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Circumventing photodamage in live-cell microscopy

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Abstract

Fluorescence microscopy has become an essential tool in cell biology. This technique allows researchers to visualize the dynamics of tissue, cells, individual organelles and macromolecular assemblies inside the cell. Unfortunately, fluorescence microscopy is not completely ‘non-invasive’ as the high-intensity excitation light required for excitation of fluorophores is inherently toxic for live cells. Physiological changes induced by excessive illumination can lead to artifacts and abnormal responses. In this chapter we review major factors that contribute to phototoxicity and discuss practical solutions for circumventing photodamage. These solutions include the proper choice of image acquisition parameters, optimization of filter sets, hardware synchronization, and the use of intelligent illumination to avoid unnecessary light exposure.

Keywords

fluorescence; live-cell microscopy; phototoxicity; photodamage; GFP

I. Introduction

Live-cell microscopy always involves a compromise between the desire to achieve the best possible image quality and the necessity to maintain cells live and happy on the microscope stage. One must always remember that fluorescence is an inherently inefficient process – emission is orders of magnitude weaker than the intensity of excitation light. Photon flux required to generate a sufficiently strong signal from typical fluorophores (fluorescent proteins or chemical dyes) is damaging to the cells. Deleterious effects of light primarily arise due to generation of chemically reactive species such as free radicals and specifically singlet and triplet forms of oxygen. As oxygen is required for respiratory-chain reactions, scavenging this molecule from the environment inevitably changes the cell’s physiology. Thus, although oxygen scavengers, for example oxyrase, have been successfully used in short-term live-cell recordings (Rodionov and Borisy, 1997; Zhai et al., 1995), this approach is not applicable when the goal is to obtain data from physiologically normal and healthy cell populations. In this case, the only practical solution for minimizing deleterious effects of light is to avoid any unnecessary irradiation. In turn, this is achieved via optimization of the hardware and proper experimental strategies.

Gradual decrease in fluorescence intensity due to light-induced degradation of fluorophores (termed ‘photobleaching’) is usually considered to be the main impediment in live-cell imaging (Fig. 1A). However, the fact is that light intensity sufficient to induce prominent photobleaching also inevitably changes homeostasis of the cell. Bleached cells are damaged! In fact, physiology of a typical mammalian cell is severely affected even under illumination conditions that cause only minimal changes in fluorescence intensity of the Green Fluorescent Protein (GFP) or similar fluorophores (Fig. 1B). However, manifestation of physiological changes can be delayed (Fig. 1C) and photodamage often remains unnoticed in shorter-term experiments.

Assessing the extent of photodamage inflicted by excessive illumination is difficult. Cells from various organisms exhibit dramatically different sensitivity to light. Embryos of *Drosophila melanogaster* and *Caenorhabditis elegans* are significantly more resilient to high-intensity illumination than mammalian cells. Wide variability in light tolerance is also common among different cell types originating from the same organism. For example, many types of transformed cells progress through the cell cycle under conditions that induce a permanent G2 block in untransformed cell lines of the same origin (Mikhailov et al., 2007). Further, even within the same population individual-cell response to excessive illumination is often heterogeneous. We find that the great majority of untransformed human cells (RPE1) complete cell division in less than 30 min under low-light illumination. Higher doses of light tend to increase the duration of mitosis and sometimes arrest mitotic progression altogether. However, a significant number of cells (~25%) still manages to progress through mitosis **with normal timing** under illumination conditions that completely arrest mitosis in ~30% of cells within the same population (Schilling et al., 2012). Thus, in their response to intense light cells follow the ‘Anna Karenina’ principle (Diamond, 1997) which originated from the opening sentence of the famous Leo Tolstoy’s novel: “Happy families are all alike; every unhappy family is unhappy in its own way.” In biological terms, the meaning of Anna Karenina’s principle is that suboptimal environmental conditions common in when cells are maintained on the microscope stage (excessive illumination, errant temperature control, unusual tonicity and/or pH of the culture media) often result in extreme variability in the behavior of individual cells within a uniform population. This artificially-induced heterogeneity in the behavior of ‘unhappy’ cells can distort the outcome of the experiment.

