OPTIMIZATION OF AN OPTOGENETICALLY ACTIVATED INITIATOR OF APOPTOSIS

by

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Abstract:

Optogenetic tools provide a window into the world of cell signaling pathways and protein-protein interactions with a precision and elegance that other biological probes can struggle to achieve. The creation of optogenetic switches for the control of cell death pathways can provide insight into the mechanisms of apoptosis and form a basis for non-invasive, next generation therapeutic strategies. Previously, we have employed Cryptochrome 2 (Cry2)/CIB, a blue light photoreceptor protein – protein dimerization module from A. thaliana in conjunction with BAX, an OMM targeting pro-apoptotic protein, for light-mediated initiation of mitochondrial outer membrane permeabilization (MOMP) and downstream apoptosis. In this work, we are further developing our light activated Cry2-BAX system (henceforth referred to as "OptoBAX") for "one click" initiation of the BAX-mediated apoptotic cascade. We also report results of experimental efforts to optimize our optogenetic switch to reduce light-independent cell death (dark activation), and to enhance experimental control of our switch by manipulating photophysical properties associated with the Cry2/CIB interaction.

Keywords: *apoptosis, programmed cell death, optogenetics, photoactivatable proteins*

Introduction:

Apoptosis is an essential and carefully controlled process of cell death that is utilized in multicellular organisms for proper development and growth. The pathway has been recognized to be a vital stage of embryonic development,[1] normal cell turnover,[2] carcinogenesis,[3] autoimmune disease progression,[4,5] and neurodegeneration.[6] Apoptosis is characterized by blebbing of the nuclear membrane, degradation of DNA by nucleases, and subsequent fragmentation and condensation.[7] From this point, phagocytic cells remove cell fragments that are left over in vivo.[8] Due to the homeostatic relevance of apoptosis, it is clear that unintentional and disruptive apoptosis can be involved in various malignancies.[9] Consequently, the control of apoptosis is of great interest as the molecular mechanisms of this pathway may prove to be potential targets for therapeutic intervention.

Apoptosis is regulated at the mitochondria by a family of evolutionarily-conserved proteins called the Bcl-2 family.[10] This family of proteins consists of members that are either pro-apoptotic or anti-apoptotic and control the process by acting on the permeabilization of the outer mitochondrial membrane (OMM). One of the many pro-apoptotic regulators is the Bcl-2 associated X protein, or BAX.[11]

BAX functions by forming oligomers with other pro-apoptotic proteins and creating openings in the OMM.[12] This opening results in the loss of membrane potential and the release of cytochrome c, an activator of the apoptotic caspase cleavage cascade **(Figure 1)**.[13] BAX is composed of nine amphipathic α-helices with a hydrophobic core and a transmembrane C-terminal α-helix that anchors itself into the OMM.[14]

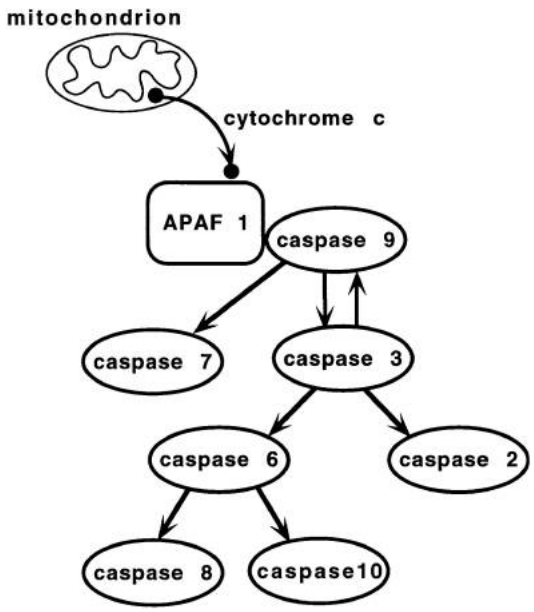

Figure 1. Schematic of caspase cleavage that occurs post-cytochrome c release from the OMM.[15]

Recent advancements in the exploitation of genetically encoded photoactivated dimers have allowed for the control of protein-protein interactions by light.[16] One such system is the Cryptochrome 2-CIB1 module found in *Arabidopsis thaliana*. Cryptochromes are a group of flavoproteins that are affected by blue light.[17] In nature, cryptochrome proteins are used in the circadian rhythms of plants and animals to regulate sleep and development patterns.[18,19] In this context, cryptochrome 2 (Cry2) travels and binds to CIB1 in its photoexcited state. Previous studies have developed a construct of Cry2 and CIB1 that are engineered to stay cytosolic.[20] This construct differs from the wildtype in that the nuclear localization sequences were mutated to convert lysine repeats to alanine repeats for cell usage.

Previous work performed by R. M. Hughes et. al. focused on the development of a Cry2-CIB1 module associated with inducing cell death by introducing BAX to the OMM. This optogenetic BAX translocates from the cytosol, where it is tethered to Cry2, to the mitochondria, where Cry2's partner protein CIB1 is tethered by a fragmented protein, Tom20, in response to blue light (470 nm) stimulation. This system allows for the light-controlled introduction of pro-

apoptotic BAX to the OMM, resulting in cell death.[21] From this point, the Cry2/CIB1 and BAX system will be referred to as OptoBAX.

Previous studies have also identified a mutation in the Cry2 sequence that extends the photocycle length of the Cry2-CIB1 module. The long-lived Cry2-L348F photocycle mutant was discovered through a genetic screen in yeast and was found to maintain a much longer bound state to CIB1.[21] Researchers found that this mutant resulted in extended activity when yeast cultures expressing Cry2-L348F were exposed to flashes of blue light; however, when exposed to light for long periods the mutant had similar activity to the wild-type Cry2.[22] Activity was measured by examining a photoactivatable Gal4 transcriptional system that utilizes the Cry2-CIB1 module. It was found that this mutation is ideal for applications in which fast turn-off of the system is not desired and light exposure must be kept brief.

In this work, we optimize a previously created Cry2/CIB1 protein dimerization module in conjunction with BAX to act as an agent of mitochondrial damage (OptoBAX) by inducing the L348F point mutation in the Cry2 protein.

