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Evaluation of the effects of antibiotics on cytotoxicity of EGFP and DsRed2 fluorescent proteins used for stable cell labeling

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The use of fluorescent markers has proven to be an attractive tool in biological imaging. However, its usefulness may be confined by the cytotoxicity of the fluorescent proteins. In this article, for the first time, we have examined an influence of the antibiotics present in culture medium on cytotoxicity of the EGFP and DsRed2 markers used for whole-cell labeling. Results showed that doxycycline negatively affected albumin synthesis in DsRed2-expressing hepatoma cells, and that both hepatoma cells and human skin fibroblasts, labeled with this protein, were characterized by the lowered growth rates. Thus, the cytotoxic effect of fluorescent markers depends on both protein used for cell labeling and on growth conditions that may cause cell stress.

Key words: stable fluorescent labeling, whole-cell labeling, fluorescent protein cytotoxicity

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INTRODUCTION

Fluorescent proteins (FPs) have become popular markers in live-cell imaging experiments since the cloning of the original green fluorescent protein (GFP) gene in the 1990s. In target cells, FPs can be expressed transiently or from DNA fragment integrated into a genome. Nowadays, scientists have an access to many different fluorescent markers, with excitation wavelength ranging from 400 nm (blue light) to 800 nm (far red) (Telford *et al.*, 2012).

Whole-cell labeling using fluorescent markers has proven to be an attractive tool in many research areas, such as studies on cell and tissue development, gene expression, cell tracking in animal models, and on kinetics of biological processes *in vitro* and *in vivo*. FPs appeared to be particularly useful in developing toolkits that enable researchers to control transfection or transduction efficiency, and to investigate gene or siRNA expression in successfully transduced cells (Teplyuk *et al.*, 2008; Koo *et al.*, 2012; van der Deen *et al.*, 2013).

However, FPs may have limited utility as a cellular label due to their reported cytotoxicity (Liu *et al.*, 1999; Baens *et al.*, 2006; Agbulut *et al.*, 2007; Tao *et al.*, 2007; Waldeck *et al.*, 2009; Strack *et al.*, 2011; Zhou *et al.*, 2011; Koelsch *et al.*, 2013). In our previous article (Samluk *et al.*, 2013) we have described generation of stably fluorescently labeled cells, hepatoma (C3A) and adult human skin fibroblasts (HSF), which were suitable to study liver cells coculture. Fluorescent markers did not affect albumin secretion by the hepatoma cells but we have noticed relatively slower growth of marker-expressing cells. Since every uncontrolled factor that may affect cell physiology is undesirable in models intended to investigate cell behavior under variable conditions or cytotoxicity of drugs, we have decided to explore potential toxicity of fluorescent markers in our modified cell lines in the absence and presence of different antibiotics: penicillin-streptomycin, a standard antibiotic cocktail used to prevent bacterial infection in cell culture and doxycycline, which is often used in tetracycline-controlled transcriptional activation to regulate transgene expression. In this article, for the first time, we have examined an influence of the antibiotics present in culture medium on cytotoxicity of the EGFP and DsRed2 — fluorescent markers used for the construction of stably labeled C3A and HSF cell lines. Results showed decreased albumin synthesis in DsRed2-expressing hepatoma cells cultured in medium containing doxycycline and lowered growth rates of both cell types labeled with this red fluorescent protein (RFP). This suggests that the cytotoxic effect of fluorescent markers depends on both protein used for cell labeling and on growth conditions that may cause cell stress.

The present study indicated that: 1. addition of antibiotics did not influence cell viability of any tested cell line; 2. fluorescent and nonfluorescent hepatoma cell lines secreted comparable amounts of albumin in medium with and without penicillin-streptomycin; 3. presence of doxycycline in culture medium reduced albumin production, especially in DsRed2-expressing cells; 4. cells labeled with DsRed2 protein displayed lag in growth even in the absence of antibiotics.

