Investigations on light-induced stress in fluorescence microscopy using nuclear localization of the transcription factor Msn2p as a reporter

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Abstract

We utilized the nuclear localization of a stress-sensitive transcription factor, Msn2p, to study light-induced stress caused by time-lapse fluorescence imaging of green fluorescent protein (GFP) in budding yeast Saccharomyces cerevisiae. A range of exposure times, light intensities and intervals between exposures were tested in order to provide guidelines for noninvasive imaging. We found that the cellular response, revealed as an enhanced nuclear shuttling of Msn2p-GFP, is induced at significantly lower light exposures than those causing observable changes in cell morphology or cell growth. However, no stress induction was observed if the accumulated photon energy per area unit used to obtain an image was maintained at 0.16 J cm⁻² or below. Above this ‘safe’ level, the stress response is determined by both the intensity and the exposure time. In particular, for a given accumulated photon energy per area unit, a high intensity applied during a short exposure causes more stress than vice versa. Interestingly, no correlation was found between the degree of stress and the absolute fluorescence signal, indicating that light-induced cellular stress in the studied system is not specifically related to GFP excitation.

Introduction

Since the introduction of the green fluorescent protein (GFP), fluorescence microscopy has evolved into one of the most powerful and widely used in vivo methods to study protein function (Shav-Tal et al., 2004; Wouters, 2006). In particular, it is now possible to follow protein localization, expression and interactions in individual cells over long time periods using automated time-lapse microscopy. However, extensive illumination may be damaging to cells due to thermal and/or photochemical effects, such as generation of reactive oxygen species (ROS) via excitation of the fluorescent tag (Dixit & Cyr, 2003; Knight et al., 2003) or intrinsic autofluorescent species (Fraikin et al., 1996; Edwards & Silva, 2001; Eichler et al., 2005; Lubart et al., 2006). Such effects will stress the cells under study, potentially rendering the information gained from time-lapse data misleading or useless. Even though the negative effects of extensive light irradiation are well known, for example from studies in the fields of skin cancer and photodynamic therapy (Oleinick et al., 2002; Almeida et al., 2004), there are so far comparatively few published reports focusing on phototoxicity induced during fluorescence microscopy measurements. Knight et al. (2003) reported elevated levels of Ca²⁺ and cell death forcontrocytes caused by cellular imaging based on confocal microscopy, while Manders et al. (2003) found that the cell-cycle progression of HeLa cells was affected by the same imaging technique. For very high light intensities, which are necessary in, for example, fluorescence recovery after photobleaching studies, it has even been shown that light scattered by illuminated cells can induce phototoxic effects on neighboring cells that are not illuminated (Dobrucki et al., 2007). Actual guidelines for noninvasive imaging using wide-field fluorescence microscopy have been proposed by Dixit and coworkers (Dixit & Cyr, 2003; Dixit et al., 2006), based on studies of mitosis in plant cells subject to varying illumination conditions, but their general applicability to different organisms is not known.
In this study, we analyzed the stress induction caused by wide-field fluorescence microscopy in budding yeast, one of the most important model organisms in basic cell biology research. Importantly, a recent library of chromosomally tagged GFP fusions (Huh et al., 2003) has considerably facilitated in vivo fluorescence microscopy studies of almost all proteins in *Saccharomyces cerevisiae*, thus highlighting the importance of quantifying light-induced stress in this organism. Previous works on light sensitivity in yeasts have mainly focused on sterilization (Takeshita et al., 2003) and treatment of fungal infections (Calzavara-Pinton et al., 2005) using visible light. To the best of our knowledge, there are no published studies on budding yeast that target phototoxicity caused by fluorescence microscopy. In order to provide guidelines for this kind of measurements, we have utilized the protein Msnp2 as a sensitive stress reporter. Msnp2 is an inducible transcription factor that is activated by a large number of stress conditions (Schmitt & McEntee, 1996; Gasch et al., 2000), including light exposure (Jacquet et al., 2003). Upon stress induction, Msnp2 rapidly localizes from the cytoplasm to the nucleus (Görner et al., 1998), where it is involved in controlling a large number of genes that respond to stress. The translocation dynamics is comparatively easy to quantify by microscopy, which makes GFP-tagged Msnp2 a technically suitable and biologically relevant sensitive reporter of light-induced stress. Here, cells transformed with an *ADH1* promoter-driven Msnp2-GFP plasmid were illuminated with blue light for GFP excitation, with a range of different exposure times, intensities and intervals between exposures. For each setting, the Msnp2 localization dynamics was registered for 50–100 individual cells over 1 h of repeated exposures to analyze the stress response in relation to the individual exposure parameters. We found that the stress response is dependent on the applied light dose and the combination of light intensity and exposure time. Furthermore, our results indicate that GFP phototoxicity is not the main source of stress.

