Imaging of Morphological and Biochemical Hallmarks of Apoptosis with Optimized Optogenetic Tools

Walton C. Godwin¹, George F. Hoffmann¹, Taylor J. Gray², Robert M. Hughes¹*

From the ¹Department of Chemistry, East Carolina University, Greenville, North Carolina, 27858; ²Department of Biology, East Carolina University, Greenville, North Carolina, 27858

Running title: Hallmarks of apoptosis via optogenetic actuators

*To whom correspondence should be addressed: Robert M. Hughes: Department of Chemistry, East Carolina University, Greenville NC 27858; hughesr16@ecu.edu; Tel. (252) 328-9730

Keywords: apoptosis, optogenetics, cryptochrome 2, BCL2-associated X apoptosis regulator (BAX), actin, cytoskeletal dynamics, mitochondrial outer membrane permeabilization (MOMP), OptoBax 2.0 system, effector molecule, cell signaling

Abstract The creation of optogenetic switches for specific activation of cell-death pathways can provide insights into apoptosis and could also form a basis for non-invasive, next-generation therapeutic strategies. Previous work has demonstrated that cryptochrome 2 (Cry2)/CIB, a blue light–activated protein–protein dimerization module from the plant Arabidopsis thaliana together with BCL2-associated X apoptosis regulator (BAX), an outer mitochondrial membrane (OMM)-targeting pro-apoptotic protein, can be used for light-mediated initiation of mitochondrial outer-membrane permeabilization (MOMP) and downstream apoptosis. In this work, we further developed the original light-activated Cry2–BAX system (henceforth referred to as OptoBAX) by improving the photophysical properties and light-independent interactions of this optogenetic switch. The resulting optogenetic constructs significantly reduced the frequency of light exposure required for the membrane permeabilization activation and also decreased dark-state cytotoxicity. We used OptoBAX in a series of experiments in Neuro-2a and HEK293T cells to measure the timing of the dramatic morphological and biochemical changes occurring in cells after light-induced MOMP. In these experiments, we used OptoBAX in tandem with fluorescent reporters for imaging key events in early apoptosis, including membrane inversion, caspase cleavage, and actin redistribution. We then used these data to construct a timeline of biochemical and morphological events in early apoptosis, demonstrating a direct link between MOMP-induced redistribution of actin and apoptosis progression. In summary, we have created a next-generation Cry2/CIB–BAX system requiring less frequent light stimulation and established a timeline of critical apoptotic events, providing detailed insights into key steps in early apoptosis.

Among the wide-ranging applications of optogenetic proteins – which include light-mediated control of transcription(1), ion channels(2), and enzymatic activity(3) – the use of light for wholesale activation or inhibition of cell signaling pathways presents a uniquely powerful approach for the determination of cell fate – acting as a rapid, non-invasive trigger for cascades of biochemical events that ultimately decide whether a cell proliferates or dies. After many years of effort, numerous cell signaling pathways critical for cell fate decisions have been elucidated, including pathways for the control of cell division(4), cell motility(5), and apoptosis(6). As a result, it is possible to identify individual proteins that serve as highly specific effector biomolecules for the initiation of signaling cascades undergirding these vital processes. Such effector molecules are ideal candidates for incorporation into light-activated
Hallmarks of apoptosis via optogenetic actuators

protein switches for control of cell signaling pathways(7–9). These switches are useful not only for the study of the effector molecules themselves, but also of biochemical events that occur in the wake of their activation. In the case of apoptosis, which can be viewed as a singular destination (“cell death”) attainable by various routes(10), numerous effector molecules exist that could serve as candidates for optical control of apoptosis, including the Bcl-2 family proteins(11). Bax, a 21-kD protein which is a key effector of mitochondrial membrane permeabilization (MOMP) in the intrinsic apoptosis pathway, is one such effector molecule with an in vivo activity profile that makes it ideal for incorporation into an optogenetic switch: phosphorylation-gated activity, predominantly cytosolic localization in the “off” state, and robust initiation of permeabilization of the outer mitochondrial membrane (OMM)(12–15). Prior work demonstrated that the incorporation of BAX into a cryptochrome 2 (Cry2) – cryptochrome interacting β helix-loop-helix (CIB) based optogenetic switch enables light activated MOMP and release of apoptotic effector proteins such as Smac-1 (16). A key step in the engineering of this switch was the implementation of a phospho-mimetic mutation in the Bax C-terminus at serine 184 (S184E), which maintains Bax in a predominantly cytosolic state by increasing the off-rate of localization at the OMM, thus eliminating one potential source of light-independent background(17). However, another potential source of light-independent (or “dark”) background for this optogenetic switch, is the dark state interaction of the Cry2 photoreceptor with the mitochondrial Tom20-CIB construct. In this work, we have created next generation iterations of the Cry2-CIB Bax system (“OptoBax”), hereafter referred to as “OptoBax 2.0”. The optimized versions of this cell death switch include longer Cry2 variants designed to reduce the dark state interaction with CIB, and incorporate a mutation that extends the photocycle of the Cry2-CIB interaction, promoting longer association times with the OMM(18). This optimized construct requires less frequent light stimulation to maintain OptoBAX and the OMM, an advantage for long timecourse experiments where blue light toxicity may be of concern(19). In addition, while the initial studies with OptoBax were conducted in HeLa cells, this study applies the OptoBax 2.0 system to the cell lines Neuro-2a and HEK-293T, demonstrating light initiated apoptosis in these cell lines analogous to that previously observed in HeLa cells. Finally, in HEK-293T cells, a combination of fluorescently-tagged proteins and commercially available dyes are used for multi-color monitoring of critical steps in apoptosis, downstream of initial mitochondrial insult (cytoskeletal rearrangement, chromatin condensation, membrane inversion, and caspase cleavage), and compared to results previously observed with a classic small molecule initiator of the apoptotic cascade. The resulting data is used to create a timeline of key apoptotic events in HEK-293T cells, in addition to providing insight into changes in actin localization and degradation of the nuclear/cyttoplasmic barrier, both key steps in early apoptosis.

Results and Discussion

Creation and validation of new optogenetic constructs.

