

ABSTRACT

PHOTOFRIN-MEDIATED ELECTROPORATION-ASSISTED PHOTODYNAMIC THERAPY OF MCF-7 HUMAN BREAST CANCER AND B16-F10 MOUSE MELANOMA CELLS

by

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Photodynamic therapy is a relatively new player on the field of cancer treatment that involves the injection of a nontoxic light-sensitive drug followed by light excitation, creating reactive oxygen species that cause intracellular damage that ultimately brings about cell death. Photodynamic therapy (PDT) has the advantage over contemporary cancer treatment methods in that it is both non-invasive and can be tailored to largely avoid damage to noncancerous cells. The drawback herein is that intravenous injections leave most of the patients' tissue prone to photodamage for long periods of time following tumor treatment. In this study, we investigated the effect of augmenting current photodynamic treatment methods with electroporation, a phenomenon involving passing an electric current through cells, known to enhance uptake of extracellular components. We hypothesized, therefore, that electroporating cancerous cells in the presence of Photofrin would increase an intracellular uptake of the photosensitizer, and would correlate to a higher degree of cytotoxicity in the cell lines. We conducted

spectrophotometric intracellular concentration assays and observed a consistent and significant increase in intracellular Photofrin content in both MCF-7 and B16-F10 cell lines in those samples subjected to electroporation and an equal Photofrin concentration compared to the non-electroporated samples. In evaluating the cytotoxicity of photodynamic therapy via the clonogenic assay, we observed a significant decrease in cell viability in MCF-7 cells when subjected to photodynamic treatment following electroporation versus the non-electroporated cells at several concentrations of Photofrin. However, the results were not always consistent, which speaks to the nature of the cell-based assay. What we also noticed was that although electroporation generally increased the intracellular Photofrin content, higher doses without electroporation yielded equal intracellular Photofrin, but often no significant cell death, suggesting electroporation may play a larger role than merely allowing Photofrin inside the plasma membrane. B16-F10 cells, despite an increased Photofrin content after electroporation, were still unresponsive to PDT. Confocal imaging gave evidence of mitochondrial localization of Photofrin in MCF-7 cells, but an indiscernible localization in B16-F10, providing further insight into ePDT's ineffectiveness with the melanoma cell line.

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THERAPY OF MCF-7 HUMAN BREAST CANCER AND B16-F10 MOUSE
MELANOMA CELLS

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INTRODUCTION

Photodynamic therapy (PDT) is a recently developed therapeutic method accruing much interest regarding the treatment of many forms of cancer. The process involves the administration of a photosensitizing drug, followed by treatment with light of a particular wavelength. The preferred wavelength of light is in the red to infrared spectrum (>600 nm), due to its proficiency at penetrating human tissue (Moore et al., 1997). When the photosensitizing drug absorbs light of the appropriate wavelength, the photosensitizer is excited to a triplet state of electron configuration, followed by photochemical transfer to ground state molecular oxygen to form reactive oxygen species that can then elicit damage to cellular structures (Oleinick et al., 1998).

The advantages that photodynamic therapy confers to cancer patients make it a potential replacement for other forms of cancer treatment. One advantageous aspect of photodynamic therapy is the ability to isolate its cytotoxic effect. Traditional radiation and chemotherapy are somewhat indiscriminate to the types of cells within the body that they inadvertently damage during treatment. The injected photosensitizer in photodynamic therapy, however, is non-toxic to cells unless excited by light within its absorbance spectrum to create cytotoxic oxygen radicals. Limiting light exposure to photosensitized noncancerous tissue is more feasible than limiting ionizing radiation or antineoplastic drugs to noncancerous tissue. This limiting factor to its lethal effect also provides somewhat of a disadvantage, as noncancerous tissue may stay photosensitized for up to two months (Oleinick et al., 1998).

Various experiments have shown that uptake of photosensitizer has discrepancies between targeted tumor cells and normal tissue; for example, the ratio of Photofrin[®] uptake in mammary cancer cells to liver cells is 0.3:1 (Moore et al., 1997). Due to this discrepancy, administration of photosensitizer requires a great deal of caution, as photoexcitation, and therefore, photosensitization associated cell damage, will occur in all cells containing the photosensitizer that are exposed to light. In cells with a much higher uptake of photosensitizer than the targeted tumor cells, photosensitivity will be substantially higher.

To enable tumor cells to better uptake photosensitizer, electroporation of the tumor is being considered for eventual clinical implementation. Electroporation involves stimulation of cells involving short electric, high powered pulses, which causes the plasma membrane to become more permeable to external agents (Powell and Weaver, 1985). The use of electroporation in conjunction with PDT has been shown to produce a much higher cytotoxic response *in vitro* than with PDT alone (Figure I) (Labanauskiene et al., 2007). A higher uptake of photosensitizer by the tumor cells implies a lower dose of photosensitizer to induce desired cytotoxic effects by PDT, and consequently, normal cells would have less exposure to the photosensitizer.

The exact mechanism of how this damage is manifested may rely upon where the photosensitizer localizes within the cell. Localization highly depends on the chemical nature of the photosensitizer: hydrophobicity, type and number of charges, charge-to-mass ratio, type and number of ring and core constituent, and whether the uptake of the photosensitizer occurs by diffusion or is facilitated by endocytosis (Oleinick et al., 1998). Membrane localization tends to be the main effective means by which photosensitizers

induce their cytotoxic effects, as merely entering the cell may not allow reactive oxygen species, which have a short lifespan and consequently narrow effective range, to inflict lethal damage (Oleinick et al., 2002).

The cell's fate after PDT, whether it is apoptosis, necrosis, or some other fate, is largely influenced by cell line, the dose of photosensitizer, dose of irradiation, incubation period, the particular photosensitizer, and the localization of the photosensitizer within the cell (Moore et al., 1997; Dellinger, 1996). Apoptosis and necrosis are the predominantly observed fates of cells subjected to PDT, however cell cycle arrest and a process called autophagy have also been observed (Piette et al., 2003; Kessel and Reiners, 2007). Cell cycle arrest generally occurs after low doses of PDT, where damage to microtubules could initiate the phosphorylation of anti-apoptotic Bcl-2 by CDK-1 (Piette et al., 2003).

Currently, Photofrin[®] is the principle photosensitizer in clinical use for tumor treatment, and is the only photosensitizer approved for clinical use by the Food and Drug Administration. As a "first-generation" photosensitizer, it poses many problems which make it less than optimal, including its inefficient absorbance at wavelengths of light at >600nm (Figure II), its chemical complexity, and its effect of making patients photosensitive for 1-2 months after treatment (Moore et al., 1997; Oleinick et al., 1998; Piette et al., 2003). However, because it is the only FDA-approved photosensitizer, it has been widely studied and is most likely to achieve a more immediate diversified application.

In this study, we focused on two cancer types that could potentially benefit from the implementation of photodynamic therapy: breast cancer and melanoma. Breast cancer

affects one in eight women and often requires surgical lumpectomy, and in some cases a complete mastectomy (Solomayer et al., 2000). Photodynamic therapy provides an alternative to the noninvasive options of radiation and chemotherapy, with potentially fewer deleterious side effects. In this study, we used the MCF-7 human epithelial breast cancer cell line, a weakly invasive metastatic cell line taken from a biopsy of a 69 year old Caucasian woman, that like most breast cancer expresses estrogen receptors, as an *in vitro* analog (Lacroix and Leclercq, 2004). Melanoma results from uncontrolled proliferation of melanocytes, melanin-producing skin cells, that is the leading cause of death from skin disease. In all current cases of melanoma, surgical removal is required, making a localized noninvasive procedure such as photodynamic therapy a welcomed alternative (Markovick et al., 2007). Furthermore, the superficial location of melanoma provides a greater ease with which to apply greater energies of light involved in photodynamic therapy. B16-F10 mouse melanoma cells provided the *in vitro* analog in this study.

Previous research in our lab has demonstrated an effectiveness of photodynamic therapy on MCF-7 cells, with additional cell death when assisted with electroporation (Figure III). These results also indicate increased effectiveness as length of light exposure increases. B16-F10 melanoma cells, however, were not observed to undergo the same cytotoxic effects of photodynamic therapy as the MCF-7 cells (Figure V). Odd, however, was the observation that Photofrin alone in higher doses reduced cell viability (Figure IV). As photosensitizers used in photodynamic therapy are nontoxic, cell death should not have been observed with these treatments (Moore et al, 1997). A potential fault in these findings may have been the viability assay used. The MTT assay used in our lab's

previous studies is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to non-water-soluble formazan crystal by metabolically active cells. The shortcoming of this assay is that it provides a relatively short-term ascertainment of cell viability. Xue et al. made an observation that apoptosis was not initiated in Pc 4 photodynamic therapy of MCF-7 cells until 24 hours after treatment, enforcing the notion that an assay measuring long-term viability is needed to discern an effective cytotoxicity and be an accurate predictor of behavior of cancerous cells *in vivo* (Xue et al., 2001). Therefore, in the current study we ascertained the viability of MCF-7 cells and B16-F10 cells post-photodynamic treatment with the clonogenic assay in order to determine trends of cell viability we had seen with the short-term MTT assay, and potentially determine an effectively lethal Photofrin and light dose.

Furthermore, we wanted to assess an intracellular localization of Photofrin in both MCF-7 and B16-F10 cell lines. A possible explanation for the ineffectiveness of photodynamic treatment of B16-F10 cells in the previous study was an aversion of these cells to uptake Photofrin. Consequently, we needed to establish a correlation between the amount of Photofrin MCF-7 cells were able to uptake and their observed mortality. Numerous studies have determined uptake and intracellular localization of photosensitizers via confocal imaging and/or fluorometric or spectrophotometric analyses (Chwilkowska et al., 2003; Kramer-Marek et al., 2005; Marchal et al., 2007; Tamosiunas et al., 2005; Woodburn et al., 1998). In this study, we chose to assess the intracellular uptake of Photofrin via spectrophotometric absorbance. We chose to further explore specific subcellular localization of Photofrin within MCF-7 and B16-F10 cells through confocal imaging.

MATERIALS AND METHODS

Cell Culture

Both MCF-7 and B16-F10 cell lines were cultured at 37°C, 5% CO₂ in RPMI 1640 medium containing 2.1 mM L-glutamine and phenol red, supplemented with 10% fetal bovine serum, 10 µg/ml insulin, and 10 µg/ml gentamicin (RPMI complete medium). Cells were passaged by performing 5 minute incubation at 37°C, 5% CO₂ with 0.1% trypsin, then harvested by centrifugation and reseeded into the appropriate vessel.

Intracellular Photofrin Concentration

All work was done illuminated by a 60 W incandescent bulb filtered by a red fabric in order to reduce incident light potentially exciting Photofrin before light treatment.

Cells were removed from flasks via trypsinization, centrifuged, and resuspended in RPMI complete medium. Cells were counted using a hemocytometer with trypan blue exclusion, then diluted to 1.3×10^6 cells/ml for a final treatment concentration of 1.0×10^6 cells/ml. This cell suspension was placed into either 2.5 ml microcentrifuge tubes or 4mm gap electroporation cuvettes, followed by the addition of a 100µg/ml Photofrin solution in Phosphate Buffered Saline (PBS). The microcentrifuge tubes were allowed to incubate at 37°C, 5% CO₂ for 10 minutes, immediately followed by centrifugation. The cuvettes were electroporated at 100V with five 1ms pulses and immediately placed on ice for 10 minutes. Contents of the cuvettes were transferred to microcentrifuge tubes and centrifuged. All samples were washed with PBS, centrifuged, and resuspended in 400 µl

methanol. Samples were placed on ice and a sonicator was applied for 2 minutes to mechanically disrupt the cell membrane, then centrifuged. The supernatant was transferred to new microcentrifuge tubes and centrifuged again to remove remaining debris. Samples were placed in 100 μ l aliquots into a 96-well plate and the absorbance of each well was read at 405 nm.

Intracellular concentration was also measured after timed incubation with Photofrin. Cells were harvested as previously described, diluted to 4.0×10^5 cells/ml for a final treatment concentration of 2.0×10^5 cells/well. This cell suspension was placed in wells of a 6-well plate in a 2 ml final volume of RPMI complete medium with added solution of 1mg/ml Photofrin in PBS for a final concentration of 12.5 μ g/ml or 50 μ g/ml Photofrin. The plates were placed in a light-resistant box, which was placed in an incubator at 37°C, 5% CO₂, and allowed to incubate for 12, 24, or 48 hours. Following the incubation period, the wells were washed with PBS and trypsinized, followed by centrifugation. The cell pellet was washed again with PBS, centrifuged, and resuspended in 400 μ l methanol. This suspension was sonicated and read spectrophotometrically as mentioned previously.

Clonogenic Assay

All work was done illuminated by a 60W incandescent bulb filtered by a red fabric.

Cell survival after electroporation-assisted photodynamic treatment was determined by clonogenic assay. Cells were harvested and counted as previously described, and diluted to 8.3×10^5 cells/ml, giving a final treatment concentration of

6.5×10^5 cells/ml. Cell suspension was placed into a 2.5 ml microcentrifuge tube or 4mm gap electroporation cuvette and incubated or electroporated while exposed to 0, 6.25, 12.5, 25, 50, or 100 $\mu\text{g/ml}$ Photofrin, and centrifuged as described previously. Cells were resuspended in PBS and diluted to 5,000 cells/ml and plated in wells of a 6-well plate at 500 cells per well in 2ml of RPMI complete medium. Each well received light treatment using a 635 nm laser at 20mW, lasting 60, 120, 180, 240, 300, or 360 seconds, followed by an incubation period of between 5 and 10 days. Following the incubation period, the medium was removed and a solution of 6% glutaraldehyde, 0.5% crystal violet was applied to each well, fixing and staining resulting colonies. This solution was removed and the wells were rinsed and allowed to dry. Surviving colonies were then counted under a microscope.

Confocal Imaging

Cells were harvested from approximately 90% confluent flasks via trypsinization. Cells were diluted to 1.0×10^6 cells/ml and incubated with 25 $\mu\text{g/ml}$ Photofrin for 10 minutes at 37°C, 5% CO₂ in RPMI complete medium. Cells were centrifuged, supernatant was removed, cells were washed with PBS, centrifuged again, and resuspended in RPMI complete medium. Cells were allowed to incubate for 30 minutes at 37°C, 5% CO₂ with 200 nM MitoTracker Deep Red or 20 $\mu\text{g/ml}$ acridine orange. Cells were then centrifuged, washed with PBS, resuspended in RPMI complete medium, and transported to the microscope in a dark container. Cells were plated on a depression slide and imaged with a Carl Zeiss LSM 510 laser scanning microscope. Cells were illuminated with a 488 nm laser to view cells stained with acridine orange and Photofrin.

Images were captured using a long pass filter attenuating wavelengths shorter than 505 nm for one channel, and a band pass filter transmitting light between the wavelengths of 505 and 560 nm captured a second channel. For cells stained with Photofrin and Mitotracker, long pass filter attenuating light of wavelengths shorter than 585 nm was used. Mitotracker localization was determined via illumination with a 633 nm laser, and Photofrin localization was determined via illumination with a 543 nm laser.

RESULTS

Intracellular Photofrin Concentration

Cell membranes were sonically broken and the cytosolic contents read spectrophotometrically to confirm the uptake of Photofrin into the cell, and to verify an increased uptake with Photofrin exposure in conjunction with electroporation. MCF7 cells showed a significant ($P < 0.05$) uptake of Photofrin versus both the control and the electroporated control at all doses tested (Figure 1). When exposed to 12.5 $\mu\text{g/ml}$ Photofrin, there was not a significant increase in the Photofrin uptake when electroporated vs. the non-electroporated control; however, a significant increase in Photofrin uptake was observed when electroporated while exposed to 25, 50, and 100 $\mu\text{g/ml}$ Photofrin, compared to the non-electroporated treatments.

