# Will you turn the light off for me?

**R. Elsaesser**[1], **P. Holroyd**[2] and **H. Wolff**[3]

[1] Carl Zeiss Microscopy, Carl-Zeiss-Promenade 10, 07745 Jena, Germany
[2] Carl Zeiss Microscopy, Kistlerhofstrasse 75, 81379 Muenchen, Germany
[3] Carl Zeiss Microscopy, Koenigsallee 9-21, 37081 Goettingen, Germany

One thing that microscopists and mythical vampires have in common, is that both of these species spend their days in dark, cool places and that they are in constant fear of bright light. For generations, light microscopists have done whatever they could to protect their precious samples from energy in the form of visible and invisible light. Unfortunately, it is commonplace and inevitable that you have to expose your sample to light if you want to obtain a light microscopic image. Therefore, in the past decades, microscopy instrument manufacturers have undertaken great efforts to develop sensitive and gentle imaging instrumentation. However, the microscope user must have a lot of knowledge to successfully reduce light exposure while scanning and imaging samples. Not only the process of image generation involves light, also several other steps in the entire workflow do so, such as sample preparation, staining and transfer to the microscope. All of these exposures to light could not only potentially affect the fluorescent labelling quality (e.g. by photobleaching), but might also reduce the viability of living specimens due to phototoxicity.

However, knowledge is very limited regarding what kind of light exposure actually harms fluorescent samples the most. Is it the ambient light during sample preparation? Or the exposure to sunlight when the sample is carried unprotected (or even in a box) from the lab to the imaging facility? Or is exposure on the imaging instrument itself the only harmful instance? And if so, what kind of instrument and illumination are most critical?

This chapter provides fluorescence microscopists with an idea how much influence the various kinds of light exposures during the imaging workflow will have on the viability of the specimen or its fluorescent labelling and staining quality. A range of recent types of imaging instruments will be compared and some advice will be given on how to minimize unavoidable light exposure.

**Keywords:** phototoxicity; photobleaching; fluorescence microscopy; light exposure; sample preservation; gentle imaging

## 1. Introduction

Exposing biological samples to light will always – again to repeat: always - have some effect on them. The question is, if these effects are minor or severe, reversible or irreversible and most importantly to what extent they will influence the outcome of a given experiment. Therefore, it is critical for every scientist to have at least a good estimation in which order of magnitude the effects are that microscopic imaging will have on experimental findings and how to minimize these. This is certainly not an easy task, as there are many parameters to take into consideration.
The good news is that for both unwanted aspects of exposing samples to light – phototoxicity and photobleaching - that are addressed in this chapter, there are ways and measures to reduce detrimental effects. Notably some kinds of exposures might be less critical than expected.

## 2. Light is energy

Before we approach the topics of phototoxicity and photobleaching in the context of biological experimentation, we have to find a standardized way to interpret and compare how much light is reaching our specimen. On a global scale this measure is the so called *solar irradiance*. It is the power per area received from the sun measured by an instrument in a certain wavelength range of electromagnetic radiation. The SI unit of solar irradiance is Watt per square meter ($W/m^2$).

Although several atmospheric and other parameters will influence the exact number, the irradiance of the sun (when it is at the zenith) at ground level on a cloudless day is around 1 $kW/m^2$. As one square meter is completely out of range for experiments with cells, let´s convert this into 1 $nW/\mu m^2$.

In comparison, recommended light levels in offices or schools are approximately 1000 lux, but will typically be lower than that. In areas where detailed tasks have to be carried out or fine work has to be done, recommendations go as high as 2000 lux [1]. Lux measures luminous flux per area unit, namely lumen per square meter. In that respect, lux is analogous to solar irradiance but it weights the power at each wavelength according to the luminosity function (a standardized model for human visual brightness perception). Therefore, there is no simple translation of lux into solar irradiance or vice versa, one always has to calculate it for a certain wavelength range. However, for the sun, we can use a very approximate conversion of 0.0079 $W/m^2$ per lux**.** As the peak of the luminosity function is at 555 nm (human eyes are most sensitive at this wavelength), we can calculate that we expect about 683.000 lux with the solar irradiance on a cloudless day (1 $nW/\mu m^2$) at exactly this wavelength. At other visible wavelengths this value will be much lower

and outside the visible spectrum (for humans) it will be zero. These calculations serve as a rough estimate that the irradiance (and therefore the energy) is approximately 100 times lower in a work environment compared to sunlight. For the moment, we shall neglect the fact that the spectrum differs greatly between the sun and light bulbs (or LEDs) in an office and that different proportions of the spectrum will have distinct effects.