MATERIALS AND METHODS

The C3A human hepatoma cell line (ATCC No. CRL-10741), adult human skin fibroblasts (HSF) isolated from dermis, and their fluorescently labeled derivatives were cultured under standard conditions in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 1% nonessential amino ac-

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Abbreviations: A1AT, α1-antitrypsin; HSF, FIBRO, adult human skin fibroblasts; CI, Cell Index; DsRed2, *Discosoma* sp. red 2 fluorescent protein; DOX, doxycycline; DMEM, Dulbecco's Modified Eagle Medium; FACS, fluorescence-activated cell sorting; FPs, fluorescent proteins; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; C3A, HEPA, human hepatoma cells; MTT, methyl thiazol tetrazolium colorimetric assay; PEN-STREP, penicillin-streptomycin; ROS, reactive oxygen species; RFP, red fluorescent protein

ids, with/without the presence of antibiotics: penicillin-streptomycin (PEN-STREP; 100 units/mL-100 μg/mL) or doxycycline (DOX; 10 μg/mL).

Generation of fluorescent cell lines has been described elsewhere (Samluk *et al.*, 2013). Briefly, both C3A (HEPA) and HSF (FIBRO) were transduced with lentiviral vectors (Pluta & Kacprzak, 2009) that enable stable expression of the fluorescent markers: enhanced green fluorescent protein (EGFP) and *Discosoma* sp. red 2 fluorescent protein (DsRed2). The resulting fluorescent cell lines: HEPA-EGFP, HEPA-DsRED, FIBRO-EGFP, and FIBRO-DsRED displayed markedly strong and uniform fluorescent emission in high percentages of cells: 90–95% as judged by flow cytometry (fluorescence-activated cell sorting (FACS)) using FACSCanto II instrument (BD, Warsaw, Poland).

For most experiments, labeled and unlabeled HEPA and FIBRO cells were plated in 24- or 96-well plates at a total density of 1.25×10^4 cells per cm^2 (HEPA) or 1.9×10^4 cells per cm^2 (FIBRO) and cultured for 16 days. Every fourth day viable cells were counted in hemocytometer using Trypan Blue Exclusion method.

Cells viability was assessed in 14-day cultures using methyl thiazol tetrazolium (MTT) colorimetric assay standard protocol with the MTT reagent (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) purchased from Sigma-Aldrich (Poznan, Poland) and microplate reader Synergy HT (BioTek, Winooski, VT, USA). A *P* value of 0.05 or lower was considered as statistically significant (Student's *t*-test).

Concentration of the human serum albumin secreted by the HEPA cells was measured in the medium, collected from the last two days of the 10-day culture, using a sandwich enzyme-linked immunosorbent assay (ELISA) with the quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) and microplate reader Synergy HT. The amount of albumin was expressed in nanograms per 10^4 C3A cells. A *P* value of 0.05 or lower was considered as statistically significant (Student's *t*-test).

On the 14th day of the experiment, HEPA cells were harvested from T25 culture flasks for flow cytometric analysis. Immunostaining of the cells was performed using a standard protocol. Cells were trypsinized, fixed with 4% formaldehyde and permeabilized using 0.1% Triton X-100 solution in phosphate buffered saline. Prepared single-cell suspensions were incubated with primary and, if applicable, secondary antibodies. The following antibodies were used for FACS: goat α-human Albumin-FITC (Bethyl), goat α-human A1AT-FITC (Bethyl), mouse α-human CD54-PE (BD), mouse α-human CK18 (Abcam, Cambridge, UK), and rat α-mouse IgG-PE (BD). Immediately after staining samples were analyzed with FACSCanto II flow cytometer. Unstained specimens, cells incubated with appropriate isotype immunoglobulins, or with corresponding secondary antibodies only were used as controls. FACSDiva software (BD) was used for data acquiring and analysis.

Cells status (expressed as Cell Index, CI) was monitored for 116 hours using real-time cell analyzer xCELLigence RTCA SP (ACEA Biosciences, Inc., San Diego, CA, USA). Sixty two hours after plating on RTCA SP 96-well plate (at a total density of 8×10^4 cells per cm^2) HEPA cells were exposed to three light-darkness (8 hours/16 hours) cycles of white light.

The 3W LED bulb used for illumination had a measured light intensity of 18 klx in 15 cm distance. Control cells were growing in darkness. RTCA software (ACEA) was used for data acquiring and analysis.