Methodology:

From the OptoBAX construct we created variations and mutants, including a sequence of Cry2, called Cry2-531-L348F, designed to extend the photocycle and recruitment of BAX to the OMM. The Cry2-531 gene was amplified by polymerase chain reaction from the full length Cryptochrome-2 gene using forward primer 172-XhoI-Cry2-F with the sequence: ggccaa CTCGAG atgaagatggacaaaaagac; and reverse primer Cry2-531-27 with the sequence: TGATATC CCCGGGC TACTTGTTGGTCATTAGAAG. The resulting DNA sequence was then cloned into

Clontech mCherry-N1 vectors using restriction digest at XhoI and XmaI sites and ligation with T7 DNA Ligase. The Cry2PHR-498 construct was recycled from previous work **(Figure 2)**.

Site directed mutagenesis was used to introduce the L348F mutation in the using forward primer Cry2-L348F-28 with sequence: ccggaatgagagag ttt tgggctaccggatgg; and reverse primer Cry2-L348F-29 with sequence: CCATCCGGTAGCCCA AAA CTCTCTCATTCCGG (Figure 2). This process was followed by DpnI digest and transformed into ultra-competent *E. coli*.

The CIB1 protein construct was recycled from previous work but includes a Tom20 anchor to the OMM and GFP.

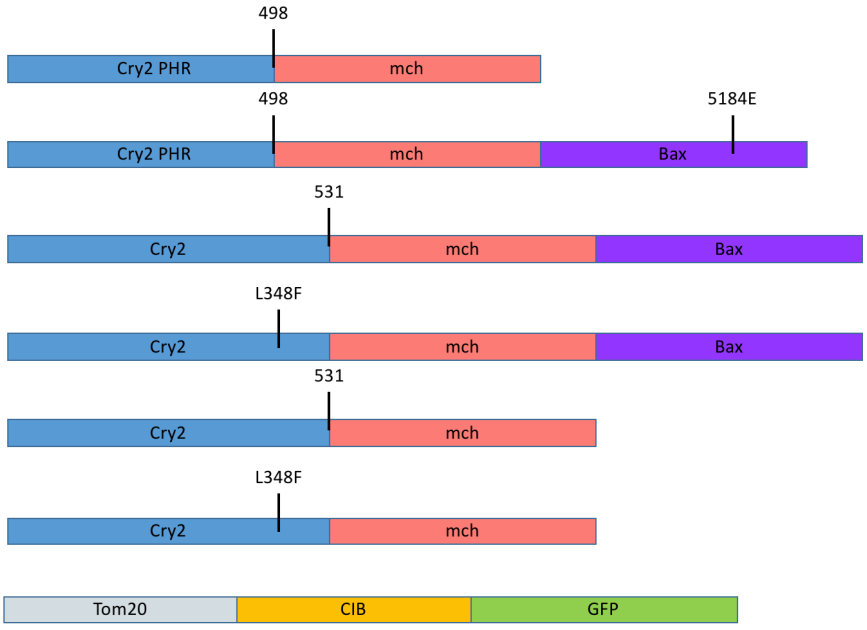

Figure 2. Diagram of the individual constructs created. From top to bottom they are: Cry2-PHR, Cry2-PHR-BAX, Cry2-531-BAX, Cry2-531-L348F-BAX, Cry2-531, Cry2-531-L348F, and Tom20-CIB. Each Cry2 protein module is tagged with (m)cherry fluorescing molecule and the CIB protein module is tagged with GFP.

Midiprep quantities of DNA of each construct were then created from *E. coli* and collected for HeLa cell transfection. HeLa cell transfections were performed, and Western Blot analyses using Rockland Anti-mCherry antibody 600-401-P16 to determine that the correct proteins were being expressed **(Figure 3)**. HeLa cell transfections were then performed with the Lipofectamine 3000 reagent from Invitrogen. These transfections were performed by combining 1,250 ng of

construct DNA, 1,250 ng of CIB1-Tom20-GFP DNA, 5 μL of P3000 reagent, and 125 μL of Opti-MEM medium in tubes. Then, 3.75 μL of Lipofectamine 3000 reagent was added to 125 μL of Opti-MEM medium for each construct in another tube. The DNA solution and Lipofectamine 3000 solution for each construct were combined and incubated for 15 minutes. This solution was then added to HeLa cells and allowed to sit overnight in the dark in a 37 °C and 5% CO_2 humidified tissue culture incubator.

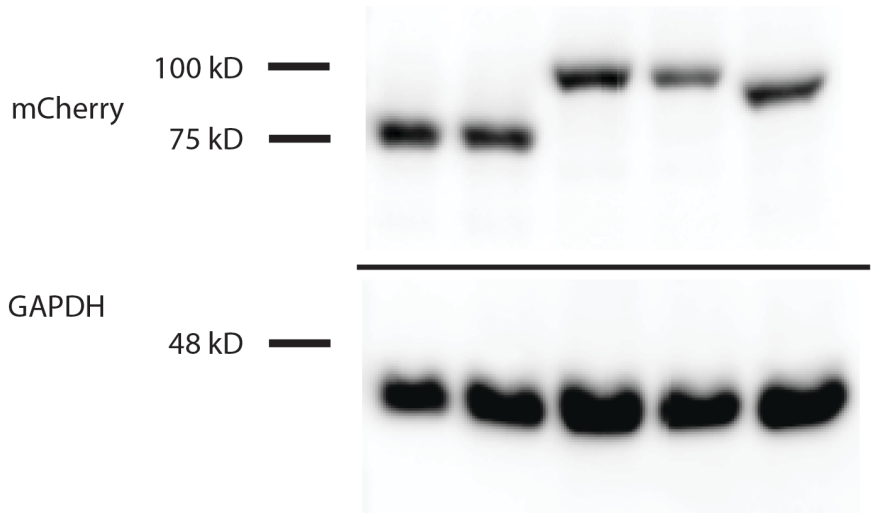

Figure 3. α-mCherry western blot of constructs. Lanes are (1) Cry2 531; (2) Cry2 531 L348F; (3) Cry2 531 Bax; (4) Cry2 531 L348F Bax; (5) Cry2 PHR Bax.