There are huge differences in the working principles and therefore required excitation intensities of different microscope systems. As the majority of observations of fluorescent specimens will still take place on a widefield microscope (e.g. for routine inspection of fluorescent protein expression, staining efficiency or to locate a position on the specimen for detailed imaging with another microscope technique), a brief examination is given on how intense the illumination is [2].

**Table 1** Absolute and relative (compared to office light) energy within 1 second of exposure. Typical energy levels for different light source types are shown. Actual values can vary.

| Illumination | Irradiance | Energy within 1 s of exposure | Relative Energy |
|---|---|---|---|
| Sunlight (cloudless day) | 1 nW/μm$^2$ | 1 nJ/μm$^2$ | 127 |
| Office (daylight spectrum) | 7.9 pW/μm$^2$ | 7.9 pJ/μm$^2$ | 1 |
| Operation room | 15.8 pW/μm$^2$ | 15.8 pJ/μm$^2$ | 2 |
| Fluorescence (Metal Halide: 543/22 nm) | 50 nW/μm$^2$ | 0.5 nJ/μm$^2$ | 63 |
| Transmitted (Tungsten Halogen: 543/22 nm; 12.2V) | 3.6 pW/μm$^2$ | 36 pJ/μm$^2$ | 5 |
| LED (peak wavelength: 535 nm) | 6.6 pW/μm$^2$ | 66 pJ/μm$^2$ | 8 |

The numbers in **table 1** are only rough estimations, and it must be clear that the light produced by a microscope light source can reach the sample in various ways and intensities due to different light trains in the systems, objectives or for instance additional blocking filters. However, we can draw a few preliminary conclusions form the data above:

1) Even very intense office illumination will only expose a sample to less than 1% of solar irradiance
2) Widefield fluorescence illumination is ~60 times more intense than office light and approximately half as intense as the sunlight
3) Light from a Halogen Lamp (usually used for transmitted light imaging) is 10 times less intense (at least) than the fluorescence excitation light from a metal halide lamp

Note: LEDs are available in various power levels (data not shown) and there are quite strong products in the blued and far red wavelength range, but especially in the green range (around 530 – 560 nm) there is still a lack of high power LEDs.

The numbers above suggest that a brief transport of a sensitive specimen in bright sunlight might be more damaging, than leaving the specimen on a desk in the lab or office for an extended time. Therefore, let´s have a look how much protection different containers provide to a sample. Shielding capacities of different materials are shown in **table 2**.

**Table 2** Shielding capacity of different materials.

| Material | Attenuation for visible light (400-800 nm) | Attenuation for near UV (350 -400 nm) | Attenuation for near IR (800-1400 nm) |
|---|---|---|---|
| Clear polystyrene sheets | ~ 10 % | ~ 10 % | ~ 10 % |
| Standard Window Glass | ~ 20 % | ~ 40 % | ~ 25 % |
| Special Insulating Glass | ~ 30 % | ~ 80 % | ~ 60 % |
| Microscope Coverslips/Slides | ~ 5 % | ~ 10% | ~ 5 % |
| Aluminium foil | 100 % | 100 % | 100 % |

Of course, these theoretical considerations do not take into account how a sample is exposed to e.g. sunlight in reality. Carrying around a sample will typically shield it during a significant proportion of the time due to shading from objects, plastic covers, or the person who carries the sample. Also, the light has to penetrate buffers or media before it reaches the cells. This can lead to light doses at the sample that are much lower than the estimated maximum (**Fig. 1**).

Nevertheless, some cell and tissue types are very sensitive to light and it is almost impossible to quantitate the effects of some ambient exposure on the outcome of experiments. Therefore, to be on the safe side and to reduced unwanted effects, closed boxes from expanded polystyrene with an inner layer of aluminium foil should be used to transport living samples when outside buildings. This will effectively protect specimens from visible light and heat (as evaporation of media sometimes is more critical than phototoxicity).

