

File S1: Guidelines to prevent photomorbidity in
live-cell imaging

as supplement to

”Preventing photomorbidity in long-term
multi-color fluorescence imaging of *S. cerevisiae*
and *S. pombe*”

Live-cell imaging microscopes are complex devices, often consisting of components from different manufacturers. Therefore, no single setup is exactly like another, which means that reproducible and optimal conditions for live-cell imaging need to be determined for each setup separately. In addition to the hardware, the experimental design and the nature of the sample are also factors that need to be considered. As mentioned by Laissue et al. [1] four main parameters of a live-cell imaging experiment - sample health, spatial resolution, temporal resolution and signal-to-noise ratio - need to be balanced. Here we present our approach to optimize the different aspects of live-cell imaging experiments and the required hardware.

Sample health

Growth conditions - Each organism shows a different dose-response relationship to excitation light. For each species, the light sensitivity is influenced by the culturing temperature and media. Generally speaking, optimal growth conditions which result in short cell cycle times make cells more resistant to excitation light. Therefore cells should be treated as gently as possible, and extra care has to be taken when fluorescence imaging is used to study stress responses or cell strains that show impaired growth.

Control for absence of photomorbidity - There are two strategies to ensure that the growth rate (GR) is not impaired by the excitation light. In the simplest case, the GR is measured in the presence and absence of the particular imaging protocol. Such a control can be easily implemented in any live-cell imaging experiment. But what can be done if the GR is impaired by the imaging protocol? In that case the researcher is faced with the question of how much the light exposure needs to be reduced to achieve the GR of the non-illuminated control. This becomes increasingly difficult in multi-color imaging, and this is where the second strategy becomes important.

In this case the researcher should measure a photomorbidity dose-effect curve for each of the required excitation wavelengths as described in the methods section. We are aware that this can lead to a substantial workload, especially when several media conditions and strains are to be tested. To reduce the amount of experiments it may be sufficient to measure the dose-effect curves under the worst growth conditions and with the strains that are most growth impaired, since it can be expected that cells will be more resistant to light under more favourable conditions. Although it is not strictly necessary to determine to absolute light intensity and dose, we recommend to acquire this data to ensure the reproducibility of your experiments over time, on other setups and by other people. Once the dose-effect curve is known, it is possible to determine the maximum light dose that can be applied without causing a growth impairment. We termed it the no-observed effect level (NOEL) as described in the methods section. We define the NOEL as the light dose at which growth rate of the fitted dose-effect curve is reduced to 98% of the GR of the non-illuminated control. In practice, a reduction of the GR by 2% is not detectable, which means that the NOEL can only be determined from the fit of the dose-effect curve. Determining the NOEL is especially useful for multi-color imaging, since we could show that the combined effect of several wavelengths on the growth rate is additive.

An additive model implies that the GR under multi-color illumination can be predicted as the product of the GRs of all single color illumination treatments:

$$GR_{multi\lambda} = GR_{\lambda_1} \cdot GR_{\lambda_2} \cdot GR_{\lambda_3} \cdot \dots \cdot GR_{\lambda_n} \quad (1)$$

With $\lambda_{1..n}$ being the individual excitation wavelengths. It is important to note, that for the absence of photomorbidity in multi-color imaging the total reduction in GR should not be larger than 2%. This means the light doses applied at each wavelength have to be even lower than the respective single-color NOEL. However, this rule only applies for fluorophores with spectrally separated excitation. If fluorophores are excited in the same spectral region, the corresponding toxicities will not act independently and synergistic effects can be expected. We did not address how far neighboring filter sets have to be separated to act independently. However, the phototoxicity measurement of cyan excitation light at different bandwidths (Figure 2D) shows no changes in photosensitivity within a bandwidth of 41 nm. For example, if excitation of two fluorophores is separated by <41 nm, like for mRuby2 (561/4 nm) and mKO κ (546/10 nm), both excitation light doses can not be regarded as independent. In this case the light doses of both fluorophores act on the same photomorbidity curve and their sum should not exceed the NOEL for that wavelength.