**Materials and methods**

**Image acquisition and stress induction**

Images were acquired using an automated epi-fluorescence microscope (Nikon TE2000 PFS) equipped with a × 60 oil-immersion objective (Plan Apo, NA 1.4) and a back-illuminated, electron-multiplying CCD camera (Ixon DV887-DCS-BV, Andor Technology). The XY-stage, the Z position of the objective, the excitation and emission filters, shutters for the light sources and the CCD camera were controlled by the iQ software from Andor Technology. GFP imaging and simultaneous stress induction were achieved using a mercury lamp in combination with excitation filter HQ470/40x, dicroic mirror 505DCLP and emission filter HQ515/30m (Chroma Corporation). Figure 1a shows a measurement of the actual excitation spectrum, recorded at the sample position using a small fiber-coupled spectrometer (Avaspec-2048). The data confirm that the light intensity is negligible outside the transmission window of the excitation filter, i.e. 470 ± 20 nm.

Figure 1b shows the exposure variables that were evaluated in the experiments, i.e. the light intensity, *I*, the exposure time, *τ* and the intervals between exposures, *τ*. The size of the field diaphragm was adjusted to match the field of view, resulting in an illumination area of \(A\sim1.45 \times 10^{-4} \text{ cm}^2\). The intensities were measured with the objective removed using a power meter (18LAB250, Melles Griot) positioned at the objective back-aperture plane and then compensated for transmission loss through the objective, which is \(c.10\%\) at 470 nm.

Table 1 summarizes the combinations of *I* and *τ* values investigated. The range of exposure times is typical for wide-field microscopy, while the intensity values were selected based on the possibilities of the microscope, such as the available ND filters, and the excitation levels necessary to obtain Msnp2 images with a reasonable signal-to-noise ratio.
Function. We confirmed that neither of these additional system of the microscope utilizes near-infrared light for its GFP excitation. We should also note that the perfect focus orbital shaker, heated to 30°C, was used. After growth from OD610 nm = 0.1–0.5 in an air contact. Perfusion of fresh medium was not allowed because that caused movements in the Z position of the coverslip. However, at the start of the experiment, the OD was low enough to allow for at least 3 h of exponential growth, which can be compared with the time of the experiment, which was 1 h. A test experiment, comparing the frequency of Msn2p nuclear localization between cells in the chamber with and without perfusion, confirmed that the stress level was not elevated when fresh medium was not supplied over the 1-h experiment time.

Results and discussion

Table 1. Exposure settings and corresponding energy doses that were compared in this study

<table>
<thead>
<tr>
<th>I (µW)</th>
<th>τs (ms)</th>
<th>2000</th>
<th>530</th>
<th>245</th>
<th>66</th>
</tr>
</thead>
<tbody>
<tr>
<td>1340</td>
<td>18.5 J cm⁻²</td>
<td>4.9 J cm⁻²</td>
<td>2.3 J cm⁻²</td>
<td>0.6 J cm⁻²</td>
<td></td>
</tr>
<tr>
<td>355</td>
<td>4.9 J cm⁻²</td>
<td>0.6 J cm⁻²</td>
<td>0.16 J cm⁻²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>164</td>
<td>2.3 J cm⁻²</td>
<td>0.6 J cm⁻²</td>
<td>0.07 J cm⁻²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>0.6 J cm⁻²</td>
<td>0.16 J cm⁻²</td>
<td>0.02 J cm⁻²</td>
<td></td>
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</table>

The values of I and τs are typical for time-lapse experiments and were selected to provide multiples of one energy dose, for example as for 0.6 J cm⁻². The energy doses, P, were calculated by P = Iτs/A, where A is the area of illumination, which was 1.45 × 10⁻⁷ cm².