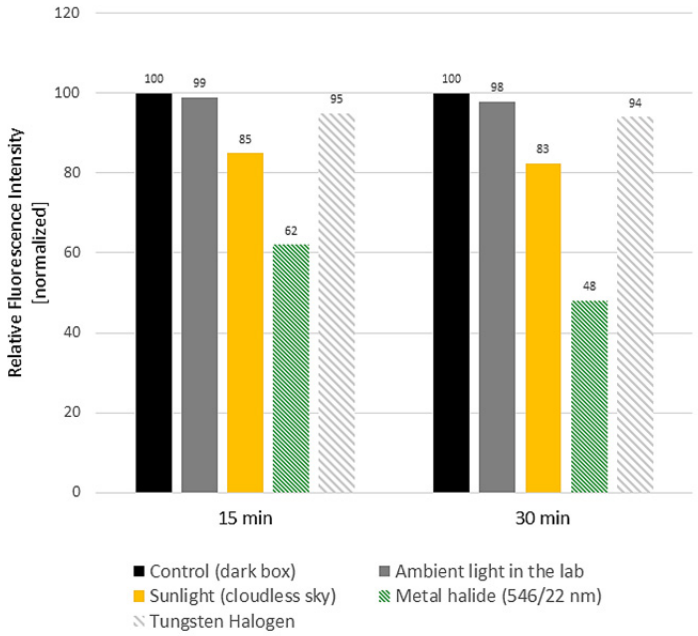


**Fig. 1** Bleaching behaviour of fixed HeLa cells expressing H2B-mRFP (in aqueous buffer). Exposure to either ambient light, sunlight, green fluorescence excitation light or a tungsten halogen lamp for either 15 or 30 minutes. Exposure to ambient light, sunlight and light from the halogen lamp was through the polystyrene lid of a glass bottom dish. Exposure to fluorescence excitation took place through the glass coverslip at the bottom of the dish. This might explain the relatively high bleach rate of the fluorescence excitation compared to sunlight.

In contrast, most fixed fluorescent specimens will not suffer significantly if left in the office unprotected for a limited amount of time (e.g. a few hours). Although it can help to prolong shelf life of fixed and embedded specimen when they are stored in a cool and dark place, there is no need to rush through staining protocols with fixed specimens or to turn off the light in the lab, while the sample, is carried to the imaging system. Light doses in laminar flow hoods or on lab work benches will typically not influence staining intensity in a measurable way. It would, for this reason, be counterproductive to e.g. shorten wash steps for immunostaining procedures only to shorten light exposure.

## 3. Phototoxicity

"Phototoxicity", in the sense of this book chapter, means the damaging effects of light on a living cell or organism. Phototoxicity is a phenomenon consisting of wavelength-dependent photophysical mechanisms that generate heat, reactive photochemical products, and DNA damage. Especially fluorescence excitation light produce free radicals, which will in turn cause a certain degree of damage. Therefore, all live imaging causes phototoxicity. However, the differences in the degree of phototoxicity can vary greatly!

To ask how much light a specimen can take before significant phototoxicity occurs is similar to asking how much exposure to sunlight a human being can tolerate before the skin reacts with a sunburn. In the case of humans, darker complexion of the skin, recent tanning of the skin or application of sunscreen would prolong such a period. Whereas certain genetic disorders, like Xeroderma pigmentosum, or exposure to photosensitizing substances, such as furocoumarins from the giant hogweed (*Herculaneum mantegazzianum*), would drastically shorten the period until the skin is damaged. The same is true for biological samples, such as cultivated cells, that are exposed to light. Is the tissue type normally exposed to sunlight in the intact organism? Are all DNA/RNA- and other repair mechanisms working properly in the cells? Have the cells been exposed to substances that either absorb or produce reactive molecules or radicals?

Beyond the specimen, the light itself influences how much damage it can do. It is common knowledge that shorter wavelengths with higher energy (e.g. UV light) are more harmful to many biological molecules then longer wavelength light. While U2OS cells (human bone osteosarcoma epithelial) withstand light intensities of ~1 nW/µm$^2$ at 640 nm for 4 minutes (~ 1,4 nJ/µm$^2$), the maximum dose at 405 nm for the same cell type is only 1 pJ/µm$^2$ [3] and therefore 1000 times lower. As another example, unlabelled Vero cells (green monkey kidney) suffer from a clearly visible reduced cell division rate after exposure to 20 J/µm$^2$ of blue (around 480 nm) light.