Initial versions of the OptoBax system were comprised of BAX as a fusion to the N-terminus of a Cry2PHR (1-498)-mCherry (BAX.Cry2PHR.mCh) fusion protein or as a fusion to the C-terminus of a Cry2PHR (1-498)-mCherry fusion (Cry2PHR.mCh.BAX), in tandem with the Cry2-interacting partner CIB in fusion with a Tom20 OMM localization domain with or without a C-terminal GFP (Tom20.CIB.GFP or Tom20.CIB)(20). While both BAX fusions were demonstrated to initiate light-mediated apoptosis in HeLa cells, the C-terminal fusion of a BAX mutant (S184E) was demonstrated to be a more effective light-activated apoptotic switch, with reduced association of BAX with the OMM due to the phospho-mimic S184E, and provide faster induction of the apoptotic cascade than the N-
terminal BAX construct (<1 h (C-term) vs. 2 – 3 h (N-term))(16). Furthermore, in the initial study, while the C-terminal BAX construct could be recruited irreversibly to the OMM after 50 minutes of repeated illumination, the N-terminal construct, after the same duration and pattern of illumination, still reverted to the cytosol, implicating the exposed C-terminus of BAX as necessary for efficient induction of apoptosis. However, despite its higher efficacy, light independent cell death was a significant drawback associated with the C-terminal construct over the N-terminal construct (22% (C-term) vs. 9% (N-term) 48 h post-transfection in HeLa cells; assessed by Trypan blue assay(16)). Taken together, these results implicate dark-state association between Cry2 and Cib, in tandem with an exposed C-terminus of BAX, as the source of undesirable levels of background cell death. In order to improve upon these initial constructs, we sought to create an OptoBax with reduced levels of light independent cell death and retention of light dependent pro-apoptotic efficacy.

Recently, optimized versions of the Cry2 optogenetic system have been reported, demonstrating, in the context of a yeast two-hybrid assay, that extending the truncated Cry2PHR from 498 to 515 amino acids or longer in length (up to 535 aa) significantly reduced the light independent background present in the optogenetic system (18). In the same report, a genetic screen also identified a Cry2 mutant (L348F) that extends the photocycle of Cry2 well beyond that of the wild type protein, prolonging the half-life of the light-promoted Cry2-CIB interaction from ~6 min to ~24 min(18). This mutation prolongs the lifetime of the light-promoted semiquinone form of FAD, the light responsive Cry2 cofactor. We hypothesized that incorporating similar changes into OptoBax might result in an improved pro-apoptotic switch, via reduced light-independent Cry2/CIB interaction, and, with incorporation of the L348F mutant, reduced light input than the original OptoBax, creating a system more easily amenable to incorporation into drug discovery platforms or into model organisms. As a result, for the OptoBax 2.0 system, two additional Cry2–Bax fusions were assembled: Cry2(1-531).mCh.Bax.S184E and Cry2(1-531).L348F.mCh.Bax.S184E, pairing them with the original Tom20.CIB.GFP and Tom20.CIB constructs (Fig. 1A). Assays of light-independent cell death (“dark background”) were subsequently performed in dually-transfected HeLa cells, using a fluorescent indicator of cell viability (Supporting Fig. 1). Versus the original OptoBax system, the OptoBax 2.0 system reduced dark background from ~36% (Cry2PHR.mCh.Bax.S184E) to ~20% (Cry2(1-531).mCh.Bax.S184E and Cry2(1-531).L348F.mCh.Bax.S184E), similar to background cell death observed with the Cry2.mCh fusions without Bax. The impact of the L348F mutant in the Cry2.mCh fusions was further validated in HeLa cells, utilizing a single pulse of 470 nm light to promote association of Cry2 with the OMM-localized CIB domain (Fig. 1B-C and Supporting Movie 1), confirming that the L348F mutant extended the half-life of association with the OMM from 6.25 (+/-0.5) min to 32.8 (+/-1.0) min. The Cry2(1-531).L348F.mCh.BAX.S184E construct was also assayed for its ability to induce apoptosis versus the Cry2(1-531).L348F.mCh fusion (Fig. 2A and Supporting Movie 2). We subsequently investigated whether the L348F mutant induces apoptosis with less frequent light stimulation vs. the non-mutant Cry2(1-531).mCh.Bax.S184E (Fig. 2B). An experiment using an interval of 10 min between blue light pulses, which is longer than the half-life of the WT construct yet significantly shorter than that of the extended photocycle mutant, was designed to demonstrate the result of less frequent light stimulation in the extended photocycle construct. As a result of this illumination sequence, the non-mutant Cry2 construct repeatedly reverts to the cytosolic state during the course of the experiment, while the L348F mutant maintains consistent localization at the OMM. Results of this experiment were assessed by manually counting the number of
apoptotic cells (based on the loss of adherent cell morphology) after one hour of simultaneous blue light stimulation and imaging on a Leica Dmi8 widefield fluorescent microscope. In this study, 65% (+/-21) of transfected cells expressing the Cry2(1-531).L348F.mCh.BAX.S184E/Tom20.Cib.GFP fusion became apoptotic vs. 12% of transfected cells (+/-13) expressing the standard photocycle Cry2(1-531).mCh.BAX.S184E construct, demonstrating that prolonging the photocycle enables more apoptosis with less frequent light input (% cell collapse and standard deviations determined from 10 replicate measurements). Some background apoptosis was observed with the fusions without Bax (Cry2(1-531).L348F.mCh and Cry2(1-531).mCh; 6 (+/-7)% and 4 (+/-5)%, respectively; determined from 10 replicate measurements). This background is attributed to the loss of mitochondrial integrity and subsequent apoptotic entry due to repeated recruitment of protein fusions to the OMM. To confirm that the observed background was a result of apoptosis, we conducted identical illumination experiments in the presence of 50 μM Z-VAD(OMe)-FMK. In the presence of this irreversible caspase inhibitor, no background apoptosis was observed for the Cry2 fusions, and OptoBAX-dependent apoptosis was completely inhibited (inset, Fig. 2B; 8 replicates per protein fusion). It should be noted that these experiments analyze apoptotic entry of transfected cells that are fully adherent at the beginning of the illumination time course; by contrast, a measure of overall cell viability in the absence of light stimulation is shown in Supporting Fig. 1.

Overall, the HeLa cell studies indicate that OptoBax 2.0 constructs possess two key improvements over the original OptoBax system: lower dark background and enhanced photocontrol. The lowering of dark background relative to the initially reported system is particularly important for the biological experiments in this paper. In the previously reported system, the higher levels of dark background present predisposed cells to rapid apoptotic entry upon light activation (< 15 minutes until cellular collapse for the Cry2PHR.BAX.S184E construct(16)). By contrast, in the current system, lower levels of dark background, and hence later apoptotic entry upon light activation (> 40 minutes until cellular collapse for the Cry2PHR.BAX.S184E construct in HeLa cells, Supporting Movie 2), provide the extended time window suitable for a more detailed dissection of the biochemical events occurring prior to, during, and post-cellular collapse.