From this point we were prepared to begin HeLa cell experiments. Background cell death experiments were performed to determine the cell toxicity of each OptoBAX construct before light exposure. Transfections were performed on HeLa cells in 6-well plates and incubated wrapped in aluminum foil to prevent light exposure. Cells were fixed with 4% formaldehyde in PBS buffer two days after transfection. Background cell death was determined by Live-or-Dye Fixable Viability stain assay at 488/515 nm (Biotium) and cells counted on an Olympus IX2-DSU confocal microscope. Background cell death rates indicated that Cry2-PHR-BAX expressing cells had the

most background toxicity and Cry2-531-BAX and Cry2-531-L348F-BAX had similar toxicity levels **(Figure 4)**.

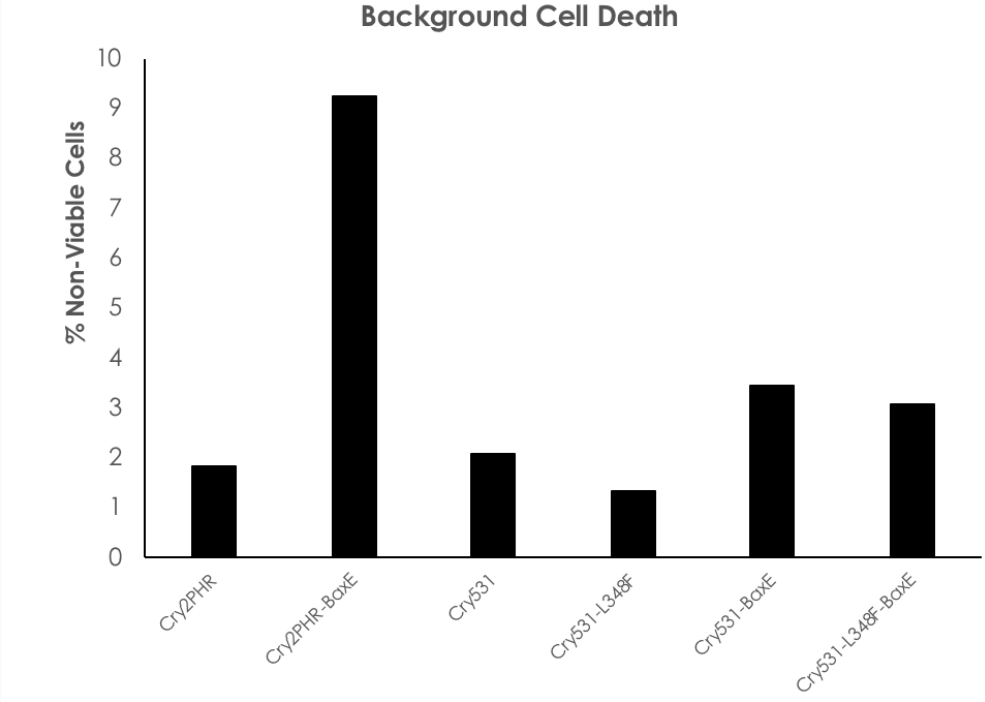

Figure 4. Percentage of non-viable cells two days after transfection. Cells were fixed and imaged using an Olympus IX2-DSU confocal microscope. At least 140 transfected cells were counted for each construct.

Light activation of the Cry2-531-mCherry construct was analyzed by fluorescent wide-field microscopy on transfected HeLa cells using a Nikon TI-E2 wide-field microscope equipped with a stage top cell incubator **(Figure 5)**.

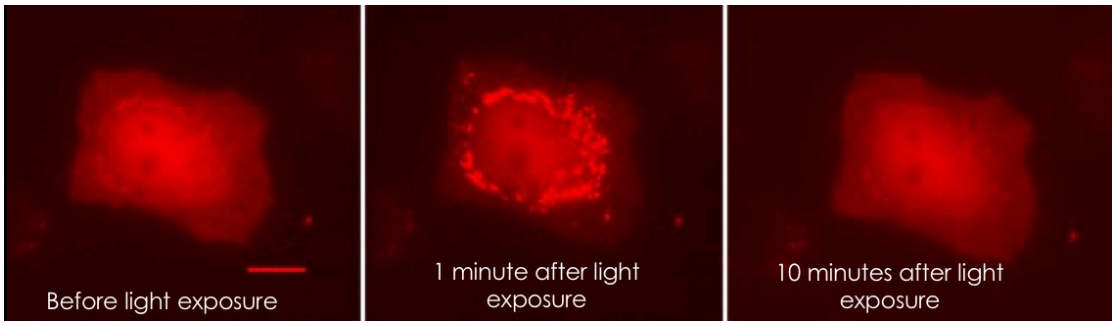

Figure 5. HeLa cell expressing Cry2-531-mCherry and Tom20-Cib before (left), 1 min post (middle), and 10 min post (right) light activation. Scale bar = 10 microns.

Light activation and photocycle lengths of the Cry2-531-mCherry-BAX and Cry2-531-L348F-mCherry-BAX were initially analyzed by fluorescent wide-field microscopy experiments on transfected HeLa cells using a Nikon TI-E2 wide-field microscope equipped with a stage top cell incubator **(Figure 6)**. Cells were initially exposed to a single 470 nm blue light pulse and then imaged for mCherry fluorescence every minute.

