Chapter 11

Optogenetic Downregulation of Protein Levels to Control Programmed Cell Death in Mammalian Cells with a Dual Blue-Light Switch

Patrick Fischbach, Patrick Gonschorek, Julia Baaske, Jamie A. Davies, Wilfried Weber, and Matias D. Zurbriggen

Abstract

Optogenetic approaches facilitate the study of signaling and metabolic pathways in animal cell systems. In the past 10 years, a plethora of light-regulated switches for the targeted control over the induction of gene expression, subcellular localization of proteins, membrane receptor activity, and other cellular processes have been developed and successfully implemented. However, only a few tools have been engineered toward the quantitative and spatiotemporally resolved downregulation of proteins. Here we present a protocol for reversible and rapid blue light-induced reduction of protein levels in mammalian cells. By implementing a dual-regulated optogenetic switch (Blue-OFF), both repression of gene expression and degradation of the target protein are triggered simultaneously. We apply this system for the blue light-mediated control of programmed cell death. HEK293T cells are transfected with the proapoptotic proteins PUMA and BID integrated into the Blue-OFF system. Overexpression of these proteins leads to programmed cell death, which can be prevented by irradiation with blue light. This experimental approach is very straightforward, requires just simple hardware, and therefore can be easily implemented in state-of-the-art equipped mammalian cell culture labs. The system can be used for targeted cell signaling studies and biotechnological applications.

Key words Optogenetics, Protein downregulation, Blue-light degron, Blue-light gene repression, Blue-OFF, Dual optogenetic switch, Optogenetic apoptosis control

1 Introduction

The development of synthetic switches for the targeted manipulation of protein levels in animal cells has facilitated the study of signaling and metabolic pathways [1–6]. A common approach is to control the expression or stability of a protein of interest with chemically induced switches. These normally consist of engineered activators or repressors, the binding of which to synthetic promoters is regulated by the presence of a chemical. For protein degradation, chemically regulated degrons have been developed

Dominik Niopek (ed.), *Photoswitching Proteins: Methods and Protocols*, Methods in Molecular Biology, vol. 2173, https://doi.org/10.1007/978-1-0716-0755-8_11,

[7]. However, chemically based switches have limitations such as toxicity, irregular spatially controlled distribution of the trigger by diffusion in cell culture or tissues, and limited reversibility [7–10]. To overcome these limitations, light has begun to be used as an inducer, and numerous optogenetic switches have been developed recently [1, 2, 11]. However, only a few have been designed for the targeted downregulation or destabilization of a protein [11, 12] and existing designs suffer from residual protein levels due to constant protein neosynthesis. Here, we present a protocol for the application of an optogenetic tool that combines blue light inducible repression of transcription and the simultaneous degradation of the target protein in animal cells [13]. We show its applicability to control apoptosis by regulating the levels of two proapoptotic proteins in mammalian cells.

1.1 Molecular Layout and Mechanism of the Blue-OFF System

The dual system designed for the simultaneous transcriptional repression and degradation of a protein of interest (POI) consists of two switches [13]: (1) the photosensitive transcription factor EL222 from *Erythrobacter litoralis* fused to the KRAB transrepressor domain [14–17] (pKM565) to inhibit transcription of the POI from a target promoter (Fig. 1); (2) the POI, in this case is either the proapoptotic proteins PUMA or BID (pPF088, pPF092), fused to the B-LID module which mediates the proteasomal degradation upon illumination with blue light [11]. The B-LID is based on the LOV2 domain of *Avena sativa* phototropin I (*As*LOV2). The *As*LOV2 contains a C-terminal Jα helix which is bound to the core domain of LOV2 in the dark but unwinds after illumination with blue light. B-LID harnesses this photoswitching mechanism to expose a RRRG degron fused to the Jα C-terminus in a light-dependent manner (Fig. 1). The proapoptotic proteins PUMA and BID are targets of the transcription factor p53 keeping the balance of cell cycle arrest and cell death upon DNA damage or other cell death insults [18–20]. Overexpression of those proteins leads to cell death. In this work we show the applicability of the Blue-OFF system for the regulation of apoptosis by reducing the levels of proapoptotic proteins constitutively expressed from a transfected plasmid in HEK293-T cells. The POI-B-LID module is cloned under the control of a synthetic promoter comprising five copies of the DNA target sequence of EL222-KRAB, namely $(C120)_5$, placed downstream of a constitutive promoter (Fig. 1). Upon illumination with 460 nm light, EL222-KRAB homodimerizes and binds via its helix-turn-helix (HTH) DNA-binding domains to the C120 sequences, thereby repressing transcription. Simultaneously, the LOV2 domain of the B-LID module exposes the RRRG degron, leading to degradation of the POI [13].