B16-F10 cell treatments showed similar results to the MCF-7 cell treatments, as all doses of Photofrin exhibited a significant uptake (Figure 2). There was a significant increase observed with the electroporated 12.5 $\mu\text{g/ml}$ treatment versus the non-electroporated that was not observed in MCF-7s. Additionally, an increased Photofrin uptake was observed with the electroporated samples with the 25, 50, and 100 $\mu\text{g/ml}$ exposures versus the non-electroporated samples, as was seen in the MCF-7 samples.

Figure 3 illustrates the measured Photofrin content of MCF-7 and B16-F10 cells after 12, 24, and 48 h incubations with Photofrin without electroporation. When incubated with 12.5 $\mu\text{g/ml}$ Photofrin, both MCF-7 and B16-F10 samples showed a relatively unchanged uptake between the three incubation periods, with the MCF-7s retaining slightly more than 0.2 pg per cell, and the B16-F10s retaining slightly less.

Incubation with 50 $\mu\text{g/ml}$ Photofrin showed greater variation between incubation periods, with an increase in Photofrin uptake and retention in both MCF-7 and B16-F10 samples after a 48 h incubation versus both the 12 and 24 h incubation uptake levels.

Photofrin Dose Response

Initially, MCF-7 cells were treated with varying doses of Photofrin with a consistent 180 s light treatment with a 20 mW, 635 nm laser, giving a total fluence of 37.4 J/m^2 . When exposed to Photofrin only, without electroporation or exposure to light (Figure 4), there was no significant decrease in the number of viable colonies counted in the clonogenic assay with any of the samples exposed to up to 25 $\mu\text{g/ml}$ Photofrin. When exposed to up to 25 $\mu\text{g/ml}$ Photofrin followed by light treatment (Figure 5), MCF-7 cells continued to show no significant decrease in cell viability. MCF-7 cells exposed to only Photofrin and electroporation (Figure 6) showed no significant decrease in cell viability even with Photofrin exposure concentrations as high as 100 $\mu\text{g/ml}$. The only significant decrease in cell viability at the administered concentrations of Photofrin could be seen when cells were exposed to Photofrin and electroporated, followed by laser treatment. As shown in Figure 7, a concentration of 12.5 $\mu\text{g/ml}$ was the lowest administered concentration of Photofrin yielding a significant decrease in cell viability, approximately 50% of the control viability. The higher doses, 25 and 100 $\mu\text{g/ml}$ Photofrin also showed significant decrease in cell viability (26% and 19%, respectively). The samples treated with 6.25 $\mu\text{g/ml}$ Photofrin showed no significant decrease in cell viability.

Light Dose Response

As the 12.5 µg/ml treatment concentration of Photofrin resulted in a significant decrease in cell viability, MCF-7 and B16-F10 cells were subjected to Photofrin exposure with electroporation, followed by light treatment (this combined treatment referred to hereafter as ePDT) with the same 20 mW, 635 nm laser for additional lengths of time ranging from 60 s to 360 s. When exposed to the light treatment alone, there was no significant decrease in cell viability between 60 s and 240 s with the MCF-7 samples, versus the control samples (Figure 8). In the following figures (Figures 9-12), the samples receiving only light exposure were used as controls. When treated with 60 s of light (Figure 9), both the samples exposed to only Photofrin and light (this treatment referred to hereafter as PDT) and ePDT samples showed significant decrease in cell viability, however, the ePDT sample did not show considerably less viability than the PDT sample (roughly 80%). With the samples treated with 120 s of light (Figure 10), the ePDT sample was the only sample with significant decrease in cell viability from the control, resulting in 71% survival. This sample also showed significantly less survival than the PDT sample's approximate 87% survival. Treatments with 180 s (Figure 11) also showed a significant decrease in viability between the control and ePDT samples (66% viability), however, there was no difference between the ePDT electroporation, Photofrin, and light treatment sample's and PDT the Photofrin and light treatment only sample's survival. When exposed to 240 s (Figure 12), there were no significant decreases in cell viability from the control.

Experiments were repeated for 60 s and 120 s timed light exposure, and an additional exposure time of 300 s was performed. When exposed to these timed

exposures, electroporation resulted in no decreased cell viability from the light exposure only (Figure 13), reinforcing previous data (figures 6, 7, 12) that indicated that electroporation alone was not lethal. When repeated with these timed exposures, the ePDT treatments showed significant decrease in cell viability from the PDT treatments with the 120 s and 300 s light exposure (74% and 75%, respectively), however, there was no decrease in cell viability with the 60 s light exposure as had been observed in the earlier experiments (Figure 9).

B16-F10 murine melanoma cells were subjected to identical experiments using 12.5 µg/ml Photofrin with varying light doses as the MCF-7 cells, with an additional timed light exposure of 360 s. Clonogenic assay data from the treatment with 60 s of light exposure (Figure 15) showed no decrease in cell viability with electroporation alone, nor with PDT or ePDT. The 120 s light exposure treatment also showed no decrease in cell viability for the electroporated samples, or the samples treated with PDT or ePDT (Figure 16). The PDT and ePDT samples treated with 180 s of light exposure also showed no decrease in cell viability, however, there was an isolated anomalous decrease in cell survival with the samples treated only with electroporation and light (Figure 17). Treatment with 240 s of light exposure yielded no difference in cell viability between the electroporated, PDT, and ePDT samples (Figure 18); however, these samples did show significant decrease in cell viability from the control. Timed light exposures were extended to include 300 and 360 s (Figures 19, 20), resulting in similar observations of no decrease in cell viability between samples treated with electroporation and light only, PDT, or ePDT. Experiments were repeated with light exposure times of 60, 240, and 300 s. When exposed to 60 s light (Figure 21), there was no decrease in cell viability with

electroporation, PDT, or ePDT as previously demonstrated (Figure 15). Results were similar with the repeated experiment with 240 s of light exposure (Figure 22), as there was no decrease in cell viability with electroporation, PDT, or ePDT versus the control. The repeated experiment with light exposure time of 300 s also mirrored the initial results of no decrease in cell viability with electroporation alone, PDT, or ePDT (Figure 23).

Subcellular Localization

MCF-7 cells were imaged while co-stained with Photofrin and either nucleic acid specific dye Acridine Orange or mitochondrial dye MitoTracker Deep Red. Images taken with AO stained cells (Figure 24) excited with 543 nm laser and filtered for wavelengths above 525 nm (green to red) showed nuclear localization with some cytosolic presence, along with lysosomal localization. When MCF-7 cells were imaged with Photofrin alone (Figure 25), most fluorescence was observed in the cytosol with brighter fluorescence around unidentifiable organellar membranes. Staining with both AO and Photofrin (figure 26) showed primarily cytoplasmic fluorescence when filtered to transmit wavelengths longer than 505 nm (Figure 26.a), and showed primarily nuclear fluorescence when filtered to transmit only wavelengths between 505 and 560 nm (figure 26.b). The band pass filter between 505 and 560 nm was used to isolate the emission peak of AO and the long pass filter ($\lambda > 505$) was used to capture the emission peak of Photofrin (610 nm). As AO displays emission peaks at both 525 and 650 nm, and has excitation and emission spectra which overlap with those of Photofrin, fluorescence due to AO was observed with both filters and Photofrin localization could not be specifically observed using this method. Using MitoTracker Deep Red, which has an excitation maximum at

644 nm, far above Photofrin's excitation maximum of 396 nm, images were captured using a long pass filter ($\lambda > 585$ transmitted) and different lasers were used for excitation of each fluorophore (633 nm for MitoTracker and 543 nm for Photofrin) (Figure 27). Figure 27.a shows the channel for MitoTracker, displaying bright fluorescence among the mitochondria. Using a lower excitation wavelength, Photofrin was excited and the fluorescence was again observed primarily in the cytosol, with some localization to organelles (Figure 27.b). Transposing the two channels reveals an overlap between Photofrin's organellar localization observed in figure 27.b and the MitoTracker's fluorescence observed in 27.a, suggesting mitochondrial localization of Photofrin in MCF-7 cells; however, not all cells observed displayed the same mitochondria-associated fluorescence with the Photofrin specific excitation, as seen in Figure 28.

B16-F10 cells were exposed to Photofrin and imaged using an excitation wavelength of 543 nm and filtered using a long pass 585 filter. Images showed primarily cytosolic localization of Photofrin within the cell, with some fluorescence observed within the nucleus, but no other organellar localization was observed, as was in MCF-7 cells.

DISCUSSION

Previous work in our lab has shown the effectiveness of augmentation of photodynamic therapy with electroporation with MCF-7 and B16-F10 cell lines. In this study, we sought to further explore the effectiveness of this treatment, as well as determine the concentration and intracellular localization of Photofrin. The initial uptake and intracellular amount of Photofrin was shown to increase under two conditions. First, with an increase in Photofrin concentration without electroporation and secondly, when electroporated while exposed to the same Photofrin concentration. Ultimately, we were able to confirm earlier results within the laboratory suggesting an increased efficacy of PDT when assisted with electroporation in the MCF-7 cell line. Prior experiments in the laboratory were unsuccessful in showing significant effectiveness of ePDT in the B16-F10 cell line. With our current parameters we were able to determine further that, PDT does not seem to significantly induce cytotoxic effects in this cell line. Confocal imaging enabled us to further explore the observed cytotoxicity of Photofrin, as it suggested a subcellular localization of the photosensitizer to MCF-7 mitochondria, however, there was no discernable subcellular localization of Photofrin to B16-F10 organelles.

Intracellular Photofrin

Intracellular Photofrin content of MCF-7 and B16-F10 cell lines was determined by spectrophotometric absorbance at 405 nm, near Photofrin's maximum absorbance of 396 nm, of the resultant supernatant of the cells' sonication in methanol after incubation with Photofrin with and without electroporation. This method allowed for the release of

cytoplasmic contents via physical membrane disruption as well as the solubility of the lipid plasma and organellar membranes in methanol. Confirming levels of Photofrin within the cells gave evidence towards Photofrin being the causality of any observed reduction in cell viability. Furthermore, determining increased intracellular levels of Photofrin after electroporation during exposure to the photosensitizer gave confirmation that electroporation indeed does increase the amount of Photofrin cells are able to uptake (Figures 1, 2), enabling a correlation to be established between amount of Photofrin entering the cells and their viability. It was also important to establish that despite the absence of cell death previously observed with B16-F10 cells that there was, in fact, Photofrin entering the cell. Determining kinetic uptake provided some insight into the relative uptake of Photofrin in MCF-7 and B16-F10 cell lines (Figure 3). In both cell lines exposed to 12.5 $\mu\text{g/ml}$ Photofrin, there was no discernable trend in uptake. However, when exposed to 50 $\mu\text{g/ml}$, both cell lines exhibited an increase between 12 h and 48 h incubations. This result conflicts with data obtained by Chwilkowska, et al., who observed a maximum uptake of Photofrin in MCF-7 cells after a 24 h incubation, followed by a decrease of intracellular Photofrin (Chwilkowska et al., 2003). The kinetic uptake results obtained in this study were conducted singly and cannot be statistically evaluated. They do, however, suggest an increase in cellular uptake of Photofrin after prolonged incubations. While this study suggests prolonged exposure to Photofrin increases its intracellular concentration, it also confirms electroporation's effect of increasing intracellular Photofrin nearly two-fold (in 25 and 50 $\mu\text{g/ml}$ doses), further suggesting a more expedient light treatment after clinical administration of Photofrin.

Effectiveness of ePDT

In the current study, in the MCF-7 cell line we saw a significant reduction in cell viability to approximately 50% using electroporation during exposure to a Photofrin dose of 12.5 µg/ml and using 180 s of a 20 mW red laser exposure (Figure 7), versus no reduction in cell viability using only Photofrin and light exposure (Figure 5). These results reinforce previous findings in our lab, which show a similar trend with cell viability levels reaching 20% when electroporated versus 80% with PDT without electroporation (Figure III). Further treatments in this study (Figures 9-12) resulted in 70% cell viability: a lower treatment effectiveness, but consistently more effective than Photofrin and light alone.

Discrepancies between our lab's previous results and the less effective photodynamic therapy treatment observed in this study could be attributed to the method in which cell viability was ascertained. The MTT assay was used to determine cell viability in our lab's previous study. Although the MTT assay is a method enabling convenient succession of multiple experiments, it has been found to provide only 77.8% true-negative results versus the clonogenic assay (Shimoyama et al., 1989) and no significant correlation with the clonogenic assay when observing chemosensitivity to some anti-cancer agents, such as vinca alkaloids, etoposide, irinotecan, and rhizoxin on human lung cancer cell lines (Kawada et al., 2002). Furthermore, Xue, et al. have demonstrated through chromatic shrinkage and fragmentation that apoptosis occurs in Pc-4 mediated photodynamic therapy in MCF-7 cells 24 hours after the treatment, increasing

the need for assays measuring cell viability at longer terms than MTT (Xue et al., 2001). Determining the effectiveness of electroporation assisted photodynamic therapy with the clonogenic assay in this study confirmed previously observed cell death determined by MTT, but not at the levels previously obtained, potentially due to the MTT assay's lower accuracy. Our results indicated that the clonogenic assay also showed that no cytotoxic effect was observed with Photofrin exposure alone at doses of even 100 µg/ml, versus a cell survival of approximately 50% shown with MTT (Figure IV). Effectiveness of the treatment was observed to increase with increasing concentration of Photofrin (Figure 7), however, using the lowest effective dose combined with increasing light doses did not yield increased cell death as had previously been seen, with a minimum cell viability of 66% at 180 s of light exposure versus a reduction to nearly 20% viability at 240 s in the previous study.

Although the effect of the electroporation assisted photodynamic therapy proved somewhat limited with MCF-7 cells, it proved practically absent with the B16-F10 line. Our lab's previous results showed no significant reduction in cell viability with B16-F10 cells using a Photofrin dose of 6 µg/ml up to 200 s of light exposure (Figure V). This lack of effectiveness was echoed in the current study even with a doubled Photofrin dose of 12.5 µg/ml and up to 360 s of light exposure (Figure 20).

Effectiveness of ePDT on MCF-7

In this study, there was a clear correlation between the PDT's effectiveness with electroporation and a resultant decrease in MCF-7 cell viability. There have been studies

on the mechanisms of MCF-7 cells' response to PDT with various photosensitizers, including Photofrin. In the MCF-7 cell line, Photofrin has been found to initially localize to the inner side of the plasma membrane, and after a 4-hour incubation, localize to the nuclear envelope and cellular compartments around the nucleus, presumably the mitochondria, with a maximum Photofrin uptake after a 24-hour incubation (Chwilkowski et al., 2003; Saczko et al., 2007). Other photosensitizers have shown preferential localization to other organelles within MCF-7 cells, such as Foscan's localization to the Golgi apparatus and the endoplasmic reticulum (Marchal et al., 2007) and NPe6's preferential localization to lysosomes (Usuda et al., 2008) in MCF-7 cells. Usuda et al. found that Photofrin mainly localizes to the mitochondria and damages Bcl-2, an anti-apoptotic protein, whereas NPe6 localizes to the lysosome and does not damage Bcl-2, resulting in less reduction in cell viability, underlining the principle that different photosensitizers induce cell death via different pathways to which MCF-7 cells are susceptible. What was observed in this study via confocal imaging was a specific localization of Photofrin to mitochondria (Figure 27) and diffusion throughout the cytoplasm and within the nucleus (Figure 28), suggesting that the cell death observed in this study was obtained by the pathways described in previous studies, namely through the damage of Bcl-2. Figures 27 and 28's dissimilarities in localization may indicate a kinetic localization or kinetic efflux associated with Photofrin's activity within MCF-7 cells.