The (S/N) ratio. The corresponding photon energy doses P = Iτs/A, shown in Table 1, span more than three orders of magnitude and encompass levels typically used in imaging of GFP-tagged yeast cells. Note that several settings yield the same energy dose, for example as for 0.6 J cm⁻², which means that the influence of I and τs can be investigated independently. Based on the dynamics of the Msn2p response, the intervals between exposures were set to τs = 20, 30 or 60 s.

Apart from the fluorescence imaging, we also recorded bright-field transmission images before and after each experimental series, using red light (λ > 600 nm), to avoid GFP excitation. We should also note that the perfect focus system of the microscope utilizes near-infrared light for its function. We confirmed that neither of these additional illumination sources affected the Msn2p stress response.

Cell strain and cultivation

Saccharomyces cerevisiae BY4741 cells (genotype: Mat a; his3Δ; leu2Δ; met15Δ; ura3Δ) were transformed with the plasmid pASMG1, which is based on vector YCpLac111 and contains an ADH1 promoter and MSN2-GFP (a kind gift from Christoph Schüller, University of Vienna). The cells were grown in a dark environment on synthetic defined medium (1.7 g L⁻¹ yeast nitrogen base without amino acids, 0.5% ammonium sulfate and 1% succinic acid, 2% (w/v) glucose, 790 mg L⁻¹ CSM-leu, pH 5.8). For comparison of wild-type and GFP-tagged cells, CSM containing all amino acids was used. After growth from OD610nm = 0.1–0.5 in an orbital shaker, heated to 30°C, the cells were directly spurted into a c. 340 μL perfusion chamber (FCS2, Bioptechs) that were mounted on the microscope. A schematic drawing of the perfusion chamber is shown in Fig. 1c. The temperature of the chamber and objective was set to 28°C to ensure that the cells were not subjected to heat > 30°C. The cells were left for 10–20 min to immobilize on a concanavalin A-precoated coverslip before imaging. During the experiment, the inlet and outlet of the chamber were left open for varying GFP intensity. Figure 2 illustrates the temporal development of Irel for a typical yeast cell exhibiting Msn2p nuclear shuttling. In order to extract a set of easily comprehensible parameters characterizing the degree of stress, we digitized each time trace by classifying time points for which Irel(t) > Irelth as time points for which Msn2p is nuclear localized. Here, Irelth is a cell-specific threshold value obtained from an estimate of the signal background and noise level for each cell and exposure setting. Apart from the fraction of cells exhibiting nuclear localization, we determined the three cell-specific parameters illustrated in Fig. 2, i.e. the first time point of nuclear localization, trelth, the duration of each localization period, trel, and the total duration of nuclear localization for each time trace, ttotal.
Influence of different exposure settings on the Msn2p stress response

To evaluate the relationship between stress induction and applied light dose, we first asked whether it is possible to image the cells, using any of the light exposure settings summarized in Table 1, every 30 s for 1 h without inducing stress. Figure 3 illustrates the Msn2p localization data for 50 individual cells at the three lowest energy doses, i.e. 0.07, 0.16 and 0.6 J cm$^{-2}$, and a constant integration time $t_c = 66$ ms. White and black squares in the figure indicate nuclear and cytoplasmic localization, respectively, and each row identifies a specific cell in the population. We first note that the individual cell responses are highly diverse, i.e. the occurrence of nuclear localization differs quite substantially from cell to cell in each population. This heterogeneity is similar to what has been observed in earlier studies of Msn2p nuclear shuttling (Jacquet et al., 2003; Garmendia-Torres et al., 2007; Cai et al., 2008) and is a typical example of ‘phenotypic individuality’ in microbial cultures (Avery, 2006). Secondly, we see that the overall occurrence of localization is similar for the two lowest energy doses, 0.07 and 0.16 J cm$^{-2}$, while the cells imaged using 0.6 J cm$^{-2}$ display much more frequent nuclear localization. We also see that the occurrence of localization increases significantly during the time course of the experiment for the highest illumination level, while this effect is not pronounced for the two lower doses. These observations indicate that illumination levels of 0.16 J cm$^{-2}$ or below do not cause additional light-induced stress above background. To further substantiate this conclusion, we performed a control experiment in which the cells were imaged only every 15 min using a light dose of c. 0.4 J cm$^{-2}$ and $t_c = 66$ ms. As shown in Fig. 3b, the fraction of cells displaying nuclear localization in the control is 5–10%, which is similar to what is observed for the two lower light doses. For 0.6 J cm$^{-2}$, however, the occurrence of nuclear localization is more frequent. This indicates that the dose level of 0.16 J cm$^{-2}$ and below does not cause any light-induced stress, for the settings $t_c = 66$ ms and $t_i = 30$ s, during 1 h of light exposures.