Moreover, the duration and intensity of the light can influence the impact of the exposure. Even if the same dose is reached, will shorter pulses of high intensity light be less or more harmful than longer exposures at lower intensity levels? There are contradicting answers to this question (based on different experimental setups), that indicate, that the answer to this depends on multiple factors, such as wavelength, the biological system and the overall exposure time and intensity levels [3] [4] [5]. Ideally, two extreme conditions (very long exposure times at very low excitation power and ultra-short high intensity pulses) are evaluated to assess what will be less harmful in the experimental setup of choice.

Some types of photodamage can be difficult to detect and/or are only revealed after a longer period of time. For instance, if cell division is disturbed, this may only be detected if the imaging experiment is long enough to include a division cycle. Therefore, the uncertainty of how much photodamage was done to a sample often influences the

interpretation of an experimental result. Standardized assays have been used to detect even low amounts of damage to samples and they should be carried out every time when phototoxicity potentially threatens quality of experimental data.

The 3T3 neutral red uptake phototoxicity assay has now for many years served as a standard alternative to animal testing [6]. Reduction of neutral red uptake by a monolayer of BALB/c 3T3 mouse fibroblasts is measured after exposure to light.

Recently, a variety of further phototoxicity assessments have been proposed to allow researchers with different equipment and possibilities to quantify phototoxic damage. As a live cell imaging variant, a cell migration and a cell protrusion assay were proposed [4] that measure the speed of cells during light exposure for 6 hours or the retraction of cell protrusions in a 30 minute timeframe, respectively.

For developing organisms a quantitative and robust assay has been introduced that uses photosensitive embryos of *Caenorhabditis elegans* with histone-marked nuclei (H2B-GFP). This organism is easy to maintain and quickly allows an estimation of how suitable the imaging equipment is to carry out experiments with results as close as possible to the natural environment [7].

It must be understood that there are huge differences in the sensitivity of cells or tissues to light exposure.

Generally, as a rule of thumb unstained or unlabelled cells are a lot more resistant to light exposure than cells labelled with chemical dyes or fluorescent proteins. This can be up to a factor of 10 or even more for some dyes [8] [5].

Also unlabelled CHO (Chinese hamster ovary), U2OS and COS-7 (African green monkey fibroblasts) cells are much more (around 5 times) sensitive than HeLa cells [3].

Maximum light doses for cell survival - dependent on wavelengths and fluorescence markers – are typically in the range of 5-200 J/cm² , which corresponds to 50 s - 2000 s of solar irradiance [8]. However, it must be pointed out, that more subtle effects of phototoxicity – not leading to immediate cell death - can occur much earlier and can influence experimental results. For example, monkey kidney fibroblasts show cytoskeletal changes after only 2 seconds exposure with green light at an intensity of 12.5 pW/$\mu m^2$ [9].

As already mentioned, phototoxicity can not be completely avoided, when specimens are illuminated with light. A balance between reasonable efforts to protect the sample on the one hand and desirable quality of conclusions on the other hand needs to be found. For this, an adequate documentation of experimental conditions is crucial, as it will not only allow others to conduct comparable experiments, but it will also help in optimizing experimental conditions.

It has previously been suggested by Laissue et al [10] to record and publish key parameters, such as biological criteria for assessing phototoxicity, wavelength of excitation light, illumination time, average power at the sample focus level, peak power of excitation light, numerical aperture of excitation and detection optics, and the photobleaching rate. This could greatly increase reproducibility of experiments with living specimens and the extraction of meaningful conclusions from them.

Photobleaching can be used as a rough indicator for photo-damage [10].

## 4. Photobleaching

Fluorescence emitted by almost all fluorescent dyes and proteins fades during observation. This phenomenon is called photobleaching (sometimes also dye photolysis). Although, in some cases, a fluorescent molecule can be switched on again after having lost the ability to emit light - or from a natural initial dark state - (photocycling), or is able to switch on and off (blinking) within a short timescale, we refer to photobleaching as an irreversible phenomenon that involves a photochemical modification of the dye.