Translation to other cell lines.

Given the common mechanism of Bax-mediated MOMP and subsequent apoptosis in mammalian cells(21), it was anticipated that this optogenetic system might readily translate to other mammalian cell lines. In order to test this assumption, the OptoBax 2.0 constructs were deployed in two additional cell lines, Neuro-2a and HEK293T (Fig. 2C). Induction of apoptosis upon recruitment of the Cry2(1-531).L348F.mCh.BAX.S184E to the OMM with light was observed in both cell lines. In particular, the reliably high transfection efficiency observed with the HEK293T cell line made further exploration of the apoptotic cascade in this cell line particularly appealing. Furthermore, in contrast to HeLa cells, no background cellular collapse was observed in one hour light activation experiments conducted with Bax-free protein fusions (Cry2(1-531).L348F.mCh and Cry2(1-531).mCh; 0/180 and 0/167 total cells counted from 6 replicates) in the HEK293T cell line. As a result, the remainder of the experiments in this work were carried out in the HEK293T cell line.

Tracking the hallmarks of apoptosis with optogenetic techniques.

The morphological and biochemical hallmarks of apoptosis include cell blebbing, loss of adhesion and cellular volume, chromatin condensation and nuclear fragmentation, cytoskeletal rearrangement, phosphatidylserine inversion, release of mitochondrial proteins (Cytochrome C/Smac/Diablo), and caspase cleavage(22).
Fluorescently labelled proteins and small molecules for tracking these key apoptotic events have become widely available in recent years (23–26). This makes practical the prospect of using a light activated system for the induction of apoptosis in conjunction with live cell compatible markers on a multi-channel fluorescent microscope equipped with a live cell incubator. To this end, several markers for key apoptotic events were selected for inclusion in multi-color imaging experiments, including a nuclear stain (Hoescht 33342) for monitoring chromatin condensation, a marker of phosphatidylserine (PS) inversion (Annexin V-FITC & Annexin V-Cy5), a cytoskeletal marker (GFP-Actin (27)), and a marker for downstream caspase cleavage (CellEvent Caspase 3/7). Our first experiment sought to simultaneously monitor chromatin condensation, cytoskeletal rearrangement, and phosphatidylserine inversion in the wake of BAX translocation to the mitochondria. The HEK293T cell line both demonstrated efficient transfection of both components of the OptoBax 2.0 system (Cry2(1-531).L348F.mCh.Bax.S184E and Tom20.CIB), in addition to a third protein, a cytoskeletal marker for actin (GFP-Actin). In the same experiment, chromatin was labeled with Hoescht 33342 and an Annexin V-Cy5 fusion was incorporated as a marker for PS inversion. These additional components provided a 4-color system for monitoring light induced BAX translocation to the OMM. The HEK293T cell line both demonstrated efficient transfection of both components of the OptoBax 2.0 system (Cry2(1-531).L348F.mCh.Bax.S184E and Tom20.CIB), in addition to a third protein, a cytoskeletal marker for actin (GFP-Actin). In the same experiment, chromatin was labeled with Hoescht 33342 and an Annexin V-Cy5 fusion was incorporated as a marker for PS inversion. These additional components provided a 4-color system for monitoring light induced BAX translocation to the mitochondria (mCherry/TxRed), cytoskeletal rearrangement (FITC/GFP), chromatin condensation (DAPI), and membrane flipping (Cy5). Using this system, the impact of BAX-mediated apoptosis on the cytoskeleton and chromatin was measured over the course of a 2 hour illumination experiment (Fig. 3 and Supporting Movie 3). In the course of the multi-color imaging experiment, the following changes were observed: redistribution of the GFP-Actin construct, indicative of cytoskeletal rearrangement; nuclear collapse and intensification of the fluorescence intensity of the Hoescht 33342 dye, indicative of chromatin condensation; and incorporation of the Annexin V-Cy5 dye, indicative of phosphatidylserine inversion (Fig. 3A).

**Actin Rearrangement**

Significant redistribution of the GFP-Actin marker from the cytosol to both the nucleus and the mitochondria occurred in the wake of OptoBax activation, with 44% (111/252) of cells clearly demonstrating mitochondrial GFP-Actin 70 min post-activation (Fig. 3B-3C). The accumulation of actin and numerous actin-binding proteins (cofilin 1; CAP1) at the OMM in the wake of small molecule induced apoptosis is often characterized as an early event that occurs well prior to BAX translocation (28–30). In our system, however, membrane permeabilization with OptoBAX precipitates downstream actin redistribution to the OMM well after BAX recruitment (~30 min post-OptoBAX activation). While the accumulation of actin at the mitochondria remains a poorly understood event in the BAX-mediated apoptosis pathway (28, 30–32), this result points to a closer relationship between cytoskeletal dynamics, Bcl-2 family translocation events, and apoptotic progression than previously described (31).

**Inhibition of Actin Dynamics**

The multi-color OptoBax experiments were repeated in the presence of compounds known to have opposing effects on actin dynamics: jasplakinolide, which inhibits actin depolymerization, maintaining the F-actin state, and latrunculin A, which inhibits actin polymerization by sequestering G-actin (33). As expected, treatment of cells with these compounds resulted in strikingly different distributions of our actin-GFP reporter (Supporting Figure 2). However, despite the differences in actin distributions, treatment with either compound delayed the entry of cells into light-activated apoptosis in comparison to the no treatment control, as assessed by both the reduction of chromatin condensation and PS externalization during a two hour timecourse (% condensed nuclei 70 min post-OptoBax activation: Jasplakinolide, 12(+/-2)% condensed nuclei (14/115 total cells); Latrunculin A, 5(+/-
1)% condensed nuclei (8/148 total cells); untreated cells, 55(+/-2)% condensed nuclei (54/98 total cells); Supporting Figure 2; assessed from 3 replicate measurements). While the redistribution of actin to the mitochondria occurred as previously described in untreated cells, the same event was infrequently observed with the jasplakinolide (3(+/-1)%; 3/115 total cells) and latrunculin A (1(+/-0.02)%; 2/148 total cells)-treated populations post-OptoBax activation. It should be noted that while these compounds delayed apoptotic onset in our system, both compounds have been demonstrated to be pro-apoptotic in cell culture over lengthy incubation periods, precluding lengthier experiments(34–36). Nonetheless, it is clear that perturbations of actin dynamics delay key components of our light-activated apoptotic cascade. It is noted that the observed correlation between Bax-induced MOMP and actin rearrangement may not be entirely causal. For example, it is possible that the rapid mitochondrial depolarization induced by small molecule-initiated apoptosis (i.e. staurosporine (STS)) triggers events that lead to actin redistribution in advance of endogenous BAX translocation. For example, in STS-induced apoptosis, mitochondrial depolarization occurs rapidly after treatment, while BAX maximally translocates to the OMM several hours post-STS treatment(37, 38). As a result, in our system, it cannot be ruled out that BAX dependent mitochondrial depolarization drives actin redistribution via a partially MOMP-independent mechanism.