Interestingly, intracellular levels of Photofrin observed in electroporated cells exposed to 12.5 $\mu\text{g/ml}$ Photofrin were identical to those levels observed in non-electroporated cells exposed to 25 $\mu\text{g/ml}$, and less than intracellular levels observed in

non-electroporated cells exposed to 50 and 100 $\mu\text{g/ml}$ Photofrin (Figure 1); however, these latter, non-electroporated samples with higher content of intracellular Photofrin showed no decrease in cell viability, whereas the electroporated sample exposed to 12.5 $\mu\text{g/ml}$ did show decreased cell viability. Although the content of Photofrin is identical in both samples, the PDT is more effective when combined with electroporation. As Usuda, et al. described, Photofrin may achieve its cytotoxic effect primarily from damaging the anti-apoptotic protein Bcl-2. The subcellular localization of Bcl-2 has traditionally been believed to be on the outer membrane of the mitochondria (Jun et al., 2007), however, there is other evidence that Bcl-2 predominantly localizes to the inner mitochondrial membrane (Gotow et al., 2000). A better access to the inner mitochondrial membrane, and therefore more Bcl-2, could potentially explain this study's observed higher effectiveness of PDT on electroporated cells, despite a similar intracellular content of Photofrin in the non-electroporated cells. Supra-electroporation is a method of passing a current through cells involving 10 to 300 kV/cm with pulses less than 1 μs , which is believed to open channels in organellar membranes rather than only opening channels in the plasma membrane achieved by conventional electroporation, which involves 0.1 to 1 kV/cm and pulses of more than 100 μs (Gowrishankar et al., 2006). However, one study provides a model which suggests that conventional electroporation can result in secondary transmembrane voltages that could open channels within organellar membranes (Esser et al., 2010). Using conventional electroporation with a field of 0.25 kV/cm with 1 ms pulses, as done in the electroporation in this study, could potentially cause these organellar channels to open, particularly effective in the mitochondria,

resulting in a larger amount of Photofrin reaching the inner mitochondrial membrane, and therefore damaging more Bcl-2 localized to this inner mitochondrial membrane.

Unresponsiveness of B16-F10

There are obviously different responses to Photofrin mediated electroporation assisted photodynamic therapy in MCF-7 and B16-F10 cells. Data from this study (Figures 1, 2) suggests that B16-F10 cells are able to uptake and retain 0.003 ng Photofrin per cell with a 12.5 µg/ml Photofrin exposure with electroporation, surpassing the retention of MCF-7 cells (0.002 ng/cell) with the same treatment, which was observed to elicit cytotoxic effects with photodynamic therapy. However, even with a slightly higher uptake than the lethal level observed in MCF-7 cells, B16-F10 cells remain viable after photodynamic therapy. One explanation for this continued viability is the melanin content of the B16-F10 cells. Melanin's primary function is to prevent much of the light of wavelengths that coincide with the excitation of Photofrin from penetrating the cell, resulting in a decreased effectiveness of PDT with cancers such as melanoma (Peeva et al., 1999). Not only does melanin serve to decrease the amount of permeating light, but it also has been found to effectively scavenge reactive oxygen species such as those critical for PDT's cytotoxic effects (Lim et al., 2004). However, PDT in B16-F10 cells has been effective with photosensitizers with a much stronger absorbance in the near infrared wavelengths or with a much broader absorbance spectrum than Photofrin (Woodburn et al., 1998; Lim et al., 2004).

Another limitation of this study's effectiveness with B16-F10 treatment is the wavelength of light treatment. Red light, used for this study, penetrates deeper into tissue

than other wavelengths of light. However, melanoma is generally located superficially, requiring less penetration than deeper tumors. Therefore, a light dose of a shorter wavelength could potentially be clinically applied. Light with shorter wavelength and higher energy has been shown to elicit greater cytotoxicity in B16-F10 cells with Photofrin, lowering the dose required to achieve 50% viability by half (Menezes et al., 2007).

Subcellular localization of Photofrin within the cell has proven to play a major role in eliciting ultimately cytotoxic cell damage, due to the short lifespan of reactive oxygen species formed after photosensitizer excitation, resulting in a very short migration of less than 0.2 μm (Oleinick et al., 2002). Confocal imaging of B16-F10 cells with Photofrin provided little insight into the specific localization and potential pathway for cell death through effective PDT. Most fluorescence from Photofrin in B16-F10 cells comes from seemingly non-specific dispersion throughout the cytoplasm, as well as some nuclear localization (Figure 29). Photofrin PDT has been shown to cause some damage to nuclear DNA, but this damage does not result in cell death, as the oxidative damage caused is usually repaired by base excision mechanisms (Woods et al., 2004). A nonspecific localization of Photofrin in B16-F10 cells does not make B16-F10 a promising candidate cell line for Photofrin PDT.

The affinity for hematoporphyrins, such as Photofrin, for lipoproteins has been observed and exploited for use in delivery of cytotoxic agents to cancerous cells, including Photofrin to MCF-7 cells *in vitro*, and elliptinium olleate in B16 melanoma in rabbit models (Somadi-Baboli et al., 1993; Chwilkowska et al., 2003). It has also been noted that in mice, high-density lipoproteins (HDL) are the chief transporters, whereas in

humans, the low-density lipoproteins (LDL) prevail (Woodburn et al., 1998). It has been shown that when Photofrin is coupled with LDL, resulting retention of Photofrin in human fibroblasts is much higher than that of those exposed to Photofrin coupled with HDL or albumin (Candide et al., 1986). B16 melanoma has also been shown to have LDL receptors with great affinity for LDL internalization and LDL analog, apolipoproteinE-containing liposomes coupled with anticancer agent daunorubicin both in culture and *in vivo* (Versluis et al.; 1996, 1998). However, the human cells' affinity for LDL-mediated transport may provide an advantage to effective Photofrin delivery and account for a slightly higher Photofrin retention at doses of 50 and 100 µg/ml observed in this study (Figure 1, 2).

CONCLUSION

The data presented in this study confirms an enhanced efficacy of Photofrin-mediated photodynamic treatment of MCF-7 cells when assisted with electroporation. This enhanced efficacy correlates to an increased amount of observed intracellular Photofrin when electroporated during exposure compared to non-electroporated cells. Although there is a similar increase in intracellular Photofrin with electroporated B16-F10 cells versus non-electroporated cells, there is no correlating decrease in cell viability. This was to be expected, as the B16-F10 cell line has proven nearly impervious to photodynamic therapy with most photosensitizers, including Photofrin, in numerous studies, including our lab's previous findings with the MTT assay. The B16-F10 line provides several impediments for effective PDT, including its melanin content and indeterminate subcellular localization. We have seen in this study, and it has been confirmed in others, the specific localization of Photofrin to MCF-7 mitochondria, which has implications towards the specific pathway of the initiation of apoptosis conferred by Photofrin mediated PDT. B16-F10 cells, however, showed mostly diffusion throughout the cytoplasm in this study, implying indiscriminate, and ultimately non-lethal, reactivity of oxygen radicals generated during Photofrin excitation.

Cytotoxicity in MCF-7 cells was also demonstrated in this study to correlate with an increase in concentration of Photofrin exposure when electroporated. Although higher Photofrin doses prove to yield a greater lethality, caution should be taken in ultimately establishing a treatment dose clinically, as non-tumor cells are also prone to the lethal effects of photosensitizers. This study further supports that electroporation potentially

provides an effective means to augment clinical Photofrin-mediated photodynamic therapy in breast cancer, by enhancing photosensitizer uptake and potentially influencing subcellular localization to coerce apoptotic initiation. In a study conducted by Tamosiunas, et al., *in vivo* application of electroporation assisted photodynamic therapy was conducted on hepatoma tumors in mice with the photosensitizers aluminum phthalocyanine and chlorine e₆, describing similar results of increased uptake of photosensitizer within cells of the electroporated tumors; in this study, however, they observed no increased photosensitivity of the tumors (Tamosiunas, et al., 2005). However, a greater selectivity of the photosensitizers to the tumor was observed, suggesting, as does our current study, that Photofrin would likely preferentially target tumor cells that have been electroporated, reducing the amount of Photofrin exposed to non-tumor cells, and therefore reducing overall photosensitivity in patients receiving PDT. Photodynamic treatments of melanotic melanomas, such as B16-F10 cells, on the other hand, will require more modifications to induce an effective cytotoxic response.

FUTURE RESEARCH

Work is currently being conducted by our lab to illustrate a dose response of B16-F10 cells to Photofrin, as the conducted 12.5 $\mu\text{g/ml}$ treatments elicited no significant decrease in cell viability, even when electroporation resulted in increased uptake. Also, further fluorescent imaging is underway to determine whether subcellular localization of Photofrin following electroporation or with higher doses is altered in the MCF-7 and B16-F10 cell lines. While Photofrin may be observed to localize to the mitochondria in both electroporated and non-electroporated cells, comparison of Bcl-2 damage by western blotting should be conducted to determine the method of apoptosis in electroporation-assisted PDT treatment. As lipoprotein coupling and internalization may also play a role in uptake of photosensitizer, and may account for the differences in cytotoxicity between MCF-7 and B16-F10 cells, an investigation of Photofrin's affinity for both HDL and LDL should be performed, as well as relative measurements of the affinity of MCF-7 and B16-F10 cells for HDL and LDL. As PDT is already being used clinically to treat tumors, future research should also focus on further *in vivo* and clinical application of locally injected photosensitizer combined with local electroporation.

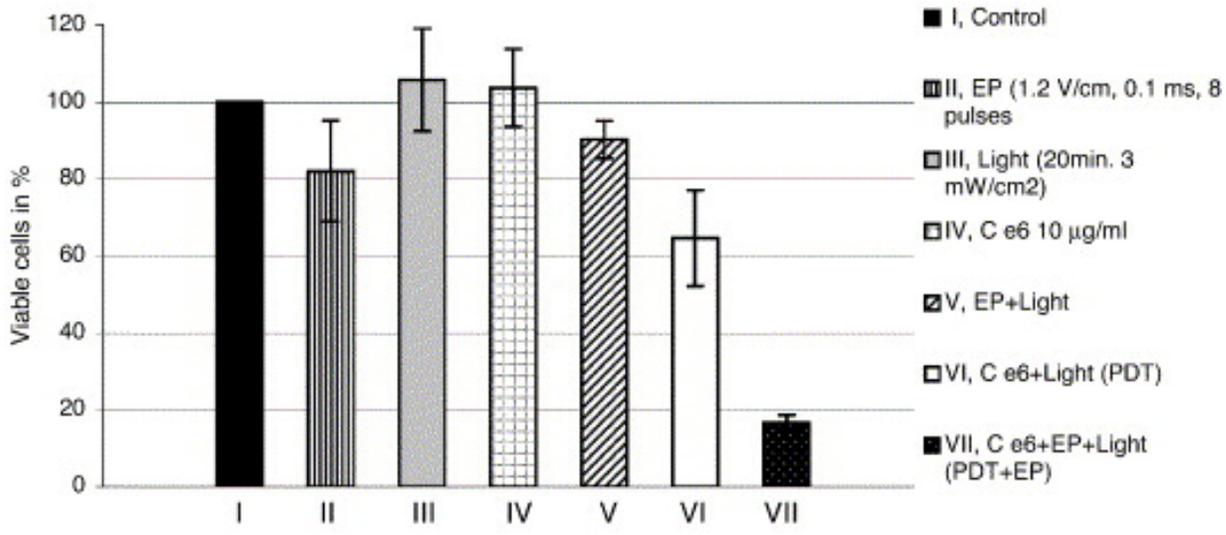


Figure I: Effect of electroporation of DC-3 Chinese hamster lung fibroblast cells with chlorine e₆-mediated PDT on cell viability (Labanauskiene et al., 2007).

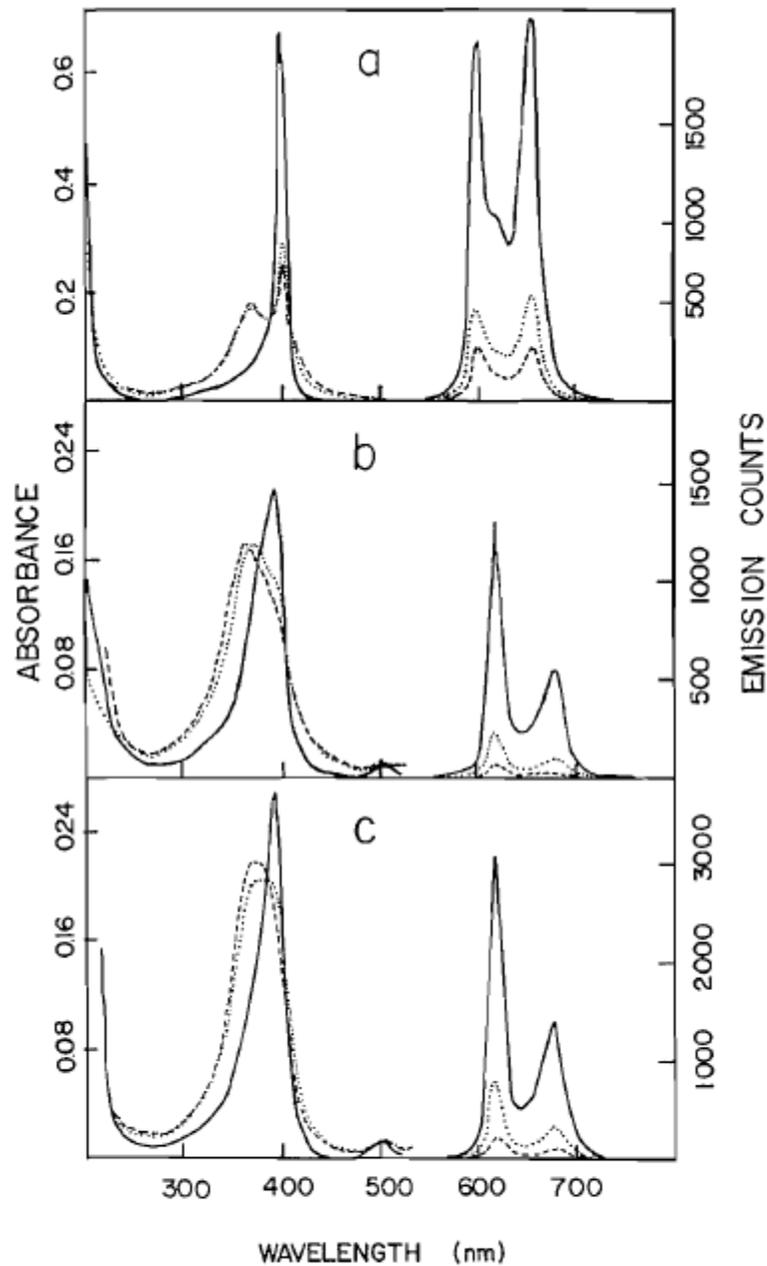


FIG. 1. Absorbance and fluorescence of Hematoporphyrin IX (—), Photofrin (·····), and Photofrin II (---). (a) pH = 1.1, (b) pH = 7.0, (c) pH = 11.6. Concentration is $1 \mu\text{M}$ and path length is 1.00 cm. Temperature is 22°C .

