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Light exposure and cell viability in fluorescence microscopy

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Summary

Test systems for measuring cell viability in optical microscopy (based on colony formation ability or lysosomal integrity) were established and applied to native cells as well as to cells incubated with fluorescence markers or transfected with genes encoding for fluorescent proteins. Human glioblastoma and Chinese hamster ovary cells were irradiated by various light doses, and maximum doses where at least 90% of the cells survived were determined. These tolerable light doses were in the range between 25 J cm^{-2} and about 300 J cm^{-2} for native cells (corresponding to about 250–3000 s of solar irradiance and depending on the wavelength as well as on the mode of illumination, e.g. epi- or total internal reflection illumination) and decreased to values between 50 J cm^{-2} and less than 1 J cm^{-2} upon application of fluorescent markers, fluorescent proteins or photosensitizers. In high-resolution wide field or laser scanning microscopy of single cells, typically 10–20 individual cell layers needed for reconstruction of a 3D image could be recorded with tolerable dose values. Tolerable light doses were also maintained in fluorescence microscopy of larger 3D samples, e.g. cell spheroids exposed to structured illumination, but may be exceeded in super-resolution microscopy based on single molecule detection.

Introduction

For many years an increase in resolution has been a main challenge in fluorescence microscopy. In addition to lateral resolution around 200 nm, axial resolution down to a few hundred nanometres has been attained by confocal (Pawley, 1990) or multi-photon (Denk *et al.*, 1990; König, 2000) laser scanning techniques or by wide-field microscopy with structured illumination and subsequent image processing (Neil *et al.*, 1997; Gustafsson *et al.*, 2008). Further resolution (down to about 20–30 nm, and thus far beyond the Abbé limit) was achieved by special techniques, e.g. stimulated emission depletion (STED) microscopy (Willig *et al.*, 2007) or super-localization microscopy based on single molecule detection, e.g. stochastic optical resolution microscopy (STORM) or photoactivated localization microscopy (PALM), as first reported by Betzig *et al.* (2006), Rust *et al.* (2006) as well as Hess *et al.* (2006). However, little attention has been drawn to the rather high light doses needed for those techniques, which could damage or even kill the cells under the microscope, and only in a few papers phototoxic effects of light in live-cell imaging have been reported (Hoebe *et al.*, 2007; Frigault *et al.*, 2009; Wagner *et al.*, 2010). According to Wagner *et al.* (2010) light doses up to 25–200 J cm^{-2} (depending on the wavelength) have been found to be nonphototoxic towards native U373-MG glioblastoma cells upon epi-illumination. These doses correspond to 250–2000 s of solar irradiance, if a solar constant of about 0.1 W cm^{-2} – corresponding to 1 nW μm^{-2} – is assumed. When using the fluorescent membrane marker 6-dodecanoyl-2-dimethylamino naphthalene (laurdan), the tolerable light dose was even smaller (about 10 J cm^{-2}), as depicted in Figure 1. This figure summarizes previous results (Wagner *et al.*, 2010) of a colony formation assay, when upon seeding of individual glioblastoma cells the percentage of formed colonies ('plating efficiency') was determined. Cell survival was defined by less than 10% reduction of the plating efficiency in comparison with nonirradiated controls (e.g. from about 80% to not less than 70%), as further described below.

Presently, measurements of cell viability are extended to further fluorescent markers of cell membranes and mitochondria, as well as to fluorescent proteins and photosensitizers. In addition to cell viability measurements upon epi- and total internal reflection (TIR) illumination, epi-illumination microscopy including laser scanning microscopy

