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Original Research Paper

UV LIGHT INDUCED FLUORESCENCE RECOVERY OF GFP AFTER PHOTOBLEACHING IN MICROSCOPY IMAGING

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ABSTRACT

Fluorescence microscopy has become one of the most important tools for biologists to visualize and study organelles and molecules in a cell. Fluorescent markers are used to visualize specific molecules. One of the most used markers is green fluorescent protein (GFP), which can be expressed along with a protein of interest. However, it is known that the intensity of fluorescence decreases with observation time. To combat this problem, researchers and companies have developed protocols and additives to mitigate photobleaching. In this study, we tested the effects of the three most used culture media on photobleaching and developed a new approach of short-wavelength fluorescence recovery after photobleaching (FRAP). Photobleaching was analyzed by comparing pixel brightness on images taken with a fluorescence microscope. We determined photobleaching of GFP-expressing cells from images taken with a fluorescence microscope by comparing pixel brightness. Statistical analysis was performed to determine the average bleaching for specific culture media. The culture media analyzed had no significant effect on the photobleaching of GFP. However, a brief UV burst (15 sec) restores > 50% of the original fluorescence and neither increases ROS nor decreases cell viability. To avoid artifacts in image analysis and interpretation, our study suggests using this simple method of GFP fluorescence recovery with UV light induced FRAP to extend the fluorescence lifetime and imaging of GFP molecules.

Keywords: fluorescence microscopy; fluorescence recovery after photobleaching, GFP, image analysis, sample preparation.

INTRODUCTION

Imaging living cells in real time with a microscope is a powerful tool in cell and molecular biology (Frigault *et al.*, 2009) and helps determine cell migration, interactions, and the movement of specific molecules and structures within the cell (Hibbs, 2000; Matsilele *et al.*, 2018; Kwon *et al.*, 2020). To determine a specific location of a molecule, it must be labeled with a marker (Vida *et al.*, 1995). The most commonly used markers to study the dynamics of molecules in living cells are members of the green fluorescent protein (GFP) family (Hein *et al.*, 2008; Scandella *et al.*, 2020). Members of the GFP family are small proteins that can be expressed in a cell and show fluorescence when illuminated with an appropriate excitatory light (Wang *et al.*, 1989).

However, it is known that fluorescence intensity decreases when fluorophores are excited repeatedly and for long periods of time. This photobleaching effect limits the observation time and quantification of the signal during an experiment (Eggeling *et al.*, 2006; Hibbs, 2004; Vanheusden *et al.*, 2020). Photobleaching is a phenomenon in which light causes photochemical changes (oxidation, bond shift, or cleavage) in the fluorophore such that fluorescence is quenched (Thorley *et al.*, 2014). The other phenomenon, although less well known, is the transition between systems to a persistent triplet state. In the triplet state, the fluorophore is unable to fluoresce, and due to the impeded transition back to the singlet (or ground) state, the decay time can be on the order of several minutes (Ishikawa-Ankerhold *et al.*, 2012). In addition to chemical destruction of the fluorophore, this process is the main cause of loss of fluorescence in fluorescence imaging procedures.

Light bleaching of labeled proteins is particularly problematic for imaging in living cells, mainly because

of the deleterious effect of high photon flux on the cells (Magidson *et al.*, 2013; Bogdanov *et al.*, 2009). Therefore, several attempts have been made to reduce this phenomenon, with varying efficacy depending on the fluorescent label, observation method, and labeled structure. However, most antifade agents developed are not suitable for live cell observation and are mostly used in fixed samples where the toxicity of the agent is not a major concern (Ono *et al.*, 2001; Longin *et al.*, 1994). To minimize the damage caused by the light due to the long observation times and to minimize the disruption of processes in the cells by the high-intensity light current, researchers have developed some techniques to combat these problems. Optimizations of microscopes and detection devices that are much more sensitive to low light currents have proven effective, but they can only improve image quality and photon-induced damage to a limited extent (Magidson and Khodjakov, 2013). Some studies have shown that the exclusion of potentially redox-active media components (such as vitamins and polyphenols) reduces photobleaching, prevents oxidative damage to the fluorescent reporters and reduces the background noise of the signal (Bogdanov *et al.*, 2009; Bogdanov *et al.*, 2012).