**Nuclear Accumulation of Actin**

In addition to actin redistribution, the nuclear accumulation of GFP-Actin was also observed in the wake of OptoBAX activation, occurring just after the appearance of mitochondrial GFP-Actin (Fig. 3C). The transition of cytosolic actin to nuclear actin has been previously described, and may be part of a general cellular stress response.(39, 40) The question of whether this freshly-nuclear localized actin binds to chromatin may also have interesting implications for actin’s role in the repression or activation of transcription of genes vital to completing the apoptotic cascade in mammalian cells, reinforcing the notion of actin as an important part of the apoptotic transcriptome, as previously observed in yeast (41–44). Furthermore, redistribution of cytoskeletal actin into the nucleus may also provide insight into the timing of disruption of the nuclear-cytoplasmic barrier – a key apoptotic event that precipitates many of the later events in apoptosis by providing access of cytoplasmic proteins to the nuclear compartment(45–47). Interestingly, both increasing chromatin condensation (Fig. 3D) and increasing phosphatidylserine inversion (Fig. 3E) occur in tandem with nuclear accumulation of actin. Both chromatin condensation and phosphatidylserine inversion peaked 70 – 90 minutes post OptoBax activation.

**Caspase 3/7 Cleavage**

In our final multi-color experiment, a fluorescent indicator of caspase cleavage was used to assess caspase 3/7 cleavage downstream of OptoBax activation (Fig. 4A). Caspase activity was observed within 1 hour of BAX recruitment, and increased steadily thereafter (Fig. 4B). While this event begins relatively early, it peaks later (> 2h post-BAX recruitment), consistent with previous western blotting assessment of caspase cleavage in the OptoBAX system.(16) By contrast, control experiments utilizing Cry2(1-531).L348F.mCh and Tom20.CIB did not activate the caspase activity indicator dye or the marker for phosphatidylserine inversion within the same time period of recruitment to the OMM and imaging (Supporting Figure 3). Furthermore, experiments with the OptoBax in the presence of 50 μM Z-VAD(OMe)-FMK showed significant suppression of caspase activity (Fig. 4B). Interestingly, while chromatin condensation is observed during the course of our experiments, significant nuclear fragmentation was not observed during the same timeframe (<1% of cells over a 120 min timecourse), suggesting that, like peak caspase cleavage, nuclear
Hallmarks of apoptosis via optogenetic actuators

fragmentation occurs later in Bax-mediated apoptosis. As a corollary to these results, longer timecourse experiments in HeLa cells utilizing a programmable LED device for cellular illumination, followed by nuclear staining and immunostaining, demonstrated nuclear fragmentation approximately 7 hours post-apoptotic induction (Supporting Figures 4 and 5).

A Timeline of Apoptotic Events

One potentially useful application of the OptoBax system, in conjunction with the foregoing markers, is the establishment of a timeline of key apoptotic events, in order to define not only the order, but also the interplay between key steps in the apoptotic cascade. In turn, the establishment of a timeline around a specific event (i.e., Bax translocation to the OMM) may provide novel insights into their interdependence. Utilizing data from our multicolor imaging experiments, a timeline of these early apoptotic events relative to OptoBax activation was assembled (Fig. 5). This timeline demonstrates that much happens within the first two hours of the apoptotic cascade, with certain events appearing to be synchronized. For example, the actin cytoskeleton, long implicated as a key player in apoptotic progression, undergoes redistribution from the cytosol to the mitochondria in addition to movement from the cytosol to the nucleus. This redistribution event generally precedes phosphatidylserine inversion. It is possible that wholesale redistribution of actin, a key structural component of the cytoskeleton, contributes to membrane instability and subsequent inversion. Interestingly, the increase in accumulation of Annexin V is also concurrent with an increase in chromatin condensation. These events (chromatin condensation and Annexin V accumulation) occur within the same timeframe as actin redistribution, implicating collapse and rearrangement of the actin cytoskeleton as a common thread between these events in the progression of apoptosis(31). Finally, previous studies of staurosporine-induced apoptosis have reported that actin redistribution to the OMM occurs well before BAX translocation. In our system, recruitment of OptoBax to the OMM precipitates the actin-OMM localization event. Staurosporine has been shown to effect multiple apoptotic pathways (intrinsic and extrinsic), which may be the basis for the timing of the BAX localization event relative to that of actin.(48) Utilization of an optogenetic system such as OptoBax for the control of a single event in the apoptotic cascade may provide a more precise way for examining these translocation events. For example, while previous studies have focused on defining the pro-apoptotic role of coflin at the OMM(49), the universal importance of the role played by coflin and other ABPs at the OMM during apoptosis remains a matter of debate(28). Future studies in our lab will attempt to resolve the complicated web of translocation events at the OMM occurring in early apoptosis using these optogenetic tools.