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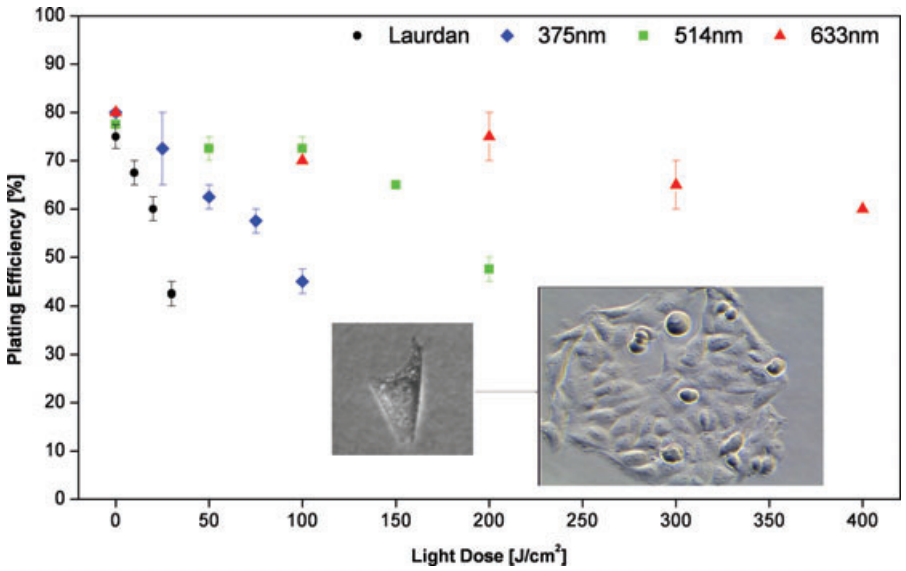

Fig. 1. Percentage of colony formation ('plating efficiency') of single nonincubated U373-MG glioblastoma cells upon epi-illumination with different wavelengths as well as U373-MG cells incubated with laurdan (8 μM; 60 min.) upon epi-illumination at 391 nm as a function of light dose. Values represent medians ± MADs. The plating efficiency at 0 J cm^{-2} is defined as 100% cell survival. Cells are regarded as viable upon less than 10% reduction of this plating efficiency, i.e. for native cells up to 25, 100 or 200 J cm^{-2} upon illumination by 375, 514 or 633 nm, respectively, and for cells incubated with laurdan up to 10 J cm^{-2} upon illumination by 391 nm. Inlay: principle of the colony-forming assay. The figure represents a summary of results reported in detail by Wagner *et al.* (2010).

(LSM) and structured illumination (Neil *et al.*, 1997) as well as TIR microscopy (Schneckenburger, 2005) were performed with two-dimensional and three-dimensional cell cultures.

Materials and methods

Cell cultures

U373-MG human glioblastoma cells (No. 300366) as well as Chinese hamster ovary (CHO-K1) cells (No. 603480) were obtained from Cell Lines Service (CLS, Eppelheim, Germany) and routinely grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics at 37°C and 5% CO_2. Part of the CHO-K1 cells were stably transfected with plasmids encoding either for a membrane-associated green fluorescent protein (pAcGFP1-Mem) or for a mitochondria-associated green fluorescent protein (pAcGFP1-Mito) using GeneJammer transfection reagent (Agilent, Waldbronn, Germany) according to the manufacturer's instructions. Subsequent to G418 selection (1 mg mL^{-1}), transfectants were analysed for AcGFP expression via flow cytometry utilizing a FACS Calibur (BD Biosciences, Heidelberg, Germany) and WinMDI software (Version 2.9, The Scripps Research Institute, http://facs.scripps.edu/software.html). Clones displaying similar expression characteristics with regard to homogeneity and strength of target gene expression (Supplemental Figure) were used for irradiation experiments and for the determination of phototoxicity of AcGFP1-Mem and AcGFP1-Mito.

Nontransfected cells were incubated for 30 or 60 min with the fluorescent dyes listed in Table 1 at the given concentration (exception: in the case of the photosensitizer protoporphyrin IX cells were incubated for 4 h with its precursor 5-aminolevulinic acid, 5-ALA). Cells were cultivated either as monolayers on glass slides or as spheroids of about 300–500 μm diameter. These spheroids were grown within individual cavities of a microtiter plate coated with agarose gels. After the addition of a cellular suspension, cell adhesion was prevented, and growth of spheroids was favoured.