In this study, we tested the effect of different culture media on photobleaching of GFP in T24- actin-eGFP cells using image data and performed pixel brightness analysis of inverted fluorescence microscope images. We also investigated the phenomenon of fluorescence recovery after photobleaching (FRAP) with short-wavelength light bursts. We used short wavelength light (λ= 330−380 nm) to induce intersystem crossing of fluorophores that entered the triplet state after prolonged exposure to excitation wavelengths (Mehdi *et al.*, 2013). We hypothesize that i) photobleaching does not depend on the cell culture media used for cell growth, ii) that we can extend the observation time of cells by using short wavelength light bursts, and ii) that ROS (reactive oxygen species) concentration and cell viability as a measure of cell toxicity are not affected with the established protocol (OECD, 2019).

MATERIALS AND METHODS

SAMPLE PREPARATION

T24-actin-eGFP cells (human muscle-invasive bladder carcinoma cells transfected with eGFP) (Veranic *et al.*, 2008) were cultured in round Petri dishes for cell culture (MatTek, United States). Cells were cultured in three different media in the HERACell CO_2 incubator at 37 °C, 5% CO_2 concentration and 100% humidity. The media used were i) basic cell culture medium ADMEM/F12 (Gibco, USA) at a ratio of 1:1, which was also used as a control, 5% FBS (fetal bovine serum) and the antibiotics streptomycin and penicillin. ii) culture medium for prolonged fluorescence observation FluoroBrite™ DMEM (Life Technologies, USA; hereinafter referred to as only FluoroBrite™). iii) basic cell culture medium ADMEM/F12 without pH indicator phenol red (hereinafter referred to as ADMEM/F12-PR), since it is known that phenol red in the culture medium increases the level of background fluorescence (Stadtfeld et al., 2005). All cells were cultured in basic cell culture medium ADMEM/F12 before experiments. Two hours before observation, the media were replaced with freshly prepared media. Three culture media were added in parallel to the corresponding cells. We performed 3 independent experiments with 6 technical replicates (parallels) each.

To measure ROS concentration and viability, T24-actin-eGFP cells were grown in a 96-well plate. Cells were first illuminated with a spectrofluorimeter (λ_{Ex}= 470 nm) Infinite® M1000 (Tecan) for 30 minutes and then washed 3 times with ADMEM/F12 medium. A solution of ROS detection dye CM-H2DCFDA (LifeTechnologies) at a final concentration of 10 µM was added for 30 min (200 µL), and then cells were washed again 3 times with ADMEM/F12. Fluorescence intensity was measured at λ_{Ex}= 503−543 and λ_{Em}= 655−675 nm with a positive control of CM-H2DCFDA in 1 mM H_2O_2.

A portion of the cells (six technical replicates) were used to measure viability with 7-AAD (LifeTechnologies). Cells were washed again 3 times with basic medium, then 100 µL of 7-AAD solution was added and incubated for 20 minutes at 37 °C. Fluorescence intensity was measured at λ_{Ex}= 503−543 and λ_{Em}= 655−675 nm (Schmid *et al.*, 1992).

IMAGE ACQUISITION

Cells were observed under a Nicon Eclipse TE300 inverted fluorescence microscope. We used an immersion objective with 100× magnification. The filter block was set to GFP wavelengths (λ_{Ex}= 450−490 nm, λ_{Em}= 505−565 nm). Images were acquired using the Nikon Eclipse E200 camera. Fluorescence intensities for calculation of ROS concentration and viability were measured using Infinite® M1000 spectrofluorimeter (Tecan, Switzerland).

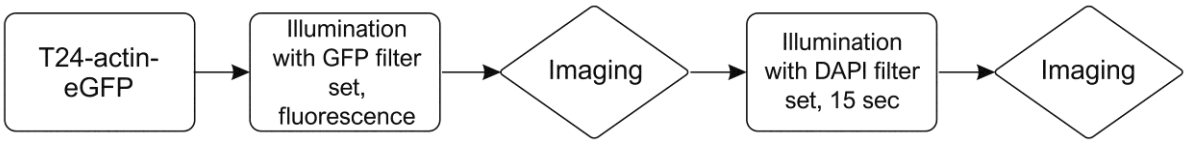

Fig. 1: *Illumination and image acquisition protocol*.