It is not uncommon for stressed cells to appear unaffected by light until they reach a certain transition point within the cell cycle. For example, most mammalian cells will initiate chromosome condensation and display other hallmarks of mitotic entry such as centrosome activation under intense illumination. However, many of these cells reverse progression through the cell cycle during mid-prophase and return to a G2-like state with completely decondensed chromatin and inactivated centrosomes. The percentage of cells that undergo cell cycle reversion is greater if the population is stressed by anti-microtubule drugs (Khodjakov and Rieder, 1999). Unfortunately, such a stage-specific effect on the cell-cycle implies that photodamage would not be detected unless the experiment is sufficiently long to include the point at which the damaging effects are manifested.

Additional complications arise from the cumulative nature of stress. Exposure to one stressful factor often increases sensitivity of the cell to other insults and unfortunately this synergism often occurs in live-microscopy experiments. For example, physical removal of the centrosome from mammalian cells has been reported to result in a cell-cycle arrest during G1 phase of the cell cycle (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). The conclusion was based on time-lapse recordings that demonstrated lack of cell-cycle progression in acentrosomal cells while cells with centrosomes within the same field of view divided repeatedly. Therefore, the result was interpreted as a solid indication that centrosomes were required to support cell cycle progression (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). However, subsequent investigations revealed that lowering the intensity of excitation light allows acentrosomal cells to progress through the entire cell cycle (Uetake et al., 2007). The reason for the G1 arrest observed in the original centrosome-removal studies was that cells already stressed by the removal of centrosome become more sensitive to intense light. Similarly, cells tend to become sensitized by treatments with various drugs, siRNA, or other environmental insults. This sensitization can be suppressed to an extent by inhibition of p38 kinase (Mikhailov et al., 2005; Uetake et al., 2007), a major component of the stress-response pathway. Germane here is that microscopy conditions that do not induce detectable deleterious effects in healthy cell populations can

become damaging when applied to cells whose health is already compromised by other types of stress. A great care must be taken in every live-cell experiment to limit light exposure to the level absolutely necessary for obtaining images of the sufficient quality. Exceeding this level is dangerous as it clouds interpretation of the experimental outcome.

II. Light Intensity and Exposure Time

Achieving the full resolution of an optical system is only possible when the ratio of useful signal to noise in the image is sufficiently high. All other factors being equal, the same number of photons can be captured by the detector during a shorter exposure time at higher intensity of excitation light vs. less intense excitation and longer exposure. In fact, these alternative sets of acquisition parameters yield comparable image quality and similar level of photobleaching in conventional imaging of fixed cells. However, in live-cell imaging phototoxicity depends not only on the irradiation dose but also on how exactly this dose was delivered. The differences are due to both photochemistry and physiological responses of the cell.

In the elementary fluorescence cycle, absorption of a photon converts the low-energy 'ground state' fluorophore into an 'excited' molecule. The life time of the excited state is short (nanoseconds) and return to the ground state is accompanied by the emission of a new, longer wavelength photon. This basic cycle is complicated by the existence of additional transitions such as decay of the excited molecule into a still energized but non-fluorescent 'triplet' state. The lifetime of triplets is relatively long (microseconds) and these molecules can absorb additional photons before their energy dissipates (Fig. 2A). Additional absorption results in a further energy increase and facilitate chemical transformations that ultimately cause destruction of the fluorophore (Donnert et al., 2007).

The existence of multiple excited states with various life times and various predispositions to chemical breakdown implies that the rate of photobleaching depends on numerous factors and in particular on whether the excitation light is delivered continuously or in pulses. Extremely short pulses (shorter than the lifetime of excited fluorophores) separated by intervals that exceed the lifetime of the non-fluorescent but photobleaching-prone triplets have been shown to substantially reduce photobleaching in single-spot scanning confocal microscopy (Donnert et al., 2007). However, implementation of this approach is difficult as relatively long pauses between the pulses are required for the dangerous triplet states to decay. These pauses would reduce the speed of a single-spot confocal imaging system by as much as 100-fold. Potential solutions to this problem include simultaneous scanning of the sample with multiple beams (Bingen et al., 2011) as well as utilizing extremely fast scanners to direct each pulse to a non-irradiated spot. With the latter approach extremely short dwell times (~50 ns) have been shown to noticeably decrease photobleaching and improve viability of brain cells imaged via multi-photon confocal microscopy (Chen et al., 2012). Thus, the use of relatively sparse but short high-intensity pulses is beneficial in certain types of microscopy.

However, the situation is reversed in wide-field and spinning-disk confocal microscopy that are most commonly used for long-term recordings of live cells. In these applications decreasing the intensity of excitation light along with the appropriate increase in the exposure time reduces phototoxicity.