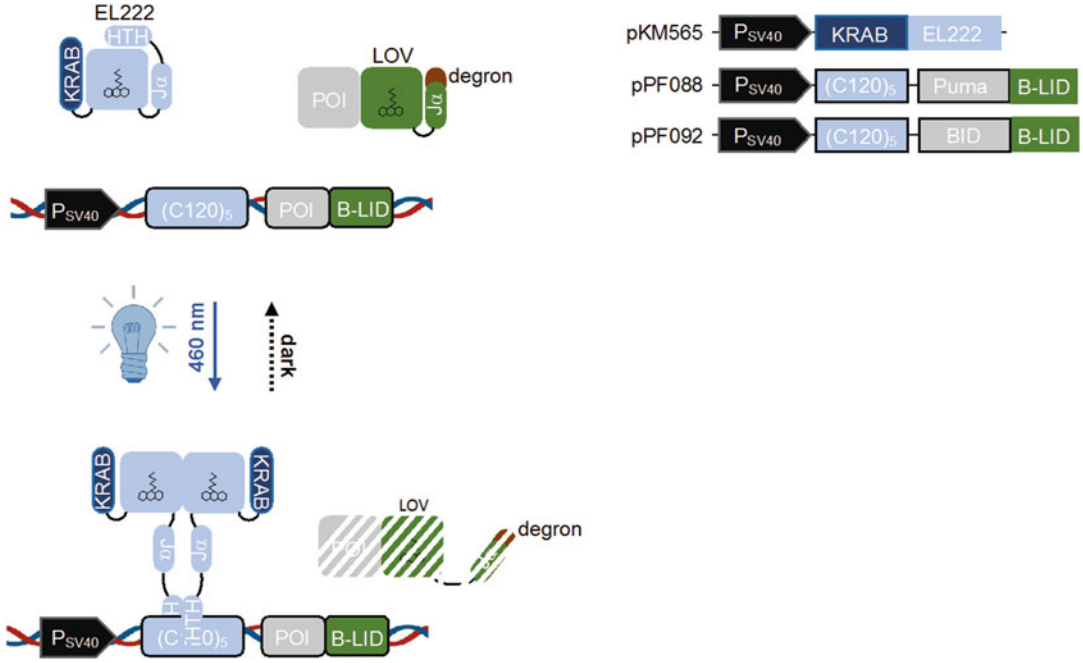

Fig. 1 Molecular design and mode of function of the Blue-OFF system for blue light-regulated downregulation of the levels of a POI. The pro-apoptotic proteins Puma and BID are fused to the B-LID system, and placed under the control of a constitutive SV40 promoter (pPF088, pPF092). The promoter sequence is followed by five copies of the EL222-binding sequence ($C120_5$). The photosensory transcription factor EL222 is fused to the inhibitory KRAB domain (pKM565). In the dark, the KRAB-EL222 fusion is not bound to the target sequence on the DNA and the B-LID system is inactive leading to accumulation of the POI. Upon blue light illumination, the Jα helix unwinds, exposing the docked degradation peptide (RRRG) which leads to proteasome mediated protein degradation. Simultaneously, the EL222 transcription factor dimerizes and binds to the $C120_5$ sequence inhibiting transcription via the fused KRAB repressor domain. Adapted from Baaske et al. [13]

1.2 Application and Experimental Considerations

This dual-controlled optogenetic switch shows highly efficient and rapid blue-light induced downregulation of protein expression and stability. These characteristics can be used to knock down essential genes in a cell, tissue or organism to study the effect of losing a given protein in an otherwise wild type context. We have previously shown a quantitative characterization of the system and its ability to control a synthetic caspase-based switch to induce programmed cell death [13]. Here, we describe a protocol to demonstrate further the applicability of the system by regulating the levels of ectopically overexpressed proapoptotic proteins such as PUMA or BID.

