching has been shown to occur in isolated FPs [24]. Furthermore, fluorescent proteins are known to induce cytotoxicity under certain excitation conditions [25]. Here, we investigated the extent of photobleaching in a three-dimensional (3D) cellular model stably expressing the mCherry fluorescent protein, which closely represents normal cellular functions and thus mimics the *in vivo* architecture of natural tissues. The cytotoxicity resulting from photobleaching was characterized during and after optoacoustic imaging sessions in a wide range of possible exposure scenarios.

2. Materials and methods

Unless stated otherwise products and chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany) or Life Technologies (Darmstadt, Germany).

2.1. Cell preparation and culture

A 3D cell culture model was developed to more closely represent normal cellular functions and mimic the *in vivo* architecture of natural tissues [26]. Murine osteosarcoma (MOS) cells were established from radiation-induced tumors as described elsewhere [27]. In short, osteosarcoma was induced by incorporating short living alpha-emitters (Th227) in a female C3H/Hhg mouse from a breeding colony established at the Helmholtz Zentrum München. Pieces of osteosarcoma were dissected under sterile conditions and placed on BD Falcon 6-well cell culture plates in a 2 mm layer of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) for 10 days. When a sufficient number of adherent cells seeded from the tumor piece, they were further passaged and finally single-cell cloned into 96-well plates. Osteoblastoid differentiation was validated by alkaline phosphatase (AP) histochemistry and tumorigeneity tested by subcutaneous transplantation into syngeneic mice. MOS cells were then transfected with pCAG Kosak-Cherry (chicken beta actin promoter, mCherry red fluorescent protein cDNA, internal ribosome entry site (IRES), puromycin resistance) using Lipofectamine 2000, and by applying 5 μg Plasmid on 10^6 cells. To enable the stable integration of the mCherry transgene, the expression construct was linearized using the ScaI enzyme before lipofectamine-mediated transfection. Successfully transfected cells were selected by growing cells for 14 days under 5 μg/ml puromycin followed by single cell cloning in 96-well plates. The expression of the mCherry protein under the CAG promoter is constitutive and very high [28]. The mCherry expression in the MOS cells was measured over more than 10 passages (approx. 40 cell divisions) after expanding a single clone and the expression of the fluorescence protein did not change over time within the range of detection sensitivity (data not shown).

MOS cells were then grown in 5% CO_2 at 37 °C in DMEM/10% FCS supplemented with 1% Penicillin/Streptomycin (PAA, Kölbe, Germany). In addition to a single cell layer, the MOS cells grow as multi-cellular spheroids that form on top of the cell layer. Spheroids up to 100 μm in diameter can be directly harvested from the flasks 3–4 days after plating the cells. To obtain larger diameter spheroids, they were manually detached and transferred into a new flask for continued growing. To reach a diameter of 1 mm, it was necessary to repeat the process two or three times to prevent the newly-formed cell layer from overgrowing the spheroid. Alternatively, spheroids were transferred into petri dishes with a hydrophobic surface (Greiner Bio-One, Frickenhausen, Germany), thus preventing formation of a cell layer at the bottom. From these petri dishes spheroids with sizes of up to 500 μm in diameter could be collected directly after 4–14 days of culture.

For the bleaching experiments, spheroids of different sizes were embedded into low melting agar (SeaPrep ultralow gelling Agarose, Lonza, Köln, Germany) with a gelling temperature of 8–17 °C. A 3% (by weight) sterile agar-solution in phosphate buffered saline was prepared by autoclaving. The dissolved agar was then kept in the cell incubator at 37 °C to prevent gelling. Standard 6-well cell culture plates were filled with 2 ml of the 3% agar and kept at 4 °C until the agar solidified. A freshly collected solution of spheroids in medium was carefully mixed at a 1:1 ratio with the 3% agar at 37 °C, and 2 ml of the solution were then pipetted into each well of the 6-well plate. The plate was then stored at 4 °C for a few minutes until the agar solidified. On top, 2 ml of fresh and warm medium were pipetted and the spheroids were allowed to recover for at least another 24 h in the incubator at 37 °C and 5% CO_2 before the agar-embedded spheroids were collected from the 6-well plates and placed into a 35 mm diameter cell culture dish with low walls (μ-Dish low, Ibidi, Munich, Germany) as shown in Fig. 1A. The dish was then filled to the top with low-melting agar to fix the spheroids in place. Spheroids placed on the outer edges of the cell culture dish served as control for indirect light exposure. Only the spheroids placed in the middle of the dish were directly exposed to the excitation laser light of the optoacoustic scanner.

2.2. Fluorescence microscopy and image analysis

Fluorescence images were recorded using a Leica DMI3000B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC360 FX camera. To record mCherry-fluorescence a filter set with excitation 580/20 nm and emission 630/60 nm was used. Fluorescence images of mCherry were taken with fixed exposure times before, directly after and 24 h after the laser light exposure sessions, thus making the fluorescence images comparable. Fluorescence intensities were analyzed from 2 to 4 single spheroids for each of the placements in the cell culture dish (Fig. 1A) using the freely available Fiji image processing software [29]. Every spheroid-containing dish exposed to laser light was accompanied by a control dish prepared and handled in the same way, except for the fact that there was no laser exposure. If the fluorescence levels after 24 h either remained at the same level as right after exposure or diminished, this was considered to be of phototoxic exposure. Otherwise, the cells were considered to be viable and healthy.

2.3. Laser exposure conditions

To investigate the fluence-dependent effects of nanosecond pulsed laser exposure, the MOS cells were stably transfected to express the mCherry fluorescent protein and agar-embedded spheroids were subjected to either unfocused or focused illumination conditions (Fig. 1B). Laser pulses of 10 ns duration were generated by a diode-pumped Nd:YAG Q-switched Laser (IS8II-E, EdgeWave GmbH, Würselen, Germany). The wavelength was tuned to the peak absorption of mCherry of 594 nm by means of a dye laser (Credo, Sirah Lasertechnik GmbH, Grevenbroich, Germany). The unfocused multimode beam, created using a liquid light-guide (Lumatec Series 2000, Deisenhofen, Germany), had a diameter of approximately 6 mm at the height of the agar-embedded spheroids. This represented typical illumination conditions in acoustic resolution optoacoustic microscopy [11] and tomography [3,10] systems, which use low light fluence levels but require long exposure