While specific studies on the physiology of cellular responses to dim vs. bright light are scarce, the notion that that illumination becomes a damaging factor only above a certain threshold level is intuitive. Most organisms are adapted to life under continuous illumination, and cells possess mechanisms that can repair a certain amount of damage inflicted by environmental factors such as radiation. As long as this protective capacity is

not overwhelmed, illumination would not trigger an emergency stress response. In fact, cultured human cells continue to undergo normal cell cycles under continuous illumination at the intensities sufficient for phase-contrast and/or DIC recordings (our unpublished observation). Unfortunately, light flux used in a typical GFP time-lapse even with a sensitive CCD camera is greater than the intensity of direct sunshine ($\sim 0.2 \text{ W/cm}^2$ vs. 0.13 W/cm^2 , Schilling et al., 2012). ‘Sunburn’ becomes unavoidable! Lower excitation intensities are not practical as they would require exceedingly long exposure times not compatible with the dynamic nature of live cells. Thus, real-life live-cell fluorescence recordings are conducted at the excitation intensity known to induce phototoxicity. This dictates special considerations in the layout of imaging systems specifically designed for life-cell experiments. Below we outline main aspects of a microscopy workstation that need to be specifically optimized to minimize photodamage.

III. Optimization of Excitation Spectrum

One of the parameters that determine the extent of photodamage in live-cell microscopy is the quality of the excitation light. Standard filter sets, well suited for fixed-cell microscopy, often are not ideal in live-cell experiments. In the latter case, circumventing deleterious effects of irradiation should take priority over maximization of total fluorescence intensity.

Standard commercial filter sets are usually designed to maximize excitation by utilizing a relatively large portion of the fluorophore’s absorption spectrum. Excitation filters with spectral width $\sim 40\text{-nm}$ are commonly offered for GFP imaging (Fig. 2B). Though these filters do offer excellent signal strength, excitation with seemingly less efficient narrow-band filters is advantageous when a large number of images need to be acquired in live cells. An example of a marked decrease in photobleaching and photodamage due to a narrower excitation filter is illustrated in Figure 2. Notice that although the quality of images recorded with a wider exciter is initially higher, the intensity of GFP signal gradually degrades throughout the course of recording (Fig. 2C). In contrast, with a very narrow exciter matched to the absorption maximum photobleaching is unnoticeable after 30 min of recording (Fig. 2D). In addition to prominent photobleaching, the cell followed with a wider excitation filter becomes photodamaged as manifested by the retractions of the initially extended lamella (Fig. 2C). In contrast, no change in the cell morphology occurs when GFP is excited with almost-monochromatic light. Therefore, imaging conditions are not stressful in this case.

Several mechanisms can contribute to the increase in photodamage observed upon wide-spectrum excitation. One is that photons with shorter wavelength are more likely to cause chemical breakdown of the fluorophore because of their higher energy. Not intuitive to a biologist is that the difference in energy between a 490-nm photon (absorption maximum for the GFP, Fig. 2B) vs. a 450-nm photon (50% of absorption maximum for the GFP, Fig. 2B) is 0.225 eV. This difference corresponds to an increase in the temperature of a single molecule by $2,300^\circ\text{C}$ (in gas). Obviously, such a prominent and localized energy increase beyond the level necessary for exciting the fluorophore is dangerous for the molecule. Another mechanism involves the difference in absorption spectra of the ground-state vs. long-lifetime triplet-state fluorophores (Rankin et al., 2011). The latter tend to absorb shorter wavelengths with higher efficiency (Fig. 2B). As absorption by the triplets leads to the formation of bleach-prone high energy states, excitation with wavelengths shorter than the absorption maximum can potentially increase photobleaching. Yet additional phototoxicity observed upon wider-spectrum excitation can arise due to collateral excitation of endogenous chromophores such as cytochromes that are always present in the cell. Photodamage of these molecules ultimately triggers stress-response pathways. Irrespective of the exact mechanism(s), empirical observations demonstrate that narrower exciters

spectrally matched to the absorption maximum of the fluorophore diminish deleterious effects of fluorescence imaging in live cells.