Experimental Procedures

Cloning

The Cry2(1-531) gene was amplified by polymerase chain reaction (NEB Q5 polymerase) from the full length Cryptochrome-2 gene using a forward primer encoding an XhoI-restriction site with the sequence: GGCCAACTCGAGATGAAGATGGACAAAA

AGAC and a reverse primer encoding an XmaI restriction site with the sequence: TGATATCCCCGGGCTACTTGTTGGTCATT

AGAAG. The resulting PCR amplicon was then digested, gel purified, and ligated into a mCherry-N1 vector (Clontech) containing BAX S184E as a fusion to the C-terminus of mCherry. Site directed mutagenesis was used to introduce the L348F mutation using a forward primer with sequence:

CCGGAATGAGAGAGTTTTGGGCTACCGG

ATGG; and reverse primer with the sequence:

CCATCCGGTAGCCAAAAACTCTCATTC

CGG. Cloning methods for constructs used in prior work (Cry2PHR.mCh.BAX, Tom20.CIB.GFP, and Tom20.CIB.STOP) have been described elsewhere.(16) The actin-GFP (pCAG-mGFP-actin; Addgene #21948) construct was a generous gift from Ryohei Yasuda(27).
Hallmarks of apoptosis via optogenetic actuators

Cell culture and transfection
Midi prep quantities of DNA of each construct were then created from E. coli and collected for cell transfection. Transfections were then performed with the Lipofectamine 3000 reagent (Invitrogen) following manufacturer’s suggested protocols. Briefly, for dual transfections in 35 mm glass bottom dishes, plasmid DNA was combined in a 1:1 ratio (1,250 ng per plasmid) in 125 uL of Opti-Mem, followed by the addition of 5 uL of P3000 reagent. In a separate vial, 3.75 uL of Lipofectamine 3000 were added to 125 uL of Opti-Mem. The two 125 uL solutions were combined and allowed to incubate at room temperature for 10 min, followed by dropwise addition to cell culture. For triple-transfections, plasmid DNA was combined in a 1:1:1 ratio (1,000 ng per plasmid), followed by an identical transfection procedure. Transfection solutions were allowed to remain on cells overnight. Cells were maintained at 37°C and 5% CO2 in a humidified tissue culture incubator. HeLa and HEK293T cell lines were maintained in DMEM supplemented with 10% FBS and 1% Pen-Strep. Neuro-2a cells were maintained in Eagle’s MEM supplemented with 10% FBS and 1% Pen-Strep.

Cell viability measurements
Background cell death experiments were performed to determine the cell toxicity of OptoBAX constructs in the absence of light exposure. Transfections were performed on HeLa cells in 6-well plates, then maintained in complete darkness for two days prior to incubation with a cell viability stain (Live-or-Dye 488/515 (Biotium). Briefly, cells were washed 1X with DPBS containing calcium and magnesium, followed by incubation at room temperature with the viability stain (diluted 1:1000 in DPBS) for 30 min. Afterwars, cells were fixed with 4% PFA in PBS for 15 min at room temperature, then washed 3 x 1 mL with DPBS, and maintained in DPBS for subsequent imaging. Images of fixed cells were acquired on a Leica Dmi8 fluorescent microscope.

Application of fluorescent dyes
Four-color experiment: Triple-transfected cells (Cry2.mCh.Bax/Tom20.Cib /GFP-Actin) in a 35 mm glass bottom dish (Mattek) were removed from incubator, cell culture media removed by pipette, and cells gently washed 1X with DPBS supplemented with calcium and magnesium. 1 mL of PBS containing Hoescht 33342 was then applied to the cells, which were returned to the incubator for 10 min. After the elapsed time, media was removed and cells were washed with DPBS (2 x 1 mL). A pre-warmed buffer containing DPBS supplemented with 10% FBS and Annexin V-Cy5 (10 μL/1 mL of buffer; ThermoFisher A23204) were added to cells, which were promptly placed on the microscope for initiation of the light-activated experiment.

Three-color experiment: Dual-transfected cells (Cry2.mCh.Bax/Tom20.Cib.STOP) were removed from incubator, cell culture media removed by pipette, and cells gently washed 1X with DPBS supplemented with calcium and magnesium. 1 mL of PBS containing Hoescht 33342 was then applied to the cells, which were returned to the incubator for 10 min. After the elapsed time, media was removed and cells were washed with DPBS (2 x 1 mL). A pre-warmed buffer containing DPBS supplemented with 10% FBS and CellEvent Caspase 3/7 Green (1 μL/1 mL of buffer; ThermoFisher C10427) were added to the cells, which were promptly placed on the microscope for initiation of the light-activated experiment.

Cell treatments with actin binding compounds
Cells were pre-treated with actin-binding compounds for one hour prior to imaging. A. Jasplakinolide (Santa Cruz Biotechnology) was diluted from a 1 mM stock in DMSO to a working concentration of 200 nM in DMEM supplemented with 10% FBS. Prior to imaging, the cells were incubated with Hoescht 33342 for 10 minutes, followed by a 1 mL PBS wash. Cells were then resupplied with media for live cell imaging (DPBS/10%FBS/200 nM Jasplakinolide/Annexin V-Cy5). B. Latrunculin A (Santa Cruz Biotechnology) was diluted from a 1 mM stock in DMSO to a working concentration of 2 μM in DMEM supplemented with 10% FBS. Prior to imaging, the cells were incubated with Hoescht 33342 for 10 minutes,
followed by a 1 mL PBS wash. Cells were then resupplied with media for live cell imaging (DPBS/10%FBS/2 μM Latrunculin A/Annexin V-Cy5).

**Cell treatments with caspase inhibitor**

Cells were pre-treated with the pan-caspase inhibitor Z-VAD(OMe)-FMK (Santa Cruz Biotechnology) for 50 min prior to imaging. The compound was diluted from a 10 mM stock in DMSO to a working concentration of 50 μM in DMEM supplemented with 10% FBS. Prior to imaging, the cells were incubated with Hoescht 33342 for 10 minutes, followed by a 1 mL PBS wash. Cells were then resupplied with media for live cell imaging (L-15/10%FBS/50 μM Z-VAD(OMe)-FMK).

**Microscopy**

Leica DMi8 Live Cell Imaging System, equipped with an OKOLab stage-top live cell incubation system, LASX software, Leica HCX PL APO 63x/1.40-0.60na oil objective, Lumencor LED light engine, CTRAdvanced+ power supply, and a Leica DFC900 GT camera, was used to acquire images. Exposure times were set at 150 ms (GFP, 470 nm), 150 ms (DAPI, 395 nm), 300 ms (mCherry, 550 nm), and 300 ms (Cy5, 640 nm), with LED light sources at 50% power, and images acquired every minute or every 10 minutes.

**Microscopy data analysis**

Analysis of microscopy data was performed in FIJI equipped with the BioFormats package. Fluorescence intensity measurements were normalized on a scale from 0 – 1 prior to being averaged. Average normalized intensities were computed from 4 – 6 fields of view on a 63x objective, with each field of view encompassing 100 (+/- 10) transfected cells. Mitochondrial residence time plots were generated by quantifying the change in fluorescence intensity at the mitochondria over time, post light recruitment. Fluorescence intensity measurements were normalized and averaged from 6 mitochondrial clusters per transfection condition. After normalizing the change in fluorescence over time, the mitochondrial association half-life was defined as the time for fluorescence intensity of the light recruited reagent to reach 50% maximum intensity at the mitochondria. Image overlays were created using the Merge Channels function in FIJI.