Images were acquired according to the protocol after 0, 10, 20, and 30 minutes of illumination with GFP filter block (λ= 450−490 nm). The illuminated cell area was always the same during this time. After the 30-minute illumination period, the filter was closed for 1 minute (the cells were in the dark), and the image was then acquired. The image was also acquired after 15 seconds of illumination with a DAPI filter block (λ= 330−380 nm). Illumination for more than 15 seconds did not improve the result. The same protocol was used at two different locations in the same Petri dish for all three selected media (Fig. 1).

SIGNAL QUANTIFICATION AND DATA ANALYSIS

Fluorescence intensity was determined from the images using AxioVision (Zeiss, version 4.8) (Wolff, 2008) based on pixel brightness. We calculated the intrinsic intensity of fluorescence by dividing the total fluorescence intensity by the surface area of the cells in the image (a.u.).

To minimize the standard error (difference in cell number) in the measurement of ROS concentration, we divided the average fluorescence intensity for each hole in the 96-well plates by an average intensity of all measured holes on the plate and subtracted the background fluorescence (GFP, medium, cells). The plots were plotted with the intrinsic fluorescence. The same analysis protocol was used to measure viability with 7-aminoactinomycin D (7-AAD).

To quantify the statistical parameters, the t-test ($p \leq 0.05$) for normally distributed data sets was applied for experiments with all culture media tested.

RESULTS

FLUORESCENCE INTENSITY AND PHOTOBLEACHING OF CELLS IN DIFFERENT CULTURE MEDIA

In pursuit of our initial hypothesis that GFP bleaching is not dependent on cell culture medium, we used human muscle-invasive bladder carcinoma cells transfected with eGFP (T24-actin-eGFP) and tested cells grown in three different media, ADMEM/F12, FluoroBrite™, and ADMEM/F12-PR (without pH indicator phenol red) for photobleaching and fluorescence renewal experiments. The pH indicator phenol red was also not present in FluoroBrite™. We investigated the time-dependent photobleaching of eGFP from cells grown in different media. Cells were grown in a CO_2 incubator in MatTek petri dishes. When confluent growth was achieved, we placed the Petri dishes in the inverted fluorescence microscope and illuminated the samples with eGFP wavelength (λ= 450−490 nm). Images were acquired at 0, 10, 20, and 30 min of illumination. The light source was then turned off and images were acquired after one minute when the cells were kept in the dark (Fig. 2). The time-dependent intrinsic fluorescence values were calculated for each parallel.

Fluorescence intensity decreased significantly after 30 minutes of illumination in all three media. In the first 10 minutes of illumination, an average of 56% of the original fluorescence intensity was lost in all three media, and this trend continued with an average decrease of 49% within 10-20 minutes. In the final 10 minutes (20-30 minutes), the average fluorescence lost an additional 32% of intensity (Table 1, acquired images 1-3). Overall, an average of 85% of fluorescence intensity was lost after 30 minutes of illumination for all three media. After 1 minute in the dark, cells regained an average of 10% of the intensity (Table 1, acquired images 4).

Table 1: *Loss of fluorescence intensity for T24 cells transfected with actin-eGFP. Cells were cultured in different media.*

Label of acquired images	Time interval (min)	Average loss of fluorescence (%)			
		ADMEM/F12	FluoroBrite™	ADMEM/F12-PR	Average[b] (%)
1	0–10	57	55	54	56
2	10–20	51	47	49	49
3	20–30	31	31	32	32
4	30–dark	−7[a]	−9	−13	−10
5	dark–DAPI	−330	−264	−235	−276

[a] Negative values represent increase in fluorescence intensity in the same experiment.

[b] Average of 2 separate experiments with 6 parallels for each of the three culture media.