Figure II: Absorbance (max 396 nm) and emission (max 625 nm) spectra of Photofrin (---). (Pottier et al., 1985)

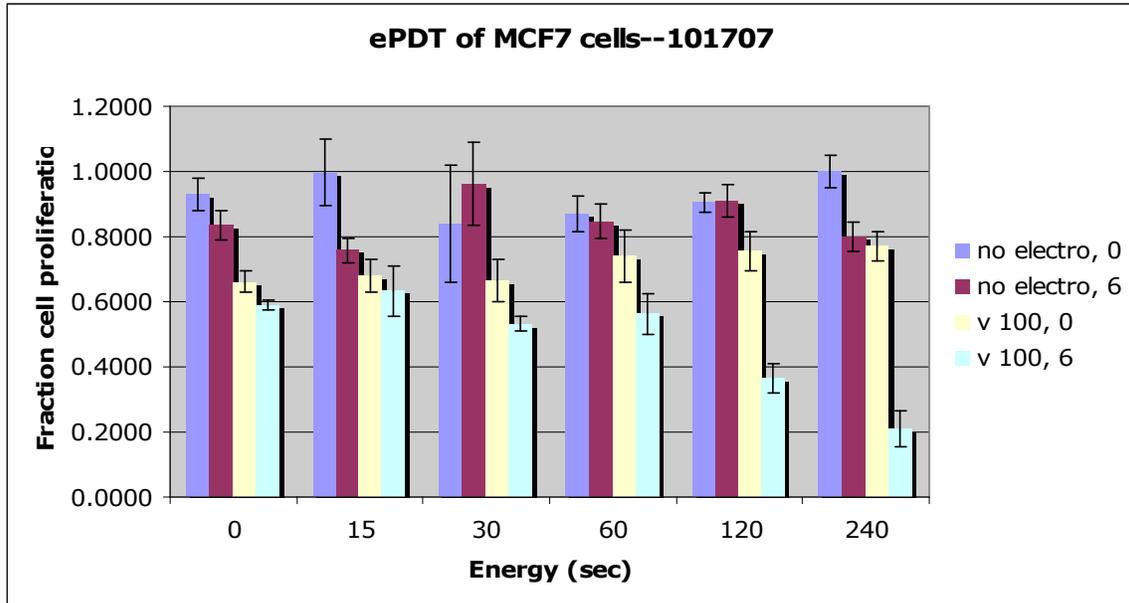


Figure III: MTT survival assay of MCF-7 cells treated with 6 ug/ml Photofrin with a 10 minute incubation, with or without electroporation of five 1 ms pulses at 100 V, treated with a 635 nm laser at 20 mW for the indicated time period. (Jenna Bone)

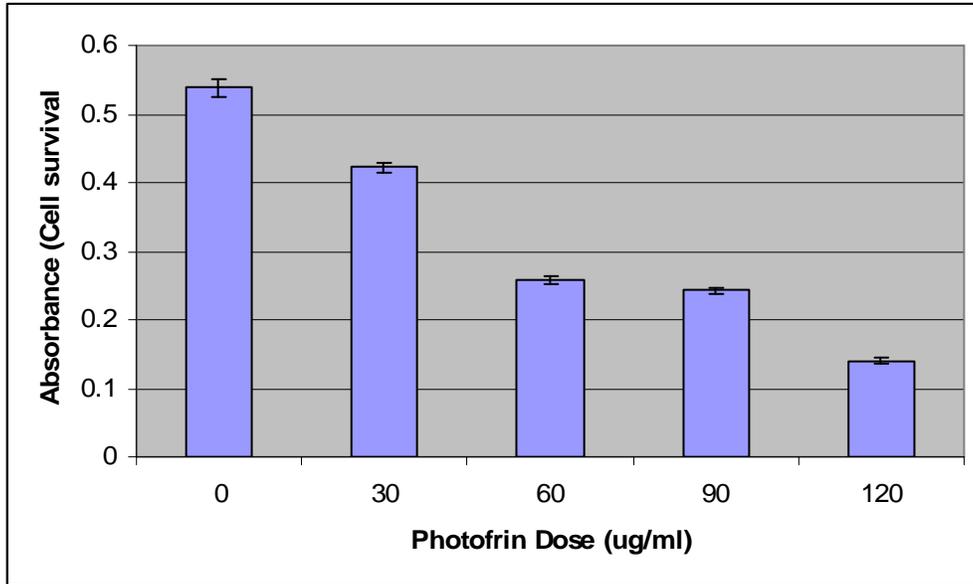


Figure IV: MTT survival assay of MCF-7 cells treated with indicated concentration of Photofrin with a 10 minute incubation without electroporation or light treatment. (Jenna Bone)

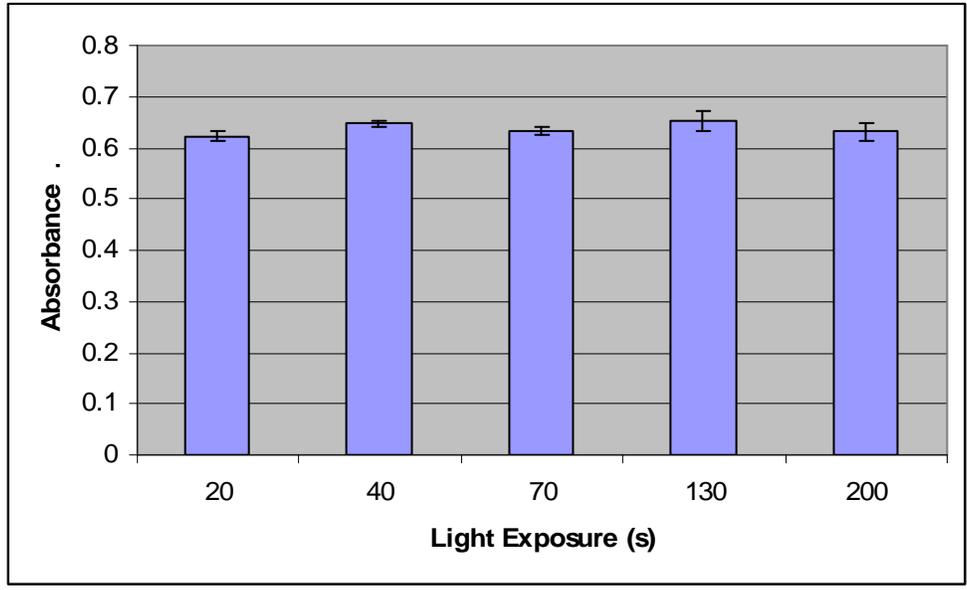


Figure V: MTT survival assay of B16-F10 cells exposed to 6 ug/ml of Photofrin with a 10 minute incubation, followed by electroporation with five 1 ms pulses of 100 V and exposed to a 635 nm laser at 20 mW for the indicated time. (Jenna Bone)

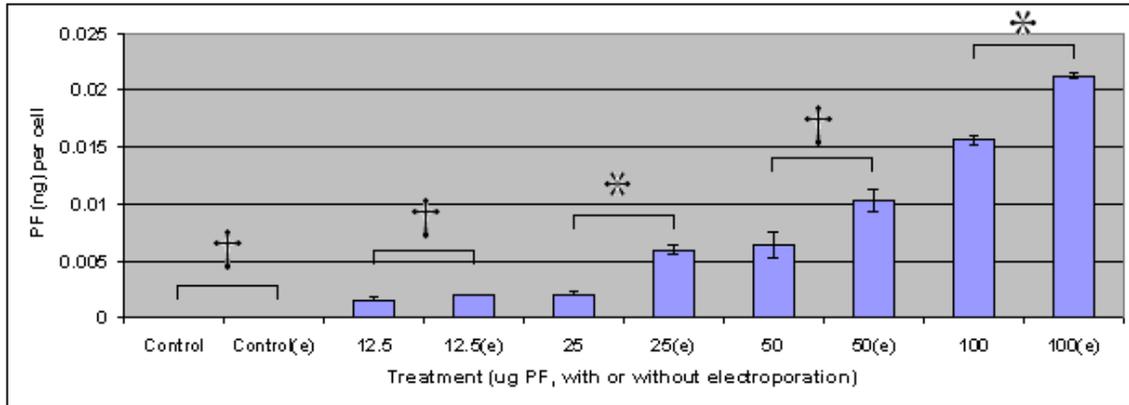


Figure 1: Photofrin content of MCF-7 cell lysates in methanol, following exposure to Photofrin with or without electroporation. Error bars represent standard error of the mean content of triplicate readings. * : difference between indicated samples statistically significant ($P \leq 0.05$). † : difference between indicated samples not statistically significant ($P > 0.05$).

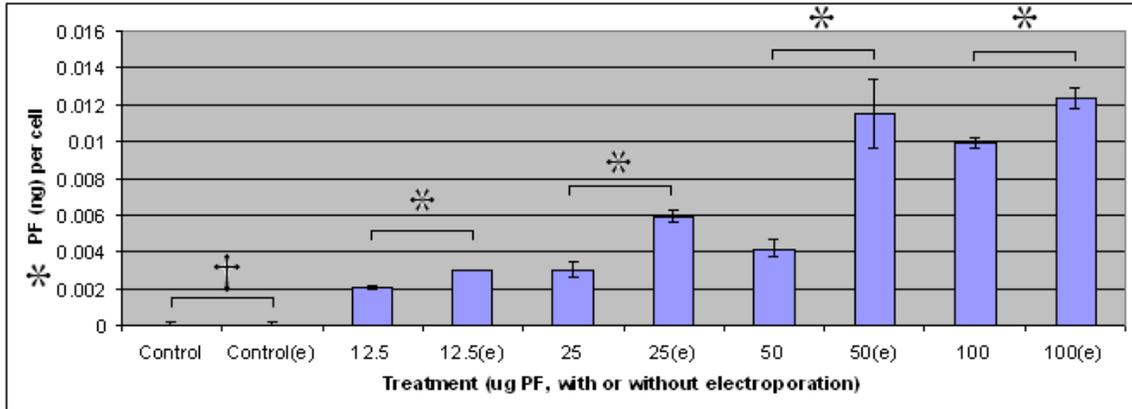


Figure 2: Photofrin content of B16-F10 cell lysates in methanol, following exposure to Photofrin with or without electroporation. Error bars represent standard error of the mean content of triplicate readings. * : difference between indicated samples statistically significant ($P \leq 0.05$). † : difference between indicated samples not statistically significant ($P > 0.05$).

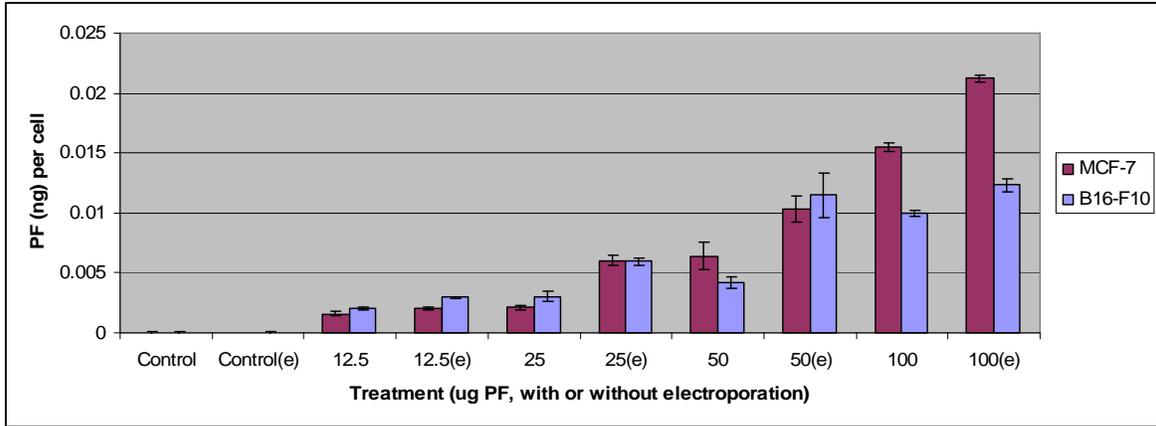


Figure 2b: Comparison of Photofrin content of B16-F10 and MCF-7 cell lysates in methanol, following exposure to Photofrin with or without electroporation. Error bars represent standard error of the mean content of triplicate readings.

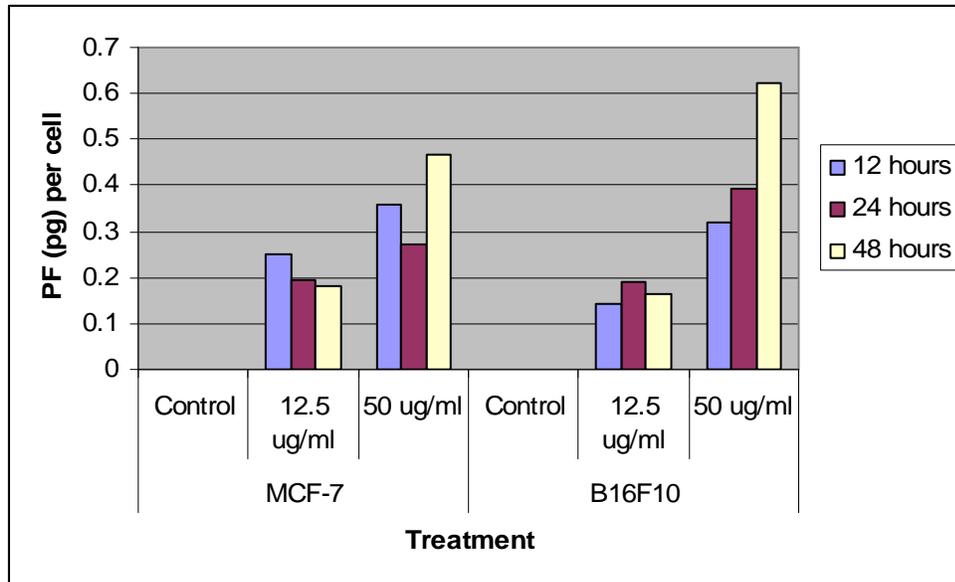


Figure 3: Photofrin content of cells following a 12, 24, or 48 incubation period exposed to 12.5 or 50 $\mu\text{g/ml}$ Photofrin.