Notably fluorescent dyes in the excited state are subject to chemical or biochemical reactions that may lead to their degradation and destruction and subsequently to a severe loss of image quality during measurement or other kinds of light exposures. As there are excellent reviews, articles and book chapters dealing with photobleaching, we will not elaborate more on it here. Nevertheless, it should be mentioned that many factors, such as the molecular environment (most importantly the presence or absence of oxygen) and the intensity and wavelength of excitation light. These factors affect the mechanism, and thus the reaction order and rate of photobleaching [11].

Prevention of photobleaching is on the one hand very important for quantitative microscopy but on the other also for obtaining high-quality images. One effective and easy way to reduce photobleaching is by decreasing the excitation time or the excitation intensity. However, this leads to a reduction of the fluorescence signal, a more noisy image and potential loss of low-contrast features. Other ways to achieve a lower bleaching rate are therefore highly desirable, even more so, as lower photobleaching will often go hand in hand with lower phototoxicity. During the fluorescence process, excited molecules can enter the triplet state where reactive oxygen species are generated. When triplet state molecules absorb light, the result is the photo-destruction of the fluorophore [12].

Molecular oxygen is one of the main actors in photobleaching. Therefore, deoxygenation of the specimen often reduces photobleaching and thereby in some cases also improves survival of the cell (of course only, if the cells or tissue generally tolerate hypoxia for a limited time period). This can be achieved by perfusing $N_2$ through the culture medium or by using biological oxygen scavengers. Molecules that are capable of quenching singlet oxygen (e.g. carotenoids, vitamin e- analogues, reduced glutathione, imidazole or histidine) usually also reduce photobleaching [13].

Although in many respects, the reduction of photobleaching in live and in dead/fixed specimen follows the same rules, there are differences and specific aspects that we have to consider in both cases (**Fig. 2**).

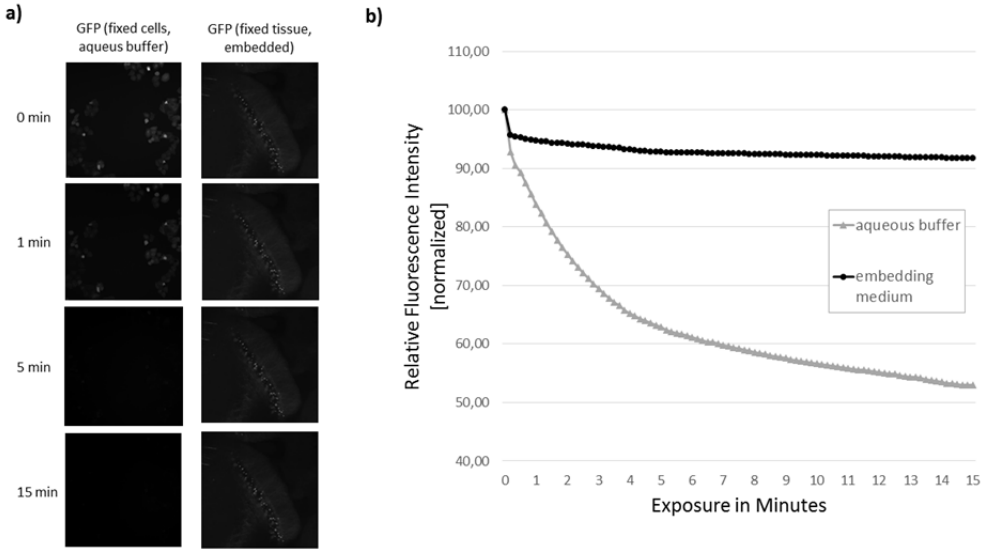


**Fig. 2** Bleaching behaviour of GFP in aqueous medium and in antifade embedding medium during continuous illumination with blue excitation light for 15 min. **A)** Images acquired at the start of the experiment and after 1, 5 and 15 minutes. **B)** Graph depicting the normalized mean intensities of image regions from a) over time. Formaldehyde-fixed GFP-expressing cells in phosphate buffered saline show a more prominent loss in fluorescence than PFA-fixed neurons in embedding media. All other experimental conditions (objective, light source etc.) were held constant.