RESULTS

In order to assess differences in a culture performance of analyzed cell lines, first we carried out cell viability tests using MTT assay (Fig. 1A). We did not observe any differences in metabolic activity between fluorescent and nonfluorescent C3A cells cultured under the same conditions. Although absorbance values for cell lines cultured in media containing antibiotics are lower than for cells cultured in DMEM alone, there were no statistically significant differences between groups of cell lines grown in tested media. Similar results were obtained for fluorescently labeled and unlabeled HSF (data not shown).

Additionally, we examined albumin production by the labeled and unlabeled C3A cells in ELISA test (Fig. 1B). In DMEM medium lacking antibiotics we observed decreased albumin secretion by the fluorescent marker-expressing cells, yet statistically significant difference can be found only between HEPA and HEPA-DsRED cells, p=0.007 (HEPA and HEPA-EGFP, p=0.07; HEPA-EGFP and HEPA-DsRED, p=0.17). There is no difference between analyzed cell lines growing in more difficult conditions, i.e. in DMEM supplemented with antibiotics. However, addition of antibiotics caused reduction of albumin production and/or secretion in all three cell lines and this effect is more prominent in the case of DOX. The difference between groups of cells grown in DMEM and DMEM withPEN-STREP is not significant (p=0.09). It is much more clear between DMEM and DMEM with DOX (p=0.0005) and DMEM with PEN-STREP and DMEM containing DOX (p=0.0002).

To measure proliferation rate of analyzed cell lines, cells were cultured in DMEM without antibiotics for 16 days and counted at multiple time points (Fig. 1C). Both HEPA and HEPA-EGFP cells showed the same growth rate, whereas HEPA-DsRED grew considerably slower, especially during the first days of the culture. On day 16th, none of the cell lines achieved 100% confluency. Late passage HSF (p20–25) used for the experiments and their fluorescent derivatives grew much slower than hepatoma cells, yet demonstrated the same tendency as observed for C3A cell lines (data not shown).

To assess the ability of HEPA, HEPA-EGFP, and HEPA-DsRED cells to express specific markers in various media, prepared single-cell suspensions were subjected to FACS analysis (Fig. 1D). We analyzed albumin-, α1-antitrypsin (A1AT)-, CD54-, and CK18-positive cells. Generally, we detected less marker-positive cells in both fluorescent cell lines populations in respect to HEPA cells. However, these differences are noticeable only in the case of albumin- and A1AT-expressing HEPA-DsRED cells cultured in the presence of DOX.

To examine possible cytotoxic effect of fluorescent proteins in the presence of white light status of the cell lines was monitored in real-time using RTCA SP instrument (Fig. 2). HEPA, HEPA-EGFP, and HEPA-DsRED cells were plated in RTCA SP 96-well plate, cultured in the CO2 incubator for 4 days and 8 hours and exposed to three 8-hour long sessions of illuminations or kept in darkness (control). RTCA SP system is based on electronic cell sensor which measure changes in electrical properties of the cell-growth surface interactions. Cell Index (CI) represents cell number, morphology, and adherence. During the first 24 hours after seeding, HEPA-DsRED cells adhered to the culture surface remarkably slower than other cell lines. Nevertheless, we were not able to observe any changes of CI in cell lines subjected to light (cycles started from 62nd hour of culture) compared to the control cells kept in darkness (for