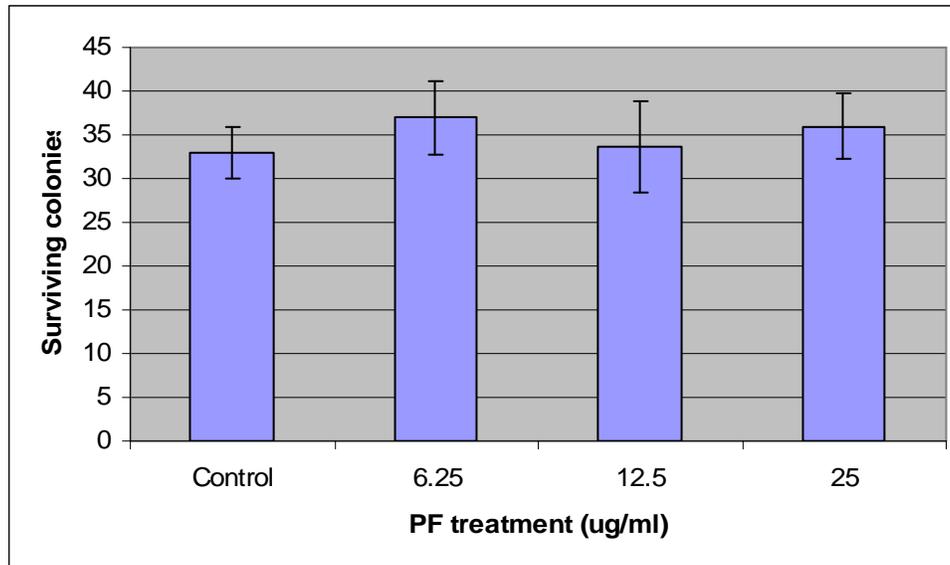


Figure 4: Surviving MCF-7 colonies after treatment with 6.25, 12.5, and 25 µg/ml Photofrin, without electroporation or light treatment. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

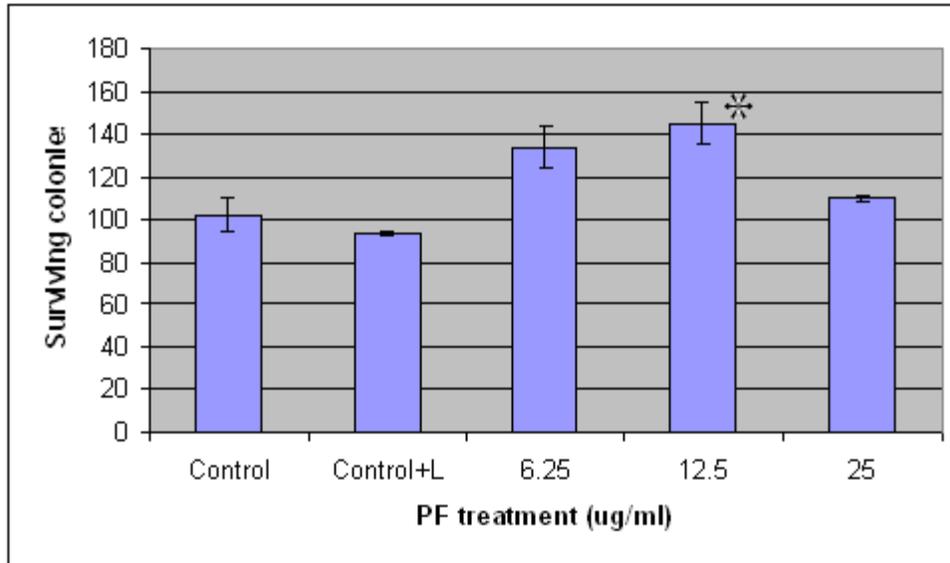


Figure 5: Surviving MCF-7 colonies after treatment with 6.25, 12.5, and 25 $\mu\text{g/ml}$ Photofrin, without electroporation, exposed to 180 s of 635 nm light at 20 mW. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

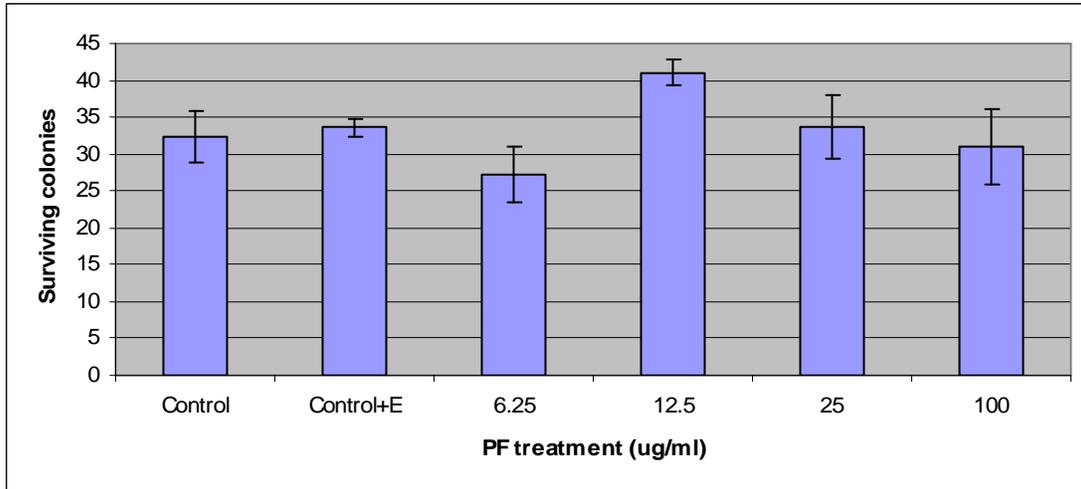


Figure 6: Surviving MCF-7 colonies after treatment with 6.25, 12.5, and 25 $\mu\text{g/ml}$ Photofrin with electroporation, without light treatment. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

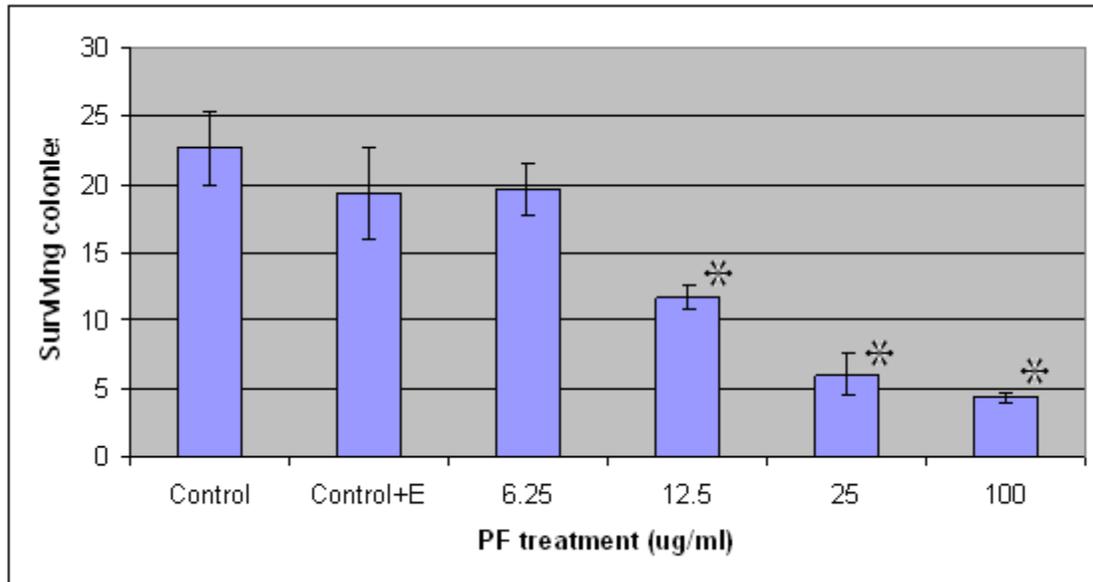


Figure 7: Surviving MCF-7 colonies after treatment with 6.25, 12.5, and 25 $\mu\text{g/ml}$ Photofrin with electroporation and exposure to 180 s of 635 nm light at 20 mW. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

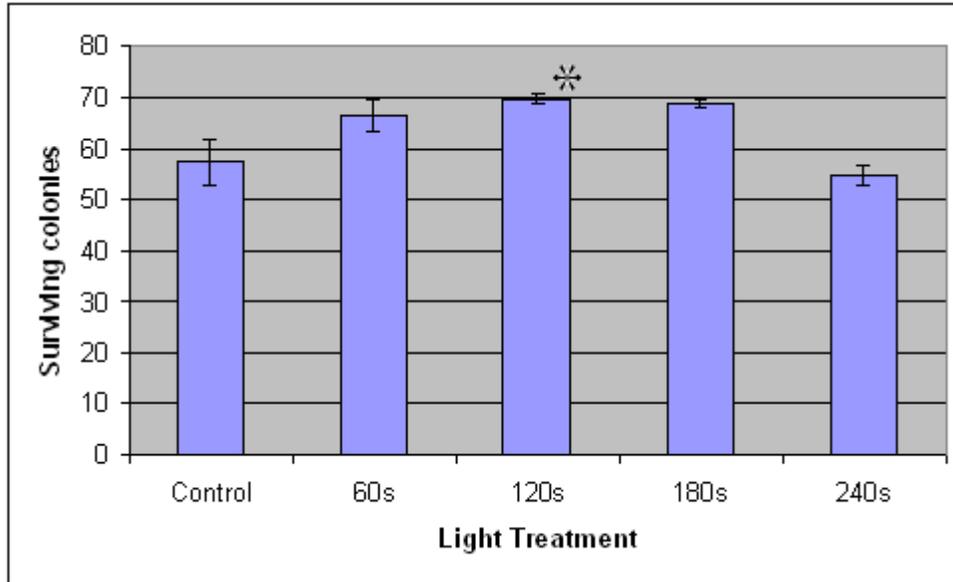


Figure 8: Surviving MCF-7 colonies after exposure to 60, 120, 180, and 240 s exposure to 20 mW, 635 nm laser over 9.6 cm² wells. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant (P≤0.05).

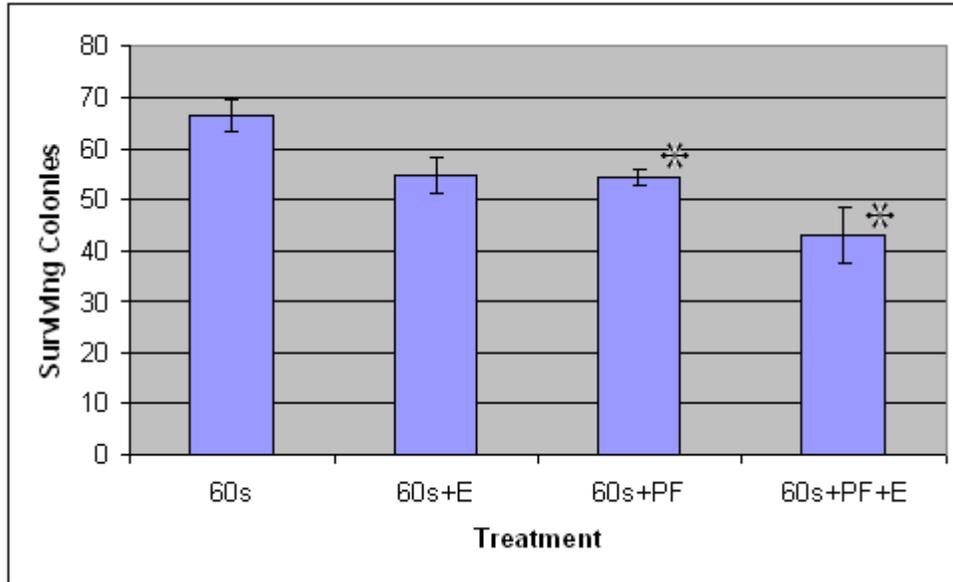


Figure 9: Surviving MCF-7 colonies after 60 s exposure to 20 mW, 635 nm laser over 9.6 cm² wells with light alone, or light in conjunction with electroporation only, 12.5 µg/ml Photofrin only, or both Photofrin and electroporation. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant (P≤0.05).

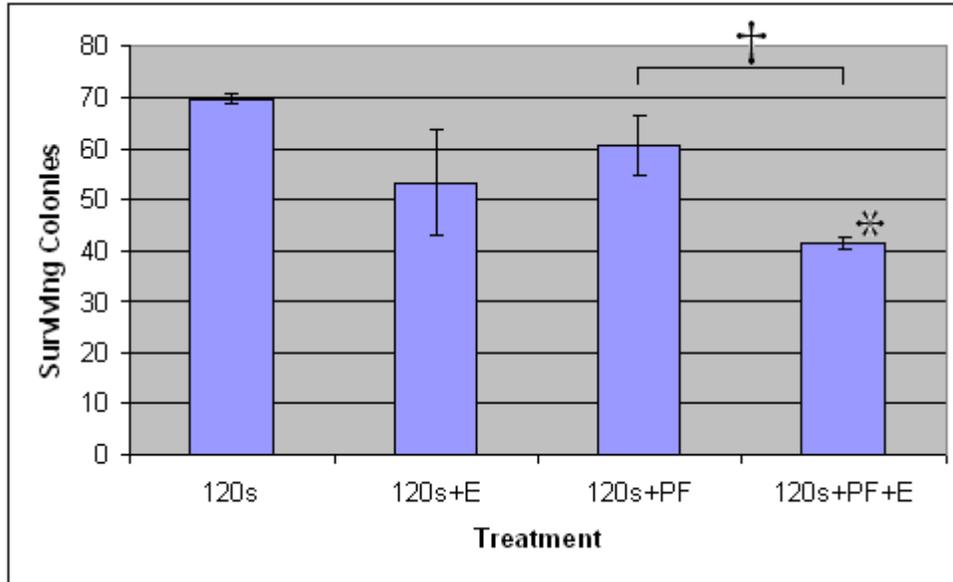


Figure 10: Surviving MCF-7 colonies after 120 s exposure to 20 mW, 635 nm laser over 9.6 cm² wells with light alone, or light in conjunction with electroporation only, 12.5 µg/ml Photofrin only, or both Photofrin and electroporation. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$). †: difference between indicated samples is significant ($P > 0.05$).

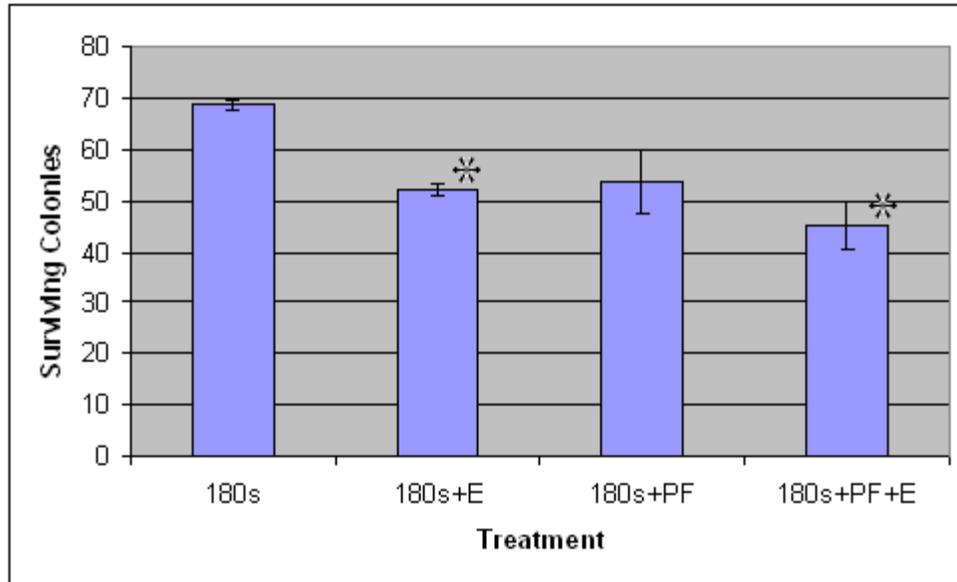


Figure 11: Surviving MCF-7 colonies after 180 s exposure to 20 mW, 635 nm laser over 9.6 cm² wells with light alone, or light in conjunction with electroporation only, 12.5 µg/ml Photofrin only, or both Photofrin and electroporation. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant (P≤0.05).