### 4.1 Photobleaching and fixed specimen

Common antifade reagents are diazabicyclo-2,2,2-octane (DABCO), *n*-propyl gallate and *p*-phenylenediamine. Although many antifade agents are highly efficient in preventing, or at least reducing, photobleaching, they have side effects. One of the most effective antifade reagents, *p*-phenylenediamine (PPD), exhibits thermosensitivity and autofluorescence, and is also toxic for cells, making it unsuitable for *in vivo* studies and live cell imaging.

Generally, the choice of dyes and staining agents for fixed samples is a lot larger than for living species, as dye toxicity will not play a big role. Therefore, the choice of fluorophores should be made according to their photostability features (in addition to spectral suitability). Usually newer generation fluorophores tolerate higher light doses and are brighter than the dyes of the early days of fluorescence microscopy. Although many labs traditionally still use FITC (Fluorescein isothiocyanate), TRITC (Tetramethyl rhodamine isothiocyanate) and DAPI (4′,6-Diamidin-2-phenylindole), these are very good examples of dyes that make it unnecessarily hard to get high contrast images after prolonged light exposures.

Especially for demanding experiments, it can make sense to optimize the combination of dye and antifade mounting medium to get the best photostability. Even with fixed specimens this optimization can make sense, as some important details of the sample could be lost during imaging, e.g. when a larger 3D volume with many z-sections is acquired.

The generation of a bleach curves (e.g. as in figure 2) for individually treated specimens can help to find ideal imaging conditions.

Notably, the extent of photobleaching in a fixed sample can be a valuable indicator of the degree of photo-toxicity endured by living samples under the same fluorescence imaging conditions. Optimizing imaging conditions with a fixed sample that has been biologically treated in the same manner as for a live experiment, can therefore be of great value when live cell imaging must be performed.

### 4.2 Photobleaching and live specimen

*N*-propyl gallate (NPG) is both photo- and thermostable and can be used for *in vivo* studies. Although NPG is very effective in retarding bleaching in many situations, it reduces the initial level of fluorescence that is observed. Whether a slower decay of fluorescence combined with initial quenching or a faster photobleaching rate at a higher base intensity is preferred, should be decided for every imaging experiment individually.

Apart from the availability of antifade mounting media for live cells, there are only a few options to optimize photobleaching properties of the sample itself. To switch from a fast bleaching fluorescent protein to a more photostable one, will usually at least require generation of new constructs for fusion proteins and in many cases even the generation of transgenic mutant or stable cell lines, which is often beyond the timeframe of a project. Therefore, for experiments on living specimens, optimization of imaging parameters typically plays a much more important role. Besides the well-

known advice for gentle imaging – reduce excitation light intensity and increase detection sensitivity – there are other aspects that have to be considered.

Additionally, measures should be taken to reduce photobleaching as early as possible, i.e. during sample cultivation, preparation, staining, transportation and other steps. However, often the reduction of light exposure during positioning of the specimen on the microscope stage, focusing and locating a region of interest on it, plays the biggest role. When living specimen are imaged, these aspects are exactly the same as for reduction of phototoxicity, and therefore are worth considering. Here are some suggestions along these lines:

- For navigation within around a sample, use light sources that emit no, or as little as possible, UV-light. Even with good fluorescence filters a few percent of UV light emitted from an HBO or metal halide lamp will reach the sample. LEDs are the best choice in this regard and they should be used not at full power, but be turned down as much as possible, so that the cells of interest are just visible.
- Use a hardware focus-device that helps find the sample carrier interface quickly and reliably. These typically emit relatively low power light in the near infrared spectrum. Such hardware focus aids can help to reduce the light burden for a sample significantly, as manually focusing with fluorescent excitation can easily take 30 seconds. If manual focus adjustment must be performed, transmitted light should be used at low power levels.

## 5. Imaging Instrumentation

Over the past 15 years, the number of imaging instrument types has increased greatly. Besides well-established techniques to perform fast and gentle imaging, such as TIRF or multi-point-scanning, new technologies like light sheet microscopy have entered the world of live cell imaging. Sometimes the use of one or the other instrument type is without alternative, but in cases where phototoxicity or bleaching is limiting to perform a specific experiment, it might be worth looking into other technologies.