Blue light illumination can have toxic effects on cells. However, the intensity and time doses needed for full activation of the Blue-OFF system (20 μmol/m^2/s for 8 h or 10 μmol/m^2/s for 24 h) have no negative effect on the cells [13]. It is worth considering when designing an experiment that higher doses might have a negative influence on growth and health. The system represses transcriptional activity and targets the protein for degradation;

however, it has no effect over the mRNA, meaning that there might be remaining expression from previously synthesized messengers. An advantage of the Blue-OFF switch is that there is no need of extra addition of FMN, the chromophore of the LOV domains, to the growth media [21]. As the photoreceptors are activated also by daylight or room light, all work should be done under green or red safe light.

1.3 Experimental Design

In this protocol, HEK293T cells are transfected with plasmids encoding the proapoptotic proteins PUMA and BID engineered into the Blue-OFF system (pPF088, pPF092, pKM565). As a negative control, the plasmids encoding for the pro-apoptotic proteins fused to B-LID without the RRRG degron (pMZ1427, pTB505) under the control of a constitutive promoter were transfected. The light treatments were performed in closed LED boxes, with a wavelength of 460 nm and an intensity of 10 μmol/m^2/s for 24 h as described below. Control cells are kept in dark for the same incubation period. Transfections for microscopy are done in duplicates. After 24 h of treatment, the cells can be directly observed under the microscope or be fixed for long-term storage.

2 Materials

2.1 Reagents, Consumables, and Kits

1. Plasmids (Fig. 1):
   (a) pMZ1203: P_{SV40}-C120$_5$-Firefly-B-LID-pA.
   (b) pMZ1427: P_{SV40}-RFP-2A-Puma-B-LIDΔRRRG.
   (c) pTB505: P_{SV40}-RFP-2A-BID-B-LIDΔRRRG.
   (d) pPF088:P_{SV40}-C120$_5$-Puma-B-LID-pA.
   (e) pPF092: P_{SV40}-C120$_5$-BID-B-LID-pA.
2. Top10 chemically competent cells.
3. Plasmid isolation kit (e.g., M&N NucleoBond Xtra Midi Kit).
4. Ampicillin.
5. LB agar.
6. LB liquid medium.
7. HEK293T cells.
8. DMEM growth medium: DMEM supplemented with 10% (V/V) FBS and 1.4% (V/V) penicillin–streptomycin.
9. Trypsin–EDTA.
10. CASY ton buffer (OLS).
11. Opti-MEM.
12. Polyethyleneimine (PEI) linear molecular weight (MW) 25 kDa (Polyscience).

13. DMSO.
14. Bottle-top filter, 500 mL, 0.2 μM pore size (e.g., VWR).
15. CASY cups (OLS).
16. 10 cm cell culture dishes.
17. 24-well cell culture plates.
18. Glass cover slides.
19. Microscopy slides.
20. Paraformaldehyde (PFA).
21. 0.2 M Tris–HCl, pH 8.5
22. PBS (10× solution): Dissolve 26.82 mM KCl, 14.7 mM KH_2PO_4, 80.34 mM $Na_2HPO_4 \cdot 2H_2O$ and 1.37 M NaCl in 1 L ddH_2O. Dilute the PBS to 1×, sterile filter and aliquot it in 50 mL Falcon tubes.
23. Mowiol (Roth).
24. Dabco (Roth).

2.2 Equipment

1. Bacterial incubator with shaking function.
2. Spectrophotometer.
3. Tissue culture hood.
4. Tissue culture incubators.
5. CASY cell counter and analyzer (OLS, CasyTT).
6. Spectroradiometer (Avatec, Avaspec-2048).
7. LED band deco Flex RGB plug and light set (Prisma Leuchten).
8. LED Boxes (LEDs: Roithner LED450-series).
9. Confocal microscope (Nikon Eclipse Ti+ C2+ confocal upgrade).