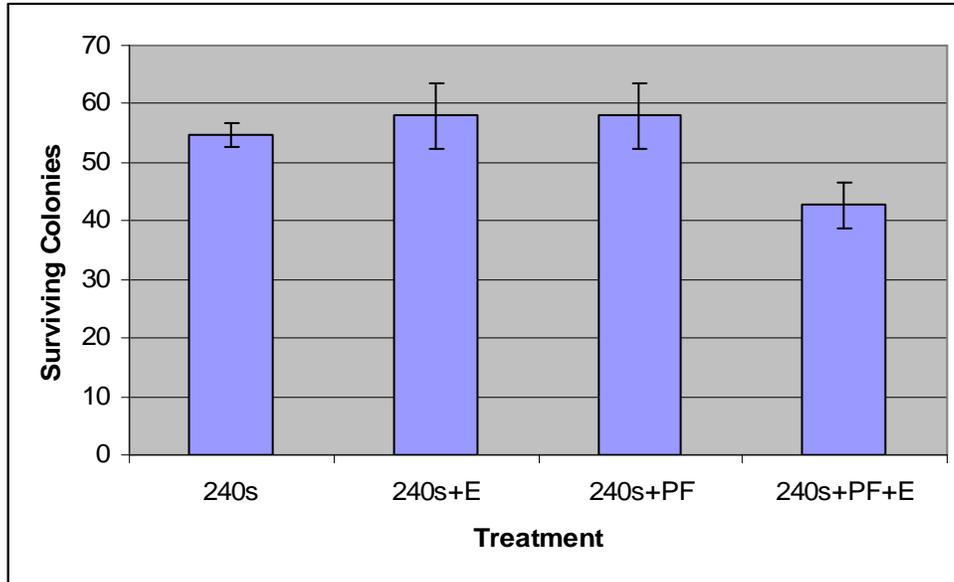


Figure 12: Surviving MCF-7 colonies after 240 s exposure to 20 mW, 635 nm laser over 9.6 cm² wells with light alone, or light in conjunction with electroporation only, 12.5 µg/ml Photofrin only, or both Photofrin and electroporation. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

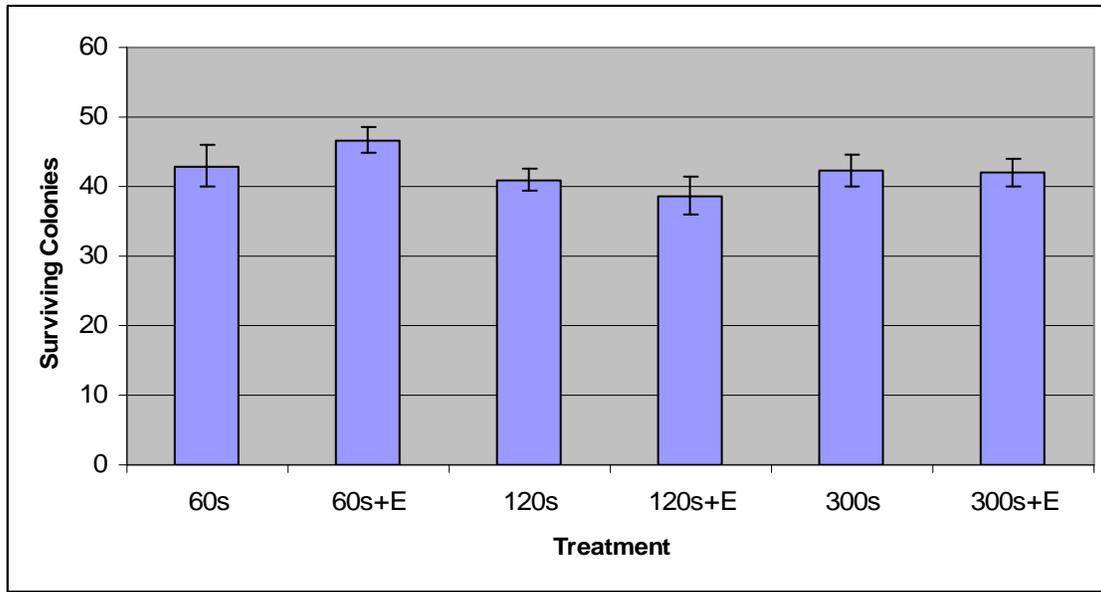


Figure 13: MCF-7 cells were treated with timed exposure to 20 mW, 635 nm laser over 9.6 cm² wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

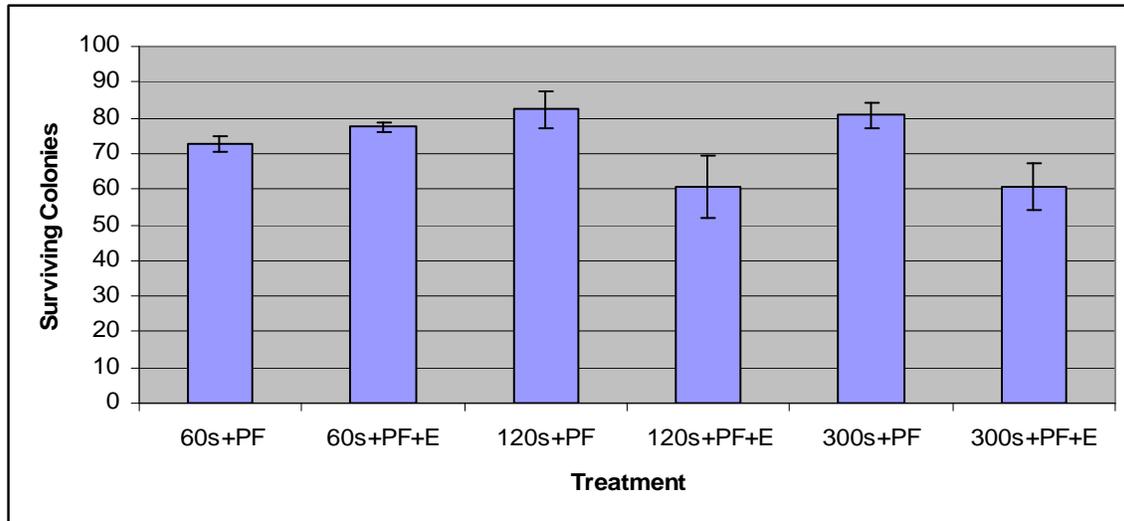


Figure 14: MCF-7 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

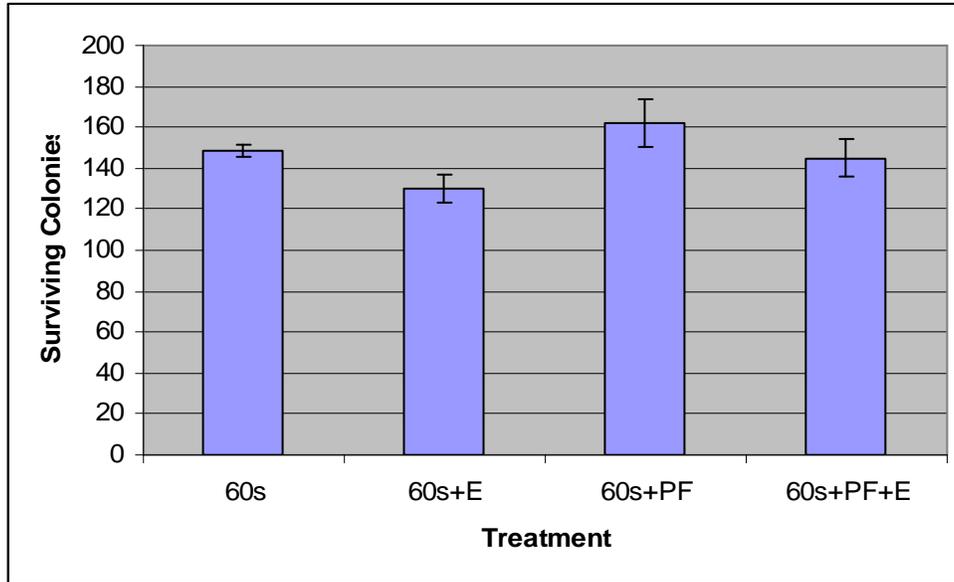


Figure 15: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

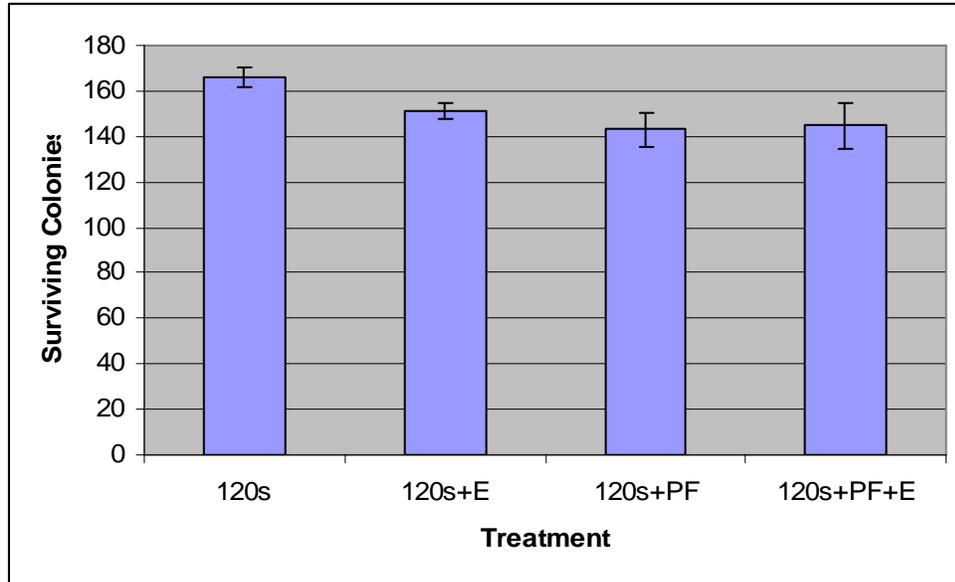


Figure 16: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

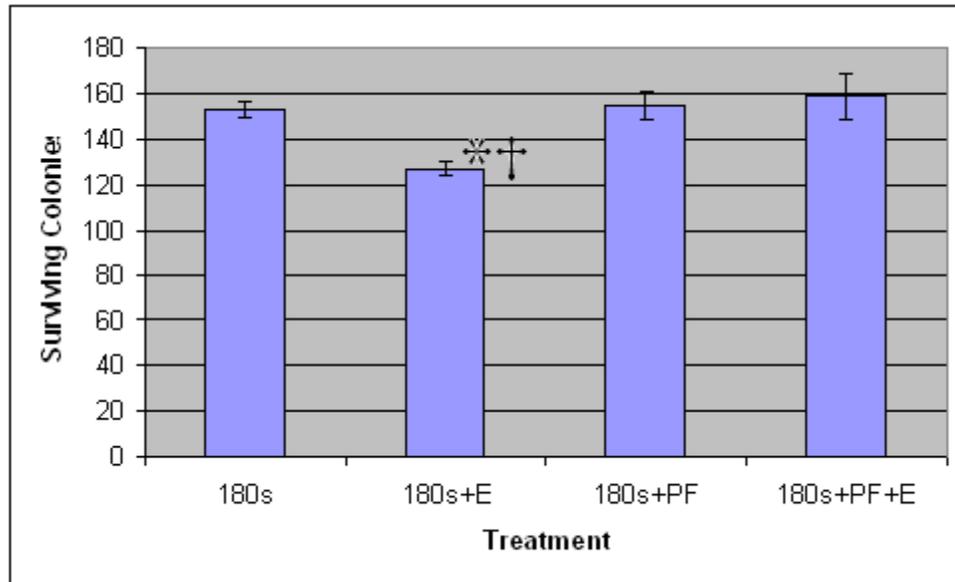


Figure 17: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$). †: difference between indicated sample and all other samples is significant ($P > 0.05$).

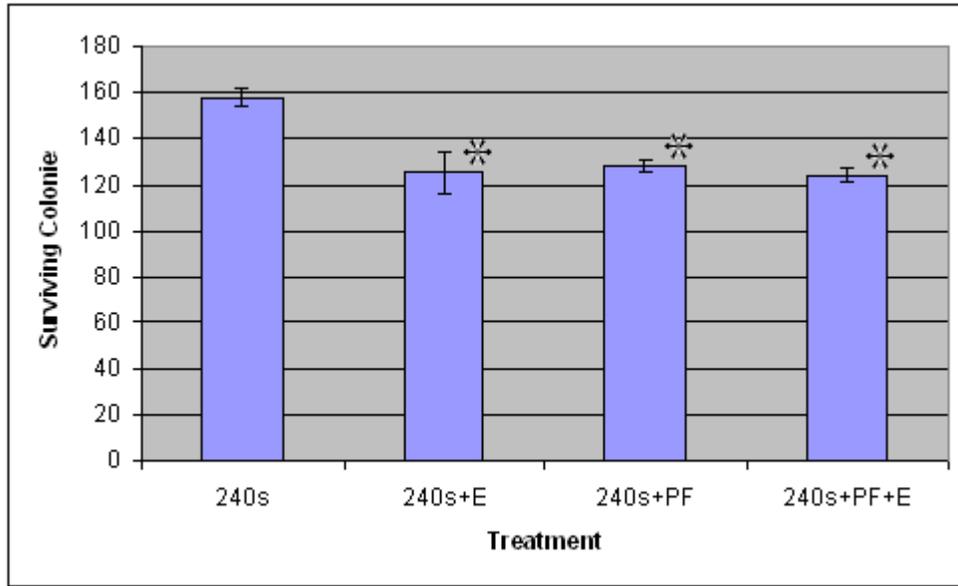


Figure 18: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$). †: difference between indicated samples is significant ($P > 0.05$).