We observed no statistically significant difference in fluorescence intensity between the different culture media (Fig. 3). There was also no statistically significant difference between the loss of fluorescence intensity due to the photobleaching effect. The photobleaching effect was observed with all three media and more than 85% of the fluorescence intensity was lost after 30 minutes of illumination. Of the three media tested, FluoroBrite™ performed the worst with the lowest fluorescence intensity during the 30-minute illumination period. The ADMEM/F12 base medium performed best with the highest total fluorescence intensity. We found no statistically significant difference between the loss of fluorescence intensity in the three cell culture media tested.

FLUORESCENCE RENEWAL AFTER PHOTOBLEACHING; EXPLOITING SHORT-WAVELENGTH ILLUMINATION

After finding that the composition of the cell culture medium had no effect on photobleaching, we concluded that most of the fluorescence loss was likely due to factors other than the destruction of the eGFP fluorophore in the cells. eGFP molecules adopting the persistent (and non- fluorescent) triplet state is another way to explain the loss of fluorescence over time (Byrdin *et al.*, 2018). A brief burst of short-wavelength light is sufficient to collapse this state and renew fluorescence intensity (West *et al.*, 2015). Therefore, we set experiments where T24-actin-eGFP cells were used to test the effect of fluorescence renewal. First, we photobleached the cells under the same conditions as in the time-dependent experiment by illuminating them with a GFP filter set (λ= 450−490 nm). After the fluorescence of the cells was no longer visible under the microscope (30 min), we illuminated the cells with a DAPI filter set (λ= 330−380 nm) for 15 seconds (Fig. 2). As a control, we used cells that were not illuminated with short wavelengths and were instead kept in the dark for the same period of time (15 seconds).

As with the photobleaching experiment, the FRAP experiment showed no statistically significant difference in the recovery of fluorescence between the cell culture media tested. In all three cases (ADMEM/F12, FluoroBrite™ and ADMEM/F12-PR), fluorescence recovery did not vary with the cell culture medium used. Using the same procedure, we tested all three cell culture media used in the first experiment. On average, 62% of the fluorescence intensity was recovered, and we measured on average a 276% increase in intensity (Table 1, image 5 and Fig. 2, bottom) in comparison to 30 min of illumination.

CELL VIABILITY AND ROS CONCENTRATION AFTER ILLUMINATION

To test the effects of illumination on cells, we measured i) cell viability with 7-AAD and ii) ROS. The viability assay as well as the concentration of ROS were determined by adding either 7-AAD or ROS reagent after 30 minutes of illumination.