2.3 Equipment Setup

2.3.1 Safe-Light Setup in the Lab

Stick the LED band to the internal surface/walls of the cell culture hood. The safe-light (green or red) can be turned on from the outside via remote control. If required, additional LED stripes can be installed all over the room. Cover all ambient light sources such as windows and/or doors with curtains or black adhesive vinyl foil to achieve full darkness.

2.3.2 LED Boxes

LED boxes were constructed and used as described in Müller et al. [8] and Ochoa-Fernandez et al. [22]. In brief, we use custom-made light boxes built out of PVC (20 cm × 20 cm × 20 cm) and equipped with an LED panel. The light boxes are additionally equipped with fans for gas exchange. Irradiation wavelength and intensity control is achieved with an Arduino microcontroller installed in the aluminum LED panel with a USB port for

programming irradiation time and pulsing. In this protocol, boxes containing blue LEDs (460 nm) were used.

3 Methods

3.1 Plasmid DNA Preparation

1. Prepare LB agar plates by mixing LB agar (40 g/L) with H_2O according to manufacturer's instructions and autoclave it.
2. Add 100 μg/mL ampicillin (from a 100 mg/mL stock solution in H_2O, sterile filtered) to the cooled-down LB agar and pour it in 100 mm petri dishes and let it solidify. The plates can be stored at 4 °C for 1 month.
3. Transform chemically competent *Escherichia coli* TOP10 cells according to Beyer et al. [23] and plate 10 and 50 μL on LB agar plates supplemented with ampicillin. Incubate at 37 °C for 24 h.
4. Inoculate 120 mL of autoclaved LB medium supplemented with 100 μg/ml ampicillin with a single colony by using a sterile pipette tip, and incubate at 37 °C for 24 h at 150 rpm.
5. Centrifuge 100 mL of the overnight culture and isolate the DNA with the plasmid isolation kit (*see* **Note 1**) according to the manufacturer's instructions. Determine the DNA concentration with a spectrophotometer.

3.2 Reagent Setup

3.2.1 PEI Solution (1 mg/mL)

1. Dissolve 200 mg of PEI in 160 mL H_2O in a glass beaker and stir. For faster dissolution heat up to 50 °C.
2. Adjust the pH to 7 with HCl until PEI is completely dissolved and fill in with ddH_2O to 200 mL.
3. Filter the PEI solution through a 0.2 μm filter in a cell culture hood and divide it into 1 mL aliquots. The aliquots can be stored at −80 °C for at least 1 year.

3.2.2 Mowiol-DABCO-Solution

1. Mix 6 g of glycerol with 2.4 g Mowiol in a 50 mL Falcon tube, incubate for 30 min while vortexing every 10 min.
2. Add 6 mL H_2O and stir solution for 2 h.
3. Add 12 mL 0.2 M Tris–HCl, pH 8.5 and heat up to 53 °C in a water bath until dissolution (approximately 2 h). Stir every 30 min with a magnetic stirring bar.
4. Centrifuge the solution for 20 min at 5000 × *g*, transfer the supernatant to a fresh tube and add 25 mg DABCO for each mL of solution.
5. Stir until complete dissolution. Prepare 500 μL aliquots and freeze them at −20 °C.

3.3 Seeding of HEK293T Cells

Estimated duration 1 h (mid-day or afternoon of day 1)

1. Start with a HEK293T 80–90% confluent cell culture in 10 cm petri dishes maintained in 10 mL DMEM growth medium. Using healthy cells is essential (*see* **Note 2**).
2. For collecting the cells, remove the culture medium, add 2 mL of trypsin–EDTA solution and incubate at 37 °C for 5 min.
3. During the incubation time prepare a 15 mL tube with 8 mL fresh DMEM growth medium (to get a final total volume of 10 mL) (*see* **Note 3**).
4. Wash away the cells from the plate by rinsing the trypsin–EDTA cell suspension 2–3 times, and pipet the suspension up and down to resuspend the cells. Transfer the cell suspension into the 15 mL tube prepared in **step 3**.
5. Sediment the cells by centrifugation (3 min, 300 × *g*, RT). Discard the supernatant and resuspend the cells in 10 mL fresh DMEM growth medium.
6. Determine the cell concentration with the CASY cell counting system or with a Neubauer cell-counting chamber.
7. Seed HEK293T cells in 10 wells of each of two 24-well plates at a density of 40,000–50,000 cells per well in 500 μL DMEM growth medium. Incubate the cells for 24 h at 37 °C with 5% CO_2 in an incubator.