Cell viability

To determine cell viability, single U373-MG cells were seeded at low density (10 cells mm^{-2}) in eight-well microslides (μ-Slide; Ibidi, Martinsried, Germany) and irradiated under the microscope with variable light doses and wavelengths on the third day after seeding. Either a continuous wave (cw) argon ion laser operated at 514 nm (Innova 90, Coherent, Palo Alto, CA, U.S.A.) or a cw helium–neon laser operated at 633 nm (Mod. 1248, Spectra Physics, Mountain View, CA, U.S.A.) or a laser diode emitting a quasi-continuous series of picosecond pulses (LDH 375 or LDH 400 with driver PDL 800-B, Picoquant, Berlin, Germany; wavelengths: 375 or 391 nm; pulse energy: 12 pJ, pulse duration: 55 ps, repetition rate: 40 MHz) was used for irradiation. CW and 'quasi continuous' light sources were not further distinguished with respect to cell viability. Epi-illumination of whole cells was

Table 1. Tolerable light doses for maintaining cell viability and maximum exposure time at solar irradiance determined by colony formation assay (CF) or neutral red assay (NR). Light doses result from epi-illumination, unless specified by 'TIR'(total internal reflection illumination). (*) Results previously published by Wagner *et al.* (2010).

Cell line	Marker	Concentration (μM)	λ_{ex} (nm)	Test system	Maximum light dose (J cm^{-2})	Solar exposure time (s)
U373-MG	–		375	CF	25 (*)	250
U373-MG	–		514	CF	100 (*)	1000
U373-MG	–		633	CF	200 (*)	2000
U373-MG	–		514 (TIR)	CF	300	3000
U373-MG	Laurdan	8	391	CF	10 (*)	100
U373-MG	Laurdan	8	391 (TIR)	CF	30 (*)	300
CHO-K1	DiA	5	488	NR	10	100
CHO-K1	DiO	5	488	NR	10	100
CHO-K1	GFP-Mem		488	NR	10	100
CHO-K1	R123	5	488	CF	20	200
				NR	5–10	50–100
CHO-K1	MTO	0.05	514	NR	50	500
CHO-K1	GFP-Mito		488	NR	5	50
U373-MG	ALA/PP IX	1000	514	CF	1	10
U373-MG	ALA/PP IX	1000	391	CF	0.25	2.5
U373-MG	ALA/PP IX	1000	391 (TIR)	CF	0.75	7.5

performed in an inverted microscope (Axiovert 200, Carl Zeiss Jena, Germany), whereas for TIR illumination of thin layers a prism-based setup (Stock *et al.*, 2003) adapted to an upright microscope (Axioplan 1, Carl Zeiss Jena) was used. Spatial coordinates of irradiated cells on a positioning table (Scan IM 130 × 100, Märzhäuser, Wetzlar, Germany) were stored. After re-incubation of the cells with RPMI 1640 medium and further cultivation until the seventh day after seeding, colony formation was evaluated. Plating efficiency, i.e. the percentage of formed colonies per seeded cells, was determined, and cell survival was defined by less than 10% reduction of the plating efficiency in comparison with nonirradiated controls. For each light dose and each condition of irradiation the percentage of colony forming cells was determined after irradiation of 20 individual cells. Medians and the median absolute deviations (MADs) were calculated from four individual experiments in each case. For CHO-K1 cells incubated with rhodamine 123 (R123) the colony forming assay was slightly modified, as described in the literature (Liang *et al.*, 1996).

In addition, cyto- and phototoxicity of various dyes towards CHO-K1 cells were determined by the neutral red assay (Repetto *et al.*, 2008), where neutral red molecules were bound within lysosomes of vital cells. Here, these dye molecules were ionized and trapped due to the acidic pH, which is actively maintained through consumption of ATP in viable and metabolically active cells. Subsequent to irradiation, cells were washed, and the dye was extracted in a mixture of water, ethanol and acidic acid (1:1:0.02). The dye was then quantified by its absorption at 570 nm (Fluostar Omega Microplate reader; BMG Labtech, Offenburg, Germany). 25–50 cells mm^{-2} were seeded at day 0 in 24-well microplates (Nunc, Langenselbold, Germany); irradiation occurred on day 2 and analysis of viability on day 4. Cell survival was calculated as the percentage of the untreated control, respectively. At least three independent experiments were performed in quadruplicate.