Signal-to-noise ratio

Two main properties influence the signal-to-noise ratio (SNR) of a fluorescence image - the brightness of the fluorescent protein (FP) and the autofluorescence in the respective imaging channel [2] [3]. To optimize the SNR the microscopist should do both, maximize the signal and also minimize the sources of noise.

Fluorescent proteins - The choice of the fluorescent protein (FP) to be used, is connected to many other aspects in this guide and one of the most important decision to be made. Oftentimes FPs are chosen based on their brightness, which is an easy and universal measure to compare the performance of different FPs. However, the choice of the FP also determines which excitation wavelengths will be used and ultimately if photomorbidity can be avoided. We therefore propose to compare FPs based on the light dose necessary to reach a certain SNR and the corresponding reduction in GR that can be expected from the photomorbidity curve. Such a comparison of some of the brightest FPs currently available can be found in Table 1 of the main text. Be aware that photomorbidity and the achievable SNR depend on the organism and the medium conditions, and as such the table can only provide guidance for work with *S. cerevisiae* and growth in SDmin.

In our opinion, the best fluorescent proteins for unstressed imaging are Citrine (YFP and its derivatives) and the orange fluorescent proteins followed by GFP and the bright red ones. Fluorescent proteins with an excitation wavelength <430 nm achieve acceptable SNR in unstressed conditions only when they are coupled to highly expressed target proteins. However, some fluorescent proteins may be excluded based on other characteristics such as their tendency to oligomerize or photobleach. Furthermore, the maturation rate of an FP can have a substantial impact on the detectable signal. How much of the protein

is available in its matured form depends on the expression level of the target protein and its turnover. It can be substantially lower than the total amount of target protein if the turnover rate of the fusion protein is similar to the maturation rate. Target proteins with a high turnover rate should be labelled with fast maturing FP. For many FPs information on the brightness and maturation rate can be found in the literature [4–7]. The expression level of proteins in yeast [8] can be used estimate the total amount of target protein to be expected. This is especially useful in multi-color imaging, when one needs to decide which of the available FPs should be fused with which target protein.

Optical filters - The choice of the right optical filters is key to obtain a high SNR. We want to emphasize that standard filter sets are not optimized for live-cell imaging, especially when novel FPs with shifted excitation and emission maxima are used. One can only harness the potential of such fluorophores when customized filter sets are used. Although custom filtersets may appear expensive at first sight, they allow the largest gains in SNR to be had for the least amount of money. Furthermore they can be easily replaced and used on another setup.

Excitation filters - Photomorbidity depends on the total light dose and is independent of the bandwidth of the excitation light. We advise to use narrow excitation filters located at the peak excitation wavelength, such that the fluorophore is excited most efficiently. Even though we found that photobleaching of the fluorescent protein is not a concern in long term imaging, narrow excitation filters were additionally shown to decrease fluorophore bleaching [9].

Emission filters - Generally it is good to gather as much of the emitted signal from the FP as possible. However, depending on the utilized cell type and growth condition, autofluorescence can be a major source of noise. Since autofluorescence spectra are rather wide, this noise becomes more prominent when wide emission filters are used (Figure S22B). We therefore advise to use narrower emission filters (bandwidth <30nm) located as close to the emission maximum of the FP as possible. This is especially important at short excitation wavelengths where background fluorescence is high. Only in cases where background fluorescence is low (long excitation wavelengths) wider emission filters may allow for higher SNRs to be reached.

Autofluorescence - The amount of autofluorescence in an image depends on the experimental setup and the specimen. Cellular autofluorescence is highest at short excitation wavelengths and for *S. cerevisiae* drops steadily until reaching a plateau for excitation wavelengths above 530 nm (Figure S22C). The cellular autofluorescence is said to depend on the composition of the media. However, the autofluorescence of the media outside the cell can quickly become the main contributor to the background. One can minimize its contribution by reducing the amount of media in the observed volume. In our case, we achieved this by growing the cells in a microfluidic channel where no media is present above or below the focal plane. In cases where imaging needs to be performed in a well plate, the media autofluorescence can be reduced by lowering the media volume.