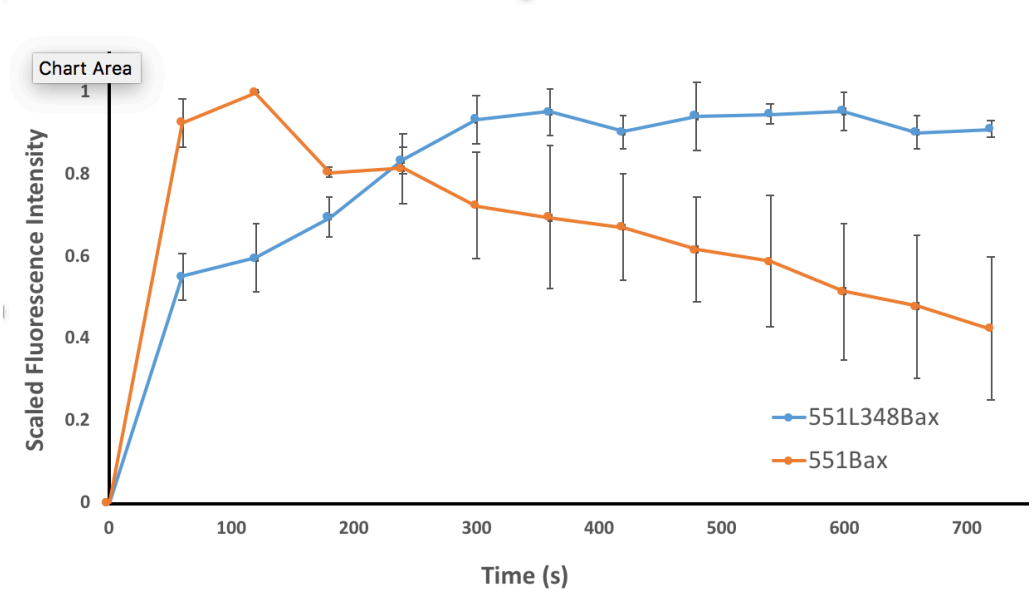

Figure 6. A graph of the scaled mCherry fluorescence intensity over time in the mutated and non-mutated OptoBAX constructs.

We repeated the experiment above using a Leica DMi8 Live Cell Imaging System. HeLa cells transfected with Cry2-531-mcherry and Tom20-CIB-GFP and cells transfected with Cry2-531-L348F-mcherry and Tom20-CIB-GFP were tested **(Figures 7 and 8)**.

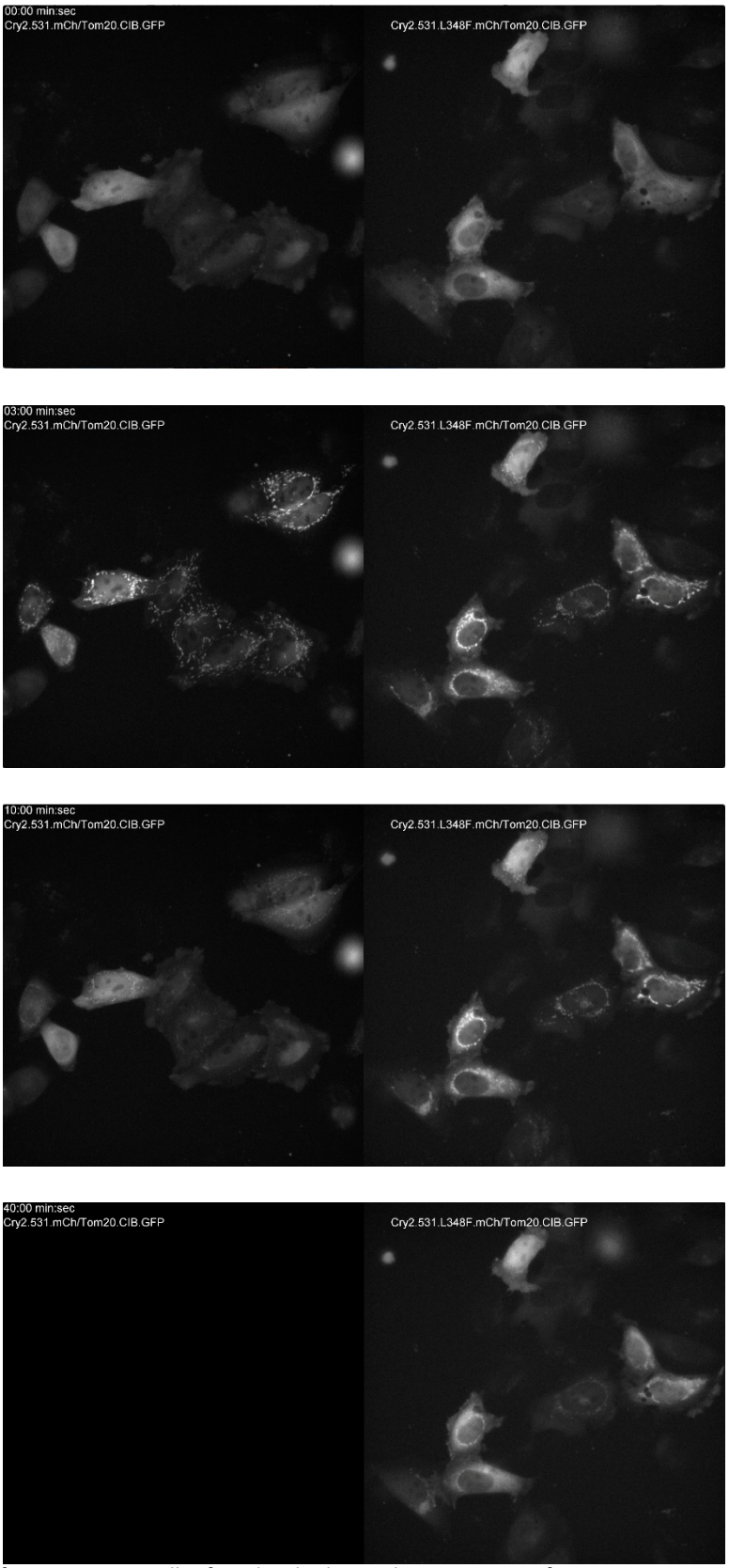

Figure 7. The four images are stills of a video displaying the progression of Cry2-CIB1 association over time after light exposure in HeLa cells expressing Cry2-531-mCherry and Cry2-531-L348F-mCherry constructs.