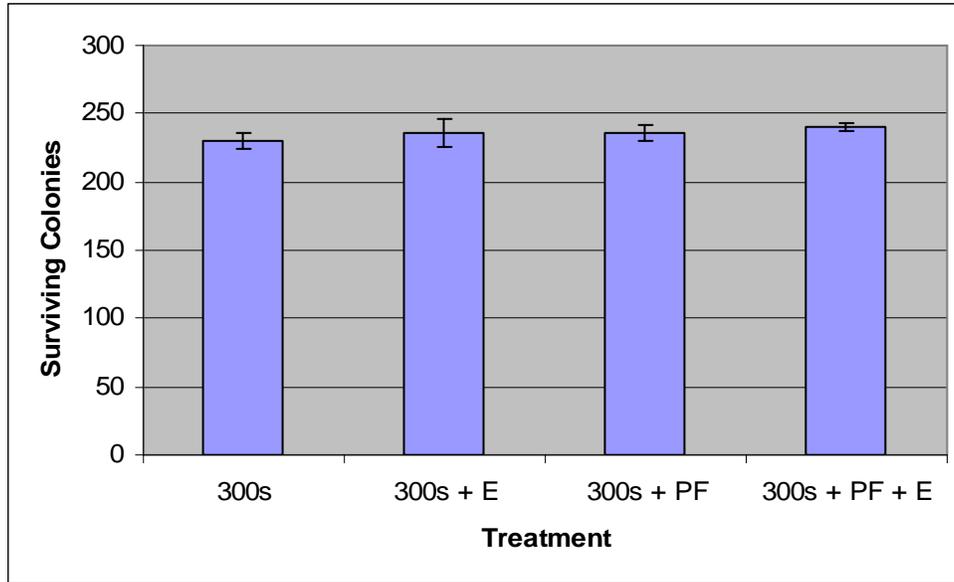


Figure 19: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

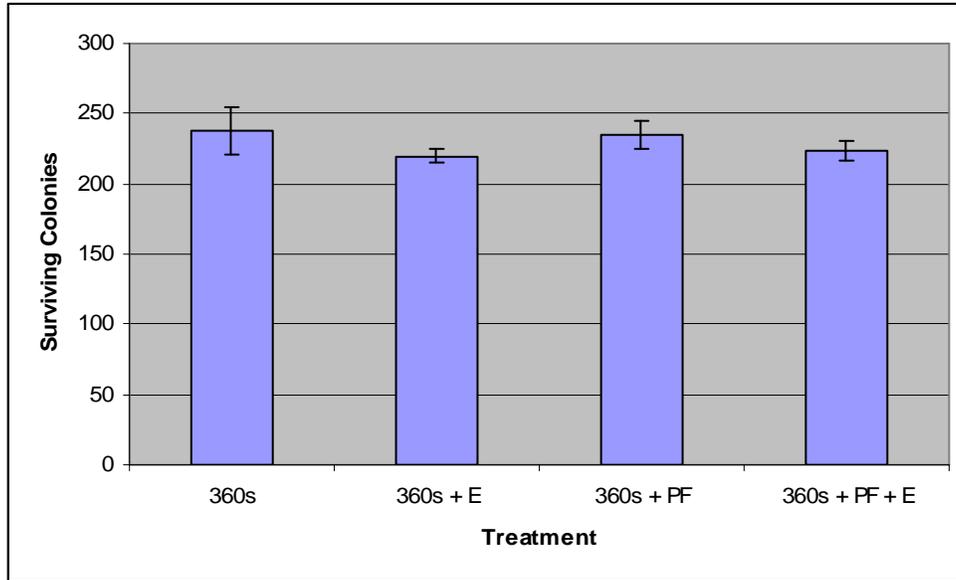


Figure 20: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

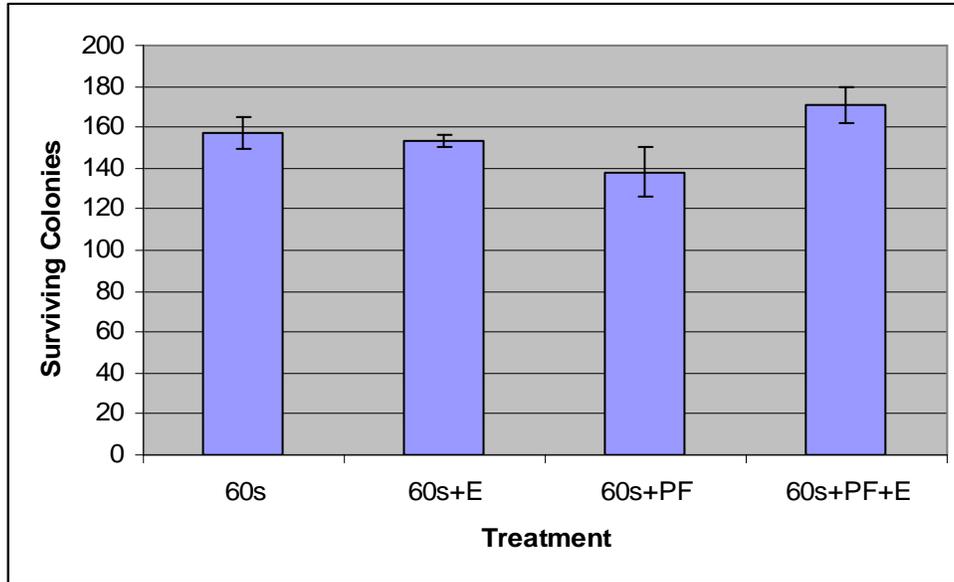


Figure 21: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$).

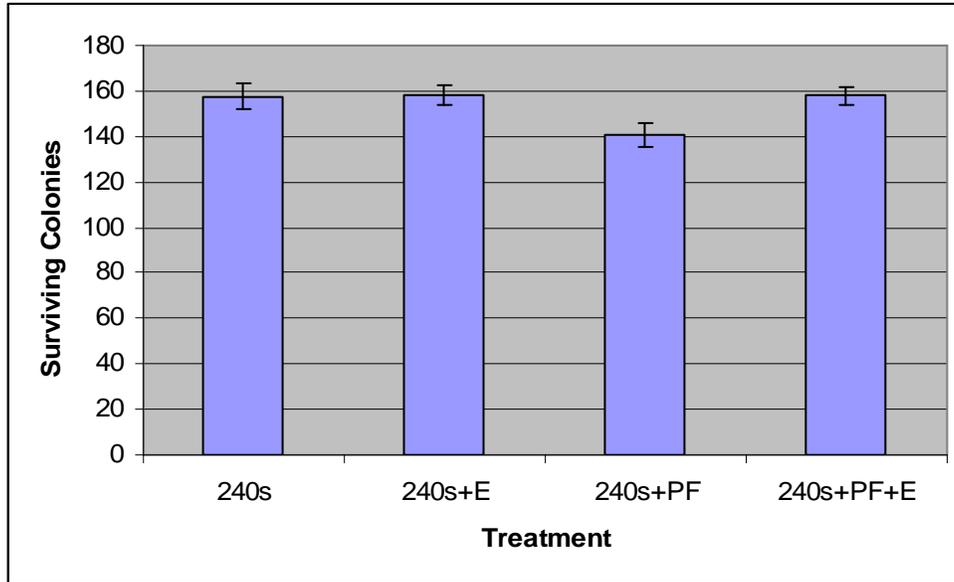


Figure 22: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$). †: difference between indicated sample and all other samples is significant ($P > 0.05$).

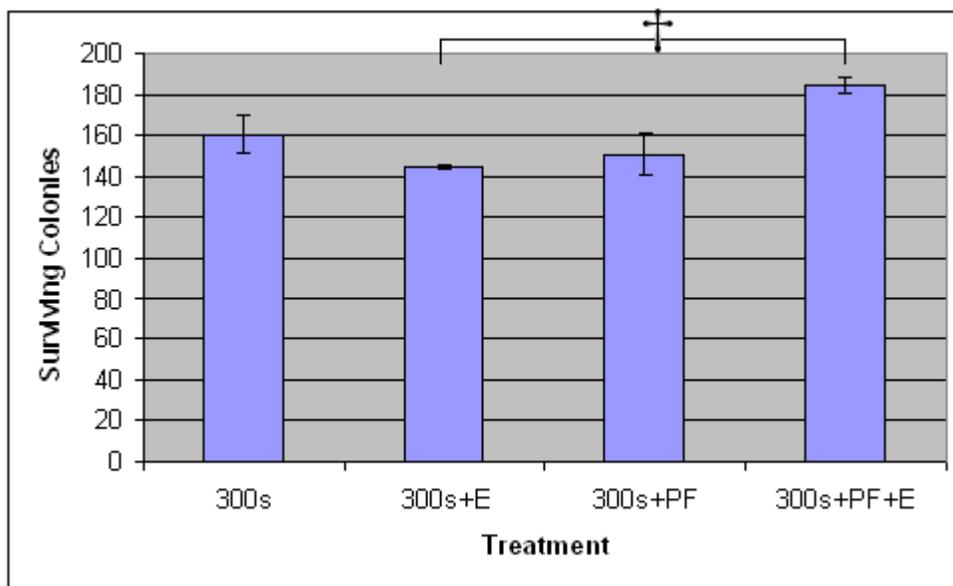


Figure 23: B16-F10 cells were treated with 12.5 $\mu\text{g/ml}$ Photofrin and were subjected to indicated timed exposure to 20 mW, 635 nm laser over 9.6 cm^2 wells with or without electroporation and surviving colonies were counted. Error bars represent standard error of the mean of surviving colonies of triplicate experiments. * : difference between indicated sample and control is significant ($P \leq 0.05$). †: difference between indicated samples is significant ($P > 0.05$).

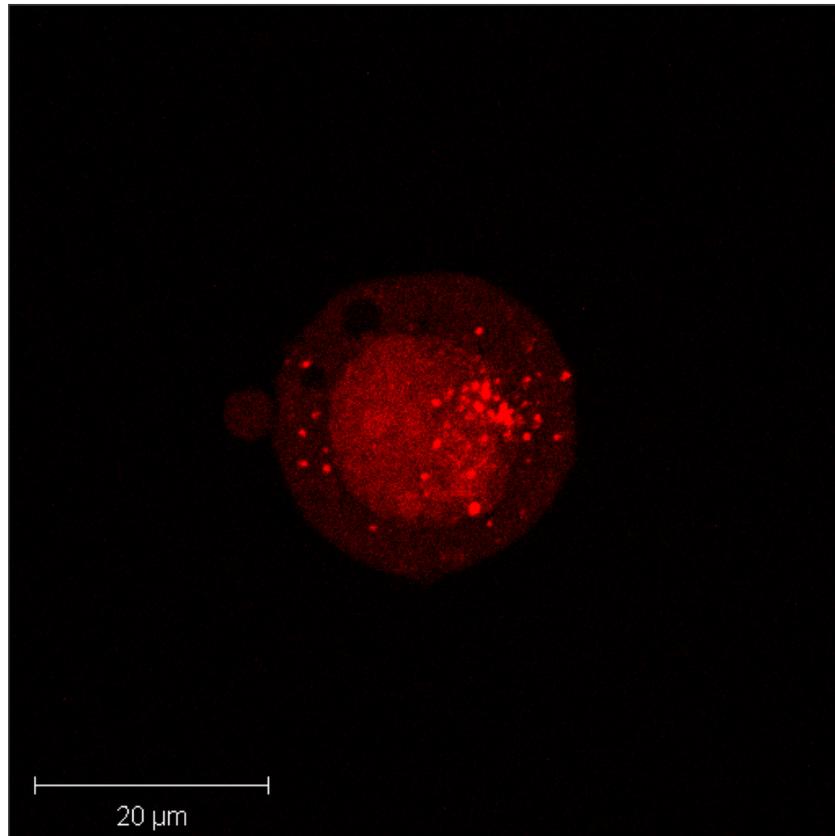


Figure 24: MCF-7 cells were stained with acridine orange and imaged with a confocal microscope, using an excitation wavelength of 543 nm and a long pass 560 filter.

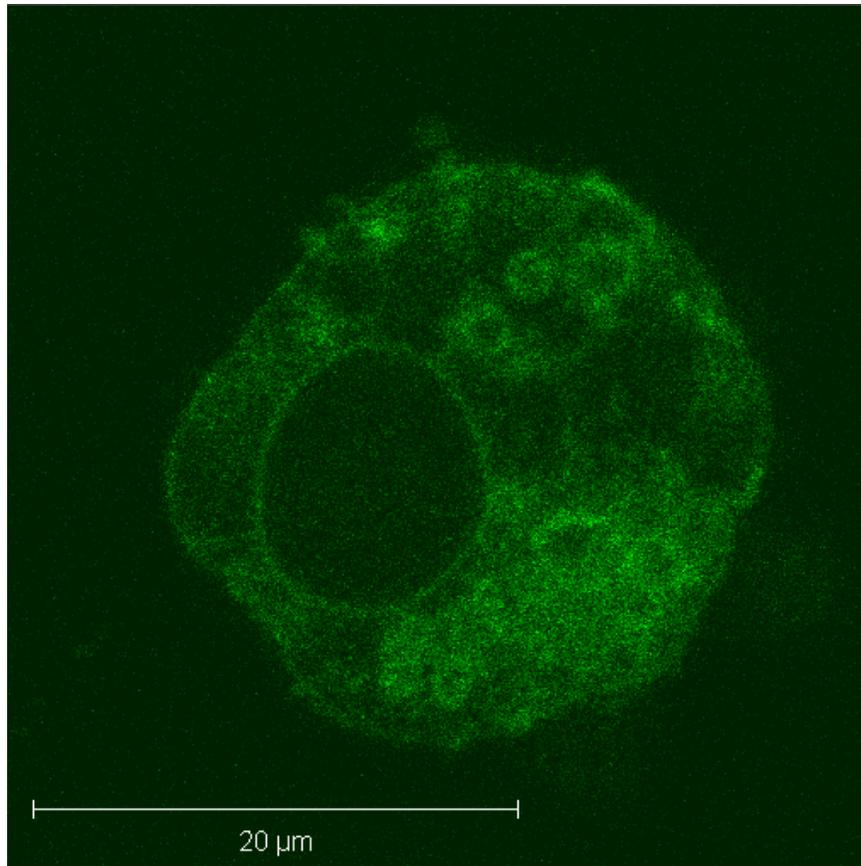


Figure 25: MCF-7 cells were exposed to Photofrin and imaged with a confocal microscope, using an excitation wavelength of 488 nm and a long pass 505 filter.

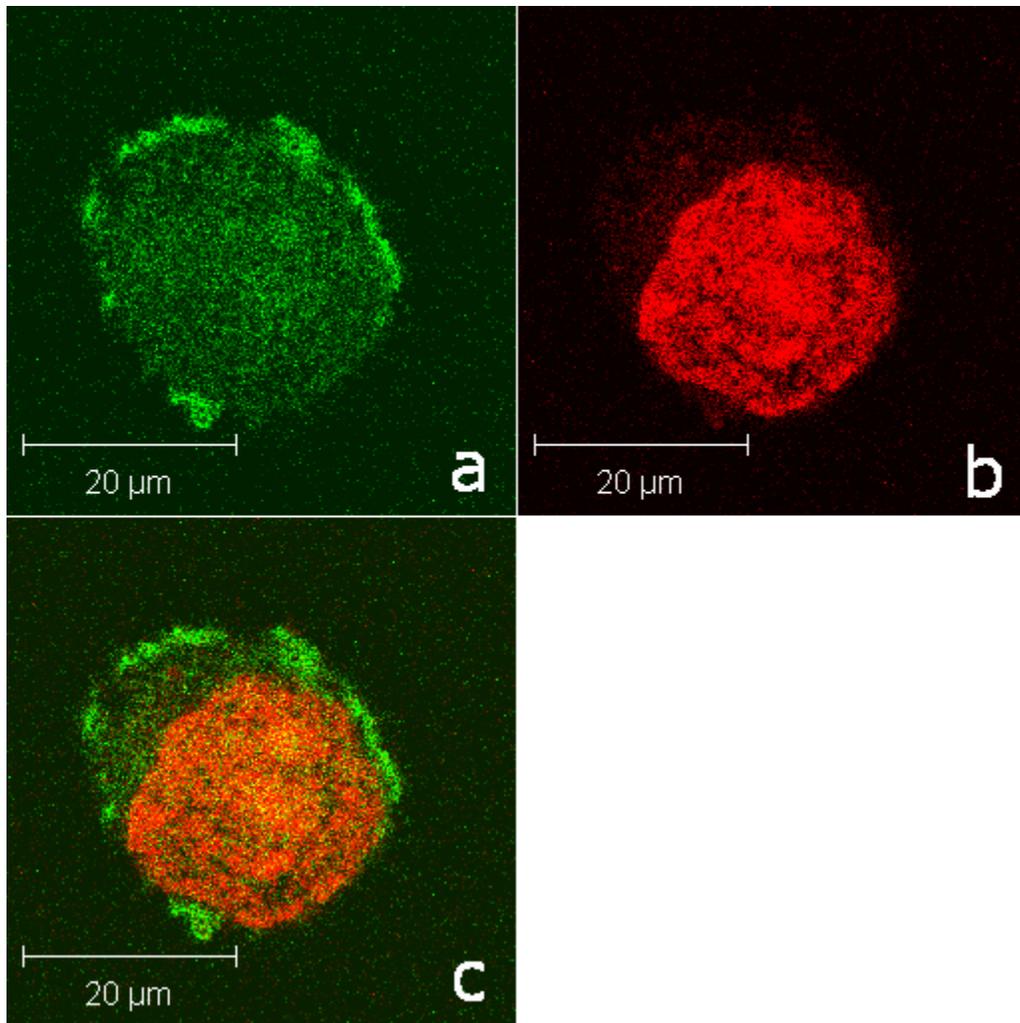


Figure 26: MCF-7 cells were exposed to Photofrin and stained with acridine orange. An excitation wavelength of 488 nm was used. Cells were imaged with two channels, A) a long pass 505 filter, and B) a band pass 505-560 filter. The two channels are superimposed in image C.