Some of the most widely used instruments, confocal laser scanning microscopes and widefield illumination microscopes, come with the disadvantage that they always illuminate the whole sample in the z-direction. Even if, in case of the confocal microscope, we only observe and acquire a single plane in z, light exposure will occur in the entire volume of the sample that has been penetrated by the laser beam. However, it has been shown that short laser pulses (in the range of 250 ps) used to excite fluorophores prolong their lifetime and lead to reduced phototoxicity [3]. However, as many labs do not have access to lasers with 250 ps pulse widths, it was proposed that laser pulses on the nanosecond timescale, such as used in a conventional point-scanning microscope, somehow simulate a “pulsing” on the timescale of the pixel dwell time. Rapid line (or spinning disc) scanning will therefore reduce photobleaching in many cases..

In addition, confocal microscopes using the so-called Airyscan principle have benefits over classical single point scanners, as they will exploit more of the emission signal while the excitation laser power can be reduced.

In the context of photobleaching and photo-toxicity, technologies like multiphoton and light sheet microscopy have the intrinsic advantage that excitation of fluorophores and as such, also irradiation of the specimen, only occurs in the z-plane which is acquired and the rest of the sample remains unaffected.

Generally speaking, a higher information content, such as higher spatial or temporal resolution, nearly always comes at the cost of increased light exposure of the specimen. It is obvious in terms of increasing temporal resolution that the acquisition of more images per second can increase the number of images as well as the shorter exposure times normally lead to a higher peak illumination.

Improving your spatial resolution by structured illumination microscopy (SIM) or STED microscopy also comes at the cost of higher light exposure. In case of SIM, this is due to the requirement of multiple illuminations to generate a high resolution image. In STED microscopy, the additional laser illumination required for increased resolution also increases the photon load on a sample. For a given experiment, it needs to be decide in which aspect to invest the photons that are to be tolerated by the specimen.

**Table 4** Imaging Instrument types with regard to photobleaching and phototoxicity.

| Instrument type | Pro | Con |
|---|---|---|
| Widefield | • Fast<br>• Very light efficient | • illuminates all z-sections at once<br>• excitation may contain UV-light when not blocked or LEDs are used |
| Structured Illumination | • resolution increase depending on technology lateral and/or axial | • several illuminations for one image required<br>• pattern might be bleached in<br>• relatively slow |
| Single Point Scanner | • short dwell times at each x/y-spot | • illuminates all z-sections at once<br>• slow |
| Multi-Point Scanner | • very fast<br>• short dwell times at each x/y-spot | • illuminates all z-sections at once |
| Airyscan | • light efficient | • illuminates all z-sections at once |
| Multi-Photon | • only illuminates z-section of interest low excitation wavelength | • High intensity laser excitation required<br>• slow |
| Localization Microscopy (PALM/STORM) | • Provides molecular resolution (lateral resolution up to 20nm)<br>• Quantitative | • illuminates all z-sections at once |
| STED | • Very high resolution | • illuminates all z-sections at once<br>• high laser powers required |
| TIRF | • only illuminates thin section at the glass interface<br>• very fast<br>• very high contrast (out-of-focus discrimination) | • no volume imaging |
| Light Sheet | • only illuminates z-section of interest<br>• very fast | • resolution usually limited |

## 6. Conclusions

There are good reasons to dim the light in an imaging lab, e.g. to improve contrast by avoiding stray light onto the objective of a “classical, non-boxed” microscope. However, it is usually not necessary to dim the light or even turn of the light completely to protect samples from ambient light, while they are sitting on a table or lab bench. Respective light doses are simply too low to induce significant photobleaching or phototoxicity.

This is in harsh contrast to the illumination light produced by a microscope light source and focussed on the sample with a high performance objective.

There is no easy answer to the question, what instrument or imaging technique will generally be the most gentle in terms of photobleaching or phototoxicity. However, when we leave other considerations like e.g. sample preparation, resolution etc. aside, then for acquisition of a specimen in 3D, light sheet microscopy sets the standard. When only imaging of surface structures or adhesion points is required, TIRF microscopy could be the method of choice.