Keeping in mind the dose level needed to induce stress, we went on to characterize the Msn2p response to applied light dose using the three parameters illustrated in Fig. 2. As can be seen in Fig. 4, the results for all three parameters are...
Again this was done by imaging the cells every 30 s for 1 h. In Fig. 5, we show the total duration of nuclear localization, which should be the most robust measure of stress according to Fig. 4, for the six different combinations. The results clearly show that a short exposure time combined with a high illumination level causes more stress than vice versa for the three investigated dose levels. Indeed, we observe a similar stress response for 0.6 J cm\(^{-2}\) obtained using \(\tau_e = 66\) ms at \(I = 1340\) µW as for the approximately four times higher light dose 2.3 J cm\(^{-2}\) obtained using \(\tau_e = 2000\) ms at \(I = 164\) µW. Furthermore, the stress response for 0.6 J cm\(^{-2}\), using \(\tau_e = 2000\) ms at 44 µW, is almost as low as for the background levels observed using \(P = 0.07\) or 0.16 J cm\(^{-2}\) and \(\tau_e = 66\) ms (Fig. 4a).

Finally, we investigated in what way the stress response depends on the accumulated light dose by varying \(\tau_e\) between 20, 30 and 60 s, and maintaining \(\tau_e = 2000\) ms and \(I = 1340\) µW. In Fig. 6, we have plotted the fraction of cells with nuclear Msn2p against elapsed time (a) and against image frame number (b). It is clear that the stress response is best related to frame number, and thus to the accumulated light dose. This indicates that each exposure generates a specific quantity of damage. We also note that the maximum fraction of cells with nuclear Msn2p, of the order 60%, is similar for the three cases (the slightly lower value for \(\tau_e = 60\) s is probably due to some shuttling events with \(t_p < \tau_e\) being missed). This value is similar to the maximum degree of nuclear localization found in Jacquet et al. (2003) and may indicate an upper level of Msn2p localization obtainable through light-induced stress.

**GFP phototoxicity and physiological changes**

The above results clearly indicate that Msn2p-GFP nuclear migration is a sensitive indicator of light stress. Importantly, within the investigated parameter range, we found that
photon doses per exposure of the order 0.16 J cm\(^{-2}\) or below did not induce an observable stress response. However, this ‘safe’ level has to be used with caution. For example, it cannot be ruled out that much more frequent imaging than at 30-s intervals could be harmful even if \( P < 0.16 \) J cm\(^{-2}\). Similarly, as the degree of stress for a given photon dose scales inversely with illumination time, we cannot exclude that high intensities applied during shorter intervals than investigated here could cause stress, even if the applied light dose is maintained \(< 0.16 \) J cm\(^{-2}\) per exposure. The intricate variation in stress response with exposure setting also points towards the question about the ultimate cause of light-induced stress. Although Msn2p is sensitive to temperature changes (Görner et al., 1998), heating due to illumination appears to be a less likely cause. The temperature increase in a yeast cell will be linearly proportional to intensity under steady-state illumination conditions, but for the short exposures and low intensities used here, we expect heating to be well below 1°C [the temperature increase of erythrocytes subject to \( > 80 \) kW cm\(^{-2}\) of focused continuous irradiation was estimated to \(< 1°C\) (Ramser et al., 2004)]. This points towards photochemical processes as the most likely cause of stress induction. Of prime concern is the possibility that light causes stress due to ROS production linked to excitation of GFP, as this would mean that the ‘safe’ illumination level could vary strongly with the expression level. Indeed, even though GFP is a relatively poor photosensitizer (Jiménez-Banzo et al., 2008), it has been used in chromophore-assisted laser inactivation studies (Rajfur et al., 2002; Jacobson et al., 2008) and it is known to generate singlet oxygen, \(^1\)O\(_2\) (Greenbaum et al., 2000; Remington, 2006; Jiménez-Banzo et al., 2008), an ROS known to cause cell damage (Davies, 2003) and induce oxidative stress response in yeast (Brombacher et al., 2006). It has also been shown that the degree of GFP photobleaching, which is typically linked to the presence of ROS, is higher if a given light dose is concentrated to a short time interval (Bernas et al., 2004), which recalls the Msn2p response in Fig. 5.