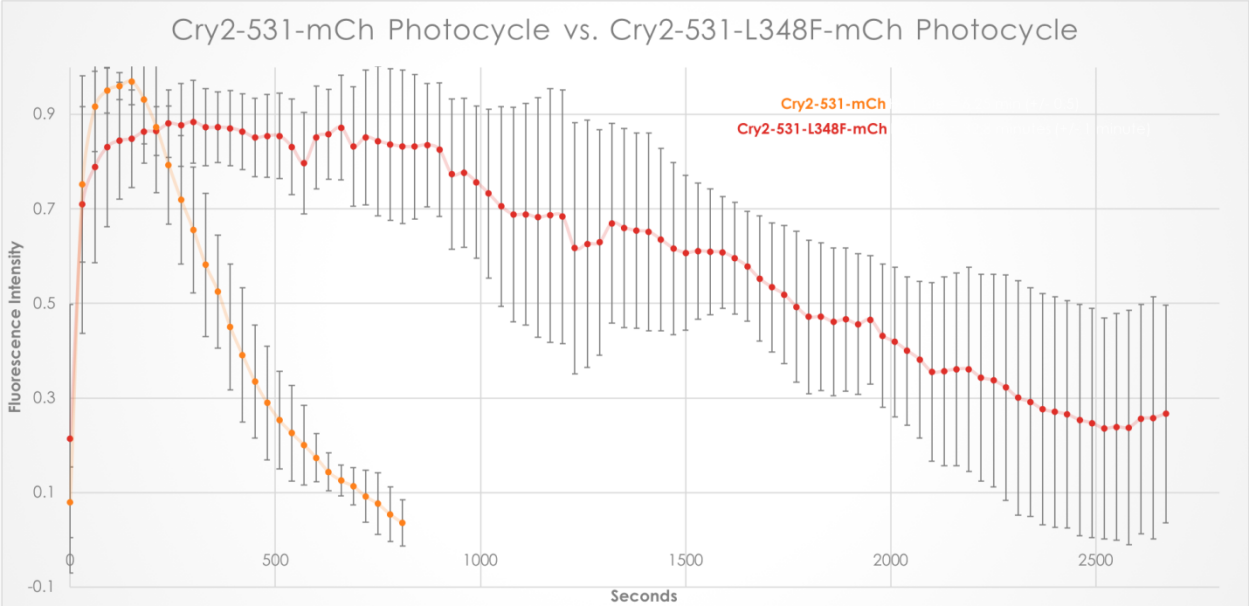

Figure 8. Graph of the fluorescence intensity of mCherry localized at the mitochondria over time for both the Cry2-531-mCherry and Cry2-531-L348F-mCherry constructs expressed in HeLa cells. The orange line represented data from the non-mutated Cry2-531-mCherry and the red line represents data from the mutated Cry2-531-L348F-mCherry.This graph represents the quantified data collected from the video represented in Figure 7.

To determine the level of optimization of the mutant on the OptoBAX construct, light versus dark cell death experiments were performed. One group of transfected HeLa cells were completely blocked from light immediately after transfection and not exposed again until fixation. A second group of cells were transfected and shielded from light over-night. Then, the cells were exposed to flashing blue light for either 5 or 15 minutes and then immediately covered again in aluminum foil to prevent further light exposure. Four hours later, all cells in both groups were fixed with 4% formaldehyde in PBS and assayed for viable cells using Live-or-Dye reagent **(Figure 9)**.

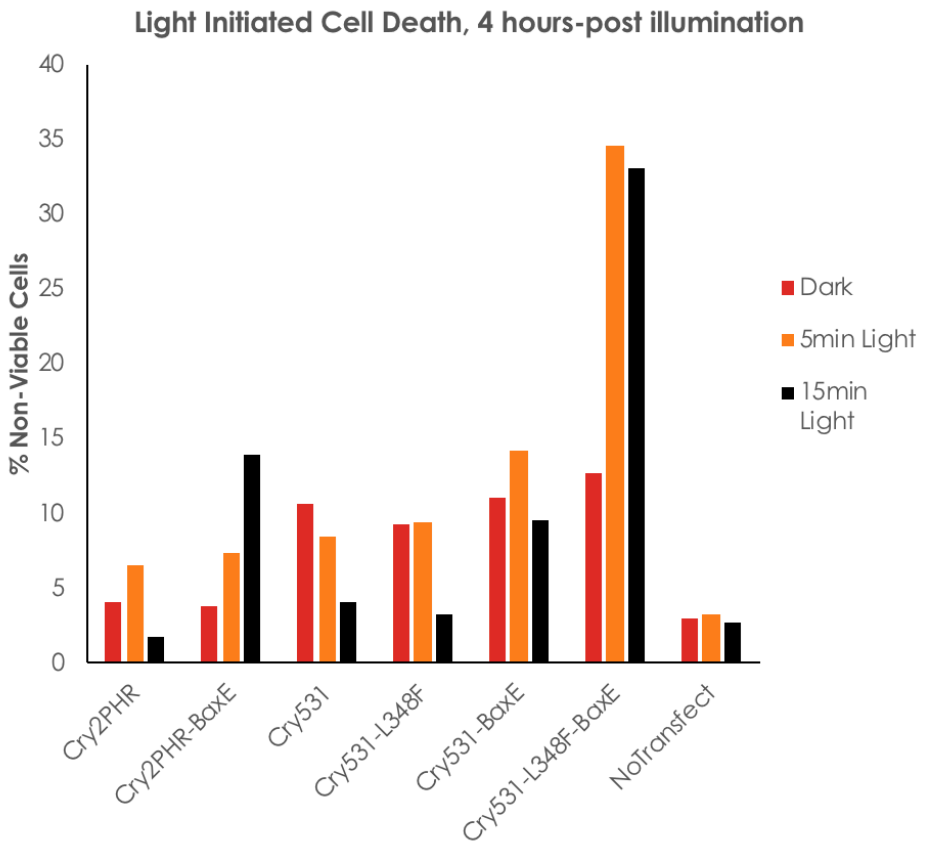

Figure 9. Bar graph of percentage of non-viable cells after blue light exposure or no light exposure.

The device used to expose cells to flashing blue light was designed and constructed in previous work by Hoffman and Hughes **(Figures 10-12)**. The device combined four wavelength ranges (440-460nm, 520-540nm, 650-670nm, 720-750nm) into a single package using Surface Mount Technology (SMT). This allowed for greater control over protein/small molecule expression. The device was constructed using Surface Mount Devices (SMD). 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 affixed 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.[23]