As a rule of thumb, all imaging techniques that selectively expose a three dimensional structure will have advantages over methods that illuminate the sample less selectively (either in x,y, z, or all of them).

There is also a variety of other technical possibilities to reduce phototoxicity and unwanted photobleaching during imaging, such as sensitive cameras, precise illumination control (spectral and temporal), field stops, IR-focus systems, protective chemical agents and many more, but there is one aspect that is often forgotten when the imaging itself is optimized: Visual inspection of the sample and locating a region of interest. While most current imaging systems are optimized to reduce the light burden for fluorescent specimen during digital imaging, the exact same systems are often still equipped with highly harmful fluorescence equipment for visual inspection. Unattenuated mercury arc lamps or metal halide lamps with low quality filter sets expose the sample to high intensity light that often also contains UV light. Moreover, human beings are typically slow in screening a sample and therefore will expose the sample for a relatively long time period. 15 - 30 seconds exposure are not uncommon, especially if multiple magnifications and fluorescent filter sets are used. 15 seconds of exposure during visual inspection and localization of a region of interest approximately correspond to a time series with 3000 time points at 50 milliseconds exposure time each with a highly efficient and precisely controlled imaging system. Therefore, to rule out the human factor in imaging could in many cases enable a big leap forward in conducting experiments that are more meaningful.

## References


[1] Gornicka GB. Lighting at work: environmental study of direct effects of lighting level and spectrum on psychophysiological variables. Eindhoven: Technische Universiteit Eindhoven; 2008; DOI: 10.6100/IR639378.

[2] Murphy CS, Davidson MD. Light source power levels. The Florida State University. Available from: http://zeiss-campus.magnet.fsu.edu/articles/lightsources/powertable.html

[3] Waeldchen S, Lehmann J, Klein T, van de Linde S, Sauer M. Light-induced cell damage in live-cell super-resolution microscopy. Sci Rep; 2015 Oct 20;5:15348. doi: 10.1038/srep15348.

[4] Mubaid F, Brown CM. Less is More: Longer Exposure Times with Low Light Intensity is Less Photo-Toxic. MicroscopyToday; 2017. doi:10.1017/26 S1551929517000980

[5] Boudreau C, Wee E, Duh S, Couto MP, Ardakani KH, Brown CM. Excitation Light Dose Engineering to Reduce Photo-bleaching and Photo-toxicity. Sci Rep; 2016; DOI: 10.1038/srep30892.

[6] Spielmann, H. The second ECVAM workshop on phototxicity testing. The report and recommendations of EVCAM workshop 42. Altern. Lab. Anim; 2000; p. 777–814.

[7] Tinevez JY, Dragavon J, Baba-Aissa L, Roux P, Perret E, Canivet A, Galy V, Shorte S. A quantitative method for measuring phototxicity of a live cell imaging microscope. Methods Enzymol; 2012; p. 291–309. doi: 10.1016/B978-0-12-391856-7.00039-1.

[8] Schneckenburger H, Weber P, Wagner M, Schickinger S, Richter V, bruns T, Strauss WSL, Wittig R. Light exposure and cell viability in fluorescence microscopy. Journal of Microscopy; 2012; Vol. 245, pp. 311–318.

[9] Knoll SG, Ahmedd WW, Saif TA. Contractile dynamics change before morphological cues during florescence illumination. Scientific Reports; 2015; 5:18513; DOI: 10.1038/srep18513

[10] Laissue PP, Alghamdi RA, Tomancak P, Reynaud EG, Shroff H. Assessing phototoxicity in live fluorescence imaging. Nature Methods; 2017 July.

[11] Bernas T, Zarebski M, Dobrucki JW, Cook PR. Minimizing photobleaching during confocal microscopy of fluorescent probes bound to chromatin: role of anoxia and photon flux. Journal of Microscopy; 2004 Sep. p. 281-96.

[12] Diaspro A, Chirico G, Usai C, Ramoino P, Dobrucki J. Photobleaching. In: James B. Pawley, editor. Handbook of biological confocal microscopy. New York; 2006. p. 690-699.

[13] Tsien RY, Ernst LWaggoner A. Fluorophores for Confocal Microscopy Photophysics and Photochemistry. In: James B. Pawley, editor. Handbook of biological confocal microscopy. New York; 2006. p. 338-348.