3.4 Transfection of HEK293T Cells

Estimated duration: 1.5 h in the morning and 30 min in the afternoon (day 2).

1. Inspect the seeded cells under the microscope. They should be uniformly and evenly distributed. The confluency should be about 30–50%.
2. Five different DNA mixes need to be prepared in Opti-MEM in a total volume of 250 μL as follows (*see* **Note 4**):
   (a) Mix 1: (the negative control) pMZ1203 = 1.875 μg and pKM565 = 1.875 μg.
   (b) Mix 2: (Blue-OFF controlled Puma) pPF088 = 1.875 μg and pKM565 = 1.875 μg.
   (c) Mix 3: (Blue-OFF controlled BID) pPF092 = 1.875 μg and pKM565 = 1.875 μg.
   (d) Mix 4: (unregulated Puma) pMZ1427 = 3.75 μg.
   (e) Mix 5: (unregulated BID) pTB505 = 3.75 μg.
3. In an additional 15 mL tube, prepare the PEI mix as follows: add 96 μL of 1 mg/mL PEI solution to 1829 μL Opti-MEM (calculate a 10% excess, in case of pipetting deviation/mistakes).

4. To produce the DNA–PEI complexes, add 250 μL of the PEI mix in two separate steps to the DNA mixes 1–5, and mix the tubes by vortexing for 10 s after each addition of the PEI mix. Incubate the tubes at RT for 10 min.
5. Add 100 μL of the transfection mix dropwise to each well of the plate seeded in Subheading 3.3, **step 7**. Transfect with each transfection mix 2 wells on the 24-well plate that will be kept in darkness (as control), and 2 wells on the 24-well plate that will be illuminated. Finally, distribute the added transfection mix evenly by gently moving the plates in "8-shape- or up-down/left-right movements," and then incubate them in a CO_2 incubator at 37 °C, 5% CO_2.
6. Four to five hours after transfection, replace the culture medium carefully in both 24-well plates with 0.5 mL of pre-warmed DMEM growth medium per well (*see* **Note 5**).
7. From now on every step should be carried out in the absence of blue/room light, that is, "darkness." Use green (530 nm) or red (660 nm) safe-light to avoid activation of the system. Directly after changing the medium (**step 6**), illuminate one 24-well plate with 460 nm light with 10 μmol/m^2/s intensity and keep the other plate in "darkness" for 16 h at 5% CO_2 and 37 °C (*see* **Note 6**).

3.5 Fixing Cells for Long-Term Storage

Estimated duration: 1 h (day 3)

1. After illumination, aspirate the medium of the wells with the transfected cells. Wash the cells once with 500 μL PBS and add 200 μL PFA (*see* **Note 7**).
2. Incubate the cells covered with PFA for 10 min on ice, and an additional 10 min at RT.
3. Remove the PFA (*see* **Note 8**).
4. Add 500 μL PBS. The cells are now fixed and the following steps can be carried in normal room light.
5. Prepare microscopy slides and add 8 μL Mowiol/Dabco to the microscopy slide.
6. Use forceps to transfer the glass slides (remove carefully the excess liquid from the glass slide with a tissue paper) with cells upside down on the Mowiol/Dabco droplet on the microscopy slide. After 30 min incubation at 37 °C the slides can be stored for more than 1 month at 4 °C (*see* **Note 9**).

3.6 Analysis of Apoptosis

Estimated duration: 0.5 h (day 3)

1. Check for cell growth under the microscope. Observe the formation of a confluent monolayer (if it does form) (*see* **Note 10**).