An added benefit of extremely narrow excitation is that it can render emission filters unnecessary. We find that rejection of the excitation light by modern dichroic mirrors is often sufficient to capture ‘clean’ spinning-disc confocal fluorescence images without a dedicated emission filter (Fig. 2E). Elimination of the emission filter increases the intensity of fluorescence because the entire spectrum of emission light is captured. A corollary is that the intensity of excitation can be lowered by several percent. Although the gain may seem insignificant, in live-cell recordings even a minute decrease in illumination intensity is advantageous particularly when a large number of images need to be acquired. Obviously, emission filters cannot be omitted when cells are labeled with more than one fluorophore.

Dependence of photobleaching/photodamage on the excitation spectrum often becomes a limiting factor in multi-color live-cell imaging. Absorption spectra of fluorescent proteins are usually wide (Chudakov et al., 2010). This feature implies that red-shifted proteins absorb wavelengths used for excitation of green-shifted fluorescent proteins. This absorption is unavoidable even when monochromatic laser light is used (Fig. 3A) and it is manifested as ‘crosstalk’ between the green and red channels (Fig. 3B). The importance of crosstalk for the interpretation of protein co-localization and quantification of signals is actively discussed in the literature. However, less attention is given to the fact that crosstalk also accelerates the rate of photobleaching for the red-shifted fluorophores. Furthermore an important property of multi-color recordings is that the severity of photobleaching for red-shifted proteins can far exceed the rate formally expected due to the absorption-spectra overlap. Comparing to single-color recordings, photobleaching of the fluorescent protein tdTomato roughly doubles in experiments that involve recording both green and red fluorescence channels (excitation with similar intensities of 488-nm and 561-nm lasers; Fig. 3D). This is significantly higher than expected as tdTomato absorbs less than 30% at 488-nm. The additional increase in photobleaching can be attributed to the same effects as increase in phototoxicity upon off-peak excitation discussed earlier in this chapter. 488-nm wavelength, optimal for GFP excitation, corresponds to the dangerous shorter-than-optimal part of the absorption spectrum for red-shifted proteins. Therefore, damaging effects of 488-nm excitation on tdTomato are more pronounced than those caused by the 561-nm ‘on-peak’ light.

Overlap between the excitation spectra of different fluorophores in a multi-color recording can be minimized by a careful selection of fluorescent-proteins pairs. For example, TagRFP-T has same spectral peaks as tdTomato (555nm) but its absorption at 488nm is only ~10% (Shaner et al., 2008). However, complete elimination of unintentional excitation is not currently possible and the additional stress inflicted by the second fluorophore needs to be considered in experimental designs. Somewhat paradoxically, in many cases a practical solution for avoiding excessive photodamage in experiments that require visualization of more than one object is to tag all objects with the same fluorophore. This approach has been proven quite beneficial when the objects are morphologically distinct (Chang et al., 1999; Tran et al., 2001) or when morphologically-similar objects exhibit distinctly different behavior (Magidson et al., 2011).

IV. Hardware Synchronization

As even fully optimized monochromatic excitation is nevertheless toxic, great care must always be taken to minimize the irradiation dose. Ideally, cells should be illuminated only during the period necessary to acquire the image. However, in real life, actual exposure time often includes a useless “overhead” due to imprecise synchronization between the various

hardware components. Relatively simple adjustments in the configuration of the imaging system help to minimize the overhead and sometime virtually eliminate it.

Acquisition of a fluorescence image is a complex routine that involves several steps: the shutter must open and then close, the detector needs to be switched from dormant state to image acquisition and then 'readout' state, and finally the image needs to be transferred to the computer memory. In a typical workstation each of these individual steps is triggered by a command generated by the computer. The commands are issued sequentially and a mechanism needs to be in place to ensure that the next command is not executed prematurely, prior to the completion of the previous step. Most commonly, software designers rely on a return code from the hardware –receiving this code by the computer confirms that the previous step has been completed and the process can advance further. This is a lengthy process particularly when acquisition routine is complex (e.g., collecting a Z-series in multiple colors). It is noteworthy that communication speed is not directly related to the computer processor clock. Somewhat paradoxically, replacing an old computer with a faster model may actually increase the overhead particularly when the workstation comprises newer and older hardware components (due to space limitations this technical issue is not discussed here).