To investigate whether GFP phototoxicity could be linked to the stress response, we utilized the fact that the GFP copy number differed significantly between cells. We thus searched for correlations between the stress parameters described above and the brightness of individual cells. We should note here that brightness means the total fluorescence intensity, i.e. including background. The latter arises because of endogenous autofluorescent species, of which in particular flavins have absorption and emission characteristics that will generate a background in GFP measurements (Kao et al., 2008) and have been identified as a source for ROS production under visible light irradiation (Eichler et al., 2005). As shown in Fig. 7, we found no direct correlation between the mean fluorescence intensity per cell at time zero and induced stress, as illustrated for the parameters \( t_{\text{first}} \) (start of nuclear migration, Fig. 7a) and \( t_{\text{total}} \) (total nuclear localization duration, Fig. 7b). This
indicates that GFP-induced ROS is not the main contributor to stress induction and that phototoxicity is either due to autofluorescent species (which contributes to c. 50 counts in Fig. 7) or to photochemical processes associated with nonfluorescent absorbing species.

In order to complement the above studies of stress induction reported by Msn2p nucleocytoplasmic localization dynamics, we also searched for possible morphological changes and growth defects that could be linked to GFP excitation. The motivation for this is that the value of Msn2p as a ‘stress sensor’ in fluorescence microscopy naturally depends on whether simpler or more sensitive reporters, in particular morphological features, of light-induced stress exist or not. Further, to gain more insight into the potential phototoxicity of GFP, we compared the Msn2p-GFP-tagged cells with cells not expressing any GFP and we analyzed growth defects and changes in intracellular cell morphology for illuminated and nonilluminated cells. First, a commercial cell viability test was applied on wild-type cells (propidium iodide, Invitrogen, a fluorescent dye that penetrates damaged plasma membranes). This test did not indicate any loss of viability, even for the highest light dose 18.5 J cm⁻² (data not shown). Second, we searched for growth defects. As illustrated in Fig. 8a, we found that for high-intensity illumination, cell cycle progression slowed down significantly during the 1-h observation time. As shown in the upper panel of Fig. 8a, wild-type unilluminated cells that are in the early S phase at the start of the experiment have almost reached the end of one cell cycle after 1 h. This is expected, because one cell cycle lasts c. 1.5 h.

Fig. 8. (a) Bright-field images of GFP-tagged (+GFP) and wild-type cells (-GFP) before and after a light exposure experiment. The cells in the right column were illuminated every 30 s for 1 h with 18.5 J cm⁻². The cells in the left column were not illuminated. A clear growth defect is seen both for GFP-tagged and wild-type cells when illuminated. Illuminated cells also have a large fraction of cells with more distinct vacuoles. (b) The bud growth of three budding cells at three different light doses. The gray lines show the growth of three unstressed cells and the black lines show the growth of the cells subjected to the light dose given in the figure, every 30 s for 1 h. The growth has been estimated by dividing the area of the bud with area of the mother, as smaller mother cells produced smaller daughter cells and vice versa. In (c) the fraction of > 100 cells with distinct vacuole is plotted. The response is almost identical between GFP-tagged and wild-type cells.
However, on illuminating the cells with a light dose of 18.5 J cm\(^{-2}\) every 30 s for 1 h, many of the cells stopped or slowed down bud growth (right panel). We found no difference in growth between GFP-tagged (bottom row) and wild-type cells (top row) that had been exposed to the same illumination sequence. In Fig. 8b, the bud growth of GFP-tagged cells is plotted for three typical cells at the three light doses: 2.3, 4.9 and 18.5 J cm\(^{-2}\), applied every 30 s for 1 h. We did not find any change at 2.3 J cm\(^{-2}\) illumination when comparing the bud growth for unstressed cells (gray lines) with the bud growth of the light stress cells (black lines). However, for 4.9 and 18.5 J cm\(^{-2}\) the growth defect is observable. This can be compared with 0.6 J cm\(^{-2}\), which is the lowest light dose where increased nuclear localization of Mns2p is detected (Figs 3 and 4). Msn2p localization is thus a more sensitive reporter for light-induced stress than cell growth.