2. Perform quantification and statistics accordingly/as needed. Note that, instead of using apoptosis/survival as readout, the rapid downregulation of protein levels can also be directly monitored (*see* **Note 11**).

4 Notes

1. For high transfection efficiencies it is recommended to use RNA-free, supercoiled DNA. For best results we use the NucleoBond Xtra MIDI Kit for DNA preparations.
2. Healthy cells are essential (viability and morphology)! Low cell viability leads to low expression levels. Ideally cells should be neither too young (passage number < 5) nor too old (passage number > 30) for best expression results.
3. Cells of a maximum of three plates can be pooled in one tube to speed up the process. In this case use only 4 mL fresh DMEM medium.
4. For each well of a 24-well plate mix a total of 0.75 μg of plasmid DNA in 50 μL of Opti-MEM, and 2.5 μL of 1 mg/mL PEI solution in 50 μL of Opti-MEM.
5. Incubation with PEI for more than 5 h might lead to decreased cell viability. However, very short incubation times with PEI decrease transfection efficiency. Additionally, the PEI solution has to be kept at pH = 7; this is essential for high transfection efficiency.
6. The system is very light-sensitive. After transfection, every step should be carried out in the absence of blue/room light, that is, always in "darkness." Use green (530 nm) or red (660 nm) safe-light to avoid activation of the system.
7. PFA is toxic! All work with PFA should be performed following the manufacturer's guidelines for proper handling. Use gloves and dispose of the liquid waste and all consumables/material which had contact with PFA under the toxic waste instructions of your institute.
8. Use a waste tube and dispose of PFA according to the toxic waste handling guidelines at your institute.
9. Fixed cells can be stored for at least 1 month at 4 °C. Expressed fluorescent proteins are still detectable with a fluorescence microscope.
10. The protocol described here was implemented for the optogenetic regulation of programmed cell death via the control of the levels of proapoptotic proteins. HEK293T cells were transfected with the Blue-OFF optogenetic switch engineered to control PUMA or BID. Incubation of the cells in the dark led