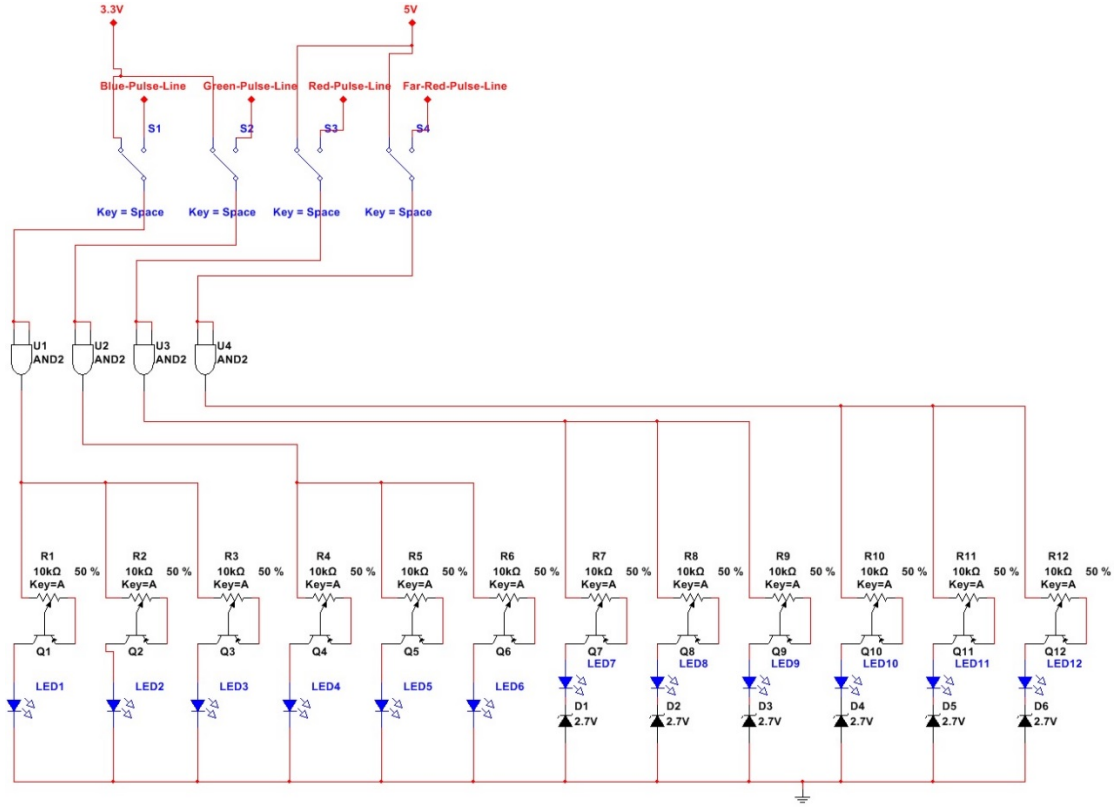

Figure 10. Schematic of circuit of LED device.

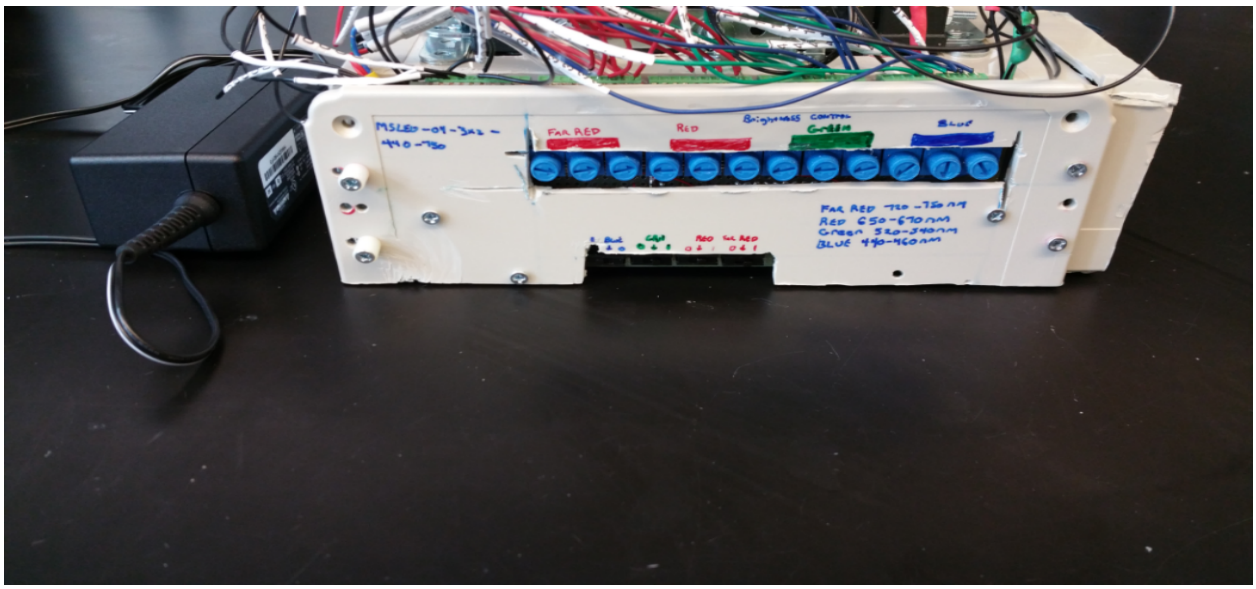

Figure 11. Case and controls of LED device.