We also observed a second effect of light exposure, namely a change in vacuolar morphology. In Fig. 8a, it can be seen that the vacuole is enlarged or more distinct for irradiated cells. This effect may be related to the fact that the vacuole morphology is highly responsive to different extracellular and intracellular environments (Li & Kane, 2009). The apparent increase in contrast could also be due to a change in the refractive index of the vacuolar interior, possibly due to an accumulation of photo-degraded products. A quantification of this morphological feature showed a similar occurrence for cells expressing and not expressing GFP, both before and after irradiation (Fig. 8c). However, it is obvious that significantly more cells exhibit an enlarged vacuole after illumination than at the start of the experiment. In Fig. 9, the fraction of cells with a changed vacuole appearance after illumination is plotted for a number of light doses. The first bar indicates the response for cells subjected to light only every 15 min, and thus serves as a negative control. Higher light doses, of the order c. 2.3 J cm\(^{-2}\), are required to detect a significant increase in stress response using a vacuolar appearance, as compared with using Msn2p nuclear localization as a reporter (Figs 3 and 4) where a stress response is indicated at much lower light doses. Furthermore, the dependence on exposure settings for a given light dose (Fig. 5) is not seen using this method. This might be due to a high noise level, as seen for the response between the lower light doses.

**Conclusions**

We have investigated a light-induced stress response in budding yeast for a set of exposure settings typical for wide-field fluorescence time-lapse microscopy measurements of GFP fusion proteins. Using Msn2p nuclear localization as a stress reporter, we registered stress at light dose levels where cell-cycle progression still appears as normal and for which no morphological effects of illumination are noticeable. When imaging the cells every 30 s, an accumulated photon energy dose per exposure of 0.6 J cm\(^{-2}\) was required to induce Msn2p stress response. For comparison, changes in growth were recorded only after using 4.9 J cm\(^{-2}\) or higher light doses and changes in morphology were seen from c. 2.3 J cm\(^{-2}\). This demonstrates that nuclear localization of Msn2p-GFP is a sensitive reporter of light-induced stress and suggests that the methodology described herein is of general value as a method of characterizing the degree of light-induced stress in various optical microscopy
applications. Importantly, however, no stress induction was observed if the light exposure was maintained at 0.16 J cm\(^{-2}\) or below. This indicates that ‘safe’ illumination levels exist, although the exact level probably varies somewhat between different systems. Further, for fixed light doses in the range 0.6–4.9 J cm\(^{-2}\) per exposure, we found a stronger stress response if a high intensity and a short exposure time were used rather than vice versa. We also found that the speed of the stress response was related to the accumulated dose rather than the elapsed time, suggesting that each exposure generates a specific quantity of stress. To reduce stress, time-lapse experiments should therefore be performed using the longest possible intervals between exposures, compatible with the studied dynamics, and using a long exposure time rather than a short one. Further, we found no indications for a variation in stress response with fluorescence intensity, indicating that GFP-induced phototoxicity is not the main source of stress in the present system. From a photochemistry point of view, it thus appears advantageous to use a high copy number of GFP molecules in time-lapse microscopy, if possible, so that the illumination levels can be kept at a minimum while retaining a high image S/N ratio. From a biological point of view, however, possible implications following protein overexpression must be considered. The overexpression of Msn2p-GFP in this study was necessary in order to detect the signal over the whole time series without too much bleaching and to allow for the wide spectra of light intensities that were used.

Acknowledgements

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References