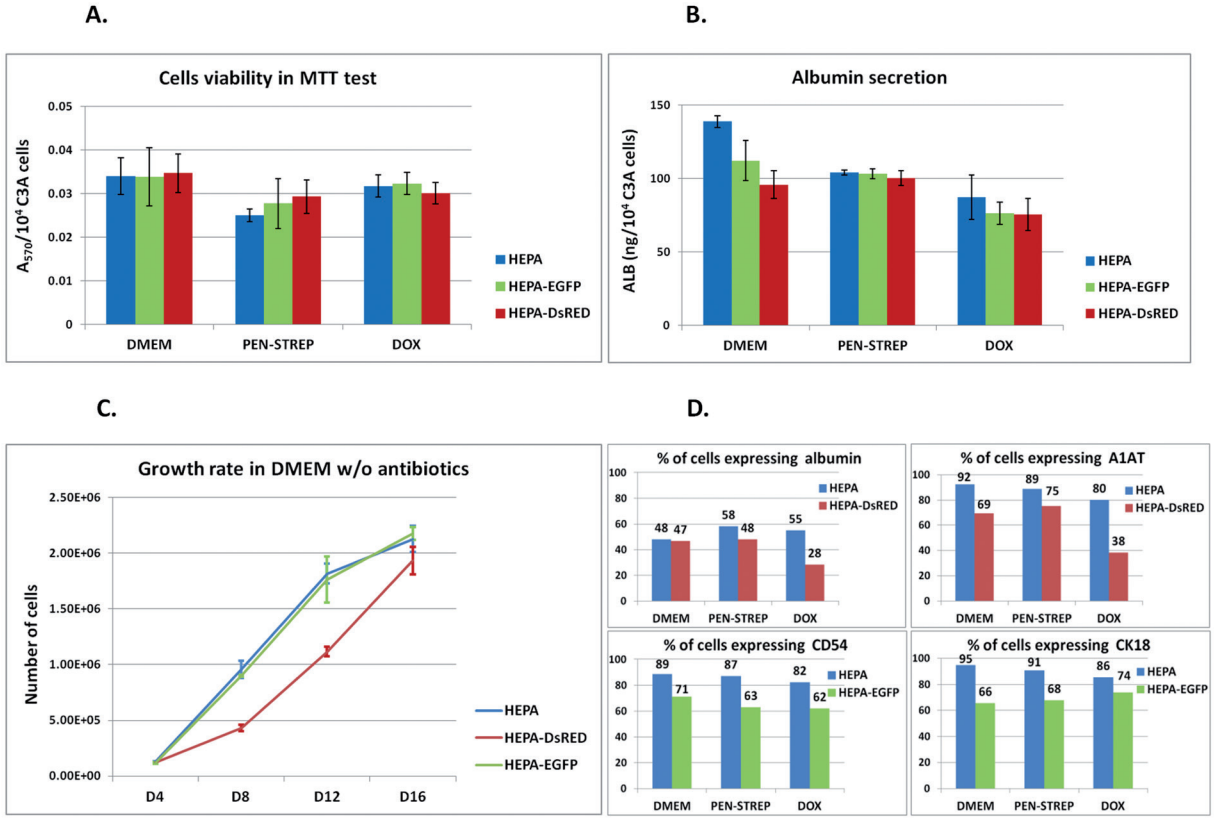

Figure 1. Performance of fluorescent and nonfluorescent C3A cells cultures in the presence and absence of antibiotics.
(**A**) MTT cell viability test on 14th day of the culture. HEPA, HEPA-EGFP, and HEPA-DsRED cells were cultured in DMEM, DMEM containing PEN-STREP, and DMEM with DOX. Results expressed in absorbance units (A) per 10^4 cells. Data were averaged from seven replicates (n=7). Standard deviation bars are indicated. A *P* value of 0.05 or lower was considered as statistically significant (Student's *t*-test). (**B**) Albumin concentration in the culture medium collected from the last two days of the 10-day culture expressed in nanograms of the protein per 10^4 cells (ELISA). HEPA, HEPA-EGFP, and HEPA-DsRED cells were cultured in DMEM, DMEM containing PEN-STREP, and DMEM with DOX. Data were averaged from three replicates (n=3). Standard deviation bars are indicated. A *P* value of 0.05 or lower was considered as statistically significant (Student's *t*-test). (**C**) Growth rates of the HEPA, HEPA-EGFP, and HEPA-DsRED cells cultured for 16 days in DMEM without antibiotics. Data were averaged from three replicates (n=3). Number of cells per well (in 24-well plate) and standard deviation bars are indicated. (**D**) FACS analysis of the cellular markers expressed by HEPA, HEPA-EGFP, and HEPA-DsRED cells cultured for 14 days in DMEM, DMEM containing PEN-STREP, and DMEM with DOX in T25 culture flasks. Upper panel: the percentage of HEPA and HEPA-DsRED cells expressing albumin and A1AT. Lower panel: the percentage of HEPA and HEPA-EGFP cells expressing CD54 and CK18. HEPA, C3A hepatoma cells; EGFP, enhanced green fluorescent protein; DsRED, DsRed2 — *Discosoma* sp. red 2 fluorescent protein; PEN-STREP, penicillin-streptomycin; DOX, doxycycline; albumin, human serum albumin; A1AT, human α1-antitrypsin; CD54, cluster of differentiation 54; CK18, cytokeratin 18.