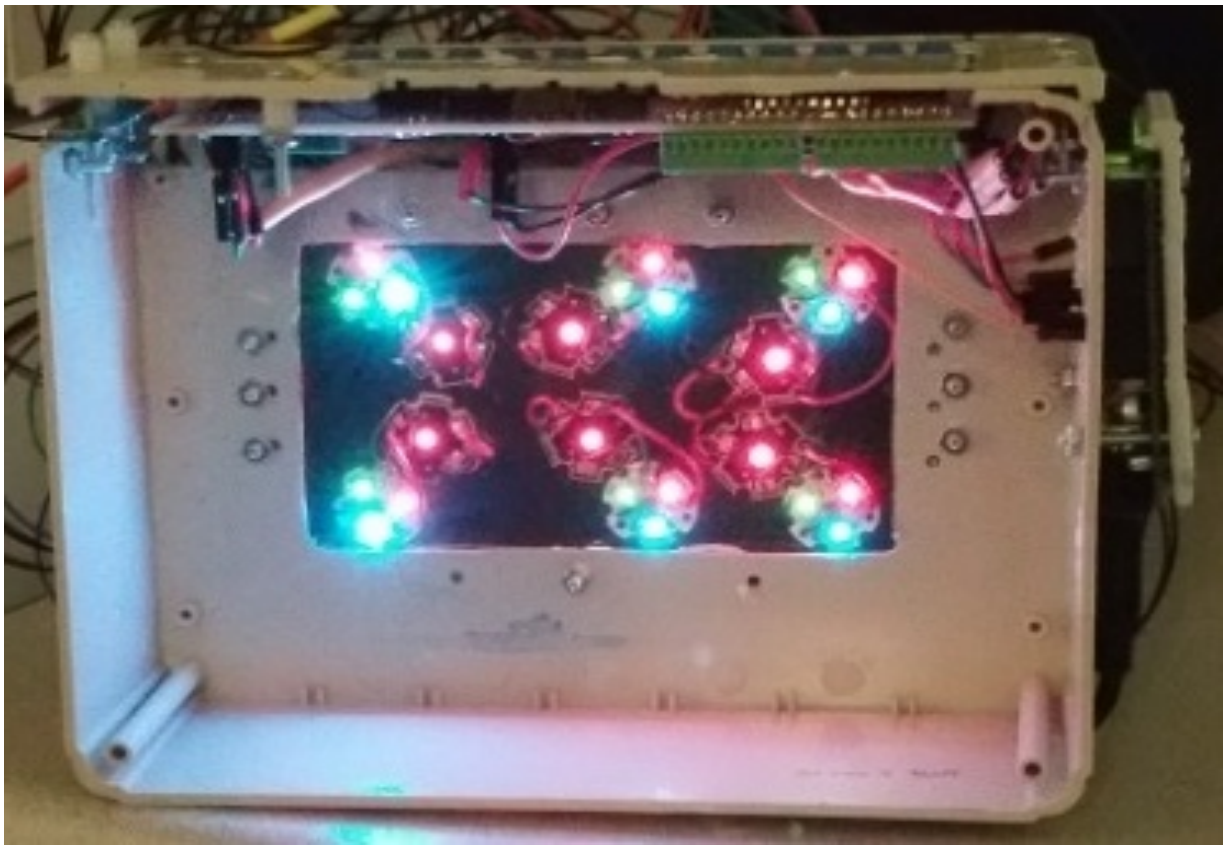

Figure 12. Interior and LED array of device.

In order to determine that apoptosis was actually occurring, caspase cleavage and nuclear degradation assays were performed. HeLa cells expressing the Cry2-531-L348F-mCherry construct were exposed to flashing blue light for five minutes and then fixed seven hours after with 4% formaldehyde in PBS **(Figure 13)**.

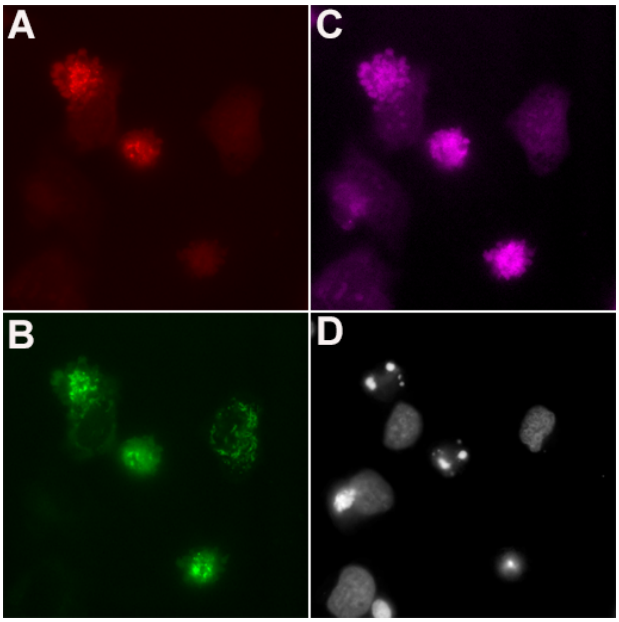

Figure 13. The four images were taken on a Leica DMi8 Live Cell Imaging System. A – mCherry fluorescence (Cry2-BAX); B – GFP fluorescence (Tom20-CIB); C – Far red secondary antibody/Cleaved caspase 3 primary antibody (immunostain); D – Hoechst 33342 nuclear stain.

Live-cell imaging was also captured of HeLa cells expressing Cry2-531-L348F-BAX and Cry2-531-L348F (without BAX) after initial light exposure. Cells were exposed to a 50 ms pulse of blue light (470 nm) and 500 ms pulse of green light to image mCherry fluorescence every 10 minutes **(Table 1) (Figures 14 and 15)**.

Construct	Apoptotic Cells	Total Cells	Percentage of Apoptotic Cells
Cry2.531.L348F.BAX	28	41	68%
Cry2.531.L348F	3	59	5%
Cry2.531.BAX	0	48	0%
Cry2.531	11	55	20%

Table 1. Data chart for live cell imaging indicating the increased cell death activity of the optimized OptoBAX construct versus the non-mutated. Other control construct data is also given.