The overhead can be quite long; particularly in those microscopy workstations that are assembled in-house from individual parts acquired with little consideration for how well these components can communicate with one another when integrated into a common imaging system. Our surveying of various instruments in our own laboratory and elsewhere reveals that a typical overhead on a mid-range research-grade imaging workstation is on the order of 100–200-ms (Fig. 4). Thus, a single fluorescent snapshot with the nominal exposure (CCD photon-accumulation time) of 50-ms can actually subject the cell to ~250-ms irradiation. In extreme cases we saw imaging systems driven by popular commercial software with overheads in excess of 1 s. Such an extreme variability in the overhead is likely to be one of the main reasons for why the extent of photobleaching/photodamage seems to be so variable in different laboratories. It is also noteworthy that overhead time remains fairly constant for a particular imaging workstation. Therefore, the contribution of overhead to phototoxicity is greater when short exposure times are desired. For example, a 100-ms overhead doubles the irradiation dose for 100-ms nominal exposures but it only adds 20% to 500-ms nominal exposures. Thus, the real-life relationship between the exposure time and light intensity is often non-reciprocal. Recordings tend to be less toxic when conducted at the longest allowable exposure time and congruently the lowest possible level of excitation light.

Overheads caused by computer/hardware communications can be eliminated by employing direct synchronization between the camera and shutters. Many research grade CCD cameras offer an 'exposure out' connectors that generate an electric signal only when the camera acquires photons. This signal can be used as a trigger to initiate shutter opening or closing at a precise time. In fact, this type of hardware synchronization is used in several high-end commercial imaging systems. Further, major manufacturers of scientific CCD cameras offer proprietary synchronization cables that allow researchers to enable direct synchronization as an after-market solution for both commercially acquired and in-house assembled workstations. We routinely employ this solution in our laboratory (Fig. 4) and can attest that it results in a marked decrease in photobleaching/phototoxicity.

The second source of overhead is due to relatively long time required for opening and closing of mechanical shutters (Fig. 4). This problem is not commonly found in confocal systems where the light is blocked by almost instantaneous Acusto-Optic Tunable Filters (AOTF). However, in wide-field microscopy mechanical shutters are common and their

limited speed is an important limitation particularly for multi-dimensional time-lapses. Fortunately, modern light sources such as light-emitting diodes (LED) now make the use of mechanical shutters in imaging workstations unnecessary. In sharp contrast to tungsten filaments and arc burners, LEDs transition between the dark and light-emitting states in less than a microsecond which is insignificant in a typical live-cell recording. Thus, fast light sources combined with proper hardware control enable complete elimination of the exposure overhead (Fig.4).

V. Avoiding Unnecessary Irradiation via Intelligent Illumination

A fundamental limitation in conventional microscopy (both wide field and confocal) is that acquiring a single image exposes every focal plane of the sample to the same amount of light. This means that phototoxicity inflicted by recording a Z-series is similar to phototoxicity inflicted by the same number of in-focus images. Unfortunately, in a typical Z-series only some planes contain in-focus images while others capture nothing but defocused 'blur'. Yet, these useless images contribute to phototoxicity by increasing cumulative exposure. Recently, several clever techniques have been introduced that help to avoid capturing images of 'empty parts' of the sample. Some of these techniques require significant modifications of the microscope's optical path. Their implementation requires a significant investment in new equipment. Others rely on computer algorithms and thus can be retroactively enabled on older instruments.

Most notable hardware-based approaches to decreasing phototoxicity include Selective Plane Illumination Microscopy (SPIM) (Huisken et al., 2004), its more recent variant termed Bessel-beam Plane Illumination (Planchon et al., 2011), and Controlled Light Exposure Microscopy (CLEM) (Hoebe et al., 2007).

In Bessel-beam Microscopy and SPIM the object is illuminated at an angle to the optical axis of the microscope by a thin light sheet. This radical departure from the classic microscope design ensures that only a single plane within the object is illuminated at any given time. The rest of the object remains in the dark. Images obtained by Bessel-beam microscopy are spectacular in both resolution and clarity, particularly when this technique is combined with structured illumination (Gao et al., 2012). Bessel-beams are expected to significantly decrease phototoxicity; however, this approach has not been extensively tested in routine investigations as specialized instruments are just becoming commercially available.

CLEM offers a way to decrease phototoxicity in classic point-scanning confocal microscopes by introducing a feedback sensor that measures the rate of photon accumulation at each diffraction-limited spot. As fluorescently-labeled objects are by definition bright, slow accumulation of the signal during exposure indicates that the spot contains no object. Therefore, a full exposure for this spot is not required. In contrast, pixels with rapid increase in signal need to be fully exposed and recorded. This clever way of attenuating irradiation dose for individual spots within the same image results in a significant decrease in cumulative exposure. However, implementation of CLEM is expensive and it is not compatible with wide-field and spinning-disc confocal modalities.