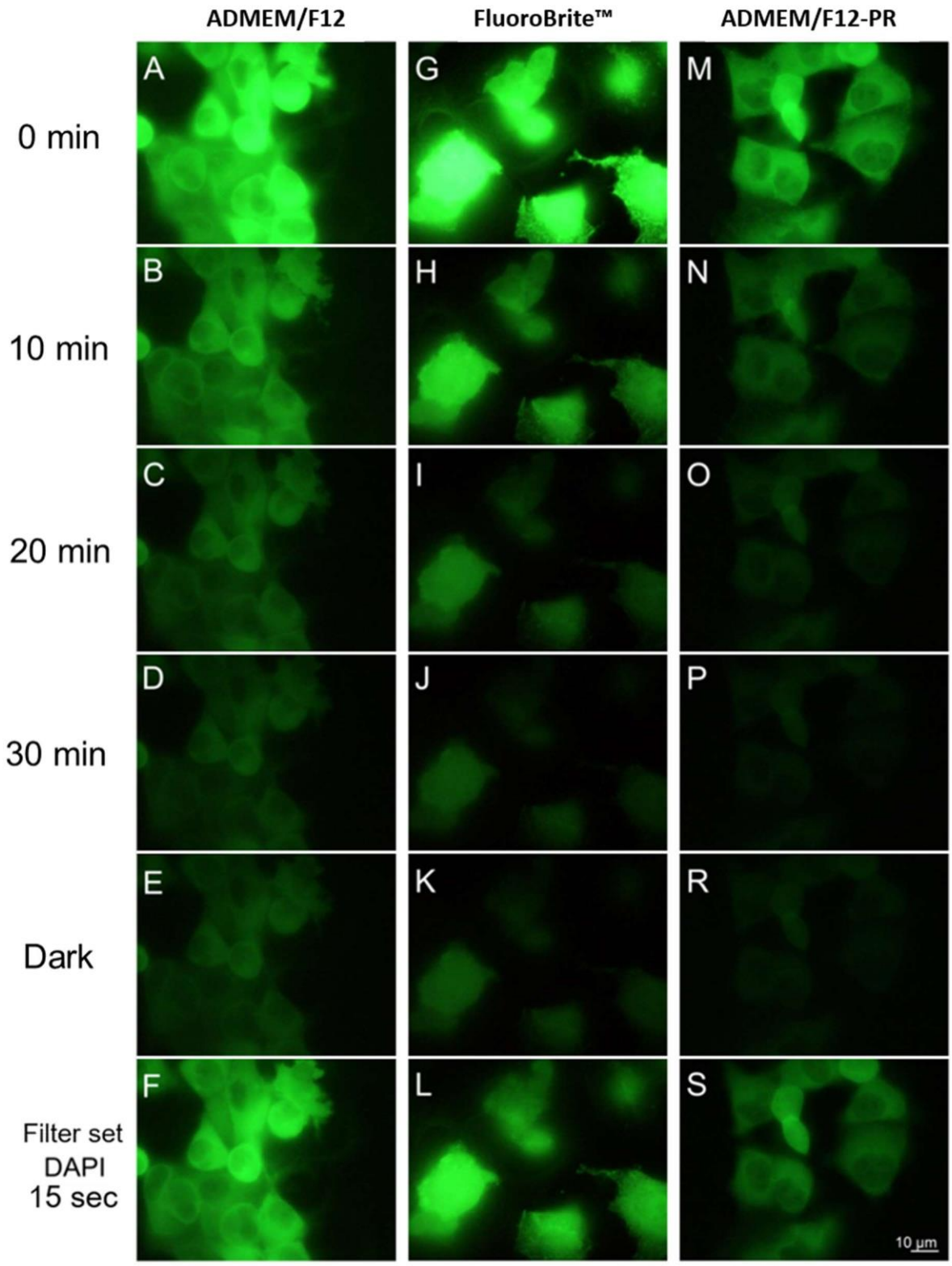

Fig. 2: *Loss of eGFP fluorescence in T24 cells transfected with actin-eGFP over 30-minute period of illumination (λ= 450–490 nm) and renewal of fluorescence intensity after 15 seconds of illumination with wavelengths provided by DAPI filter block (λ= 330–380 nm) for different culture media. A-D, G-J and M-P: Loss of fluorescence intensity over 30-minute period. E, K and R: control cells that were held in dark for 1 minute after 30-minute period of illumination with GFP filter block. F, L and S: Renewal of fluorescence intensity after 15 seconds of illumination with DAPI filter block (λ= 330–380 nm). Scale bar 10 µm.*