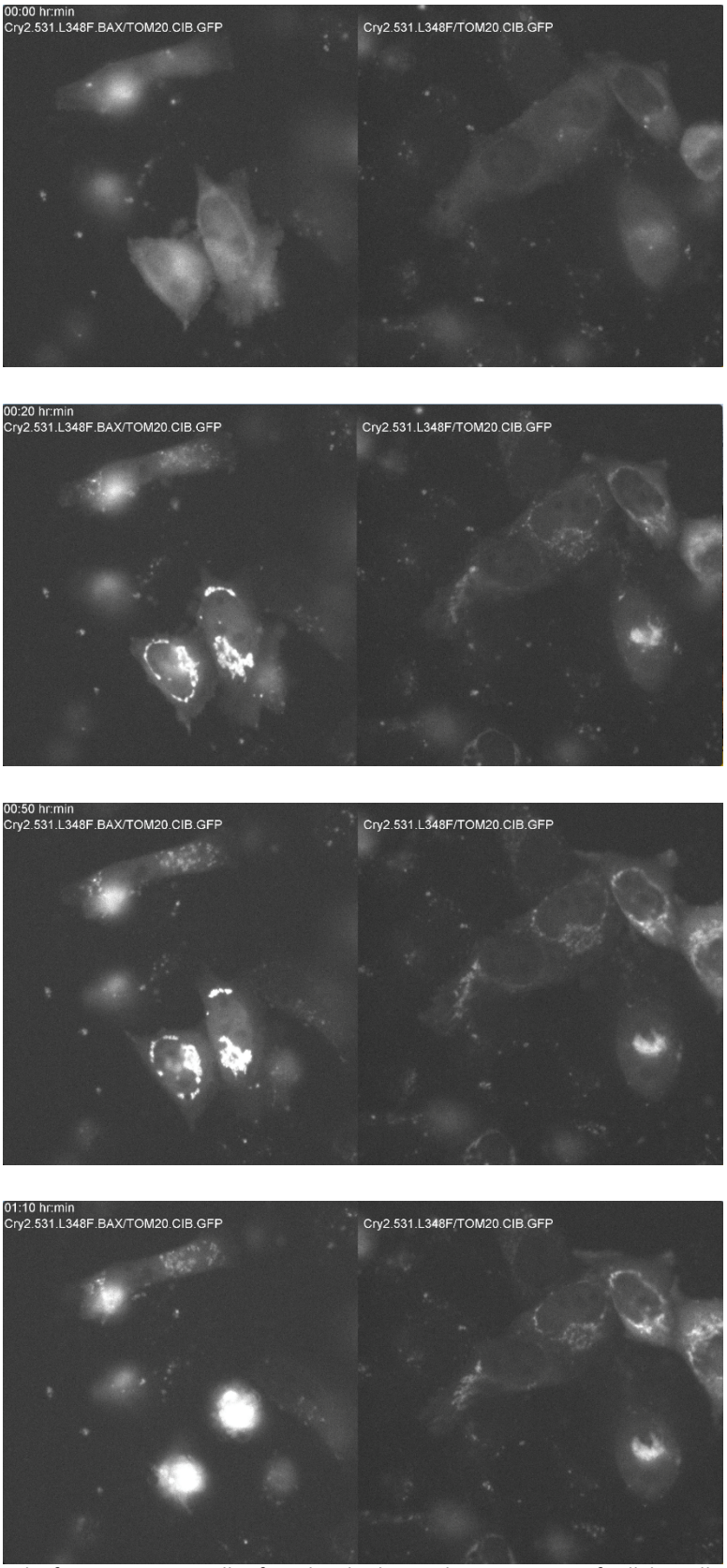

Figure 14. The four images are stills of a video displaying the progression of cellular collapse in the OptoBAX expressing cells as compared to cells not expressing BAX.

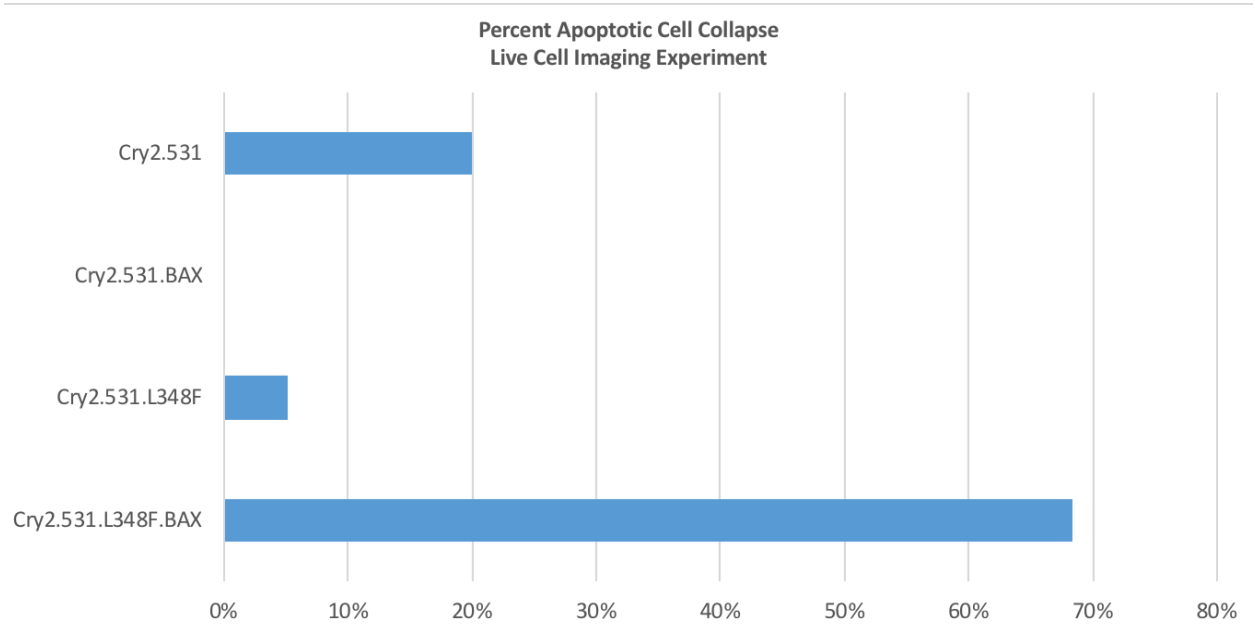

Figure 15. Bar graph of live cell imaging data indicating the increased percentage of non-viable cells of the optimized OptoBAX construct versus the non-mutated. Other control construct data is also given.

Conclusions:

The goals of this research were to identify background toxicity levels of OptoBAX construct variants, develop an optimized mutant of the original Cry2-531-BAX construct, and confirm that cells dying after short exposure to 470 nm blue light were in fact succumbing to apoptosis. It was found that the Cry531-Bax construct reduces background toxicity levels in HeLa cells compared to the Cry2PHR-Bax construct. Also, as anticipated, the L348F site mutation extends the photocycle length of cryptochrome significantly compared to the normal protein. This means that the Bax protein stays in close contact with the outer mitochondrial membrane for a longer period of time without additional light stimulation.

The Cry531-L348F-BaxE construct resulted in a higher percentage of non-viable cells after light exposure compared to after no light exposure. This result indicates that the long photocycle is successful in inducing a higher rate of apoptosis induction versus constructs with shorter

photocycles and thus shorter residence times at the mitochondria. Finally, the imaging of non-viable cells expressing Cry2-531-L348F-BAX with cleaved caspase-3 immunostain and nuclear stain revealed hallmarks of apoptosis occurring, thus confirming the system of cell death after light exposure.

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