Fluorescence microscopy

For fluorescence microscopy either a confocal laser scanning microscope (Carl Zeiss Jena; Axiovert 200 with Pascal 5 scanning head) or an upright wide-field microscope (Carl Zeiss Jena; Axioplan 1) was used. In the latter one special units for structured illumination (Schneckenburger *et al.*, 2010) and TIR of a laser beam (Stock *et al.*, 2003) were integrated. For structured illumination of larger samples, e.g. 3D spheroids, the optical setup was modified: a grid of lower periodicity (10 line pairs per mm in combination with a PX 100 piezo positioning system) was used together with an objective lens of 10−20× magnification and a resolution of 0.5−1.0 μm (lateral) as well as about 5 μm (axial), as reported by Bruns *et al.* (2009). As a light source either an argon ion laser (operated at 488 or 514 nm), a 633 nm helium–neon laser, laser diodes emitting at 375 or 391 nm (same as described above) or a light emitting diode (LED) at 470 ± 12 nm was used. Wide-field fluorescence imaging was performed either with an electron multiplying charge-coupled device (EM-CCD) camera at a sensitivity below 25 photons/pixel (corresponding to less than 10 $^{-16}$ W/pixel within a time frame of 100 ms; DV887DC, ANDOR Technology, Belfast, United Kingdom)

(Coates *et al.*, 2004) or with an integrating digital CCD camera (ProgRes C10, Jenoptik GmbH, Jena, Germany), while an integrated highly sensitive photomultiplier (Hamamatsu Photonics; Ichino-Cho, Japan) was used for laser scanning microscopy.

Results

Previously published experiments (Wagner *et al.*, 2010) revealed that U373-MG glioblastoma cells remain viable up to a light dose of about 25 J cm^{-2} at an irradiation wavelength of 375 nm, 100 J cm^{-2} at 514 nm and about 200 J cm^{-2} at 633 nm, corresponding to 250, 1000 or 2000 s of solar irradiance, respectively. Cells incubated with the fluorescent membrane marker laurdan (8 μM, 1 h) and irradiated at 391 nm (i.e. close to its absorption maximum) were more sensitive to irradiation and remained viable up to a light dose of only 10 J cm^{-2} corresponding to 100 s of solar irradiance (Fig. 1).

In this study, we extended our phototoxicity analyses to various fluorophores commonly utilized in live-cell imaging. For further membrane markers (the carbocyanine DiO and its related substance DiA), the maximum tolerable light dose was also around 10 J cm^{-2} in CHO-K1 cells, thus similar to that of laurdan in U373-MG cells. This also holds for green fluorescent proteins associated with membranes or mitochondria of CHO-K1 cells (Table 1). For the mitochondrial marker rhodamine 123 (R123) the maximum tolerable light dose in CHO cells slightly differed between 5–10 J cm^{-2} (neutral red assay) and about 20 J cm^{-2} (colony formation assay) when different tests were used. Only for the mitotracker orange (MTO) the maximum light dose was considerably higher (50 J cm^{-2}), possibly due to the rather low dye concentration needed for incubation of the cells. Maximum light doses around 10 J cm^{-2} for all other dyes again limit the exposure time to about 100 s (at solar irradiance) and typically permit recording of 10 – 20 individual high-resolution (wide field or laser scanning) images, e.g. from different cell layers (Fig. 2), as required for reconstruction of a three-dimensional image.

For more than 20 years porphyrins and related compounds have been used for fluorescence detection and photodynamic therapy of cancer (Dougherty, 1987). Understanding of their cellular accumulation and principal mechanisms has proven to be important for their clinical application. However, as depicted in Table 1 for protoporphyrin IX (PPIX; synthesized endogenously from aminolevulinic acid; Malik and Lugaci, 1987), only very low light doses of 0.25–1.0 J cm^{-2} can be used for live-cell imaging, and this limits the number of wide field or laser scanning images to a maximum of 1–5, depending on the required resolution and signal/noise ratio.

For native cells as well as for the fluorescent dye laurdan and the photosensitizer PPIX, the maximum tolerable light dose for maintaining cell viability is about three times higher upon illumination by the evanescent electromagnetic field arising upon TIR of a laser beam than upon epi-illumination. This is also depicted in Table 1 and proves some reduced cellular damage when only parts of the cell (plasma membrane and adjacent sites) are illuminated.