**Western blot**

HeLa cells were lysed post-transfection with 200 μL of M-PER lysis buffer (Thermo Scientific) plus protease inhibitors. After 10 min on a rotary shaker at room temperature, lysates were collected and centrifuged for 15 min (1000 rpm at 4 °C) and supernatants reserved. The resulting lysates were subjected to electrophoresis on a 10% SDS-PAGE gel and then transferred to PVDF membranes (20 V, overnight, at 4 °C). Membranes were then blocked for 1 h with 5% BSA in TBS with 1% Tween (TBST), followed by incubation with the primary antibody (Rockland Anti-mCherry antibody 600-401-P16) 1:1000 dilution in 5% milk – TBST overnight at 4 °C on a platform rocker. The membranes were then washed 3 x 5 min each with TBST and incubated with anti-rabbit IgG – HRP secondary antibody (abcam 6721) at 1:3000 in 5% BSA – TBST for 2 h at room temp. After washing 3 x 5 min with TBST, the membranes were exposed to a chemiluminescent substrate for 5 min and imaged with using an Azure cSeries imaging station.

**Programmable LED device**

The device (designed and constructed by Hoffman and Hughes) combines four wavelength ranges (440-460nm, 520-540nm, 650-670nm, 720-750nm) into a single package using Surface Mount Technology (SMT) and is powered with an Arduino UNO system. Surface Mount Devices (SMD) Luxeon Rebel and Luxeon Color series LEDs were soldered on 20mm mounts with a hot air gun designed for Surface Mount Devices. The LEDs/MCPCB aluminum LED bases were fixed to a 130-mm x 70 mm Rectangular 15 mm high heat sink to prevent overheating and allow for longer exposures of samples. The intensity of the light was controlled with a combination of variable resistors, PNP transistors, and zener diodes. The LED arrays are controlled using an Arduino controller to control illumination, time,
intensity, and wavelength. Light intensity was measured with a Fisherbrand digital light meter; impacts of illumination protocols on media temperature were determined with a Fisherbrand digital thermometer using type-K beaded probe.

**Cellular illumination and immunostaining**

HeLa cells were plated in 35 mm glass bottom dishes (Mattek) at a density of 2.0 x 10^5 cells per dish and maintained at 37 °C in a humidity-controlled incubator with a 5% CO_2 atmosphere in DMEM (10% FBS, 1% Pen-Strep). The following day, cells were transfected with Cry2.L348F.531.mCh.BAX and Tom20.CIB.GFP as previously reported. 24 h post transfection, cells were illuminated with 440-460 nm light using a programmable LED device prior to being returned to the incubator. At the conclusion of the dark incubation period, cells were washed 3 x 1 mL with PBS and then fixed with 1 mL of 4% paraformaldehyde in PBS at room temperature for 10 min. Cells were washed 2 x 1 mL with PBS and blocked and permeabilized for 20 min in antibody dilution buffer (1% BSA; 0.3% Triton-X-100; PBS) at 4 °C. Blocking was followed by overnight incubation at 4 °C with rabbit anti-cleaved caspase 3 antibody (Cell Signaling 9661) at 1:400 dilution in antibody dilution buffer (1% BSA; 0.3% Triton-X-100; PBS). Cells were then washed with PBS (3 x 5 min) before incubation with anti-rabbit AlexaFluor 647 secondary antibody (Life Technologies A21245) at 1:500 dilution in antibody dilution buffer. After washing cells with PBS (3 x 5 min), nuclei were stained briefly (5 min) with Hoescht 33342 (1 μg/mL), followed by image acquisition on a confocal microscope (Olympus IX2-DSU Tandem Spinning Disk Confocal; 60X objective).

**Corrected total cell fluorescence (CTCF)**

Whole cell fluorescence intensities were measured in FIJI/ImageJ (50) and quantified by the Corrected Total Cell Fluorescence Method (CTCF) (51, 52). Briefly, Individual cells were outlined in FIJI followed by calculation of area, integrated density, and the mean gray value for each cell. For each image, background measurements of fluorescence intensity were taken from regions without cells. The following formula was then used to calculate the CTCF for each cell: CTCF = Integrated Density – (Cell Area * Mean Background Fluorescence).

**Plasmids**

Plasmids generated for this publication are available through Addgene, Inc., a not-for-profit plasmid DNA repository.
Acknowledgements

The authors wish to thank Dr. Karen Litwa (ECU Brody School of Medicine) for the gift of HEK293T cells; Dr. Alex Murashov (ECU Brody School of Medicine) for the gift of Neuro-2a cells; Dr. Nathan Hudson (ECU Department of Physics) for use of the Leica Dmi8 widefield microscope.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.
References

1. Hughes, R. M. (2018) A compendium of chemical and genetic approaches to light-regulated gene transcription. *Crit. Rev. Biochem. Mol. Biol.* **53**, 453–474

2. Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G., and Deisseroth, K. (2005) Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci.* **8**, 1263–1268

3. Wu, Y. I., Frey, D., Lungu, O. I., Jaehrig, A., Schlichting, I., Kuhlman, B., and Hahn, K. M. (2009) A genetically encoded photoactivatable Rac controls the motility of living cells. *Nature* **461**, 104–108

4. Rhind, N., and Russell, P. (2012) Signaling pathways that regulate cell division. *Cold Spring Harb. Perspect. Biol.* 10.1101/cshperspect.a005942

5. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005) Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* **6**, 56–68

6. Elmore, S. (2007) Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35**, 495–516

7. Bleger, D., and Hecht, S. (2015) Visible-Light-Activated Molecular Switches. *Angew. Chem. Int. Ed. Engl.* **54**, 11338–11349

8. Stein, V., and Alexandrov, K. (2015) Synthetic protein switches: design principles and applications. *Trends Biotechnol.* **33**, 101–110

9. Mühlhäuser, W. W. D., Hörner, M., Weber, W., and Radziwill, G. (2017) Light-Regulated Protein Kinases Based on the CRY2-CIB1 System. in *Synthetic Protein Switches: Methods and Protocols* (Stein, V. ed), pp. 257–270, Springer New York, New York, NY, 10.1007/978-1-4939-6940-1_16