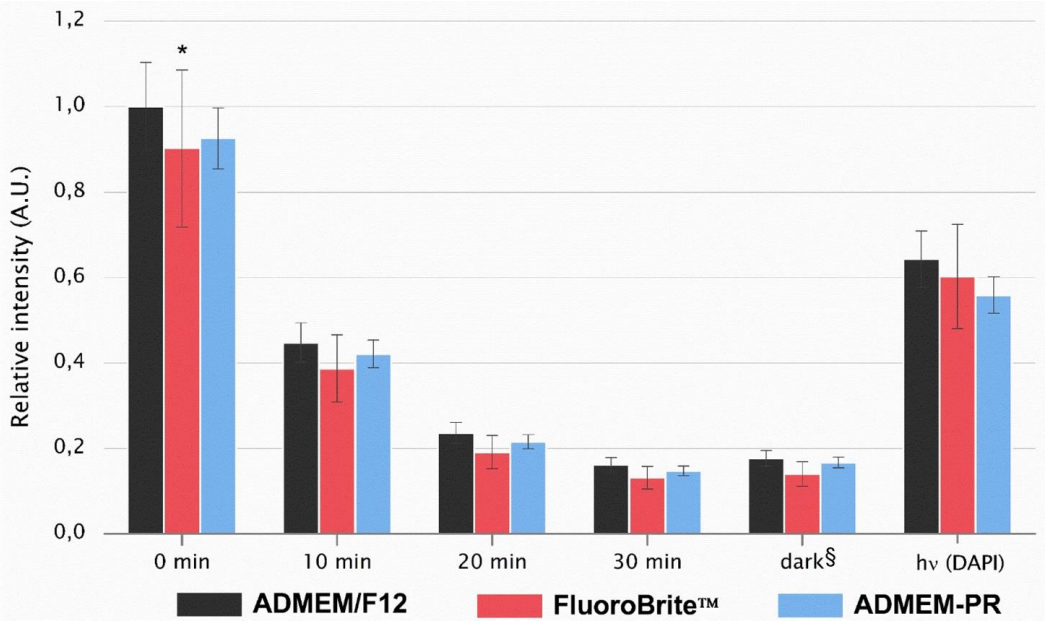

Fig. 3: *Fluorescence intensity loss and renewal after illumination with DAPI filter block. On average 62 % of fluorescence intensity is renewed. Error bars were determined on a basis of average fluorescence intensity for different parallels. (*p < 0.001). hv (DAPI)= light with λ= 330–380 nm. § cells not illuminated for 1 minute in order to show that thermodynamic relaxation is not the factor of fluorescence renewal*.

Cell viability was assayed with 7-AAD according to the manufacturer's instructions and literature (Višnjar *et al.*, 2017; Schmid *et al.*, 1992). We found no statistically significant difference between the cells that were illuminated and the control, the cells that were kept in the dark. Comparison of the percentage of live and dead cells showed a difference of less than 0.1% in both the illuminated and non-illuminated samples. Cells that were not illuminated were 99.7% viable according to our analysis, cells that were illuminated for 30 minutes were 99.0% viable, and cells exposed to both 30 minutes of illumination and 15 seconds of photo-recovery (DAPI filter block) had a viability of 99.3% (Fig. 4, orange columns).

The difference in ROS concentration was calculated using the mean fluorescence intensity of the ROS dye. There was no statistically significant difference in the concentration of ROS between cells kept in the dark and illuminated cells (Fig. 4, green columns).

From the results, we can conclude that our illumination protocol had no statistically significant effect on cell viability or cell survival potential. Illumination with GFP-specific or DAPI dye-specific wavelengths does not stress the cells in any way.

DISCUSSION

In this study, we investigated the effect of three different culture media, ADMEM/F12, FluoroBrite™ and ADMEM/F12-PR, on photobleaching of T24 cells transfected with actin-eGFP and on short wavelength induced fluorescence renewal. The culture media tested do not play a decisive role in the photobleaching of GFP. Compared to the standard ADMEM/F12 medium, the exclusion of the fluorescent indicator phenol red from the mixture does not lead to a reduction in the bleaching of GFP over time. The same results were obtained with FluoroBrite™ medium, which was reportedly formulated to reduce the effects of photobleaching (Thermo Fischer). Since the composition of the medium had no effect on fluorescence intensity, we tested another phenomenon in which the GFP fluorophore adopts the long-lived and non-fluorescent triplet state.