In comparison with high-resolution microscopy, light dose of irradiation is reduced when larger object fields are illuminated. This holds e.g. for 3D cell systems, which often describe tissue properties better than conventional 2D models, and which may not require ultimate optical resolution. An example is given in Figure 3 for a spheroid of CHO-K1 cells with a membrane-associated green fluorescent protein (pAcGFP1-Mem) imaged with a 10× magnifying objective lens. While Figure 3(a) shows a conventional fluorescence image, Figure 3(b) depicts a sectional view of one cell layer from the focal plane after structured illumination. Information from various focal planes is combined in the 3D image depicted in Figure 3(c). Although for each sectional view three structured images have to be recorded, the total light dose for imaging one plane can be limited to about 0.05 J cm^{-2} due to the large number of fluorophores located within a 5 μm layer and contributing to the fluorescence signal. Therefore, images of more than 100 focal planes can probably be recorded without damaging the cells.

Discussion

Light dose, wavelength of illumination and application of any kind of fluorophore proved to be essential parameters for maintaining cell viability in optical microscopy. Upon epi-illumination nonphototoxic light doses were up to 200 J cm^{-2} (increasing with excitation wavelength) for nonincubated cells, but decreased upon application of a fluorescent dye to about 1–50 J cm^{-2} corresponding to 10–500 s of solar irradiance. Increase of phototoxicity of native cells with decreasing wavelength may be related to an increasing number of molecular species with phototoxic properties, e.g. porphyrins (Ricchelli, 1995), flavins (Hockberger *et al.*, 1999) and possibly also nicotinamide adenine dinucleotide (NADH; Tiede & Nichols, 2006). Exogenous dyes induce further phototoxicity (depending on their concentration and triplet yield), which is most pronounced for photosensitizers, e.g. protoporphyrin IX. As reported earlier (Wagner *et al.*, 2010), thermal damage could be excluded in the range of light doses under investigation. Whether nonphototoxic light doses are the same upon cw and repetitive pulse excitation (e.g. in laser scanning microscopy) is still unclear; however, a comparison of excitation by cw and modelocked lasers gave similar cell viabilities when the wavelength and the total light dose were the same (results not shown). Also Murray *et al.* (2007) found equal amounts of photobleaching in a wide field and a laser scanning microscope, when the total dose of illumination was identical.

Maximum tolerable light doses were found to differ between epi-illumination and TIR illumination, and for native cells as well as for cells incubated with laurdan or protoporporphyrin