Dose-decreasing illumination is also being developed for wide-field and spinning disc confocal microscopy. Recently, a simple software based approach, termed Predictive-Focus Illumination (PFI) was introduced (Schilling et al., 2012). In PFI, a full-range Z-series is recorded only once at the first time point of the series. At subsequent time points Z-range is restricted to a small number of focal planes centered on the object-of-interest (OOI) whose position is estimated by a tracking algorithm (Fig. 5). OOI is defined by the user or can be automatically detected by the computer. All necessary computations (OOI detection and

tracking) are done during the lapse between consecutive time points. While the exact benefits of PFI depend on the number of OOIs and their distribution in space, this approach has been shown to decrease cumulative light exposure up to fivefold in certain types of 3-D time-lapses (Schilling et al., 2012). Noteworthy is that PFI requires no hardware modification and thus can be implemented by end users in the environment of a cell biology laboratory (software to enable PFI is freely available; see Schilling et al., 2012).

VI. Conclusions

Live-cell fluorescence microscopy is not a truly non-invasive observation technique. The number of images that can be captured before the cell becomes damaged is fundamentally limited. Sooner or later the cell is likely to change its physiological state in response to stress induced by high-intensity excitation light. This limitation needs to be considered in the design of every live-cell fluorescence experiment. For example, inexperienced investigators would browse through the sample in fluorescence mode in search of the cell to record. However, even a brief look at the cell with one's eyes takes 5–10 seconds which means that it subjects the sample to the same amount of irradiation as recording 50–100 images at 100-ms exposures. This “curiosity damage” can be easily avoided by using completely safe phase-contrast or DIC microscopy to choose the cell and position it. Understanding limitations inherent in live-cell fluorescence microscopy helps to recognize problems and avoid artifacts.

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Figure 1. Photobleaching and photodamage effects in GFP-microscopy

(A) 3-D fluorescence time-lapse (30-s intervals) recorded on a standard research-grade imaging workstation. Fluorescence intensity gradually decreases (photobleaching) under these conditions. Concurrent with photobleaching, physiology of the cell changes as evident from the formation of membrane blebs (0:30, arrow). (B) Lower intensity of the excitation light circumvents photobleaching. However, photodamage remains severe as evident from the appearance of membrane blebs at later time points (1:12, arrow). (C) Increasing intervals between time points from 30 s to 2 min further delays manifestation of photodamage. In this sequence the cell enters mitosis 5 h after initiation of the time-lapse. However, cell division is abnormally long (~3 h) and both daughter cells ultimately develop membrane blebs (13:48, arrow). (D) Further increase of time intervals (to 5 min) allows cells to remain healthy and undergo multiple cell cycles during 64-h recording (progeny of cell #2 is not shown due to space restrictions). HeLa cells constitutively expressing Centrin1-GFP to label centrioles. Fluorescence recorded in wide-field mode. Excitation light generated by a Xenon lamp (XBO-75) filtered with infra-red (KG-5) and ultraviolet (GG-420) absorption filters in addition to the GFP filters. Top rows in each panel present maximal intensity projections of the entire Z-stack. Bottom rows present individual DIC images selected from Z-stacks. Time shown as hours:minutes. Scale bars = 5 μ m.

Figure 2. Dependence of photodamage on the spectrum of excitation

(A) Various states of fluorophore excitation. (1) ground-state fluorophore; (2) singlet-state excited molecule; (3) non-fluorescent triplet state; (4) higher-energy chemically reactive states. (B) Spectral properties of GFP. (1) Absorption spectrum of ground-state GFP molecules. (2) Absorption spectrum of already excited GFP molecules. (3) Fluorescence emission spectrum. Curly bracket denotes spectral range of excitation filters used in standard GFP filter cubes. Notice that 490-nm wavelength is efficiently absorbed by ground-state but not excited molecules. In contrast, 450-nm light is absorbed by both states. (C, D) Comparison of photobleaching and photodamage induced by wide vs. narrow excitation filters. Photobleaching is prominent in (C), recorded with a standard GFP exciter (450–490 nm band-pass). Also notice that the cell retracts its leading edge in response to excessive illumination. In contrast, photobleaching as well as morphological changes are minimal in (D), recorded with a narrow 488 ± 1 -nm laser-line filter. Both recordings were conducted at 2-s intervals but neutral-density filters and shorter exposure times were used in (C) to decrease the difference in the amount of light delivered to the sample (notice that intensities are similar at 0:00 in both sequences). Time shown as minutes:seconds. (E) Effects of emission filter on image quality. (1) Standard combination of filters used in spinning-disc confocal microscopy. (2) Same object recorded with no emission filter. Although the amount of noise increases slightly, improved brightness is beneficial in single-color live-cell recording. (2') Adjusting the look-up table of (2) creates an image virtually indistinguishable from that recorded with the proper emission filter (cf. 1 and 2'). PtK₂ cells constitutively