Microscope objective - The objective plays a key role in avoiding photomor-

bility, since the SNR strongly depends on the objective numerical aperture (NA) and magnification [10, 11]:

$$SNR \sim \frac{NA^4}{\text{Magnification}^2} \quad (2)$$

Since the NA enters the equation at the power of four, it has the largest single influence on the SNR and ultimately the required light doses. Only high NA oil immersion objectives should be used for live-cell imaging of yeast. These usually come at magnifications of either 40x, 60x or 100x and do not differ significantly in NA. A 60x or 100x objective of the same NA as a 40x objective will require 225% or 625% of the light dose to reach the same SNR as a 40x objective. Furthermore, 60x and 100x objectives lead to finer axial sectioning than 40x objectives. In practice, the optical slice thickness of a 40x oil objective is similar to the thickness of haploid *S. cerevisiae* or *S. pombe* cells ($\sim 4 \mu m$). Therefore fluorescence imaging of a single focal plane is sufficient to image the complete cellular volume with a 40x objective. In contrast, the finer axial sectioning of 60x and 100x objectives requires the collection of z-stacks to image the whole volume of the cell, which multiplies the required light exposure. If the added spatial resolution of a 60x or 100x objective is not required, 40x immersion objectives will deliver the best SNR and most efficient use of the available light dose.

Pulsed illumination - We did neither observe a reduction in photomorbidity nor an increase in SNR when using pulsed blue light (200.000 Hz, 50% duty cycle) compared to constant wave illumination (Figure S12, Figure ??). This is in contrast to earlier reports, which indicated that pulsed illumination can lead to background fluorescence suppression as well as lower photomorbidity in widefield microscopy [12].

Spatial resolution

XY-resolution - Depending on the experimental question it may not be necessary to acquire images at the highest possible resolution. For example, quantification of an evenly distributed cytosolic protein does not require diffraction limited imaging. Lowering the XY-resolution by binning of pixels on the camera, will decrease the light dose required to reach a certain SNR. Of course, the XY-resolution is closely related to the choice of microscope objective (see above).

Multi-position imaging and tiling - Acquisition of large overview pictures usually requires some overlap between adjacent images, to allow stitching of the images during post-processing. This means that the cells on the edge of each image will receive twice as much light as the ones in the center, and cells in the corners may receive up to four times the original light dose. Taking into account that the area of the sample that is illuminated by the excitation light is often substantially larger than the field-of-view of the camera, this may apply to the majority of cells in the image. Therefore, acquisition of overview pictures should be avoided whenever possible. Even during multi-position imaging one should

ensure that neighbouring positions are sufficiently separated to avoid multiple exposure of the same cells.

Temporal resolution

The overall light dose determines the extend of photomorbidity. The imaging interval, light intensity and exposure time can be freely chosen within the available light dose for unstressed imaging. However, depending on the required temporal resolution and the microscope hardware, these parameters need to be optimized.

Hardware delays and exposure time - In most microscopes the excitation light source and the acquisition camera are only synchronized through the microscope software. In this case, the time that the sample is exposed to excitation light is longer than the set exposure time of the camera, leading to an illumination overhead in the range of 100-500 ms [9, 13]. Hardware triggering is a way to limit these delays to a few milliseconds, and we strongly recommend to upgrade live-cell imaging microscopes with hardware triggering. In setups without hardware triggering, the duration of hardware delay should be measured e.g. using a high speed video recording from a camera. To minimize the additional light dose delivered during hardware delays, the exposure time should be at least 10x hardware delay.

Excitation light intensity - Photomorbidity is solely determined by the cumulative light dose and is independent of the light intensity. This is in contrast to previous reports of stronger induction of adverse effects at high light intensities [14, 15]. However, in case your microscope setup suffers from hardware delays (see above) you may observe stronger apparent photomorbidity at higher light intensities.

Imaging interval - The imaging interval should be chosen based on the speed of the cellular process that needs to be observed. In multi-color imaging it can be beneficial to image certain channels less frequent than others. For example, brightfield images may be acquired at high frequency to allow precise cell tracking over time, whereas fluorescence channels monitoring slow cellular processes can be imaged at lower frequencies.

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