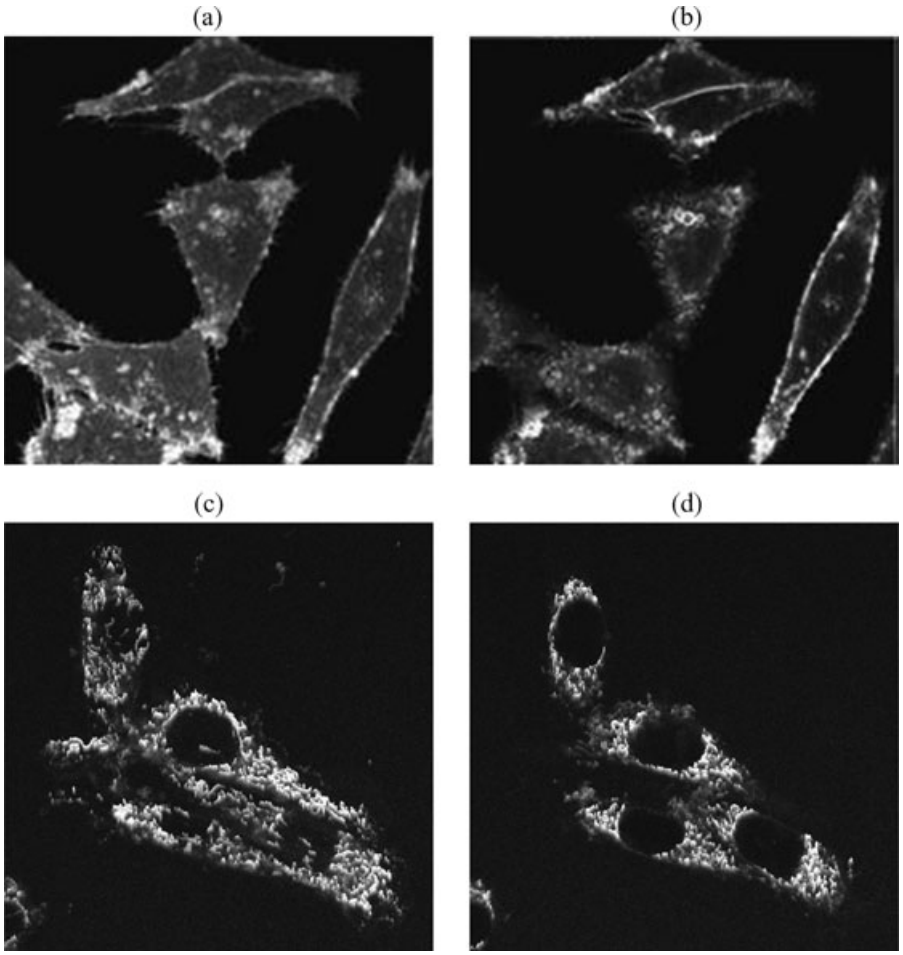

Fig. 2. Confocal images of planes of CHO-K1-GFP-Mem cells (a, b) and CHO-K1 cells incubated with the mitochondrial marker rhodamine 123 (5 μM; 30 min.) (c, d). Figures 3(a) and (b) as well as Figures 3(c) and (d) are shifted by $\Delta z = 2.1$ μm between one another. High-resolution fluorescence microscopy with a 100×/1.45 oil objective lens (excitation wavelength: 488 nm; detection range: $\lambda \geq 510$ nm; image size: 92 μm × 92 μm). For each fluorophore images from 13 focal planes were recorded with a tolerable light dose of 10 J cm^{-2}.

IX were three times higher upon TIR than upon epi-illumination. This most probably results from the low penetration depth of the evanescent electromagnetic field of about 100 nm upon TIR illumination in comparison with whole cell illumination. Also an intracellular distribution of phototoxic molecules and the corresponding site-specific photodynamic efficacies may play a role. Previously, a relation between intracellular location and photodynamic efficacy of protoporphyrin IX has been reported (Sailer *et al.*, 2007). It remains an open question, so far, why sensitivity towards TIR and epi-illumination differed by the same factor for endogenous molecules in native cells and membrane-associated dye molecules (e.g. laurdan). Lower phototoxicity upon TIR illumination in comparison with epi-illumination is favourable for single molecule detection in layers of about 100 nm thickness. In this case, however, a power density of irradiation around 50−100 W cm^{-2} (corresponding to 5−10 mW on a sample area of 0.01 mm^2) is required to record about 100 fluorescence photons per second from one molecule. Assuming that the maximum tolerable light dose at a very low dye concentration of 10^{-8}−10^{-10} M is similar to that of native cells and amounts to about 300−600 J cm^{-2} (see Table 1), exposure times should be limited to a few seconds in super-resolution microscopy based on single molecule detection, e.g. STORM or PALM.

In comparison with super-resolution microscopy, microscopy at lower resolution requires less focusing and, therefore, lower irradiance. This permits longer exposure times, e.g. upon repetitive measurements of kinetic processes or upon imaging of three-dimensional samples, when its planes are examined successively, as depicted in Figure 3. However, for recording each plane by laser scanning or structured illumination techniques the whole sample is exposed to light, and its dose sums up over numerous