expressing α -tubulin-GFP to label microtubules. Scale bars = 5 μ m. Spectra are adapted from (Rankin et al., 2011).

Figure 3. Disproportionate increase in photobleaching observed in multi-color recordings
(A) Absorption spectra of GFP (dashed line) and tdTomato (solid line). Arrows and arrowhead mark the efficiency of absorption for the wavelengths commonly used to excite these fluorophores. Notice that tdTomato absorbs ~27% of the 488-nm light. **(B)** Human RPE-1 cell expressing CenpA-GFP and Centrin1-tdTomato. DIC image is shown in (1). Panels (2) and (3) present the green (488-nm excitation / 525-nm emission) and red (561-nm excitation / 617-nm emission) fluorescence channels. Notice that CenpA-GFP localizes inside the nucleus while Centrin-tdTomato targets centrioles in the cytoplasm. Thus, FRET between these proteins is not possible. Image in (4) shows red fluorescence induced by 488-nm excitation. The presence of GFP signal in the red channel is due to the wide emission spectrum of this fluorophore (bleeds through). tdTomato signal is due to excitation of this protein by the 488-nm light. **(C)** Selected frames from time-lapse recordings of cells similar to one shown in (B). Top row illustrates stability of Centrin-tdTomato fluorescence in a single-color recording. Bottom row illustrates a prominent increase in photobleaching of Centrin-tdTomato when both the green and red channels are recorded. **(D)** Quantification of photobleaching. Curves 1 and 2 correspond to the top and bottom rows in panel (C). Curve 3 is theoretical prediction of tdTomato photobleaching in a two-color recording that accounts for the partial excitation by the 488-nm laser light. As evident from these curves the extent of photobleaching observed in the experiment is much greater than the theoretical expectation.

Figure 4. Actual exposure times for different types of synchronization and different light sources
 In all cases, nominal exposure time is set to 100-ms in the commercial software package that controls the imaging workstation. Actual exposure time is measured by a photo-detector placed near the focal plane of the objective lens. The output of the detector is recorded by an oscilloscope. Curve 1 represents a standard configuration where excitation light generated by a mercury burner is shuttered with a mechanical shutter and the hardware (CCD camera and shutters) is synchronized via computer control. As evident from this example, the actual exposure exceeds the nominal by almost two fold (190 vs. 100 ms). Curve 2 is recorded with the same hardware but the shutter and camera are directly synchronized (details in the text). In this configuration, unnecessary exposure is limited to the time required to close the shutter. Notice that the shutter begins to open when the camera is already in the charge accumulation mode and it closes after charge accumulation is completed. For curve 3, the mercury burner and mechanical shutters were replaced with a light-emitting diode (LED). In this case, due to fast on/off time of the LED, the actual exposure is virtually the same as the nominal exposure time.

Figure 5. Example of an adaptive illumination technique

(A) Selected frames from a 3-D time-lapse of centrosome movements during cell division in human (HeLa) cells. These cells become rounded during mitosis and the centrosomes move extensively in all three dimensions. Approximately 50 Z-planes need to be captured at every time point to ensure that both centrosomes remain in focus throughout the recording. Only few of these planes contain information pertinent to the centrosome behavior (white boxes).

(B) Predictive-focus illumination dramatically decreases the number of necessary exposures. In this technique, short Z-series centered on the predicted positions of the objects-of-interest are recorded at every time-point. Notice that when the objects separate in Z, their positions are tracked individually. Top and bottom halves of each panel present X-Y and X-Z maximal projections of 3-D volumes. Time is in minutes:seconds. Bar = 10 μ m.