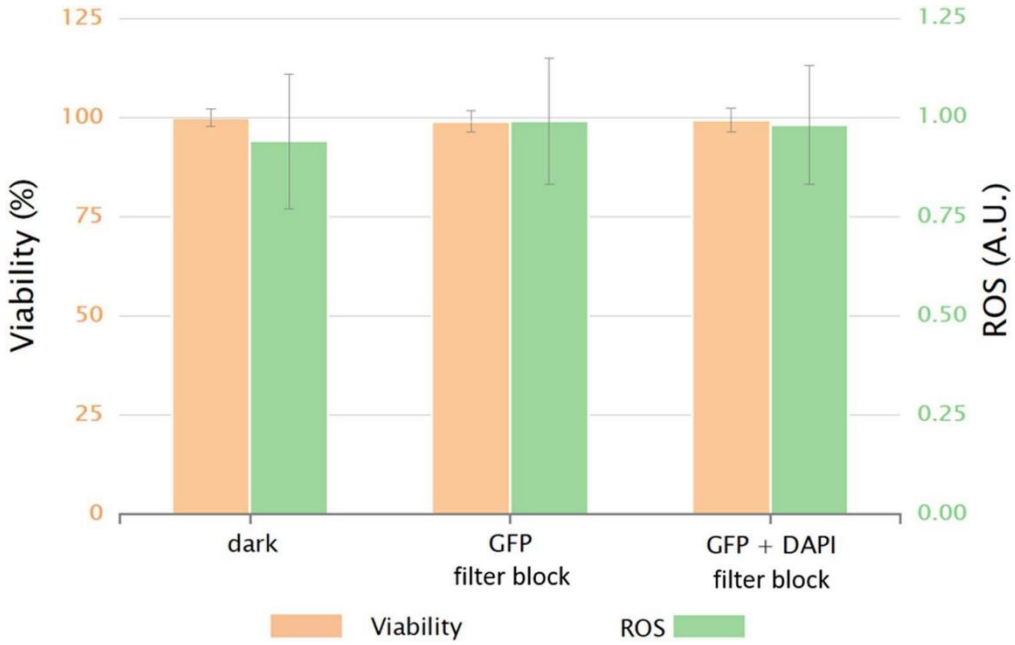

Fig. 4: *Cell viability in % (orange columns) and ROS concentration (green columns) in cells a) kept in dark, b) after 30 minutes of illumination with GFP filter block (λ= 450–490 nm) or c) 30 min of illumination with GFP filter block and 15 sec with DAPI filter block (λ= 330–380 nm). Cells kept in dark are control. Green: ROS concentration measured in illuminated and nonilluminated cells in 6 parallel measurements. Error bars were determined on a basis of average fluorescence intensity for 6 different parallels in each of two independent experiments.*

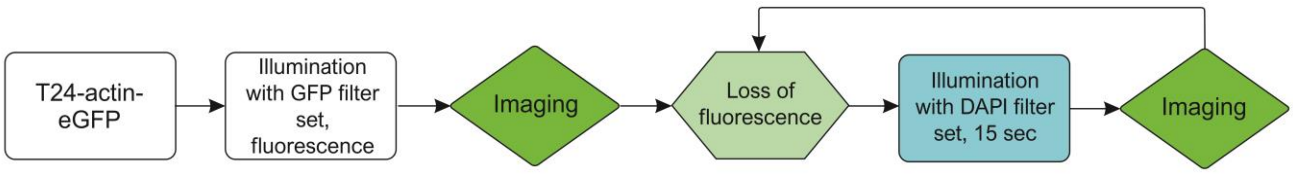

Fig. 5: *Proposed protocol for long term illumination and observation of eGFP transfected cells. Following illumination and observation / imaging, 15 seconds of fluorescence renewal illumination with short wavelengths (we propose DAPI filter block in case of eGFP cells) is applied. Fluorescence intensity is renewed, and observation / imaging is continued.*

The fluorophore in GFP can assume one of three quantum states: the ground state, the excited state, or the dark state "I" in which the fluorophore does not fluoresce. Each time a photon is absorbed, the probability of a molecule adopting the dark state I increases. The dark state I is a triplet state of the molecule (the total spin is 1 with the three allowed values 1, -1 and 0). There is an energy gap between the ground state and the triplet state (state I), which allows this state to be permanent and long-lived. However, upon absorption of a photon of suitable energy (in our case λ= 330−380 nm), this state can collapse back to the ground state and the fluorescence properties of the molecule revert (Ishikawa-Ankerhold *et al.*, 2012). The triplet state can also collapse by thermal relaxation, but the half-lives are much too long to be useful for our research ($\tau > 5$ min). A burst of short-wavelength light can collapse this state and restore the fluorescence properties of GFP.

Cell viability and ROS concentration measurements have shown that short bursts of UV light do not affect cell viability and do not cause oxidative stress. We therefore developed a protocol that can be used for long-term live cell monitoring (Fig. 5). The protocol consists of sequential illumination of cells with short-wavelength light when the fluorescence intensity falls

below the desired brightness threshold. Since fluorescence decreases linearly with time, we can expect the useful observation time to increase from 30 minutes to 42 minutes. 12 minutes is a time that is certainly not negligible when studying cellular biological processes.

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