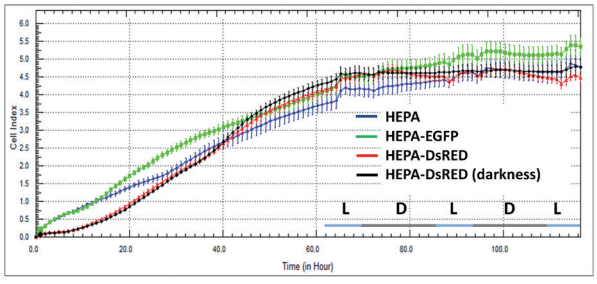

Figure 2. Cells status monitored by real-time cell analyzer (RTCA) instrument.
HEPA, HEPA-EGFP, and HEPA-DsRED cells were cultured for 116 hours in DMEM without antibiotics. Sixty two hours after plating on RTCA SP 96-well plate (at a total density of 8×10^4 cells per cm^2) HEPA cells were exposed to three light-darkness (8 hours/16 hours) cycles of white light. Control cells were kept in darkness (only HEPA-DsRED control is shown in figure). Results expressed in Cell Index units. Data were averaged from eight replicates (n=8). Standard deviation bars are indicated. L–D, light-darkness cycles.

better clarity of the picture only HEPA-DsRED control is shown in Fig. 2).

DISCUSSION

Previously, we had documented the usefulness of the stably labeled cell lines to study coculture models (Samluk *et al.*, 2013). At the same time, we had noticed slower growth of the cells expressing fluorescent proteins in medium containing antibiotics (doxycycline (10 μg/mL) in combination with ciprofloxacin (10 μg/mL)) and postulated possible cytotoxicity of these markers. On the other hand, we had not found any differences in albumin secretion between fluorescent and nonfluorescent C3A cell lines growing under those culture conditions. Slower growth rate of labeled cell lines was responsible for lower accumulation of albumin in the culture medium. Here, we have performed several experiments to check whether the expression of fluorescent markers affect culture performance in the absence and presence of two sets of antibiotics: penicillin in

combination with streptomycin — a cocktail of antibiotics routinely used in tissue culture to prevent bacterial infection and doxycycline which is used as transcriptional activator to regulate transgene expression from tetracycline-controlled promoters (however, DOX functions in the Tet-based system at levels below those required for bacteriostatic activity — 1 μg/mL (Pluta *et al.*, 2007)).

Firstly, we observed that the expression of fluorescent marker and the presence of antibiotic in culture medium did not affect cells viability (Fig. 1A). This suggests that potential cytotoxic effect of both factors is rather negligible. Secondly, in ELISA tests, we have confirmed our previous observations that in media containing antibiotics labeled and unlabeled C3A cells secreted similar amounts of albumin per calculated cell number (Fig. 1B). Moreover, in DMEM supplemented with standard set of antibiotics (PEN-STREP), HEPA, HEPA-EGFP, and HEPA-DsRED cells secreted similar levels of the protein when compared to the medium with no antibiotic addition. However, the presence of DOX resulted in significant reduction of albumin accumulation in the culture medium for all three cell lines.

We have also decided to investigate the differences in cellular expression of the other proteins characteristic for the C3A cells using flow cytometry. Albumin- and A1AT-positive cells in HEPA-DsRED population and CD54 and CK18 in HEPA-EGFP population were analyzed (Fig. 1D). Overall, we detected slightly lower number of cellular marker-positive cells in both fluorescent cell lines populations when compared to HEPA cells. This might partially result from the need of compensation of the overlap of emission spectra from antibody-conjugated fluorophores and fluorescent markers. Nevertheless, these differences are apparent in the case of albumin- and A1AT-expressing HEPA-DsRED cells cultured in the presence of DOX (Fig. 1D upper panel). The discrepancy in results for albumin in DOX-containing medium between FACS and ELISA (compare Fig. 1D upper right panel and 1B) can be explained by the fact that in cytometric analysis we tracked down only cells that contain more than minimal levels of albumin detectable for given antibody, whereas in ELISA assay we measured amounts of protein accumulated in medium for two days of culture.

Hence, slightly lower albumin production indicated on cellular level might not be clearly visible in long-term accumulation-based bulk assay. There is also a possibility that cellular protein accumulation does not necessarily reflect the magnitude of its secretion.

In DMEM without antibiotics both cell types expressing DsRed2 protein grew slower than nonfluorescent and EGFP-expressing cells (Fig. 1C). Additionally, when we counted fibroblasts for growth rate analysis, there were, on average, two times more dead FIBRO-DsRED cells (ca. 8.5%) than FIBRO and FIBRO-EGFP cells (data not shown).Therefore, our results indicated that RFP is more cytotoxic than EGFP and this phenomenon has been reported earlier (Tao *et al.*, 2007; Waldeck *et al.*, 2009). Fluorescent markers can act as photosensitizing agents. FPs absorb visible day-light and, predominantly RFPs, emit active electrons producing reactive oxygen species (ROS) leading to photokilling processes in eukaryotes and prokaryotes (Waldeck *et al.*, 2009; Waldeck *et al.*, 2012). To check whether this negative influence of DsRed2 production on albumin and A1AT synthesis as well as on proliferative capacity of the cells can be attributed to light-induced ROS production, we have monitored C3A cells status in real-time with the exposure to light-darkness cycles. We did not observe any differences between fluorescent and nonfluorescent cell lines nor between cells incubated in darkness and those treated three times for 8 hours with white light (Fig. 2). Thus, the extensive illumination did not cause cell death during the course of the experiment (54 hours). Instead, we observed significantly slower rate of adherence of the HEPA-DsRED cells for the first 24 hours after seeding. This fact can shed light on the mechanism of a lag in growth showed by the DsRed2-expressing cell line (compare Figs. 1C and 2).

There are also other mechanisms of cytotoxicity attributed to the expression of FPs. Expression of the GFP has been linked, for first time, to the induction of apoptosis by Liu and coworkers (1999). More recently, it was shown that cytotoxic effect of RFPs, DsRed and its variant DsRed-Express2, is caused by the inhibition of translation of B-cell lymphoma-extra large (Bcl-xL), a member of the Bcl-2 family of pro-survival proteins (Zhou *et al.*, 2011). At the same time, other results suggested that cytotoxic mechanisms of RFPs from different species may be varied. It was also indicated that toxicity of FPs may stem from their aggregation (Strack *et al.*, 2011). In addition, there are results showing that GFP interferes with signaling through the NF-κB pathway (Koelsch *et al.*, 2013), inhibits polyubiquitination (Baens *et al.*, 2006), and can affect actin-myosin interactions in muscles by binding to myosin (Agbulut *et al.*, 2007). FPs cytotoxicity often appears to be cell-specific. Our results showed that cytotoxicity of EGFP is minimal in C3A and HSF cells, while DsRed2 exerts negative effect on proliferative capacity of both cell lines and on production of albumin and A1AT in C3A cells growing in more challenging conditions (presence of DOX).

CONCLUSIONS

In this study, for the first time, we have analyzed the influence of the antibiotics present in a culture medium on cytotoxicity of the FPs, EGFP and DsRed2, in C3A and HSF cells. Addition of DOX, in opposite to PEN-STREP, resulted in lower albumin secretion by both fluorescent and nonfluorescent hepatoma cells, suggesting that this antibiotic generates grater cellular stress. The difference between unlabelled and DsRed2-producing C3A cells was apparent in DOX-containing medium when cells were subjected to flow cytometric analysis. HEPA-DsRED cells accumulated markedly lower levels of albumin and A1AT than their nonfluorescent counterparts. Expression of RFP, but not EGFP, affected cell culture also in medium lacking antibiotics, leading to slower growth of both C3A and HSF cells as well as lower albumin secretion by hepatoma cells. One possible mechanism for RFP cytotoxicity in our C3A and HSF cells is light-induced ROS generation. Illumination of cultured cells occurs periodically during routine cell culture manipulations. However, we were not able to confirm this hypothesis in our electronic-based real-time cells status analysis.

In summary, as far as the use of fluorescently labeled cells to study protein production or to analyze various cellular processes is concerned, not only cell-specific cytotoxicity of different FPs should be taken into consideration, but also side effects of growth conditions, such as use of antibiotics that might cause cellular stress.

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