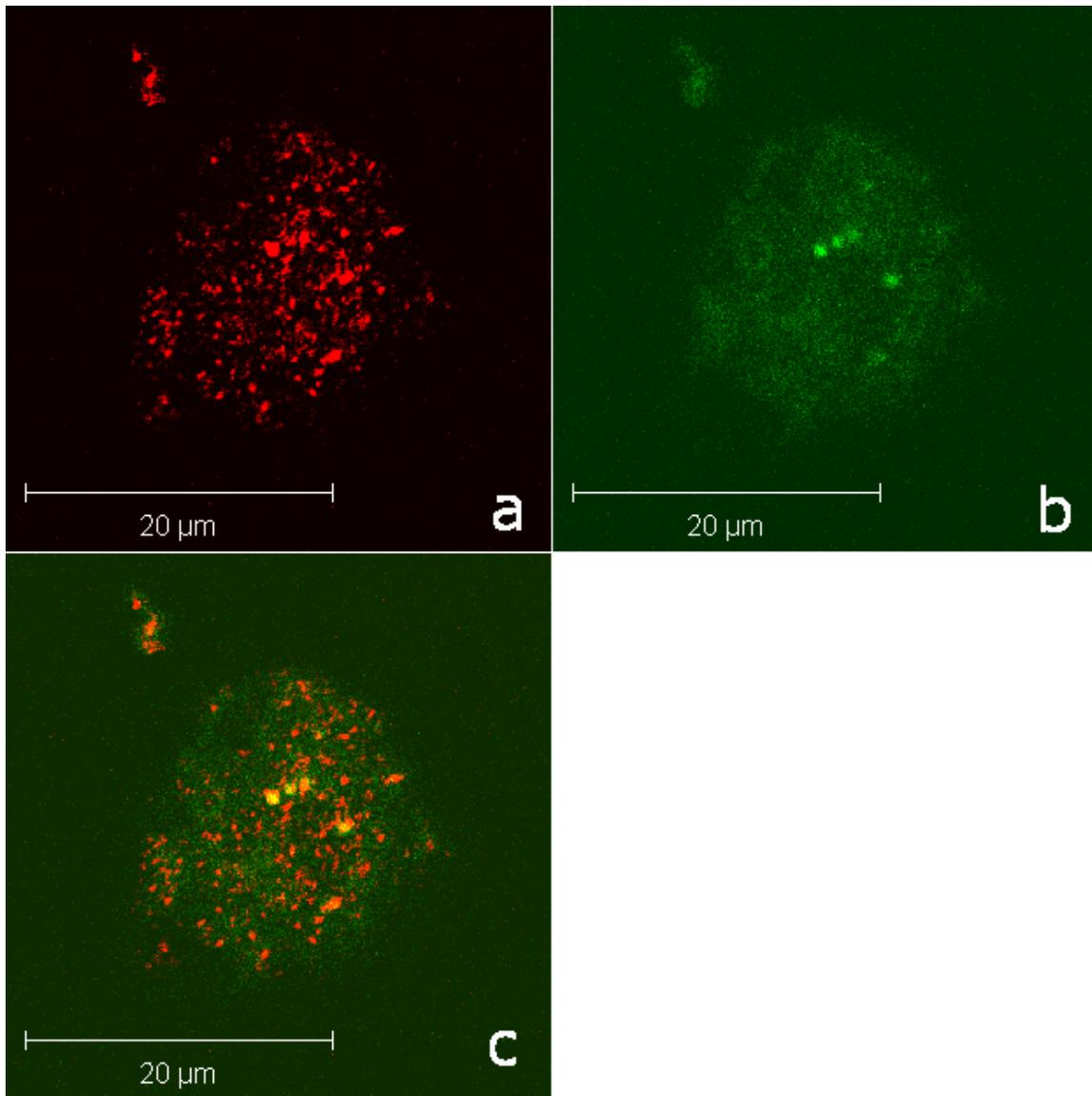


Figure 27: MCF-7 cells were incubated with Photofrin and then exposed to MitoTracker Deep Red, and filtered using a long pass 585 filter. A) MitoTracker was excited with a wavelength of 633 nm, and B) Photofrin was excited with a wavelength of 543 nm. Channels are superimposed in image C.

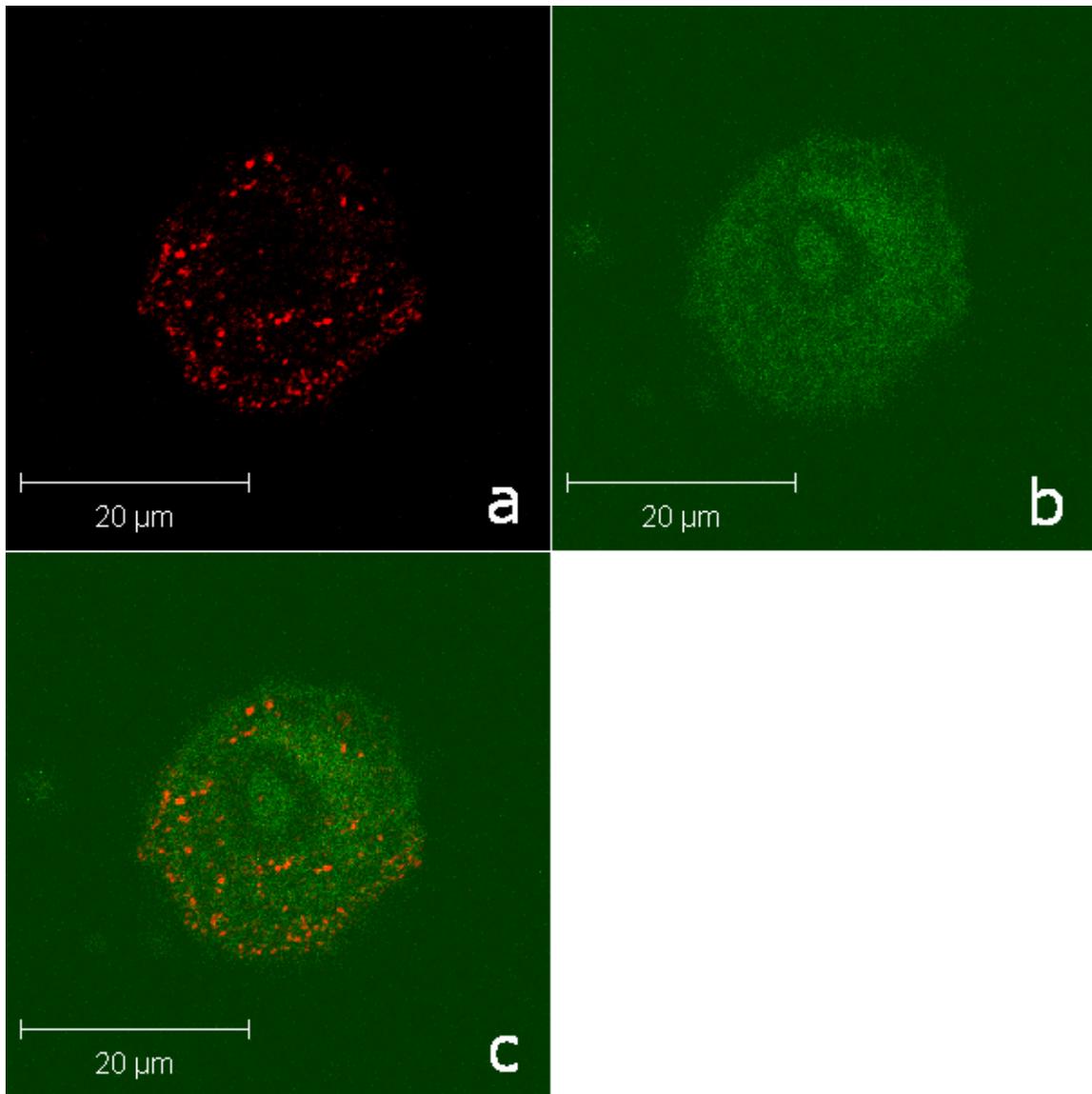


Figure 28: MCF-7 cells were incubated with Photofrin and then exposed to MitoTracker Deep Red, and filtered using a long pass 585 filter. A) MitoTracker was excited with a wavelength of 633 nm, and B) Photofrin was excited with a wavelength of 543 nm. Channels are superimposed in image C.

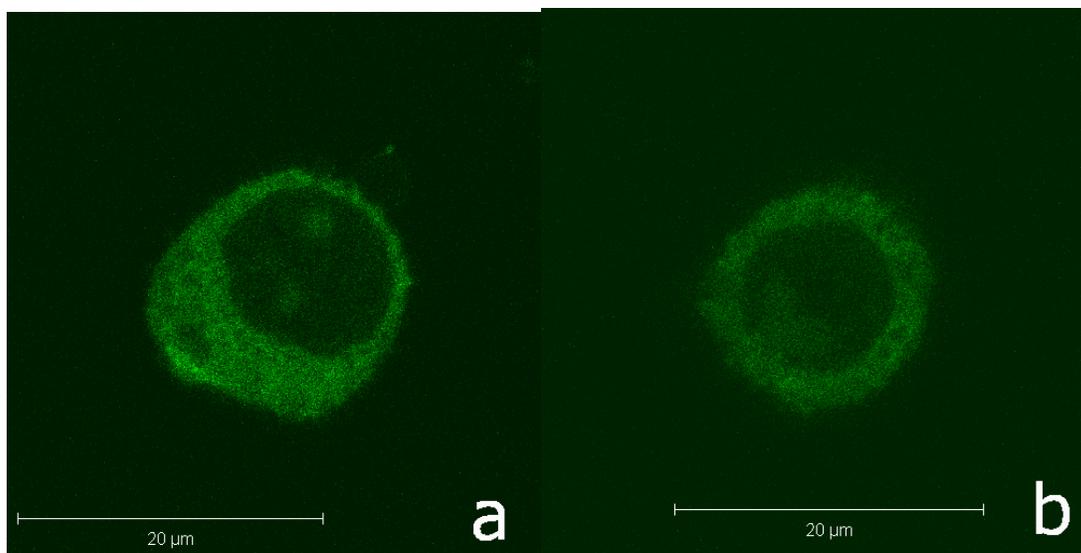


Figure 29: B16-F10 cells were exposed to 25 $\mu\text{g/ml}$ Photofrin at a density of 1.0×10^7 cells/ml and imaged with a confocal microscope, using an excitation wavelength of 543 nm and filtered using a long pass 585 filter.

Cell line	Photofrin Dose ($\mu\text{g/ml}$)	Light Dose (s)	Electroporation	% Survival	Std. Err.	P value	Significant
MCF-7	6.25	0	-	112	4.16333	0.474	-
MCF-7	12.5	0	-	102	5.23874	0.917	-
MCF-7	25	0	-	109	3.78594	0.563	-
MCF-7	0	0	+	104	1.20185	0.736	-
MCF-7	6.25	0	+	85	3.75648	0.384	-
MCF-7	12.5	0	+	127	1.73205	0.090	-
MCF-7	25	0	+	104	4.25572	0.820	-
MCF-7	100	0	+	96	5.03322	0.838	-
MCF-7	0	180	-	91	0.88192	0.318	-
MCF-7	6.25	180	-	131	10.0167	0.068	-
MCF-7	12.5	180	-	142	9.64365	0.026	+
MCF-7	25	180	-	107	1	0.387	-
MCF-7	0	180	+	85	3.38296	0.486	-
MCF-7	6.25	180	+	87	1.85592	0.415	-
MCF-7	12.5	180	+	51	0.88192	0.019	+
MCF-7	25	180	+	26	1.52753	0.006	+
MCF-7	100	180	+	19	0.33333	0.003	+
MCF-7	0	60	-	116	3.1798	0.169	-
MCF-7	0	120	-	122	0.88192	0.049	+
MCF-7	0	180	-	120	0.88192	0.062	-
MCF-7	0	240	-	95	2.02759	0.607	-

Table 1: Percent MCF-7 cell survival following indicated treatments, where n=3 and P value is compared to control samples. Control samples were untreated.

Cell line	Photofrin Dose ($\mu\text{g/ml}$)	Light Dose (s)	Electroporation	% Survival	Std. Err.	P value	Significant
MCF-7	0	60	+	82	3.52767	0.070	-
MCF-7	12.5	60	-	82	1.45297	0.027	+
MCF-7	12.5	60	+	65	5.50757	0.021	+
MCF-7	0	120	+	77	10.3333	0.190	-
MCF-7	12.5	120	-	87	5.78312	0.199	-
MCF-7	12.5	120	+	60	1.20185	0.000	+
MCF-7	0	180	+	76	1.1547	0.000	+
MCF-7	12.5	180	-	78	6.11919	0.072	-
MCF-7	12.5	180	+	66	4.70225	0.008	+
MCF-7	0	240	+	106	5.68624	0.610	-
MCF-7	12.5	240	-	106	5.50757	0.600	-
MCF-7	12.5	240	+	78	3.84419	0.051	-

Table 2: Percent MCF-7 cell survival following indicated treatments, where n=3 and P value is compared to control samples. Control samples were treated only with equivalent amounts of light as treated samples.

Cell line	Photofrin Dose ($\mu\text{g/ml}$)	Light Dose (s)	Electroporation	% Survival	Std. Err.	P value	Significant
B-16-F10	0	60	+	87	6.9282	0.065	-
B-16-F10	12.5	60	-	109	11.4649	0.323	-
B-16-F10	12.5	60	+	97	9.2376	0.701	-
B-16-F10	0	120	+	91	3.60555	0.065	-
B-16-F10	12.5	120	-	86	7.54983	0.061	-
B-16-F10	12.5	120	+	87	10.2686	0.132	-
B-16-F10	0	180	+	83	3.21455	0.006	+
B-16-F10	12.5	180	-	101	6.48931	0.832	-
B-16-F10	12.5	180	+	104	9.70109	0.612	-
B-16-F10	0	240	+	79	8.74325	0.026	+
B-16-F10	12.5	240	-	81	2.60342	0.003	+
B-16-F10	12.5	240	+	79	2.96273	0.002	+
B-16-F10	0	300	+	103	9.86577	0.609	-
B-16-F10	12.5	300	-	102	5.696	0.524	-
B-16-F10	12.5	300	+	104	2.51661	0.177	-
B-16-F10	0	360	+	92	5.23874	0.372	-
B-16-F10	12.5	360	-	99	9.64365	0.899	-
B-16-F10	12.5	360	+	94	6.69162	0.489	-
B-16-F10	0	60	+	97	3.1798	0.646	-
B-16-F10	12.5	60	-	88	12.4677	0.261	-
B-16-F10	12.5	60	+	108	8.51143	0.303	-
B-16-F10	0	240	+	100	4.3589	0.964	-
B-16-F10	12.5	240	-	89	5.4569	0.091	-
B-16-F10	12.5	240	+	100	4.04145	0.963	-
B-16-F10	0	300	+	90	1	0.172	-
B-16-F10	12.5	300	-	94	10.2686	0.512	-
B-16-F10	12.5	300	+	115	3.66667	0.079	-

Table 2: Percent B16-F10 cell survival following indicated treatments, where n=3 and P value is compared to control samples. Control samples were treated only with equivalent amounts of light as treated samples.

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