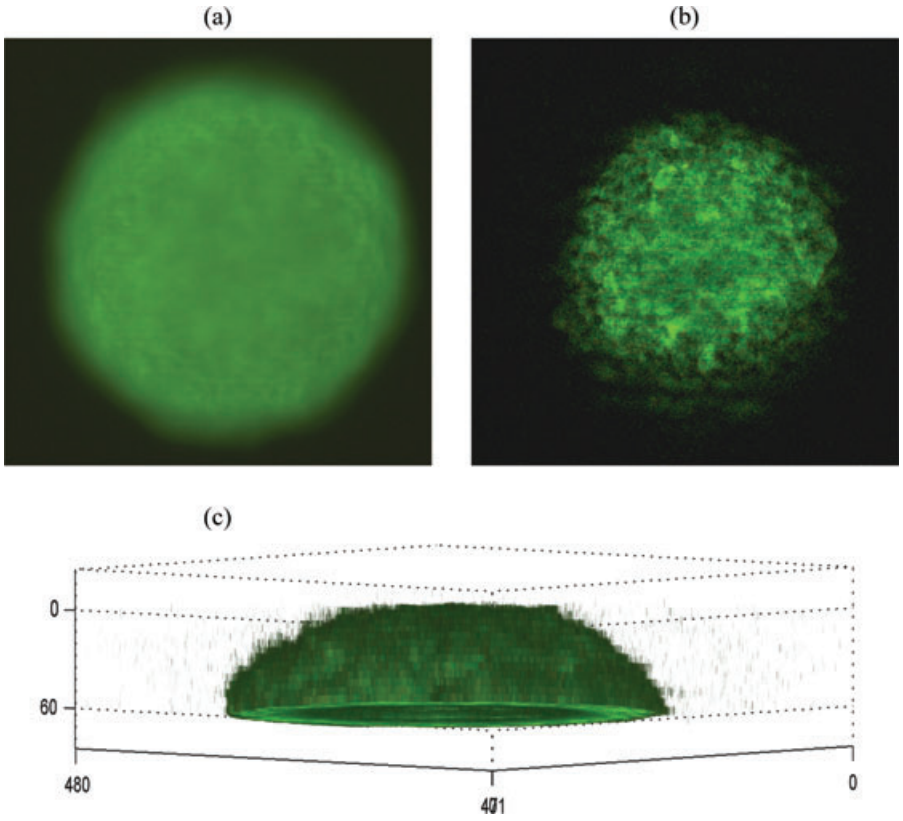

Fig. 3. Spheroid of CHO-GFP-Mem cells: (a) conventional fluorescence image; (b) sectional view from the focal plane (thickness: 5 μm) after structured illumination and (c) reconstruction of a 3D image from 14 focal planes (excitation wavelength: 470 ± 12 nm; detection range: $\lambda \geq 510$ nm; image size: 400 μm $\times$ 400 μm). The total light dose for recording each focal plane is 0.05 J cm^{-2}.

experiments. This problem may be overcome by single plane illumination microscopy (SPIM) (Huisken *et al.*, 2004; Santi, 2011), where the sample is illuminated by a light sheet in perpendicular direction to the observation path. This light sheet can be generated with a typical diameter of 1−10 μm by a cylindrical lens or by scanning a laser beam. Thus, only those planes are exposed to light, which are examined simultaneously, and even upon successive measurements of multiple planes light exposure of the whole sample is similar to light exposure during a single plane experiment and, therefore, comparably small. This permits prolonged exposure times until the maximum tolerable light dose (as depicted in Table 1) is attained.

Light exposure has also been minimized in epi-illumination (e.g. laser scanning) microscopy by nonuniform illumination, where excitation light was adapted to the local concentration of fluorophores (Hoebe *et al.*, 2007, de Vos *et al.*, 2009). In particular, by using an electronic feedback system, illumination was reduced in regions of high fluorescence as well as in the background between fluorophores. This controlled light-exposure microscopy (CLEM) reduced phototoxicity and photobleaching considerably without affecting image quality. In this context, however, it should be mentioned that almost no photobleaching was observed at light doses considered in this paper.

Conclusion

Present investigations may be regarded as a first step towards a quantitative description of cell viability in fluorescence microscopy under different light exposure. Measurements include various fluorescent dyes and excitation wavelengths as well as a comparison between TIR and epi-illumination. SPIM and CLEM are suggested as appropriate methods to reduce cell damage in 3D microscopy. Mechanisms of cell damage are still rather unknown, but possibly related to phototoxic or photosensitizing properties of endogenous molecules as well as exogenous fluorescent markers. Integral light dose has been found to be a key parameter for maintaining cell viability. However, some preferential damage by short, high power light pulses or some recovery upon prolonged exposure times cannot be excluded. Finally, cell viability in nonlinear, e.g. multi-photon microscopy has not been addressed by the present experiments.

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Supporting Information

Additional Supporting Information may be found in the online version of this paper:

Supplemental Figure. (a) Flow cytometric analysis of CHO-K1, CHO-K1-AcGFP1-Mem and CHO-K1-AcGFP1-Mito cells (number of cells vs. AcGFP fluorescence intensity per cell). Both transfected clones reveal homogeneous and strong expression of the respective fluorescent marker protein. When compared to green autofluorescence of parental CHO-K1 cells, the transfected clones reveal approx. 1000-fold elevated

mean fluorescence values. Correct targeting of the respective fluorescent marker ((b) cellular membranes for CHO-K1 AcGFP1-Mem, (c) mitochondria for CHO-K1 AcGFP-Mito) was ascertained by fluorescence microscopy.

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