10. Green, D. R. (1998) Apoptotic pathways: the roads to ruin. *Cell* **94**, 695–698

11. Adams, J. M., and Cory, S. (1998) The Bcl-2 protein family: arbiters of cell survival. *Science* **281**, 1322–1326

12. Westphal, D., Kluck, R. M., and Dewson, G. (2014) Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis. *Cell Death Differ.* **21**, 196–205

13. Westphal, D., Dewson, G., Czabotar, P. E., and Kluck, R. M. (2011) Molecular biology of Bax and Bak activation and action. *Biochim. Biophys. Acta - Mol. Cell Res.* **1813**, 521–531

14. Lalier, L., Cartron, P.-F., Juin, P., Nedelkina, S., Manon, S., Bechinger, B., and Vallette, F. M. (2007) Bax activation and mitochondrial insertion during apoptosis. *Apoptosis* **12**, 887–896
15. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J.* **17**, 3878–3885

16. Hughes, R. M., Freeman, D. J., Lamb, K. N., Pollet, R. M., Smith, W. J., and Lawrence, D. S. (2015) Optogenetic Apoptosis: Light-Triggered Cell Death. *Angew. Chemie - Int. Ed.* 10.1002/anie.201506346

17. Nechushtan, A., Smith, C. L., Hsu, Y. T., and Youle, R. J. (1999) Conformation of the Bax C-terminus regulates subcellular location and cell death. *EMBO J.* **18**, 2330–2341

18. Taslimi, A., Zoltowski, B., Miranda, J. G., Pathak, G. P., Hughes, R. M., and Tucker, C. L. (2016) Optimized second-generation CRY2-CIB dimers and photoactivatable Cre recombinase. *Nat. Chem. Biol.* 10.1038/nchembio.2063

19. Godley, B. F., Shamsi, F. A., Liang, F.-Q., Jarrett, S. G., Davies, S., and Boulton, M. (2005) Blue light induces mitochondrial DNA damage and free radical production in epithelial cells. *J. Biol. Chem.* **280**, 21061–21066

20. Hughes, R. M., Freeman, D. J., Lamb, K. N., Pollet, R. M., Smith, W. J., and Lawrence, D. S. (2015) Optogenetic Apoptosis: Light-Triggered Cell Death. *Angew. Chemie - Int. Ed.* **54**, 12064–12068

21. Dewson, G., and Kluck, R. M. (2009) Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. *J. Cell Sci.* **122**, 2801–2808

22. Saraste, A., and Pulkki, K. (2000) Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc. Res.* **45**, 528–537

23. Gelles, J. D., and Chipuk, J. E. (2017) Robust high-throughput kinetic analysis of apoptosis with real-time high-content live-cell imaging. *Cell Death Dis.* **8**, e2758

24. Gelles, J. D., and Chipuk, J. E. (2016) Robust high-throughput kinetic analysis of apoptosis with real-time high-content live-cell imaging. *Cell Death Dis.* **7**, e2493

25. Zeng, W., Wang, X., Xu, P., Liu, G., Eden, H. S., and Chen, X. (2015) Molecular imaging of apoptosis: from micro to macro. *Theranostics.* **5**, 559–582

26. Puigvert, J. C., de Bont, H., van de Water, B., and Danen, E. H. J. (2010) High-throughput live cell imaging of apoptosis. *Curr. Protoc. cell Biol.* Chapter 18, Unit 18.10.1-13

27. Murakoshi, H., Lee, S.-J., and Yasuda, R. (2008) Highly sensitive and quantitative FRET-FLIM imaging in single dendritic spines using improved non-radiative YFP. *Brain Cell Biol.* **36**, 31–42

28. Rehklau, K., Gurniak, C. B., Conrad, M., Friauf, E., Ott, M., and Rust, M. B. (2012) ADF/cofilin proteins translocate to mitochondria during apoptosis but are not generally required for cell death signaling. *Cell Death Differ.* **19**, 958–967

29. Wang, C., Zhou, G.-L., Vedantam, S., Li, P., and Field, J. (2008) Mitochondrial shuttling of CAP1 promotes actin- and cofilin-dependent apoptosis. *J. Cell Sci.* **121**, 2913–2920

30. Tang, H. L., Le, A.-H. P., and Lung, H. L. (2006) The increase in mitochondrial...
association with actin precedes Bax translocation in apoptosis. Biochem. J. 396, 1–5

31. Desouza, M., Gunning, P. W., and Stehn, J. R. (2012) The actin cytoskeleton as a sensor and mediator of apoptosis. Bioarchitecture. 2, 75–87

32. Boldogh, I. R., and Pon, L. A. (2006) Interactions of mitochondria with the actin cytoskeleton. Biochim. Biophys. Acta. 1763, 450–462

33. Fenteany, G., and Zhu, S. (2003) Small-molecule inhibitors of actin dynamics and cell motility. Curr. Top. Med. Chem. 3, 593–616

34. Martin, S. S., and Leder, P. (2001) Human MCF10A mammary epithelial cells undergo apoptosis following actin depolymerization that is independent of attachment and rescued by Bcl-2. Mol. Cell. Biol. 21, 6529–6536

35. Morton, W. M., Ayscough, K. R., and McLaughlin, P. J. (2000) Latrunculin alters the actin-monomer subunit interface to prevent polymerization. Nat. Cell Biol. 2, 376–378

36. Posey, S. C., and Bierer, B. E. (1999) Actin stabilization by jasplakinolide enhances apoptosis induced by cytokine deprivation. J. Biol. Chem. 274, 4259–4265

37. Tsuruta, F., Masuyama, N., and Gotoh, Y. (2002) The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria. J. Biol. Chem. 277, 14040–14047

38. Smaili, S. S., Hsu, Y. T., Sanders, K. M., Russell, J. T., and Youle, R. J. (2001) Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential. Cell Death Differ. 8, 909–920

39. Pendleton, A., Pope, B., Weeds, A., and Koffer, A. (2003) Latrunculin B or ATP depletion induces coflin-dependent translocation of actin into nuclei of mast cells. J. Biol. Chem. 278, 14394–14400

40. Meijerman, I., Blom, W. M., de Bont, H. J., Mulder, G. J., and Nagelkerke, J. F. (1999) Induction of apoptosis and changes in nuclear G-actin are mediated by different pathways: the effect of inhibitors of protein and RNA synthesis in isolated rat hepatocytes. Toxicol. Appl. Pharmacol. 156, 46–55

41. Gourlay, C. W., and Ayscough, K. R. (2005) A role for actin in aging and apoptosis. Biochem. Soc. Trans. 33, 1260–1264