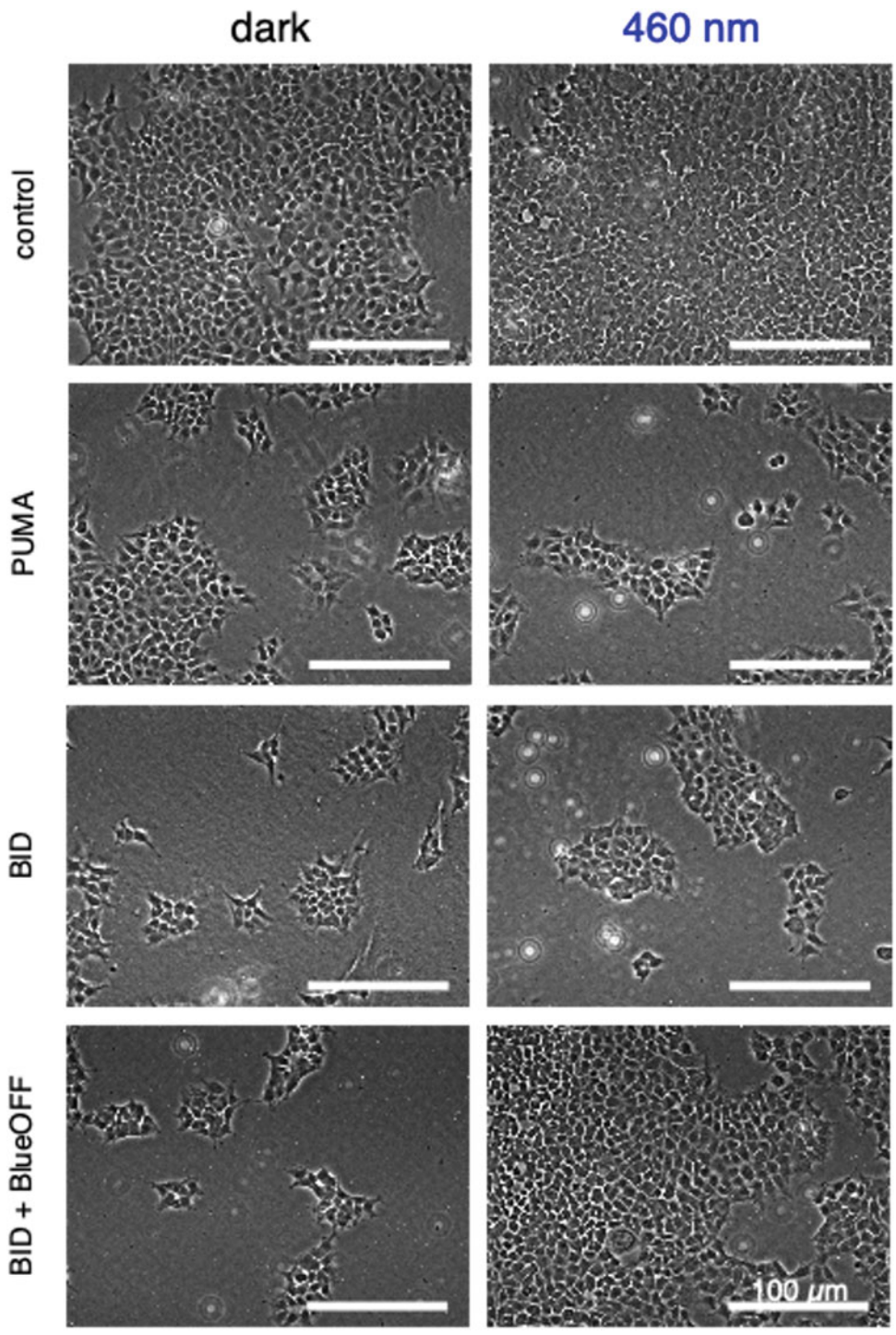

Fig. 2 Control of programmed cell death. Representative results of HEK293T cells transfected with control (pMZ1203) forming a uniform monolayer in darkness and under blue illumination. Constitutive expression of PUMA or BID (pMZ1427; pTB505) in darkness and blue light, and of PUMA-Blue-OFF and BID-Blue-OFF (pPF088 + pKM565; pPF092 + pKM565) in darkness leads to increased cell death. In contrast to this, cells transfected with PUMA-Blue-OFF or BID-Blue-OFF show a higher survival rate, observed as a uniform monolayer, upon blue light illumination

to a high cell death rate, in contrast to control cells transfected with pMZ1203 (Blue-OFF controlling FLuc) which formed a uniform monolayer (Fig. 2). Cells transfected with the light-dependent PUMA or BID systems showed as expected a higher survival rate (uniform monolayer) when illuminated with blue light. The results open up novel perspectives for the targeted regulation of programmed cell death in animal cells with applications in fundamental research such as the study of apoptotic and carcinogenic cellular mechanisms [24, 25]. Additionally, the high spatiotemporal resolution of the system might be of advantage for the establishment of cellular patterns in tissue engineering approaches.

11. To analyze the rapid downregulation of protein levels in a quantitative manner, one can use reporter genes (e.g., luciferases, phosphatases, or fluorophores) instead of proapoptotic proteins [13].

Acknowledgments

We thank J. Andres, L. Koch, and T. Blomeier for experimental support and fruitful discussions, and R. Wurm, M. Gerads, and J. Müller for valuable experimental support. This work was supported by the German Research Foundation (DFG) (grant ZU259/2-1 to M.D.Z. and under Germany's Excellence Strategy CEPLAS—EXC-2048/1—Project ID 390686111 to M.D.Z., BIOSS—EXC-294 and CIBSS—EXC-2189—Project ID 390939984 to W.W.) and the European Commission—Research Executive Agency (H2020 Future and Emerging Technologies (FET-Open) Project ID 801041 CyGenTiG to J.A.D. and M.D.Z.).

Author contributions: P.F. designed the system and performed the experiments, analyzed the data, and wrote the protocol. P.G. and J.B. designed the system. J.D. designed experiments and analyzed the data. W.W. designed the system and experiments, and analyzed the data. M.D.Z. designed the system and experiments, analyzed the data, and wrote the protocol.

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