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Phototoxicity in live fluorescence microscopy, and how to avoid it

Jaroslav Icha [1)†], *Michael Weber* [2)*,†], *Jennifer C. Waters* [2)] *and Caren Norden* [1)*]

Phototoxicity frequently occurs during live fluorescence microscopy, and its consequences are often underestimated. Damage to cellular macromolecules upon excitation light illumination can impair sample physiology, and even lead to sample death. In this review, we explain how phototoxicity influences live samples, and we highlight that, besides the obvious effects of phototoxicity, there are often subtler consequences of illumination that are imperceptible when only the morphology of samples is examined. Such less apparent manifestations of phototoxicity are equally problematic, and can change the conclusions drawn from an experiment. Thus, limiting phototoxicity is a prerequisite for obtaining reproducible quantitative data on biological processes. We present strategies to reduce phototoxicity, e.g. limiting the illumination to the focal plane and suggest controls for phototoxicity effects. Overall, we argue that phototoxicity needs increased attention from researchers when designing experiments, and when evaluating research findings.

Keywords:
light sheet fluorescence microscopy; live imaging; photobleaching; phototoxicity; photodamage; reactive oxygen species; selective plane illumination

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[1)] Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany
[2)] Department of Cell Biology, Harvard Medical School, Boston MA, USA

***Corresponding authors:**
Michael Weber
michael_weber@hms.harvard.edu
Caren Norden
E-mail: norden@mpi-cbg.de

Abbreviations:
CLEM, controlled light-exposure microscopy; **GFP,** green fluorescent protein; **LSFM,** light sheet fluorescence microscopy; **NA,** numerical aperture; **ROS,** reactive oxygen species; **TIRF,** total internal reflection fluorescence.
[†] These authors contributed equally.

Introduction

Live imaging using fluorescence microscopy is a very popular and well-established tool in biology. Imaging processes in a living specimen is a powerful experimental approach that provides insights into the inner workings of a cell or into tissues during developmental processes. However, fluorescence microscopy requires the use of strong light to excite fluorophores in the sample, and therefore is a potentially invasive experimental method. Excitation light exposure can have significant phototoxic side effects in many live samples from cells to organisms [1–8]. As a result, the reliability and reproducibility of the obtained image data and the biological conclusions drawn from them can be negatively affected. This is especially true of imaging with high spatial or temporal resolution, which is often required to analyze and understand dynamic cellular processes. In many cases, the phototoxicity effects are not immediately apparent when samples are inspected visually and thus require more sophisticated controls. Therefore, to obtain reliable data from live imaging microscopy experiments, it is critical to ensure that the effects of imaging on the sample are minimal and well understood.

Here, we summarize various approaches to becoming aware of, and being able to limit, phototoxicity that occurs during live fluorescence microscopy. We review possible modifications to conventional imaging buffers, discuss advantages and disadvantages of different fluorescent tags, and cover diverse illumination approaches that at the same time need to be combined with efficient fluorescence signal detection. Limiting illumination of the sample to the focal plane is highlighted as a very powerful strategy for reducing phototoxicity. Overall, the implementation of these strategies should reduce photodamage in living samples. This in turn will enable the collection of more reliable and reproducible image data in high resolution, and the observation of samples for extended periods of time. Although many potential difficulties that can occur during live imaging experiments are discussed in this review, we want to emphasize that when such experiments are carefully designed and controlled, they can deliver unprecedented information about cellular and developmental processes.

Reactive oxygen species are the main cause of phototoxicity during live imaging

Phototoxicity in fluorescence microscopy can originate from different sources. Organic molecules such as flavins and porphyrins, naturally present in cells, absorb light at visible wavelengths, and subsequently become degraded once they react with oxygen [9–11]. This process produces reactive oxygen species (ROS). ROS, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, can damage cells in various ways. They can directly oxidize DNA and potentially cause mutations, or they oxidize proteins and unsaturated fatty acids in lipids rendering them non-functional [12]. In addition, excess ROS can oxidize enzyme cofactors, or globally change the redox state of the cell cytoplasm and mitochondria [12].

Additional sources of ROS are the fluorophores introduced into cells as labels or probes. When fluorophores are in the excited state, they can react with oxygen and become degraded. This is commonly referred to as photobleaching. During fluorophore photobleaching, ROS are produced in a way similar to the process occurring during degradation of the naturally present light absorbing molecules [2, 13]. The resulting ROS may cause phototoxicity. Thus, photobleaching and phototoxicity are closely connected. Nevertheless, they are two distinct phenomena, and under some circumstances phototoxicity can occur without photobleaching and vice versa.

The photosensitizing effect has been shown for most fluorophores, including the regularly used fluorescent proteins and organic dyes such as Acridine Orange, Rhodamine 6G, Fluo-4AM, and Laurdan [2, 10, 13, 14]. Such reactions of excited fluorophores with oxygen must be accepted as a constraint in most live imaging experiments, because oxygen is typically required for respiration of the samples, and cannot be removed from the imaging media. Taken together, ROS production and degradation of endogenous molecules are the main contributors to phototoxicity during live fluorescence microscopy.

Living systems have developed multiple ways of detoxifying ROS. These systems are naturally present in cells, and include endogenous antioxidants and ROS scavengers. The most abundant endogenous ROS scavengers (also called antioxidants) are glutathione, ascorbic acid, tocopherol, among others. These small molecules are complemented by the enzymatic antioxidants, mainly superoxide dismutase, glutathione peroxidase, and catalase. This combined system of ROS scavengers has a limited capacity to detoxify ROS that form as a byproduct of excitation light illumination, and therefore phototoxicity can be further reduced by supplementing the imaging medium with additional antioxidants [20]. The recommended additives are ascorbic acid [5, 13, 21] and the flavonoid rutin [22], which can buffer ROS to some extent. However, the benefit of ascorbic acid needs to be tested in every setup [23]. In summary, changing the composition of imaging buffers can reduce ROS toxicity.

To further optimize the conditions for fluorescence imaging, some molecules can be withdrawn from the media before the experiment, at least in cell culture. It was shown that removing the vitamins riboflavin or pyridoxal from imaging medium lowered phototoxicity and GFP bleaching [20, 24]. Importantly, the absence of vitamins does not affect cell survival for at least 5 days [24]. Commercial additives and media for live imaging based on the ideas described above are also available. In conclusion, natural and artificial ROS scavengers and antioxidants can counteract the damage to the sample caused by illumination. For a discussion on how the photosensitization effects of fluorophores vary with subcellular localization, refer to Box 1.

Sample morphology is not a reliable readout for phototoxicity

The first step to counteract phototoxicity is to recognize it. Various predominantly morphological indicators of

Box 1

Photosensitizing fluorescent proteins: Intracellular localization of fluorophores matters

The extent to which fluorophores increase photosensitivity of a live sample depends on their localization in the cell. This can be demonstrated with fluorescent proteins that were designed to exploit the photosensitizing effect to kill cells, e.g. KillerRed [15] or miniSOG [16]. These proteins were optimized for high ROS production upon illumination. The high ROS production of KillerRed is explained by presence of a water-filled channel leading from the protein surface directly to the chromophore [17, 18]. This enables electron transfer from an external moiety to the chromophore and subsequent reaction with oxygen to form a superoxide radical. The exposed chromophore is a special feature of KillerRed, whereas standard fluorescent proteins have the chromophore typically buried in their hydrophobic core. Practical experience from work with photosensitizing fluorescent proteins shows that membrane-associated and mitochondrially localized KillerRed are the most potent inducers of cell death, which occurs due to membrane lipid oxidation and apoptosis induction, respectively [15, 19]. In contrast, KillerRed localized to cytoplasm is a much less potent inducer of cell death, likely because the ROS generated cannot reach critical levels there (unpublished observation: information on Evrogen company website). This implies that illumination conditions safe for a particular subcellular localization of a fluorophore might become toxic when another structure in the cell is labeled, even with the same fluorophore.

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Table 1. Instances of subtle phototoxicity

Affected process without morphological changes	Model system	Microscopy method	Reference	Countermeasure
Anomalous leukocyte activation and adhesion in the skin	Syrian golden hamster	Wide-field (intravital)	[26]	More sensitive detection
Reduction of the dynamic range of calcium pulses in neurons in brain slices	Rat	2-photon laser scanning	[21]	Lower illumination intensity, ascorbic acid
Reduction of the dynamic range of calcium pulses in neurons in brain slices	Rat	2-photon laser scanning	[40]	Not tested
Slower migration of growth cones of motor neurons	*C. elegans*	Laser scanning confocal	[41]	Not tested
Occurrence of calcium transients in chondrocytes	Cow	Laser scanning confocal	[5]	Lower illumination intensity, ascorbic acid
Long range interphase chromosome movements stopped	Chinese hamster ovary cells (CHO DG44)	Wide-field	[39]	Filtering the UV and blue wavelengths out of the lamp excitation light, lower illumination intensity
Slowing down of sperm flagellum beating, increase in intracellular Ca^{2+} concentration	Human	Wide-field	[27]	Pulsed illumination
Loss of mitochondrial membrane potential, slowing down of cell cycle	Human retinal pigment epithelium cell line	None	[28]	Lower illumination intensity
Reduction of the dynamic range of calcium pulses in neurons in brain slices	Rat	2-photon laser scanning	[38]	Pulse splitting (creating more pulses of lower intensity from one larger pulse)
Slowing down of cell cycle	*S. cerevisiae*	OMX	[29]	Lower illumination intensity
Slowing down of embryonic development	*C. elegans*	Spinning disk confocal	[30]	Lower temporal resolution
Slowing down of embryonic development	*C. elegans*	Spinning disk confocal	[31]	Lower temporal resolution, using light sheet microscopy instead
Slowing down of embryonic development	*C. elegans*	Spinning disk confocal, wide-field	[7, 25]	Longer exposure with lower intensity, finding phototoxicity threshold and staying below it
Reduction of the dynamic range of calcium pulses in neurons in brain slices, change in kinetics of voltage responses	Rat	2-photon laser scanning	[37]	Pulse splitting (creating more pulses of lower intensity from one larger pulse)
Longer time spent in mitosis	Human fibrosarcoma cell line (HT1080)	Wide-field	[1]	Pulsed illumination
Slowing down of microtubule growth	HeLa cells	Wide-field	[13]	Longer excitation light wavelength, pulsed illumination, ascorbic acid
Relaxation of cellular contractility	African green monkey kidney cells (Cos-7)	Wide-field	[32]	Longer excitation light wavelength, lower illumination intensity
Changed protrusive activity, loss of mitochondrial membrane potential	Chinese hamster ovary cells (CHO-K1)	Laser scanning confocal	[33]	Pulsed illumination
Slowing down of cell cycle	Human retinal pigment epithelium cell line	Wide-field	[34]	Culture at low (3%) oxygen concentration, Trolox, longer excitation light wavelength
Loss of mitochondrial membrane potential, mitochondria fragmentation and changed dynamics	*S. cerevisiae*	Wide-field	[35]	Lower illumination intensity, lower temporal resolution
Changed dynamics of aggregates of NEMO, a membrane-associated protein regulating NF-κB pathway	Human embryonic fibroblasts	Spinning disk confocal	[25]	Lower illumination intensity, using very sensitive camera
Slowing down of neuronal migration	Zebrafish	Spinning disk confocal	[36]	Using light sheet microscopy instead
Slowing down of cell cycle	Zebrafish	Spinning disk confocal	This article	Using light sheet microscopy instead

phototoxicity in live imaging experiments have already been established, e.g. membrane blebbing, vacuole formation, mitotic arrest, nuclear fragmentation and eventually cell death [2, 6]. However, long before cells start to display such morphological changes they might already be negatively affected by the illumination (Table 1). Phototoxicity can take many forms, including slowing down of the cell cycle and other processes, changes in cytoplasmic Ca^{2+} levels, and loss of mitochondrial membrane potential (Table 1). In practice, such subtle phototoxicity has a higher potential to influence experimental conclusions, as its effects on cell behavior are not immediately obvious. They may often go unnoticed without the appropriate controls to account for them. In one striking example, the particles of membrane-associated protein NEMO seemed to move by active transport. However, careful controls revealed that these particles moved by restricted diffusion, and that the active transport was a phototoxicity artifact [25]. Phototoxicity that occurs during short experiments, in which the imaging time window is too short for the damage to the sample to become apparent, is more likely to pass unnoticed. In conclusion, the fact that the sample has normal morphology and its fluorescence did not bleach are not sufficient indicators of sample health during imaging. Thus, performing careful controls and taking active measures against phototoxicity are essential to obtaining reproducible quantitative data, as documented in (Table 1).

Living samples show different sensitivity to light exposure

As discussed, illuminating living specimens during an imaging experiment can induce the formation of ROS, and living organism's ROS scavenging mechanisms provide limited protection from phototoxic effects. Throughout evolution, these mechanisms have adapted to organism's individual habitat and type of metabolism. However, the resistance to generated ROS is still hard to predict, and needs to be tested experimentally. For example, *Drosophila* and *Caenorhabditis elegans* appear to be more resistant to laser light exposure than zebrafish embryos, cultured cells, or corals [6, 42]. In addition, the fact that HeLa cells were found to be more light-resistant than the U2OS cells [13] shows that even different human cell lines can vary in their resistance toward illumination.

Furthermore, in experimental conditions like the presence of mutations or exposure to drugs, the specimen's sensitivity to light exposure can increase. An example of this additive effect was shown by an imaging experiment with mammalian cells lacking centrosomes. Early studies suggested that this condition resulted in cell cycle arrest [43]. However, another study later demonstrated that with a reduced light exposure during imaging, such cells progressed through the whole cell cycle [44]. Thus, to keep phototoxicity effects to a minimum, testing the cell's or organism's individual sensitivity to light exposure should be included when designing an experiment. Frequently, live cell imaging using fluorescence microscopy will demand compromises. On the one hand, a certain level of light exposure is required to excite fluorophores and provide enough signal to achieve the signal-to-noise ratio and the spatial and temporal resolution that is necessary to analyze the process of interest. On the other hand, the goal should always be to assure data quality and reproducibility by minimizing the energy load for the specimen.

Limiting illumination and fluorophore excitation to the focal plane can reduce phototoxicity

The most sensible way of gentle fluorescence excitation in living samples is to selectively illuminate only the focal plane of interest (Fig. 2A). However, common fluorescence microscopes, such as wide-field or confocal microscopes, utilize epi-illumination. Here, the excitation light is delivered to the focal plane along the detection axis and is absorbed by the specimen above and below said plane. Consequently, phototoxicity is induced throughout the preparation [45–47]. When z-stacks are recorded during 3D (volumetric) imaging, the entire sample is exposed to light for every plane that gets imaged, and phototoxicity increases by a factor equal to the number of imaged planes. To circumvent this issue, three illumination schemes achieving selective sample illumination have been developed: Total internal reflection fluorescence (TIRF), two-photon excitation and light sheet illumination (Fig. 2A). These techniques differ with regards to technical implementation, applicability, and challenges.

TIRF microscopy

In TIRF, a laser beam is positioned at the edge of the objective's back aperture or scanned along it, resulting in an oblique illumination of the coverslip. When the objective's numerical aperture (NA) is high enough, the laser beam is totally internally reflected at the interface between coverslip and sample medium with a lower refractive index. This generates an evanescent wave that propagates in the axial direction away from the coverslip. The evanescent electromagnetic field decays exponentially and can penetrate 100–300 nm into the sample [48]. Only fluorophores present within this depth are excited, while the rest of the sample is not exposed. Thus, TIRF microscopy is ideal for processes that take place at or close to the plasma membrane, e.g. exocytosis or focal adhesion turnover [49, 50]. TIRF microscopy has been shown to be less phototoxic than wide-field microscopy [10, 14]. An obvious caveat of this technique is that it is restricted to a single plane, and cannot be applied for imaging deeper into the sample or for volumetric imaging. An approach called highly inclined thin illumination (HILO) combines a TIRF-like illumination at sub-critical angle with the capability of imaging multiple planes in close proximity to the coverslip [51].

Two-photon microscopy

Two-photon microscopy is a laser-scanning, non-linear microscopy technique that aims to limit fluorescence excitation to the focal volume [52, 53]. To achieve this, a laser pulsed at high frequency generates a high photon density at its focal point. This can result in near-simultaneous absorption of two photons by a

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fluorophore, leading to so-called two-photon excitation. The utilized laser emits light of approximately twice the wavelength that would be required for single-photon excitation of the fluorophore. Therefore, fluorophore excitation outside of the focal volume with its high photon density is reduced. In addition, the longer wavelength of the excitation light results in decreased light scattering in biological tissue, thereby increasing the penetration depth. In combination with the inherent optical sectioning, two-photon microscopy is especially popular for in vivo imaging deep inside larger living organisms [54].

Having said that, phototoxicity of two-photon excitation is even more difficult to assess and control than that of conventional linear illumination. Although two-photon fluorescence excitation is limited to the focal plane, the sample is still exposed to high doses of light above and below this plane, carrying the risk of linear damage. Especially in pigment-rich tissue, such as skin or retina, single-photon absorption and heating are the dominant sources of photo-induced damage. This can result, for example, in cavitation and morphological changes [55, 56]. Linear damage is reduced by using shorter laser pulses. It has been demonstrated that splitting a pulse into shorter sub-pulses reduces photobleaching in live *C. elegans* larvae and photodamage in rat hippocampal brain slices [38]. However, two-photon microscopy utilizes high peak intensities from the start. Whereas peak excitation intensities can reach 10 $nW/\mu m^2$ in wide-field microscopy, they might approach 10 W (a factor of 1,000,000,000) for similar imaging results in two-photon microscopy [57]. Not surprisingly, photobleaching rates in two-photon microscopy were shown to be higher than in single-photon excitation at power levels typically used in biological imaging, likely caused by higher-order photon interactions [58]. In addition to linear damage, two-photon excitation results in non-linear damage mechanisms, which are major contributors to phototoxicity in cells [59, 60]. The near-simultaneous absorption of two photons triggers ROS formation and can induce direct DNA damage and plasma formation [61, 62]. Overall, the increased performance in deep tissue imaging remains the major asset of two-photon microscopy, with the reduction of phototoxicity being much less tangible [37, 63, 64].

Light sheet fluorescence microscopy (LSFM)

Another technique that limits light exposure of the specimen to the focal plane is light sheet illumination. In LSFM, the sample is illuminated with a light sheet from an objective oriented perpendicular to the detection axis. Fluorescence is only excited around the focal plane of the detection objective, and the collected light is recorded with a camera. Although LSFM often requires modifications in the experimental design and has illumination-specific challenges, it is the only fluorescence microscopy technique enabling volumetric imaging without exposing the sample to light beyond the focal plane. It therefore reduces the energy load on the specimen, while at the same time provides optical sectioning [65]. Thus, LSFM is considered to be the gentlest fluorescence microscopy technique available to date [66, 67].

For this review, we performed two qualitative comparisons of photobleaching (Fig. 1A and B) and phototoxicity (Fig. 1C and D) between confocal and LSFM setups. In the first set of experiments, cultured cells containing a marker for growing microtubule tips mEmerald-EB3 were imaged for 15 minutes at two different systems (Fig. 1A and B). While the EB3 signal quickly faded in the spinning disk microscope, little photobleaching of the sample was detected in the LSFM setup.

In a second experiment, we compared a published dataset of cell cycle phase lengths of zebrafish retinal neuronal progenitors acquired in several confocal setups [68] to data acquired in LSFM (Fig. 1C and D). Compared with the latter, the cell cycle phase's length in the confocal dataset was prolonged with the G1 and S phases being most affected, likely due to phototoxicity in the confocal setups. The imaging conditions in both experiments were similar and differences in sample mounting were also not responsible for the observed differences between the setups, as shown previously [36]. Therefore, light sheet illumination likely reduces both photobleaching and phototoxicity in living samples. Similar observations were made while imaging *C. elegans* embryogenesis, which could be observed at 30 times higher time resolution in the light sheet microscope due to its lower phototoxicity compared to the spinning disk confocal [31]. Other study found that spinning disk imaging is inherently more phototoxic than wide-field microscopy [7]. It should be noted, though, that samples and processes can vary in their sensitivity to light, and for samples that are more resistant to light, the use of spinning disk confocal microscopy is not inherently problematic. For example, the process of *Drosophila* dorsal closure occurred with similar kinetics when observed with LSFM or spinning disk confocal microscopy [69].

Light sheet illumination greatly reduces phototoxicity, however, depending on how the light sheet is created, the degree of improvement varies. Two major approaches of generating a light sheet exist: in one system, a static sheet can be created by sending an expanded laser beam through a cylindrical lens [70]. In another system, a laser beam that is scanned across the field of view within the exposure time of the camera generates a scanned or "virtual" light sheet [71]. A scanned light sheet is the more flexible technique, because the dimensions of the sheet are easily adapted to the field of view. In addition, the use of a single laser beam allows the researcher to implement interesting beam shaping options, e.g. Bessel beam illumination, as well as the combination with two-photon excitation, confocal line detection, or spectral detection [72–75]. However, when high acquisition speed needs to be achieved, the line-by-line illumination utilized for creating a scanned light sheet requires high peak power. As mentioned before, high peak intensities can overburden the intrinsic ROS scavenging mechanisms of living organisms, and result in photo-induced damage [76]. Reports about the impact of beam shaping, e.g. Bessel or Airy beam illumination, on photobleaching rates have been conflicting: one study found higher bleaching rates with beam shaping while another study stated the opposite [77, 78]. For many applications in light-sensitive living organisms, a static light sheet remains the gentler solution. Compared with a scanned light sheet – and assuming the same sheet thickness, using the same total exposure time and resulting in an identical signal-to-noise ratio – a much lower peak intensity is continuously spread across the entire field of view for a longer time, resulting in lower phototoxicity [66, 67, 79].

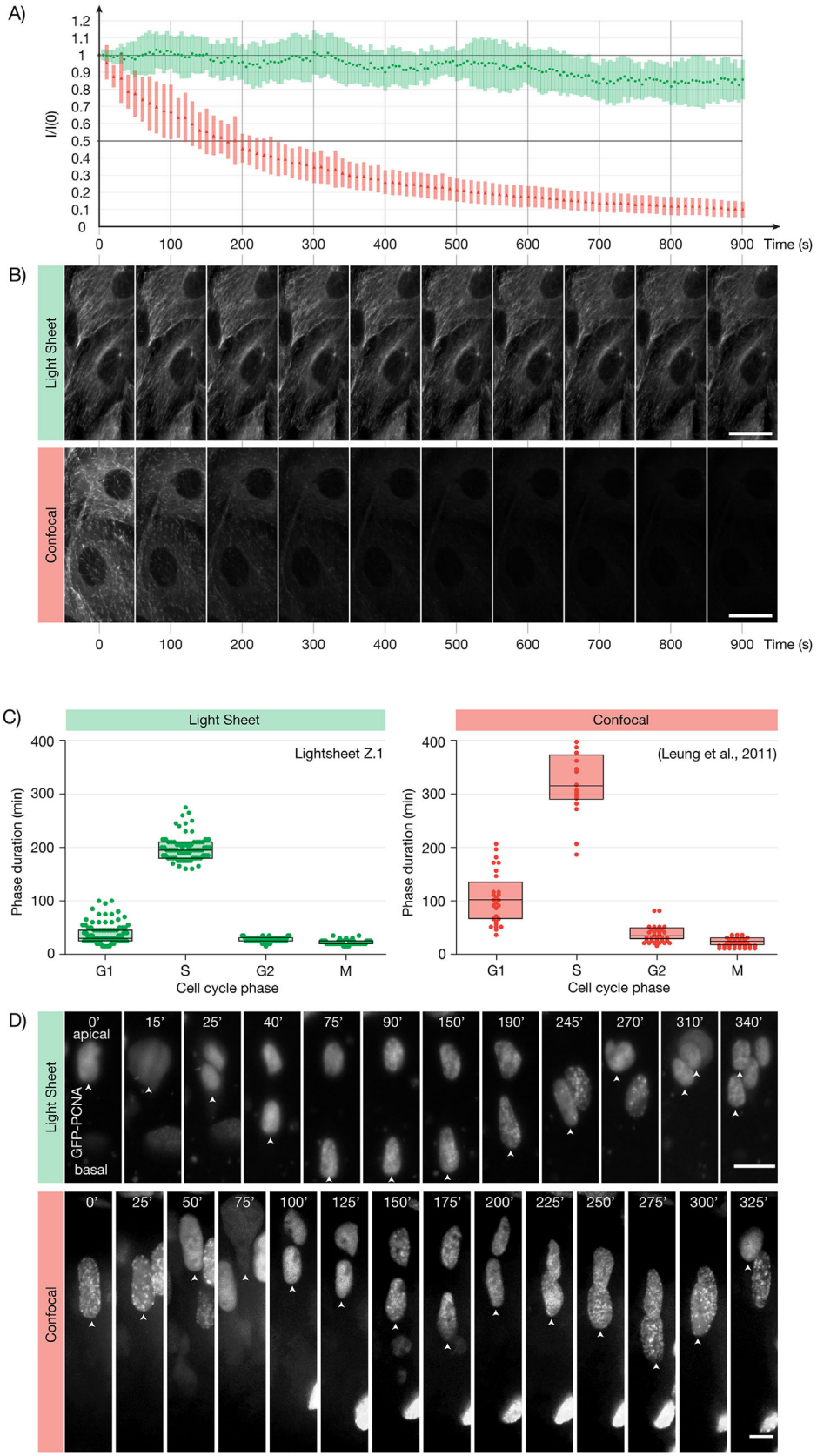


Figure 1. Reduced photobleaching and phototoxicity by selective illumination of the focal plane. Comparison of photobleaching during volumetric imaging using light sheet and spinning disk confocal microscopy. LLC-PK1 mEmerald-EB3 cells were grown on glass and kept at 37°C until imaging. Transferred to Gibco Leibovitz's (1×) L-15 Medium with 5% FBS and Pen/Str and kept at room temperature during imaging. Volumetric image data recorded with 0.7 μm z-steps over a course of 15 minutes. Laser excitation 488 nm, emission filter BP 525/50. Imaged on two different microscopes with settings adjusted for similar initial signal-to-noise ratio. Light Sheet (green): Nikon 40×/0.8 W objective, ASI diSPIM arm, scanned light sheet, single-sided illumination and detection, Hamamatsu Orca Flash4.0 sCMOS camera, 30 ms/plane, 5 s interval, 60 planes/time point, 12,000 planes in total. Confocal (magenta): Nikon 40×/1.2 W objective, Nikon Ti inverted microscope, Yokogawa CSU-X1 scan head with Borealis modification, Hamamatsu Orca-ER, 300 ms/plane, 10 s interval, 11 planes/time point, 1,100 planes in total. **A:** Average intensity (I) normalized to intensity at time point 0 (I_0) of 10 small random regions of interest in different cells and planes. Intervals around mean indicate standard deviation. **B:** Maximum intensity projections of a 30 × 80 μm region of interest. Scale bars: 20 μm. Cell cycle phases of retinal neuronal progenitors in the zebrafish retina are prolonged when imaged in a confocal microscope. The nuclei were labeled by GFP-PCNA, and the imaging of retinas started around 28 hpf. Images were taken every 5 minutes for 12–16 hours at 28.5°C. Light sheet imaging was performed using the Lightsheet Z.1 (Zeiss). A 40–70 μm z stack was acquired with 1-μm steps in a single view, dual-sided illumination mode. Images were taken using the 10×/0.2 illumination objectives, a Plan-Apochromat 40×/1.0 W detection objective (Zeiss) and the two PCO.Edge 5.5 sCMOS cameras. The spinning disk and point scanning confocal setups are described in [68]. A 30-μm z stack was acquired with 1-μm steps. **C:** The top and bottom of each box indicate upper and lower quartiles; the horizontal line represents the median. Light sheet: The mean cell cycle length G1 = 38 min, S = 199 min, G2 = 27 min, M = 22 min. Confocal: A single data point from S phase dataset (660 min) was omitted in the graphic representation of the dataset for more illustrative plotting. The mean cell cycle length G1 = 108 min, S = 337 min, G2 = 40 min, M = 25 min. Confocal data adapted with permission from [68]. **D:** Selected time points from the recording. Time is given in minutes. Scale bar Light sheet: 10 μm. Confocal: 5 μm. Confocal data adapted with permission from [68].

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Lattice light sheet microscopy is an interesting middle ground that generates a light sheet by dithering a lattice of Bessel beams, allowing the reduction of peak intensities compared with a scanned light sheet, while providing increased depth of penetration and reduced scattering at the same time [76]. Independently of the way the light sheet is created, it reduces photodamage when compared with common illumination strategies in fluorescence microscopy.

Until now, light sheet illumination has been implemented in several microscope configurations to accommodate a variety of specimens from single cells to entire embryos [80]. It has enabled imaging of biological processes at all scales, from fast intracellular events to whole embryo development [31, 81–86]. Looking forward, we anticipate a continuing development and spread of LSFM in the field of live imaging.

A reduced peak intensity has a positive impact on sample health

Independent of the type of fluorescence microscopy, a straightforward way to reduce the energy load on the specimen is to reduce overall light exposure. In a study of zebrafish craniofacial bone development, phototoxicity-induced shape changes could be suppressed simply by doubling the interval between time points [3]. In another study, scientists imaged pre-implantation mouse embryos and again, doubling the interval between time points greatly improved the viability of embryos [87]. Similarly, lowering the initial illumination intensity by a factor of 100 made it possible to monitor dynamic behavior of chromosomes over the entire cell cycle in yeast without compromising sample health increasing the temporal resolution at the expense of signal-to-noise ratio and spatial resolution [29].

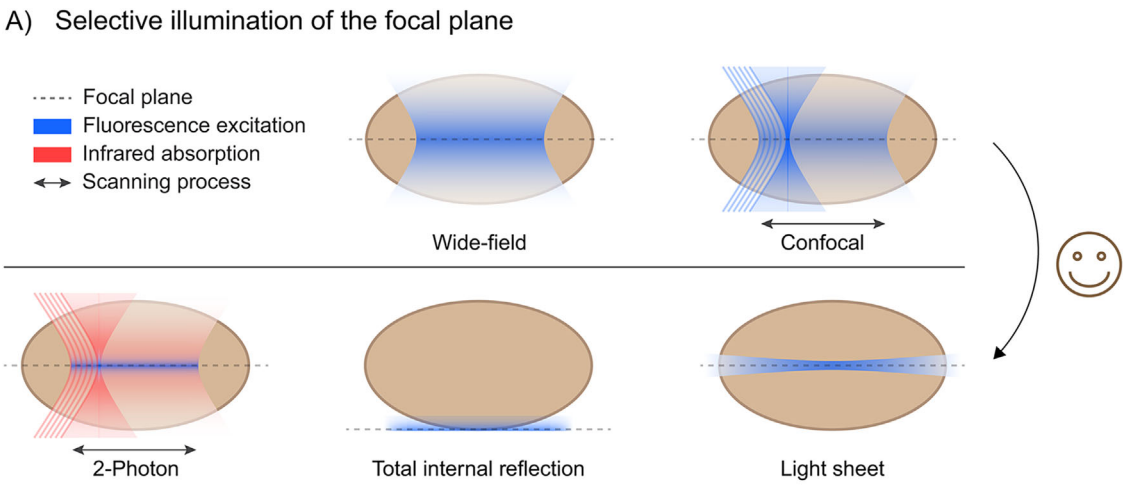
Alternatively, with the same total light exposure, phototoxicity in living organisms can be reduced by lowering the peak intensity and extending the exposure time accordingly (Fig. 2C). Studies investigating calcium waves in rat brain slices [21], tobacco cell mitosis [2], and *C. elegans* embryonic development [7] convincingly showed that living samples can make better use of their limited intrinsic ROS detoxification mechanisms when light is delivered at a lower rate.

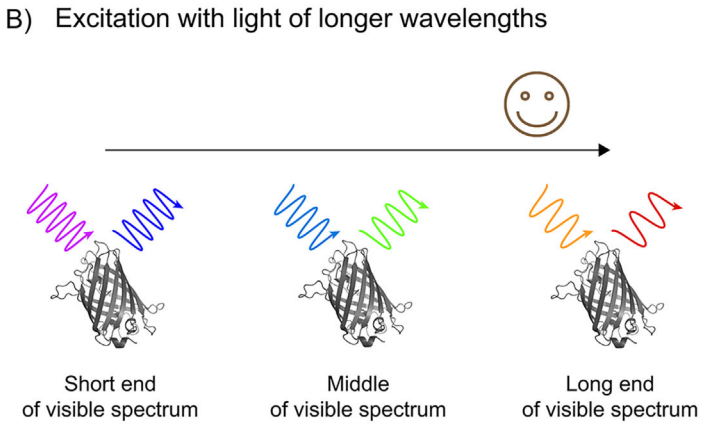
Understandably, a long exposure time decreases the maximum acquisition frequency, which might be unacceptable in imaging experiments where a high temporal resolution is required. Furthermore, it carries the risk of motion blur when imaging a moving object. However, as reducing the peak intensity and extending the exposure time do not require any modifications of existing microscopy hardware, they are easy to implement.

Pulsed illumination can reduce phototoxicity

A more advanced modification that can reduce phototoxicity in live fluorescent microscopy is pulsed illumination (Fig. 2D). Instead of constantly illuminating the specimen during the exposure time, light is delivered in pulses, with short temporal breaks in the microsecond range. The peak intensity and overall light exposure remain the same; only the exposure time is extended to accommodate the temporal breaks. Why pulsed illumination may reduce phototoxicity can be explained by taking a closer look at the fluorescence time scales. Completion of an entire fluorescence cycle (excitation from the ground state, vibrational relaxation, and emission while returning to the ground state) takes about 10 nanoseconds. However, instead of returning to the ground state and emitting light, excited fluorophores can also undergo intersystem crossing to an excited triplet state. Compared with the normal singlet state, the triplet state is much longer-lived. In vitro fluorescence correlation spectroscopy of a GFP mutant has shown that the triplet-state lifetimes of fluorophores can be in the microsecond range [88]. Continued illumination thus increases the chances of photon-induced chemical damage and covalent modifications of excited fluorophores, the most common source of photobleaching [89–91]. Temporal breaks in illumination give excited fluorophores in the triplet state time to return to the ground state, thus reducing the rate of fluorophore destruction. Studies using a pulsed LED for wide-field microscopy have shown that this method can lead to increased health of human sperm [27], reduced apoptosis [92], and a return to normal rate of mitosis in cultured cells [1]. Pulsed illumination also improved the metabolic state and viability of rat brain slices [9]. In another study, phototoxicity in live cell imaging was reduced by implementing pulsed illumination on a point scanning confocal microscope [33]. Because pulsed illumination may transition fluorophores into a dark or "off" state, in which they avoid entering the triplet state, the level of fluorescence emission is increased while photobleaching rates are reduced [93]. Furthermore, the period between two pulses may provide enough time for heat to dissipate away from the excitation site. Interestingly, some studies indicate that confocal microscopes scanning at a higher speed, such as resonant scanning or spinning disk confocal microscopes, may show less photobleaching. The temporal illumination pattern of such approaches mimics pulsed illumination, and the reduced photodamage could arise from a combination of photoactivation, – conversion and – switching of fluorophores. In the context of light sheet microscopy, this could argue for using a scanned light sheet over a static one. So far, details on the processes that change photodamage with pulsed illumination remain sparse. In addition, results suggest that pulsing is only beneficial in high excitation power levels as used in two-photon microscopy [73]. Nevertheless, maintaining the same total light exposure but pulsing the illumination has potential for reducing phototoxicity and can be implemented in standard confocal microscopes.

A technically elegant solution to reducing the energy input during laser scanning confocal microscopy is controlled light-exposure microscopy (CLEM, not to be confused with correlative light and electron microscopy) [94]. In CLEM, the laser illumination of a point is controlled via a feedback loop and stopped as soon as a pre-defined





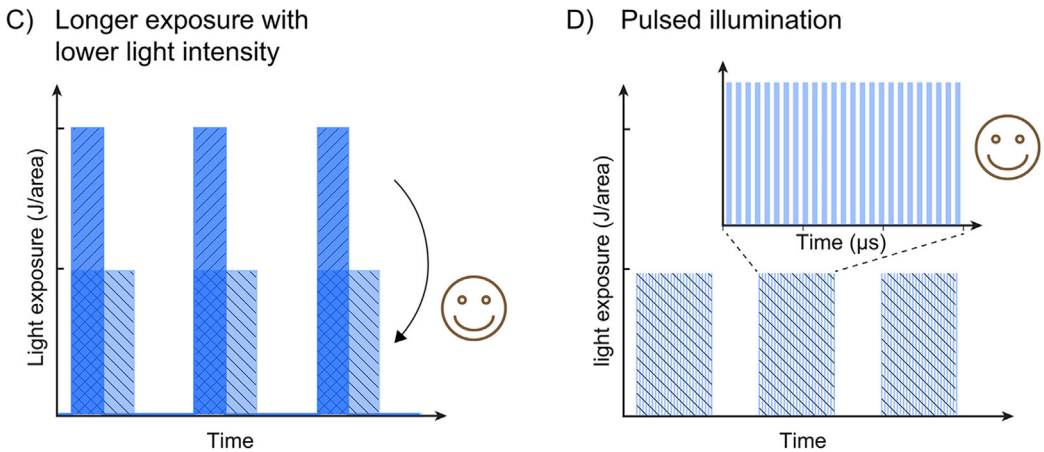


Figure 2. Illumination strategies for reducing phototoxicity. **A:** Selective illumination of the focal plane. In wide-field and confocal microscopy, the sample is exposed to light also above and below the focal plane. The same is true for two-photon microscopy but here longer wavelengths are used and fluorescence excitation is mostly limited to the focal plane. In total internal reflection and light sheet microscopy, the specimen is only exposed to light in the focal plane. Selective illumination is especially beneficial for specimens that are much thicker than the focal plane. **B:** Illumination with light of longer wavelengths. Using fluorophores with excitation shifted toward the longer end of the spectrum can reduce photodamage in live fluorescence microscopy. **C:** Longer exposure time with lower light intensity. Reduced peak light intensities and compensating with increased illumination time can lower photodamage. **D:** Pulsed illumination. Light pulses in the µs-range instead of constant illumination with the same overall light dose can reduce phototoxicity in live imaging.

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amount of fluorescence is collected by the detector. In areas of higher fluorophore density, the exposure is greatly reduced, hence minimally impacting the image quality. Every point's individual light dose is recorded to compensate for variations in exposure time per pixel. CLEM reduced photobleaching and phototoxicity 2- to 10-fold in U2OS and HeLa cells [95]. The major requirement for, and at the same time a limitation of CLEM, is the need to use a point scanning microscope.

Illumination with longer wavelengths decreases phototoxicity

There are big differences among commonly used excitation light wavelengths when it comes to phototoxic effects (Fig. 2B). In general, using longer wavelengths for fluorescence excitation is better for sample health. Blue light is more harmful compared with red or infrared light, due to its higher energy and the higher absorbance of biomolecules at blue wavelengths [2, 4, 11]. Light of 405 nm, used for the excitation of common DNA dyes, is considered unsuitable for live cell imaging. Even modest shifts in illumination from 488 to 514 or 546 nm appear to improve sample health during imaging [11, 13, 34]. However, it should be noted that the red- and far-red fluorescent proteins are less bright compared with green and, in particular, yellow and orange fluorescent proteins. Therefore, using a red fluorescent protein in an experiment will require higher intensity illumination. Ultimately, the least phototoxic option needs to be determined empirically. For further discussion see Box 2.

An important point to keep in mind is that components of conventional microscopes, such as lenses, mirrors, and detectors, are often designed for the visible spectrum, and perform worse for infrared wavelengths. Thus, the sample health that is gained by switching to longer wavelengths could potentially be foiled by the need to increase light exposure to compensate for a lower efficiency of the microscope components in that spectral range. For gentle fluorescence microscopy of live organisms, red- and far-red fluorophores should be favored, but performance of the microscope in that part of the spectrum also needs to be considered.

Efficient detection is important for gentle microscopy

The ideal fluorescence microscopy experiment that includes a living specimen yields images with a signal-to-noise ratio sufficient to analyze and interpret the process of interest, while at the same time causes as little photodamage as possible. Irrespective of how the sample is illuminated detection efficiency is pivotal to achieve this goal. A loss of fluorescence signal on the detection side needs to be compensated with either longer exposure time or higher excitation power – both of which result in higher energy load for the specimen.

Box 2

The right choice of fluorescent proteins can reduce phototoxicity

When choosing a fluorescent protein for an experiment, its brightness (quantum yield times extinction coefficient) should not be the sole considered parameter. In addition, high photostability (low photobleaching) should be prioritized. In some cases, the total number of photons obtained from a dimmer but more photostable protein can be higher than for its brighter relative.

As the photostability of fluorescent proteins has been measured extensively [96–99], this information should be used when planning imaging experiments. However, one needs to consider that the typically listed parameters of fluorescent proteins measured in vitro [98] are not necessarily transferred to in vivo applications. A quantitative comparison of fluorescent proteins in vivo was performed in yeast and in *C. elegans*. Both studies demonstrated that the relative performance of fluorescent proteins in brightness and photostability differs from in vitro studies and is distinct for each organism [100, 101]. Therefore, published numbers on the properties of fluorescent proteins should be confirmed in the model organism of choice under experimental conditions [102].

The photosensitizing effect, i.e. the amount of ROS produced upon illumination, of different fluorescent proteins can also be compared, however, this parameter is not always assessed in studies that introduce novel fluorescent proteins. The photosensitizing effect was measured for several red fluorescent proteins and GFP in bacteria and yeast, showing that the DsRed variants were less phototoxic than GFP. This study additionally revealed very high photosensitizing effect of the popular fluorescent protein mCherry [103]. Overall, even well-established fluorescent proteins should be carefully tested and compared with available alternatives.

A promising alternative to imaging fluorescent proteins is luminescence microscopy [104, 105]. Compared to fluorescence, luminescence imaging removes the use of excitation light, thus eliminating phototoxicity and also lowering the background due to the lack of autofluorescence. Newly developed luminescent proteins approach the brightness of fluorescent proteins and are available in several spectral variants [106], however spectral unmixing is necessary to separate the signals. In the future, luminescence microscopy might be a viable option for long term live imaging of various cellular structures, provided the chemiluminescent substrate can be delivered and maintained at sufficient concentration in the specimen.

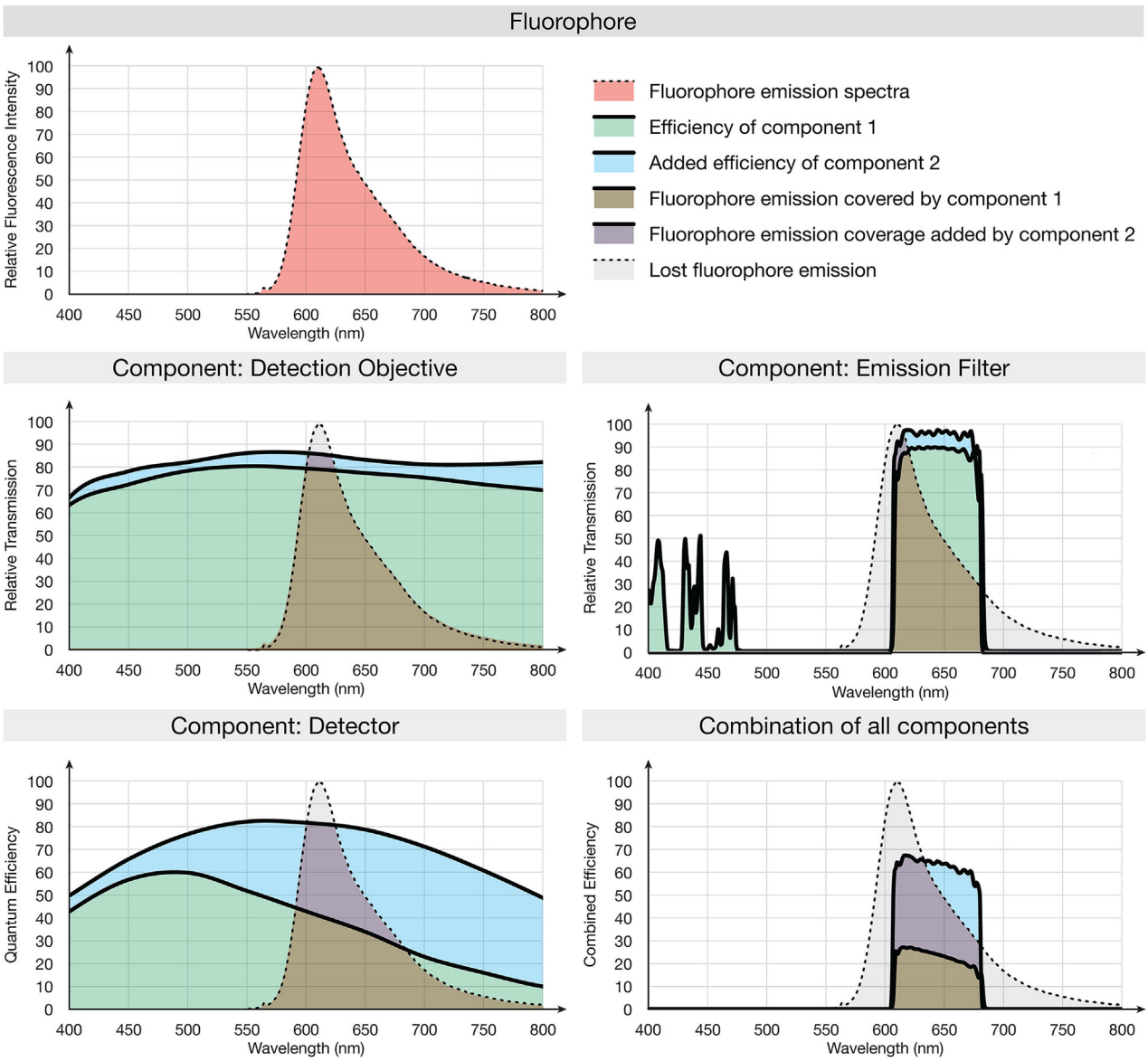


Figure 3. Contribution of microscope components to fluorescence detection efficiency. Selection of three commercial microscope components in two versions each, with their respective transmission/detection efficiency and fluorophore coverage. "Combination of all components" shows the multiplied efficiencies of options 1 and 2. Fluorophore example spectra: mCherry. Detection objectives: Olympus XLUMPLFLN-W 20×/1.0 (1) and Zeiss Plan-Apochromat 20×/1.0 W (2). Emission filters: Chroma HQ 645/75 (1) and Chroma ET 645/75 (2). Detectors: Andor Clara with Sony ICX285 interline CCD (1) and Hamamatsu Orca Flash4.0 v2 with sCMOS (2). Efficiency data retrieved from official datasheets or via a distributor.

The overall detection efficiency of a light microscope is a result of the numerical aperture, the transmission of the detection objective, the transmission of the dichroic mirrors, emission filters, and additional components in the detection path, as well as the quantum efficiency of the detector (Fig. 3). In addition, autofluorescence, bleed-through between channels, as well as detector noise, should be as low as possible to increase the signal-to-noise ratio. The detection path of a wide-field microscope consists of only a few optical components and is very light efficient. More complex detection paths that involve additional components, such as scan mirrors, pinholes, or additional beam shaping optics lead to increased light loss [93]. Many microscope component suppliers provide specifications to decide which parts provide the best efficiency for a given application, e.g. transmission curves for objectives and filters or quantum efficiency curves for detectors and cameras. These parameters need to be taken into account when planning an experiment [99, 107, 108]. A frequently overlooked aspect when trying to optimize the detection efficiency of a fluorescence microscope is background fluorescence. It can be minimized by using clean glass coverslips, clean objectives, a bandpass

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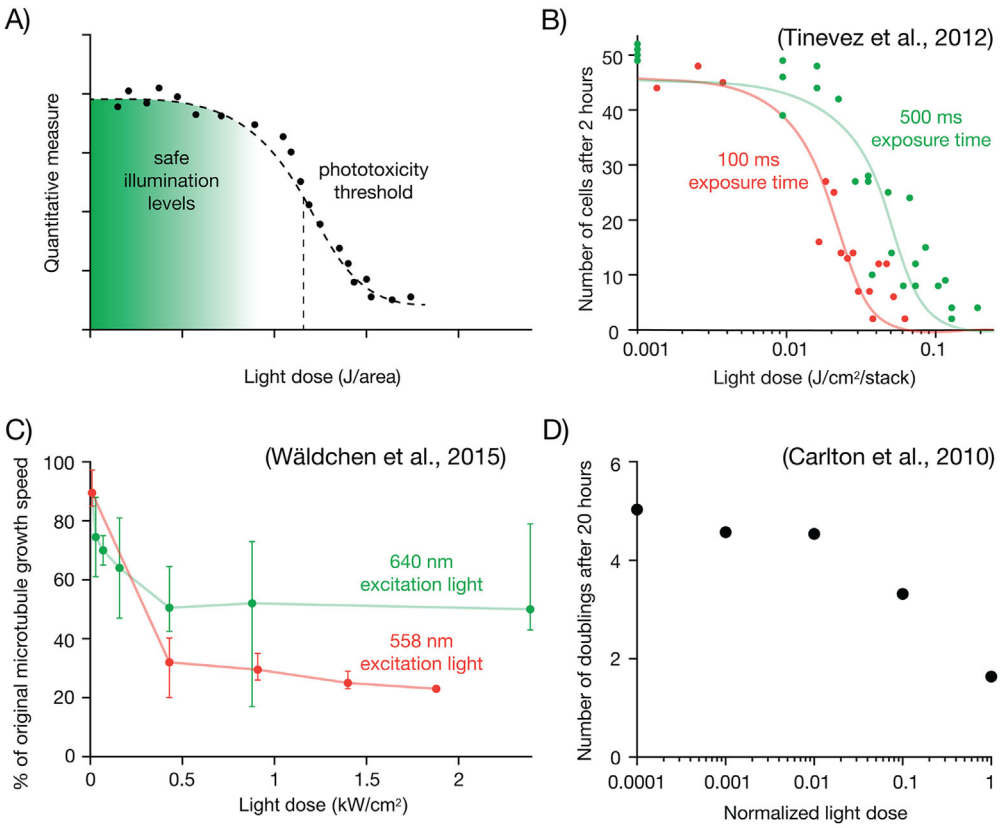


Figure 4. Finding safe levels of illumination from phototoxicity curves. **A:** A theoretical phototoxicity curve. Measuring the decrease of an example quantitative parameter in the living sample with increasing illumination should reveal a phototoxicity threshold. Depending on the parameter choice, it can also increase with increasing illumination. **B:** Phototoxicity curve adapted with permission from [7]. The first two hours of *C. elegans* development were imaged with different illumination intensities. At the end of the time interval the number of cells (N cell) in the embryo was counted as a proxy for health. Plotting the dependence of the number of cells on illuminating light dose gives sigmoidal phototoxicity curves, in which the safe light doses, phototoxicity thresholds and toxic light doses can be identified. The image shows two phototoxicity curves, acquired on the same wide-field system with the same total light exposure, differing only in the exposure time per slice. The *C. elegans* embryos can tolerate more than twice as much light, if it is delivered over longer exposure times as seen from the higher phototoxicity threshold of the green curve. **C:** Phototoxicity curve based on data from [13]. Deceleration of microtubule growth detected from EB1-YFP movement depends on the irradiation intensity at 558 nm (red) and 640 nm (green) (median ± interquartile range). Solid lines connecting the data points were used to guide the eye. **D:** Phototoxicity curve based on data from [29]. The number of yeast cell doublings observed during a 20 hour period is plotted as a function of excitation light intensity. Normalized intensity values of 1 or 0.1 lead to decreased division rate, whereas intensities of 0.01 or lower did not affect the speed of *S. cerevisiae* cell cycle.

fluorescence filter, and minimally fluorescent medium. This maximizes the signal-to-noise ratio and allows for a reduction in excitation power [109]. Even with every aspect of the microscopy optimized, it is important to perform controls that reveal the amount of phototoxicity, as discussed in the next section.

Monitoring phototoxicity in live fluorescence microscopy

Phototoxic effects of illumination might be apparent from inspecting the morphology of the sample. However, as discussed above, in many cases the sample physiology is affected long before any visible signs of toxicity occur (Table 1). Therefore, the optimal control experiment to detect phototoxicity, including its subtle manifestations, is not trivial. A good quantitative estimate of illumination toxicity can be extracted from measuring changes of a given quantitative sample parameter with growing illumination intensities. Plotting the dependence of that quantitative parameter on the illumination intensity will yield a phototoxicity curve, from which the safe levels of light exposure and the phototoxicity threshold for illumination can be extracted (Fig. 4A) [7, 25]. This phototoxicity threshold reflects the point at which the capacity of ROS scavenging systems in the sample is exhausted. Such phototoxicity curves were obtained by following the slowdown of cell division rates in the early stages of *C. elegans* embryonic development (Fig. 4B) [7]. Subsequently, that assay was used to compare different microscope setups and to optimize the imaging conditions [7]. In other studies, the quantitative parameters measured were the deceleration of microtubule growth (Fig. 4C) [13] or slowing down of the cell cycle in yeast (Fig. 4D) [29]. Ideally, such parameter would be connected to the actual research question, but it could also be one of the sensitive readouts shown in (Table 1), such as the time spent in mitosis [1], which was also suggested by others [11, 110].

A promising approach for the future, at least for cells in culture, is the use of convolutional neuronal networks to classify the subtle phototoxicity effects from transmitted light images [111].

Monitoring photobleaching is generally an unsuitable strategy to estimate phototoxicity. Observing no photobleaching in the sample is a good sign, however, in many live samples new fluorescent proteins are constitutively produced, making photobleaching an even more unreliable readout for phototoxicity. Performing controls for phototoxicity is essential for ensuring that the acquired image data reflect a healthy condition of the sample. Unfortunately, phototoxicity is hard to predict, and thus appropriate controls need to be performed for each novel experiment. Overall, suc controls should become a routine for researchers performing live fluorescence microscopy.

Conclusion and outlook

Whenever light is used to examine living samples, there is a risk that an excess of illumination may trigger experimental

Box 3

Reference card for reducing phototoxicity during live imaging

Experimental design

- Define the information you need from your sample:
  - Field of view, spatial resolution and sampling – How large is your structure of interest? What's the smallest structure that needs to be resolved? Do you need 3D image data? Is optical sectioning required?
  - Temporal resolution/sampling – How fast are the events of interest expected to be?
- Optimize your sample preparation
  - Choose fluorophores with excitation in the (far-)red spectrum and avoid the blue range, but double-check the microscope performance in that spectral range. Check for autofluorescence and try to avoid the parts of the spectrum that show high autofluorescence. Try to increase the spectral distance between fluorophores to reduce bleed-through.
  - Select the best sample mounting and medium. Avoid colored media that introduce background signal. Investigate whether you can use oxygen scavengers.
- Choose and optimize the right microscope
  - Choose the microscope based on the information you want to record from your specimen.
  - Choose the best objective for your experiment. Higher NA for the required field of view and the best transmission in the spectral range of interest should be preferred to increase the signal. Oil immersion objectives can provide the highest NA, but are only recommended for live imaging of thin samples or when using TIRF microscopy. Correct refractive index matching is important for live imaging inside thicker samples, where water, glycerol, or silicon oil objectives can outperform oil immersion objectives. Objectives with longer working distance but reduced NA might be required for deep tissue imaging. The higher depth of field of objectives with lower NA can be used to capture more information in a single image, if depth information is not a priority.
  - Check the specifications and quality of the filters used in the microscope. The filters should match emission spectra of the fluorophore as well as possible. The transmission should be close to 100%. Filters that show visible delamination of the coating or other damages should be replaced.
  - Check the quantum efficiency of the detector in the desired spectral range. Most detectors perform well in the green range but their efficiency declines toward the infrared. Multiple detectors with different quantum efficiency peaks could be combined for multi-color imaging. For most applications, sCMOS cameras are a superior choice over EMCCD cameras.
  - Check whether illumination "tricks" can be used or implemented, e.g. longer exposure with lower peak intensity or pulsed illumination.
  - If you need imaging techniques that require high energy input (confocal, super-resolution, etc.), think about repeating the experiment with a less intrusive setup (TIRF, light sheet, wide-field) as an additional control.
  - Use transmitted light instead of fluorescence lamp to set up an experiment and to find the region of interest in your sample for imaging. Later, use the transmitted light to monitor the health of your sample during imaging.

Controls before, during and after the experiment

- Record example data under realistic conditions and test your analysis pipeline. Adjust the acquisition settings or change the microscope, if necessary.
- Check your sample's health with transmitted light under your experimental conditions on the same microscope. Record a non-illuminated control region with transmitted light only in parallel to your experiment.
- Check for changes in cell cycle length, occurrence of membrane blebbing, delayed hatching, etc., between controls and experiment. Monitor the viability of the samples after the imaging experiment is completed, e.g. if cells will divide or embryos will survive.

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artifacts. In the worst case, this results in non-representative and irreproducible data. While most scientists are well aware of this problem, they often only consider those phototoxicity effects that are obvious and manifest as changes in the morphology of the observed specimens. This review highlights that safe levels of illumination cannot be judged solely by the specimen's morphology. Physiology of the living specimens can be altered already at much lower illumination levels, even when the specimens may seem healthy. Subtle phototoxicity invisible at the morphological level has the capacity to change the kinetics of cellular and developmental processes, leading to data misinterpretation. Here, we have summarized some effective strategies for minimizing phototoxicity, most of which are based on adjusting the illumination of the specimen. One recommended approach is to limit illumination to the focal plane. Furthermore, we suggest appropriate experimental controls that help with interpreting the live imaging data.

The problem that phototoxicity poses for live imaging experiments has also been highlighted by several recent journal editorials and technology features [112–115]. Thus, it is likely that in the future journal editors, reviewers, and all scientists using fluorescence microscopy will increasingly scrutinize live imaging data for phototoxicity effects. We hope that the readers will be encouraged by this review to adopt novel imaging techniques and to put more emphasis on the appropriate controls for sample health during imaging. For a discussion on these issues, see Box 3.

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