42. Gourlay, C. W., and Ayscough, K. R. (2005) The actin cytoskeleton: a key regulator of apoptosis and ageing? Nat. Rev. Mol. Cell Biol. 6, 583–589

43. Gourlay, C. W., and Ayscough, K. R. (2005) Identification of an upstream regulatory pathway controlling actin-mediated apoptosis in yeast. J. Cell Sci. 118, 2119–2132

44. Zheng, B., Han, M., Bernier, M., and Wen, J. (2009) Nuclear actin and actin-binding proteins in the regulation of transcription and gene expression. FEBS J. 276, 2669–2685

45. Strasser, C., Grote, P., Schauble, K., Ganz, M., and Ferrando-May, E. (2012) Regulation of nuclear envelope permeability in cell death and survival. Nucleus. 3, 540–551
46. Kihlmark, M., Rustum, C., Eriksson, C., Beckman, M., Iverfeldt, K., and Hallberg, E. (2004) Correlation between nucleo-cytoplasmic transport and caspase-3-dependent dismantling of nuclear pores during apoptosis. *Exp. Cell Res.* **293**, 346–356

47. Faleiro, L., and Lazebnik, Y. (2000) Caspases disrupt the nuclear-cytoplasmic barrier. *J. Cell Biol.* **151**, 951–959

48. Nakamura-Lopez, Y., Sarmiento-Silva, R. E., Moran-Andrade, J., and Gomez-Garcia, B. (2009) Staurosporine-induced apoptosis in P388D1 macrophages involves both extrinsic and intrinsic pathways. *Cell Biol. Int.* **33**, 1026–1031

49. Chua, B. T., Volbracht, C., Tan, K. O., Li, R., Yu, V. C., and Li, P. (2003) Mitochondrial translocation of coflin is an early step in apoptosis induction. *Nat. Cell Biol.* **5**, 1083–1089

50. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat Meth.* **9**, 676–682

51. Karpova, T. S., McNally, J. G., Moltz, S. L., and Cooper, J. A. (1998) Assembly and function of the actin cytoskeleton of yeast: relationships between cables and patches. *J. Cell Biol.* **142**, 1501–1517

52. Cooper, J. A. (2008) *Corrected Total Cell Fluorescence method*

53. Haar, L. L., Lawrence, D. S., and Hughes, R. M. (2019) Optogenetic perturbation of the biochemical pathways that control cell behavior. *Methods Enzymol.* **622**, 309–328
Footnotes

Funding was provided by the East Carolina University Division of Research, Economic Development and Engagement, Thomas Harriot College of Arts and Sciences, and the Department of Chemistry.

The abbreviations used are: Cry2, cryptochrome 2; CIB, cryptochrome-interacting beta helix-loop-helix; MOMP, mitochondrial outer membrane permeabilization; OMM, outer mitochondrial membrane.
Figure 1. Redesign of optogenetic actuators and characterization of extended photocycle mutant L348F A. Optimized versions of OptoBax created for reduction of light-independent cell death and extension of photocycle. Western blot (α-mCherry; inset) lanes are (1) Cry2.531.mCherry; (2) Cry2.531.L348F.mCherry; (3) Cry2.531.mCherry.Bax; (4) Cry2.531.L348F.mCherry.Bax; (5) Cry2PHR.mCherry.Bax. B. Demonstration of elongated photocycle in the Cry2 L348F mutant. Images shown are before, 5 min, and 10 min post activation with a 150 ms pulse of 470 nm light. C. Plot of normalized fluorescence data from the experiment shown in 1.B. Error bars represent standard deviations from 6 replicate measurements of light initiated recruitment using the method described in Hughes, et al(53). Scale bar = 10 microns.
Figure 2. Minimal light application required for apoptotic cell death and application to other cell lines A. OMM recruitment of OptoBax constructs with (Cry2.531.L348F.Bax/ Tom20.Cib.GFP) and without (Cry2.531.L348F/ Tom20.Cib.GFP) the proapoptotic Bax fusion in HeLa cells. Cells illuminated with a 150 ms pulse of 470 nm light every 10 minutes over a two hour time course. B. Quantification of cellular collapse over the one hour time course experiment; Error bars represent standard deviations from a minimum of 10 replicate measurements per condition. *P<0.05 for all pairwise comparisons (One-way ANOVA). C. Inhibition of Cry2.531.L348F.Bax occurs in the presence of 50 μM Z-VAD(OMe)-FMK. C. Demonstration of light induced cellular collapse with OptoBax (Cry2.531.L348F.Bax/Tom20.Cib.GFP) in HEK293T and Neuro-2a cell lines over a two hour time course (470 nm; 150 ms pulse; images acquired every minute). Scale bar = 10 microns.
Figure 3. Four color experiment A. Multi-color imaging (Leica Dmi8 widefield microscope equipped with OKOLab stage-top incubator) of mitochondrial recruitment, chromatin condensation, actin rearrangement, and phosphotidylserine exposure in HEK293T cells. B. Overlay of actin/mitochondria after 2 hours of imaging. C. Timelapse of actin rearrangement and accumulation at mitochondria. D. % chromatin condensation versus time for chromatin marker (Hoescht 33342) and E. fluorescent intensity changes for PI marker (Annexin V-Cy5). Images acquired every minute over a two hour time course. Error bars represent standard deviations from 4 replicate measurements (Fig. 3D) and 6 replicate measurements (Fig. 3E) per condition. Scale bars = 10 microns.
Figure 4. Three color experiment A. Multi-color imaging (Leica Dmi8 widefield microscope equipped with OKOLab stage-top incubator) of mitochondrial recruitment, chromatin condensation, caspase cleavage in HEK293T cells. B. Fluorescence intensity versus time for caspase activity marker (CellEvent 3/7). Images acquired every minute over a 2.5 hour time course. Cells were either treated with 50 μM Z-VAD-FMK (dark circles) or untreated (empty circles) for 50 min prior to the experiment. Error bars represent standard deviations from 4 replicate measurements. Scale bar = 10 microns.
Scheme 1. Timeline of apoptotic events

- Light activated mitochondrial recruitment
- Actin mitochondrial localization
- Detectable caspase 3/7 cleavage
- Peak membrane inversion
- Peak chromatin condensation
- Time (h)

0 1 2 3 4
Imaging of Morphological and Biochemical Hallmarks of Apoptosis with Optimized Optogenetic Tools
Walton C Godwin, George F Hoffmann, Taylor J Gray and Robert M Hughes

J. Biol. Chem. published online October 3, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.009141

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts