The Effectiveness of Suffruticosol B. in Treating Lung Cancer by Laser Trapping Technique

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Abstract

The biophysical effects of Suffruticosol B. on lung cancer cells were studied. The physical and mechanical changes due to the treatment were statistically analyzed based on areas measured from the images captured for each cell once free, trapped, and released. Measurements were conducted on the untreated A549 cells for comparisons. The result showed that the treatment damages the internal structure of the cells, resulting in the collapse of the spherically A549 cells as the average area indicated a relative increase with the length of the treatment period. The cells' response indicated that the elastic properties of the carcinoma cells had been reduced due to the treatment by Suffruticosol B. Changing the trap's power and time constant predicted interesting behavior for the A549 cells' relaxation rate. We have shown that Oligostilbene can play a role an effective in treating cancer.

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Abstract

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Keywords: Suffruticosol B., Oligostilbene, Optical Trapping, Lung Cancer, Radiation

Introduction

Cancer is a condition in which a group of abnormal cells grows uncontrollably by disregarding the normal rules of cell division. The fate of normal cells is constantly determined by signals indicating whether they should divide, differentiate into another cell or die. These signals cause cancer cells to become autonomous, causing them to grow and multiply uncontrollably, resulting in a tumor[1] [1]. There were 10 million cancer-related deaths worldwide in 2020, the leading cause of death[1,2] [1,2]. In Unites State the number of cancer deaths per 100,000 persons is 161.4 and lung cancer accounts for most of these[3] [3]. In developed and developing countries alike, lung cancer remains the leading cause of cancer death, accounting for approximately 20% of all cancer-related deaths worldwide (1.6 million deaths annually) and putting an estimated 1.8 million new cases at risk every year [4][4].

Some research studies have shown that radiotherapy used in combination with chemotherapeutic drugs can significantly improve control of local tumor and patient survival rate[4-8] [4-8 3-7]. Despite the fact that chemo-radiotherapy is superior to radiotherapy for treating locoregional diseases and improving survival, its efficiency is less than maximal, with only about a 15% to 20% 5-year survival rate[9] [9]. The effectiveness of standard chemotherapeutic agents such as cisplatinum and taxanes has been improved by their use in combination with one another, selected for their own antitumor activity. As a consequence of this toxicity associated with conventional chemo-radiotherapy with these agents, the amount of safe RT can often be reduced. Thus, new strategies that increase antitumor activity and, at the same time, reduce toxicity to normal tissues during chemo-radiotherapy are needed. Thus, the search for antitumor drugs that increase the radio-sensitivity of the tumor cells is its way.

One approach is the use of the Laser Trapping (LT) technique which was invented by Professor Ashkin in the early 1980s and is considered one of the greatest discoveries of the twentieth century[10,11][10,11]. This advanced technique utilizes radiation force to capture, translate and manipulate microscopic particles[12-17] [12-17]. With LT, we can conveniently trap and manipulate dielectric particles as small as a few nanometers in diameter and as large as a few micrometers in size[12,13] [12,13]. LT has the potential to help determine a better therapeutic ratio based on the cellular level, to eradicate radiation-induced tissue toxicity while advancing the sterilization of the cancerous cells[7,8] [7,8]. This technique provides unique means to control the dynamics of small particles and manipulate biological samples at the micron level[17,18] [17,18]. In the physical and biological sciences, this experiment has played a fundamental role. Our group has demonstrated that LT can be used to determine both ionization energy and charge at the single cell level for BT20 and 4T1 breast cancers, neuroblastomas, and RBCs[7,8, 16-20] 16-20 [7,8, 16-20].

In recent studies, researchers have discovered that certain traditional Chinese medicines (TCMs) that were used for treating cancer carry antitumor agents that increased tissue sensitivity to radio waves and protected normal tissues from radiation damage due to radiation[4-8] [4-8]. This new approach is combined with TCMs for single cell ionization and is extended to multiple in 4T1 breast carcinoma cells[9] [9]. The cell line was treated with a naturally occurring compound, 2-Dodecyl-6-methoxycyclohexa-2, 5-diene-1, 4-dione (DMDD) extracted from the root of *Averrhoa carambola* L. produces a significant reduction in the Threshold Radiation Dose in multiple cell ionization was observed and related to the chain effect of ionization by the radiation field[8] [8]. It is well known that the botanical plant (Paeonia suffruticosa) has antitumor, antioxidative, and other health benefits. It has been shown in previous studies that Paeonia suffruticosa, a naturally occurring plant lignin, inhibits the growth of liver, esophageal, and lung cancer cells[21,22] [21,22]. In this study, we use a Suffruticosol B, a naturally occurring oligostilbene isolated from peony seeds[21,22] [21,22]. It is an antitumor agent that is part of the highly regarded 3,5,4'-trihydroxystilbene family of molecules[23-27] [23-27].

Radiotherapy relies on the fact that healthy cell much more easily undergoes DNA repair than cancer cells. The cell cycle controls genome integrity to prevent genetic changes from being passed to subsequent

generations[21,28] [21,28]. Several studies have demonstrated that malignancies are associated with the deregulation of cell cycle checkpoints, including those in G1/S and G2/M phases[29] [29]. Thus, cell cycle arrest provides an opportunity for DNA repair to occur, hence inhibiting replication of the damaged template[30] [30]. Although, a recent study shows that the active fractions in Suffruticosol B are cytotoxic towards various cancer cell lines such as MCF-7, MDA-MB-231, HeLa, A459, and CaOV3 since induced G2/M phase cell cycle where cells are more radiosensitive[21,22] [21,22].

In previous studies of this compound, antioxidant and anticarcinogenic activities were associated with it. In a laser trap, cancer cells can be exposed to electromagnetic radiation pressure that may operate as a radio-sensitizer so that these bioactive compounds can act as radio-sensitizers. In this paper, by using laser trapping techniques, we present our study on the effects of the Suffruticosol B treatment of human lung carcinoma (A549) compared to the untreated cells.

2. Methods

2.1 Culturing and Treating of Cells

The cell line used in this study were the human lung carcinoma cell line A549, purchased from the American Type Culture Collection (ATCC Manassas, VA, USA). The cells were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 100U/ml penicillin and streptomycin. The cells were incubated in a humidified atmosphere with 5% CO_2 at 37^0C. Cells were harvested by trypsinization and were diluted in medium to a density of 4.5 x 10^4 cells/ml. 100µl of diluted cells was added to each well of a 96-well tissue culture-treated plate (Corning Costar). After overnight incubation, cells were treated with 100µM Suffruticosol B for three and six hours, respectively. We then prepared samples from the untreated, 3hr treated and 6hr treated cells on depression slide, to measuring the response of the cells to laser trapping (LT).

2.2 Laser Trap Design

The design for the complete set-up of the experiment is shown in fig.1. In the current experimental setup, a top-power infrared laser (=1064 nm) is paired with a high numerical aperture (NA) inverted microscope, and a computer-controlled digital camera. The laser was a linearly polarized infrared diode laser (LS) for which the power was controlled by a half-wave plate (w) and polarizer combination (p). A beam expander was used to expand and align the laser beam, and a pair of two lenses (L1, L2) with some focal lengths were used to resize the beam. At the trap port of the inverted microscope (Olympus IX 71), four optical mirrors directed the beam to the dichroic mirror (DM). The dichroic mirror is angled at 45^0 such that the reflected light makes an angle normal to the incident light. The aligned beam would then be reflected into the back of the objective lens (OL) that has a good magnification and numerical aperture. Two lenses (L3, L4) are positioned such that the laser trap is on the focal plane of the microscope. Computer based piezo-driven stage (PS) is attached to the microscope. The stage is used to locate and manipulate the collimated sample for trapping, and a camera is used to take live 2D images by halogen lamp (HL) for 2D contrast analysis.

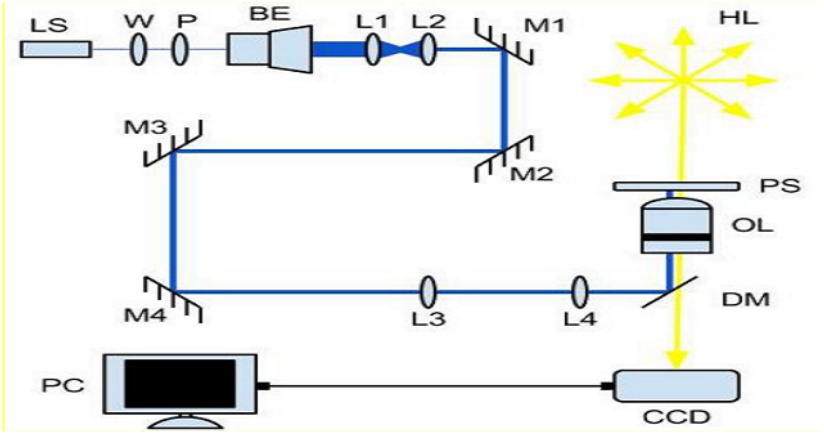

Fig. 1. An illustration of the laser trap experimental set-up. The laser has a wavelength 1064 nm with maximum power 8 Watts and is controlled by a half-wave plate (W) and a polarizer (P).

2.3 Experimental Procedure

From the three prepared samples (2.1), the untreated cancer cell was placed on a dispersion slide and mounted on the PS of the microscope. Using the digital camera, we took three consecutive images of the cell when free, lying on the bottom of the slide. In many cases, the cells adhered quickly to the coverslip of the slide and needed tapping to be separated for trapping. When the laser port on the microscope is opened, the gate gets opened and the cell becomes trapped. A laser gate at the laser port was shut before taking three consecutive images of the cell before releasing it from the trap. As soon as the cell was released, we took images of it every second for a minute. Similarly, the procedure was repeated for 3 and 6 hour treated samples. As part of our research, we compared the effects of laser traps of various strengths on cells that are kept inside the trap, released, and relaxed. To alter the pressure of the trap, the laser power was varied from 300mW until 2700mW by a 600mW variation at the laser port right outside of the microscope. Only 30% of this power is applied to the trapped cell, which is outside the microscope's objective lens. For better averages, we have conducted a measurement for ten different cells at each power setting Images of the cells from the three samples are presented in Fig. 1.

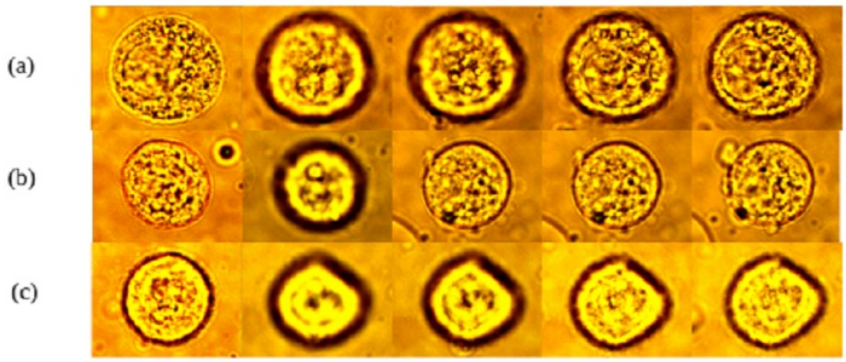

Fig. 2. (a) 3hours treated A549 cell at 2700mW, (b) 6 hours treated A549 cell at 2700mW. (c) Untreated cell at 2700mW

The images in the 1st row (a) are of a cell after the 3-hr treatment with Suffruticosol B, in the 2nd row (b) are from 6hr treated cell, and in the 3rd row (c) are from an untreated lung cancer cell. In these, from left to right, the first is the free cell, the second is the trapped cell, and the next three are sequential images of the relaxation at about 3 second intervals.

3. Results

3.1 The physical Properties of the Free Cell

The physical properties of the untreated and treated free cells were examined by statistical analysis of the mean area. The mean area of these cell groups (untreated and treated) was determined from the image captured for each free cell using Image-Pro Plus 6.2, an image processing, enhancement, and analysis software. The area of the cell was measured in pixels square and converted into micrometers square using a conversion factor of 5.28529 and reported in Table 1. The response of untreated and treated cells with Suffruticosol B. was investigated. The distribution of cell size according to four essential statistical parameters (mean, standard deviation, minimum, median, and maximum) can be found in Table 1. As a result of the treatment with Suffruticosol B, the average area of the A549 lung cells increases.

Table 1 : The values of essential statistical parameters of the area of the untreated and treated A549 lung cells free and trapped at 300 mW.

Free Cell

Treatment periods (hours)	# of cells	Mean area (μm^2)	Standard deviation (μm^2)	Min. area (μm^2)	Media area
0	50	186.1	47.2	92.2	186.4
3	50	304.8	79.6	141.9	294.5
6	50	333.9	88.2	168.3	325.3
Trapped Cell	Trapped Cell	Trapped Cell	Trapped Cell	Trapped Cell	Trapped Ce
0	50	134.2	36.3	68.3	124.0
3	50	208.8	66.7	76.9	294.5
6	50	242.2	104.6	83.8	223.2

The results are displayed in Fig. 3(a) using a histogram. Figure 3(a) displays the mean area of the cells that were not treated with Suffruticosol B (red). These cells have an average area of about 186.2±47.2 μm^2. Whereas the cells treated with Suffruticosol B for three hours (blue) is 304.8±79.6 μm^2 which is greater than the untreated. The change in the average area due to the treatment is observed for the cells treated to six hours (magenta) with its average area of 333.9±88.2 μm^2.

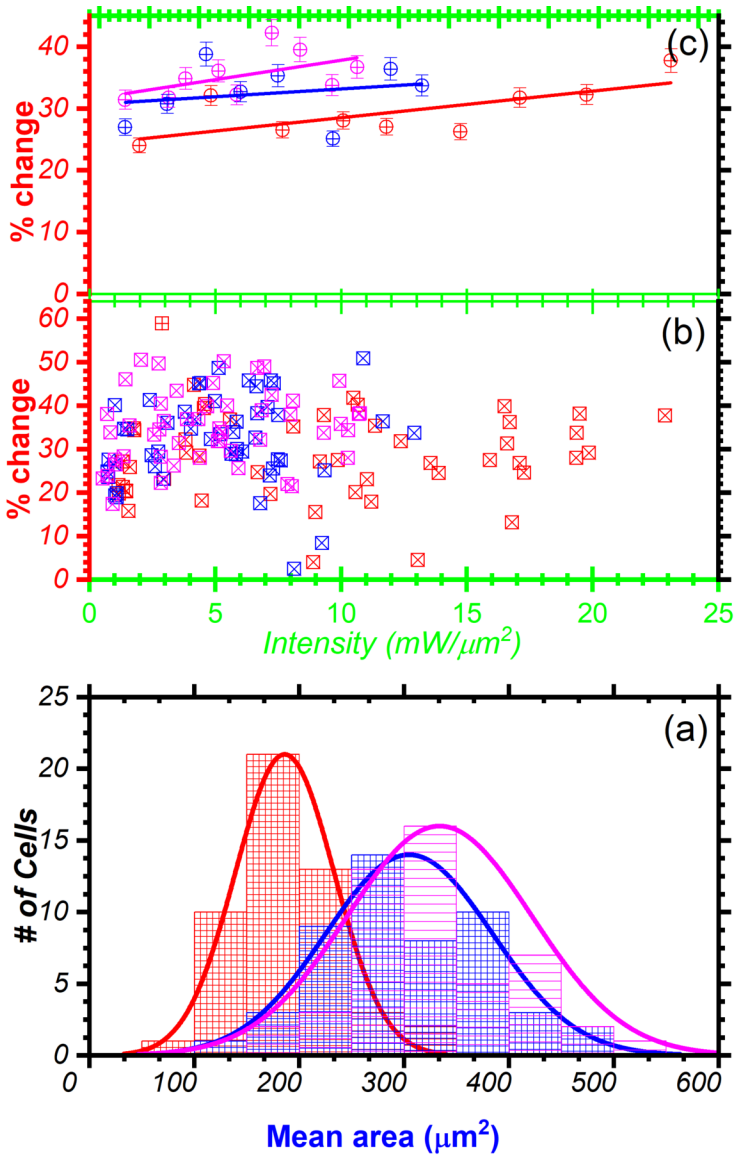

Fig. 3. (a) A size distribution of the mean area of untreated(red), 3-hr treated (blue), and 6-hr treated(magenta). (b) The percent change of the free cells as a function of the intensity. (c) the reduced percent change of the cells as a function of intensity for a free cell. In all Fig 3a-c traces of the behaviors were made using a fourth order polynomial approximations.

Based on the images from both the free (A_f) and trapped (A_t) states of the cells, we measured the mean area of each cell. We then calculated the average values for $A_t$and A_f at a given power setting from the measurements, we made for the ten different cells. Using these two average values we determined the relative change in the mean area of the cell at a given power using, $\%A = ((A_f\text{-}A_t)/A_f)$ x100. Similarly, we calculated the amount of intensity falling on the trapped cells, using power per mean area of the cell. In Fig. 3(b), the raw data for the percentage change in the area of cancer cells that are untreated, 3 hours treated, and 6 hours treated is displayed while in Fig. 3(c), the relative percent changes in the area are displayed. The average cell area increases with treatment in this figure (Fig. 3(c)). In either case, this may indicate that the spherical cells have been flattened and have become larger or that the mitochondrial division has occurred

after the mitotic phase, resulting in fewer daughter cells.

3.2 The Physical properties of the A549 Cell with Laser Trap

The comparative analysis in relative size changes due to direct laser trapping of the untreated and treated A549 cells were also conducted. We first analyzed the relative changes in the trapped cells concerning the corresponding free cells. Following the release from the trap, the relaxation rates of these same cells are then examined and studied based on their area as well. The size analyses of the images captured for the trapped cells are analyzed with the same procedure of free cells (Fig.3(c)).

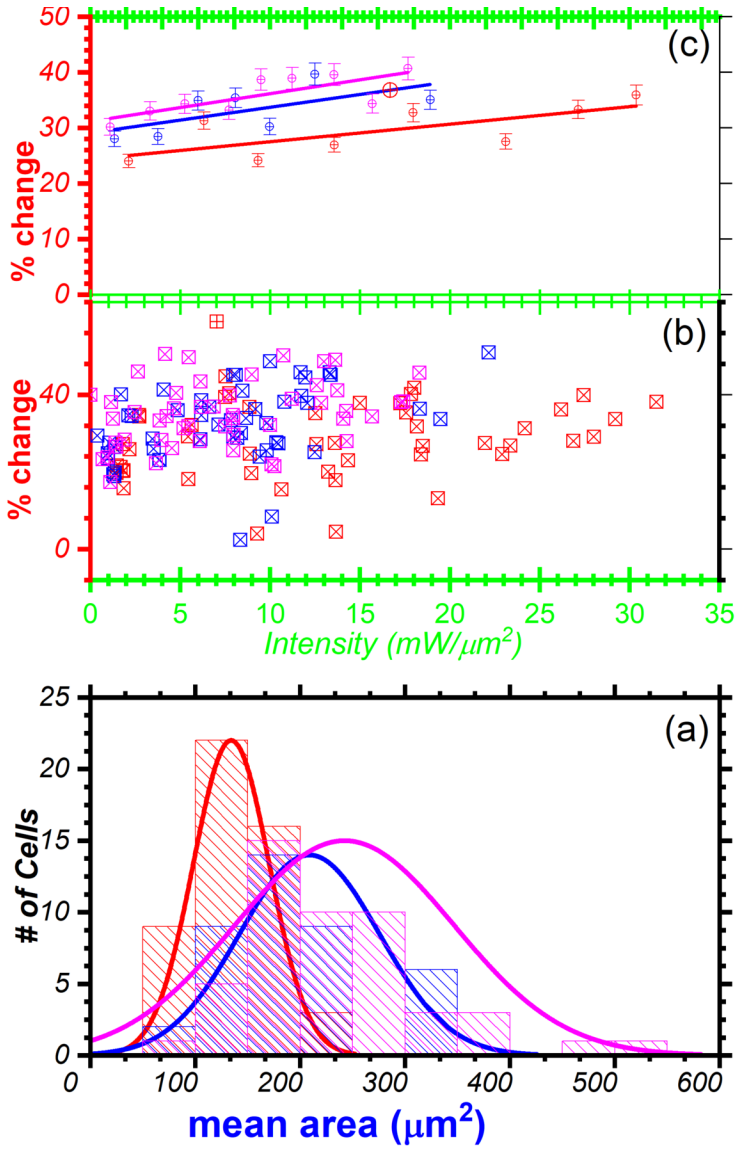

Fig. 4. (a) A size distribution of the mean area of untreated(red), 3-hr treated (blue), and 6-hr treated(magenta). (b) The percent change of the trap cells as a function of the intensity. (c) the reduced percent change of the cells as function of intensity for a trap cell. In all Fig 4a-c traces of the behaviors were made using a fourth order polynomial approximations.

Table 2: The values of essential statistical parameters of the area of the untreated and treated A549 lung cells with laser trap at 300mW

	Trapped Cell Mean area (μm^2)	Trapped Cell Mean area (μm^2)	Trapped Cell Mean area (μm^2)
	Untreated	Treated	Treated
Power (mW)	0	3hr	6hr
300	183.3	253.5	280.9
900	207.9	319.1	347.2
1500	215.8	339.3	357.5
2100	234.7	340.9	344.5
2700	261.9	398.3	422.6
	Free Cell Mean area (μm^2)	Free Cell Mean area (μm^2)	Free Cell Mean area (μm^2)
300	186.1	304.8	333.9
900	225.9	357.1	364.4
1500	245.1	359.7	374.4
2100	281.9	361.7	387.7
2700	297.8	399.1	440.6

The results are displays in Fig. 4(a) using histogram. Figure 4(a) displays the mean area of the cells that are not treated with Suffruticosol B. These cells have an average area of about (red) 186.1±47.2. The area of cells treated with Suffruticosol B for 3 hours (blue) have 304.8±79.6 which is greater than the untreated. The change in the average area due to the treatment can also be observed for the cells treated to 6 hours(magenta) with its average area of 333.9±88.2.

For the Free Cells regression, we obtained the following: Contribution of treatment on cell size: B=22.140, t=8.176, p<.001; Contribution of Power strength on cell size: B=.39, t=5.041, p<.001. For the Trapped Cells regression, we obtained the following: Contribution of treatment on cell size: B=21.637, t=6.777, p<.001; Contribution of Power strength on cell size: B=.043, t=4.678, p<.001. Results reveal that both treatment length and power strength have a significant impact on cell size, however more so when the cells are Free.

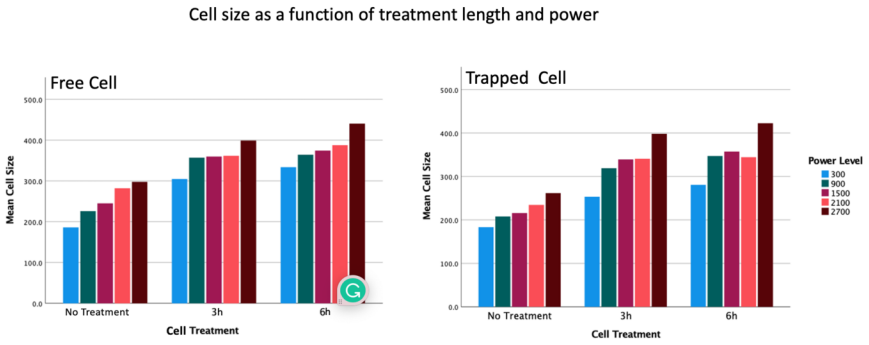

Figure 5. Cell size as a function of treatment length and power. (a) mean size of the free Cell at various at different treatment times. (b) mean size of the Trapped cell varies at different treatment times.

Fig. 5 clearly illustrates that in comparing the relative sizes of the untreated and the treated cells, the change in percent in the mean area of cells was significantly greater with higher treatment periods. The result in Fig. 3 and 4 shows that the length of the treatment affects how A549 cells respond to the laser intensity. As the treatment time increases, the relative changes in the cells tend to increase for laser treatments with intensity. To change the trap pressure, the laser power was varied from 300mW to 2700mW by a 600mW variation at the laser port right outside the microscope during our laser trapping experiment. Only 30%

of this power is directed at the trapped cell, which is located outside the objective lens of the microscope. We measured the area of ten different cells at each power setting, including the laser power that fell on the surface of the cell's area, and calculated the intensities for each trapped cell.

4. **Discussion**

The observation for the intensity range studied shows that for untreated A549 cells, the change in the cells becomes larger as the intensity of the trap increases. This is consistent with the generally expected elastic properties of cancer cells compared to free cells. However, the results show that the relative change from untreated to 2h treated is greater than the relative change from 2hr treated to 6hr treated at the same power. This indicates that Sufffruticosol. B. affects significant A549 cells since there are alive, damaged, and dead cells due to the treatment.

When the laser port is opened**,** and power is applied to the treated cancer cells during the laser trapping experiment, dead cells are easily ejected within a fraction of a second. The slide with untreated shows very little cell death compared to treated slides. This suggests that the drug we use to treat the cells causes cell death. In a recent study**,** researchers demonstrated that. Sufffruticosol. B. inhibit proliferation of human breast cancer cell lines MCF-7 and MDA-MB-231 through the induction of $\frac{G_2}{M}$arrest and apoptosis [21]. Cell cycle arrest provides an opportunity for DNA repair to occur, hence inhibiting replication of the damaged template.

The time rate of an average area for the untreated and treated of five different powers of the trap Sufffruticosol are shown in Fig. 6(a-c). These three graphs are analyzed in terms of the mean area as a function of time. Fig 6a represents the time rate at which the untreated cell is released from each power (300mW-2700mW) while Fig. 6(b) and 6(c) show the release of treated cells from these powers. In Fig 6, red signifies a power of 300mW, blue represents 900mW, green represents 1500mW, magenta represents 2100mW, and black represents 2700mW for each treated and untreated cell. We calculated the total time interval of each cell, using $t = t_f - t_i$. t_f and t_i are the time taken for the final and the initial relaxing cells; the time interval per frame was found using$R = C\left(\frac{t_f - t_i}{N}\right)$. where C=0,1,2, 3... (each frame number) and N is the total number of the frame. However, this time rate of the mean area A_t is the measurement of the mean area of the relaxing cells in one-second intervals immediately after the laser power is cut off. For A_f, we used the average mean area for the free cell, as previously discussed. Each data point represents the cell released from every five powers calculated using the average of the mean areas (for A_t and A_f) for ten different cells.

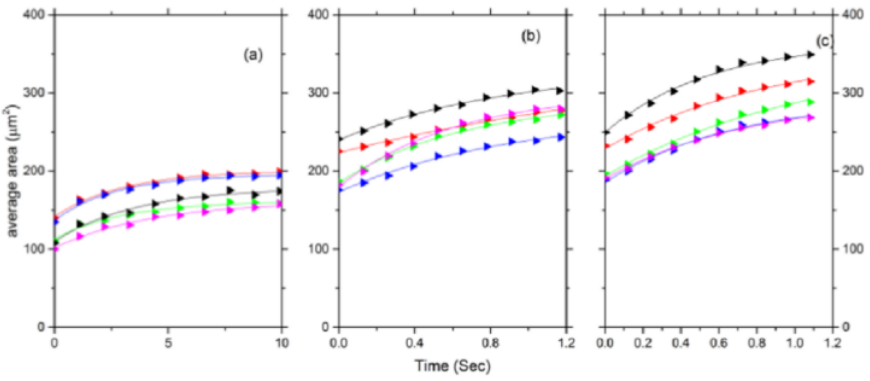

Fig. 6. A size distribution of the mean area of the cells as a function of the time for untreated (a), 3hr treated (b) and 6hr treated (c) at 300mW(red), 900mW(blue), 1500mW (green), 2100mW (magenta) and 2700mw (black) powers.

Using the laser power as a function of the relaxation rates of the cells in the sample, we have defined and calculated the time constant for these behaviors. According to Fig. 7, a function was used to calculate the

time constant based on the points for the relative change in the area of the maximum. The program Origin 6.2 was used to fit the data points in Fig. 7. An exponential decaying functionf $(t) = A_0 + A_1 e^{-kt}$ was founded as shown by the solid lines. The time constant ($\tau = \frac{1}{k}$) is defined as the time for which the relative change in the maximum area immediately after the cell is released from the trap (as predicted by a function f (t = 0)) is reduced by one-third. We then determined a time constant by solving a function for each power. The average of the three as a function of the trap power is plotted in Fig. 8. The data points and the fitting curve shown in red are for the untreated inner lung carcinoma cell, and those shown in blue and black are for 3hr, and 6hr treated human lung carcinoma cells respectively.

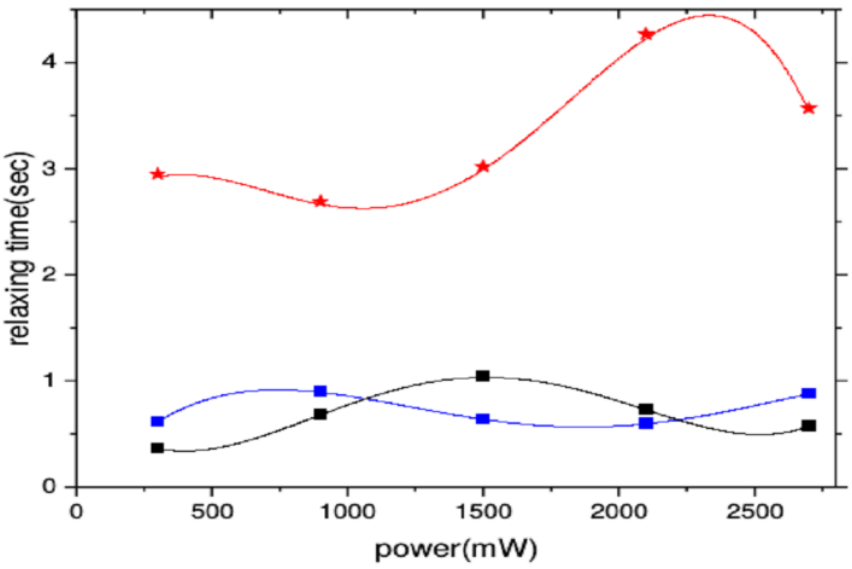

Fig.7. The relaxation time constant as a function of power for untreated (red), 3hrs treated (blue), and 6hrs treated (black).

Fig. 7 illustrates how changing the trap's power and time constant predict interesting behavior for the Relaxation rate of the A549 cells. Despite an increase in power, the samples show that the time constant first decreases before reaching a minimum value and then increases as power increases. From the three samples, we found that the lung cell relaxation increased with increasing trap power up to a certain limit. The time constant appears to increase when the power is increased beyond these limits, which means the cell relaxes gradually with increased power. From the three graphs in Fig. 7, we may predict that the time constant reaches a minimum value of 2.6s at about 900mW for the lung cells from the untreated sample minimum value of 0.4s at about 280mW from the 3hr treated sample, and a minimum of 0.6s at about 280mW.

A reasonable explanation for the fast relaxation rates (decrease in the time constant) up to this limit in these cases could be the inevitable heating effect resulting from increasing the power of the trapping laser[31] [31]. The wavelength of the infrared laser (1064nm) can penetrate the water molecules more strongly at relatively high powers, which could increase thermal vibrations within the lung cells, which would possibly lead to an increase in lung size[7,8,16] [7,8,16]. The heating process, however, would compromise the integrity of the cytoskeleton of lung cells, which is largely responsible for lung cells' viscoelastic properties. The untreated sample appears more susceptible to this effect than a treated sample. A recent study shows that treating normal cells with Suffruticosol B has significantly less toxicity[32][32].

Further, at extreme powers, we have observed the cell membranes rupture as they become charged and are ejected from the trap. At extremely low power, the results in Fig. 7 predict a higher time constant for the

lung cells from the treated sample as expected. Cell treatment with Suffruticosol B will provide a better therapeutic ratio.

Since the A549 lung cells are from a single patient, using statistical methods to track systematic variability in individual results and synthesize single-subject designs based on experimental replications presents a challenge. Thus, the next step will be to obtain these from multiple patients, who would improve this study's experimental technique to find more accurate and better predictive results. In the experimental technique, we used one trap to deform a single cell with a limitation in the number of cells we needed to study. The more cells are studied, the better predicting the mechanical properties in the specific sample. One can improve this limitation by creating multiple traps using an acoustic-optical deflector[33-36] [33-36]. Such a device allows trapping multiple cells simultaneously, and we can increase the number of cells studied per sample.

5. Conclusions

In this study, Suffruticosol. B. extracted from Paeonia suffruticosa, commonly used for anti-tumor treatment, has been applied to cultured human lung carcinoma cells (A549). An intensity gradient laser trap was used to analyze the similarities and differences between untreated, 3 hours treated, and 6-hour treated cells. Cells inside and outside the trap are measured by their relative sizes. This cell line responded interestingly to the treatment, which indicates that the duration of treatment had significant effects on its biophysical properties. We have used LT to show that the antitumor compound Suffruticosol. B. increases the radio sensitivity of the tumor cells.

Author Contribution: All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization* , D.B.E and M.S.; *Methodology* , D.B.E., M.S.; *Investigation* , M.S.; *Formal Analysis* , H.T.C and M.S.; *Resources* , Y.G. and D.B.E; *Writing - Original Draft* , M.S., D.B.E. and H.T.C.; *Writing - Review & Editing* , H.T.C; *Visualization* , D.B.E, M.S., and H.T.C.; *Supervision* , D.B.E and H.T.C.; *Funding Acquisition* , H.T.C.

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Disclosures. The authors declare that there was no commercial or financial relationship during this study that could be construed as a potential conflict of interest. The authors declare no competing interests.

Data availability. All data are available in the main text.

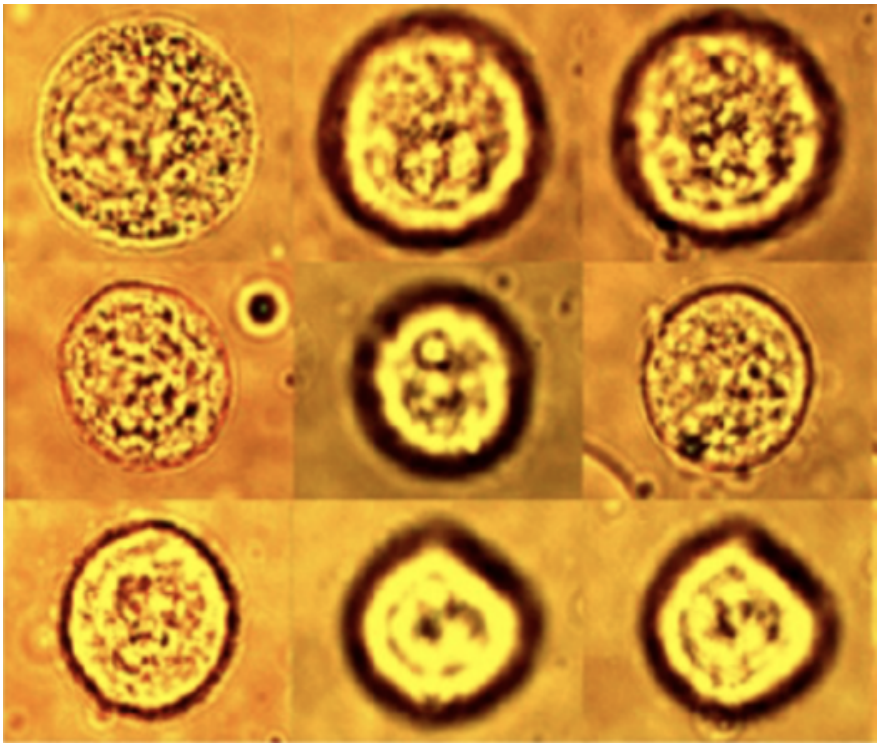

Fig. 8. Preview of thumbnail image display on the author submission page

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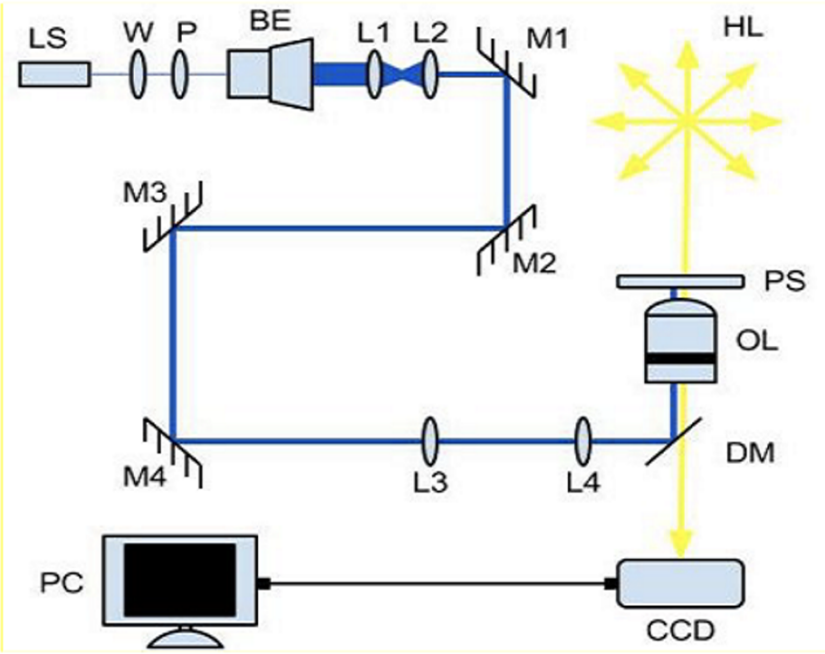
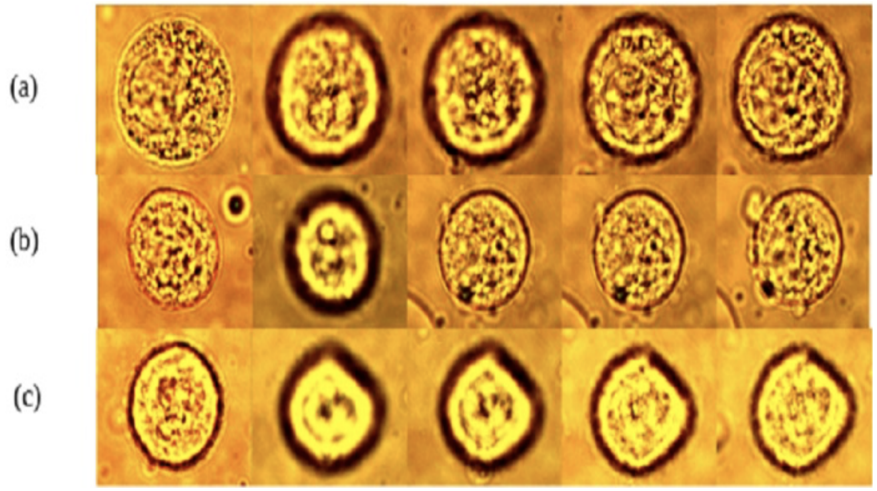
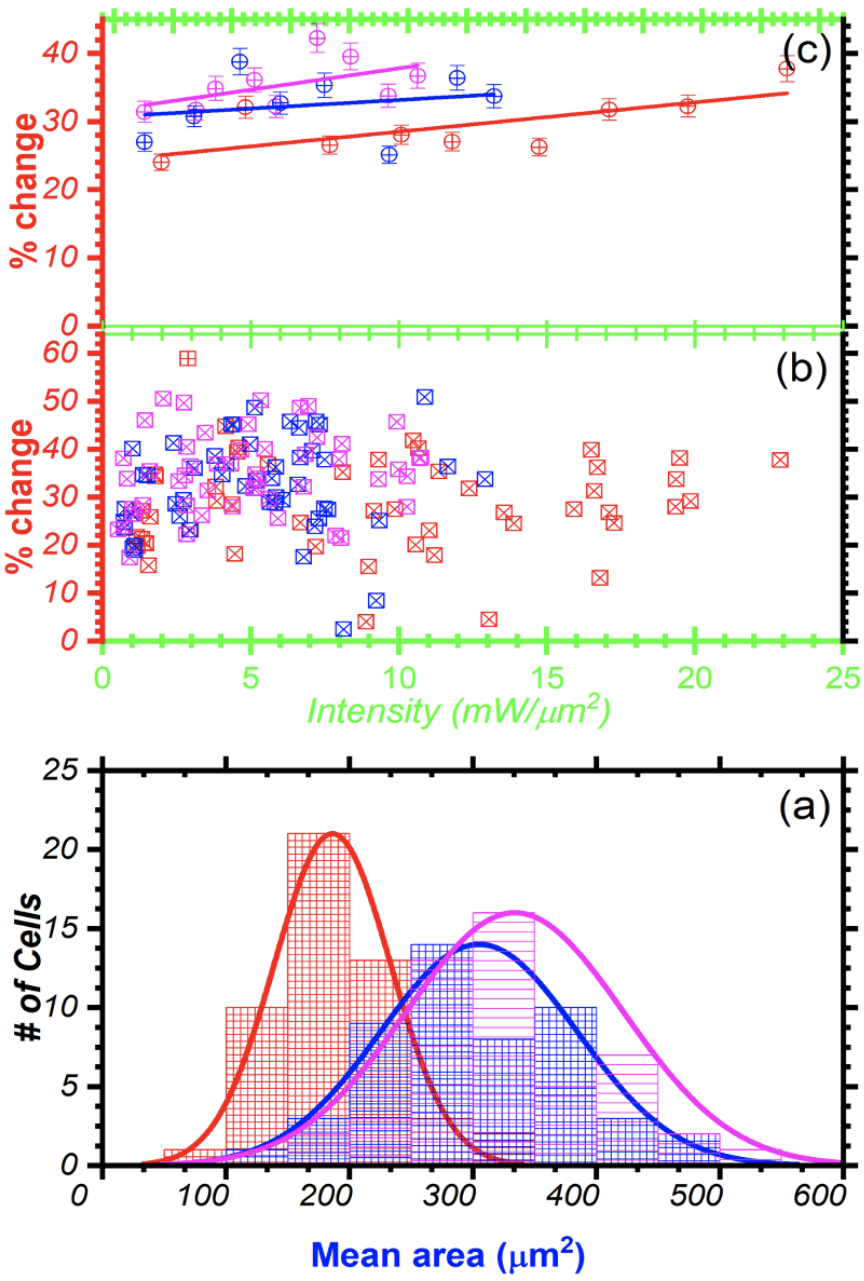
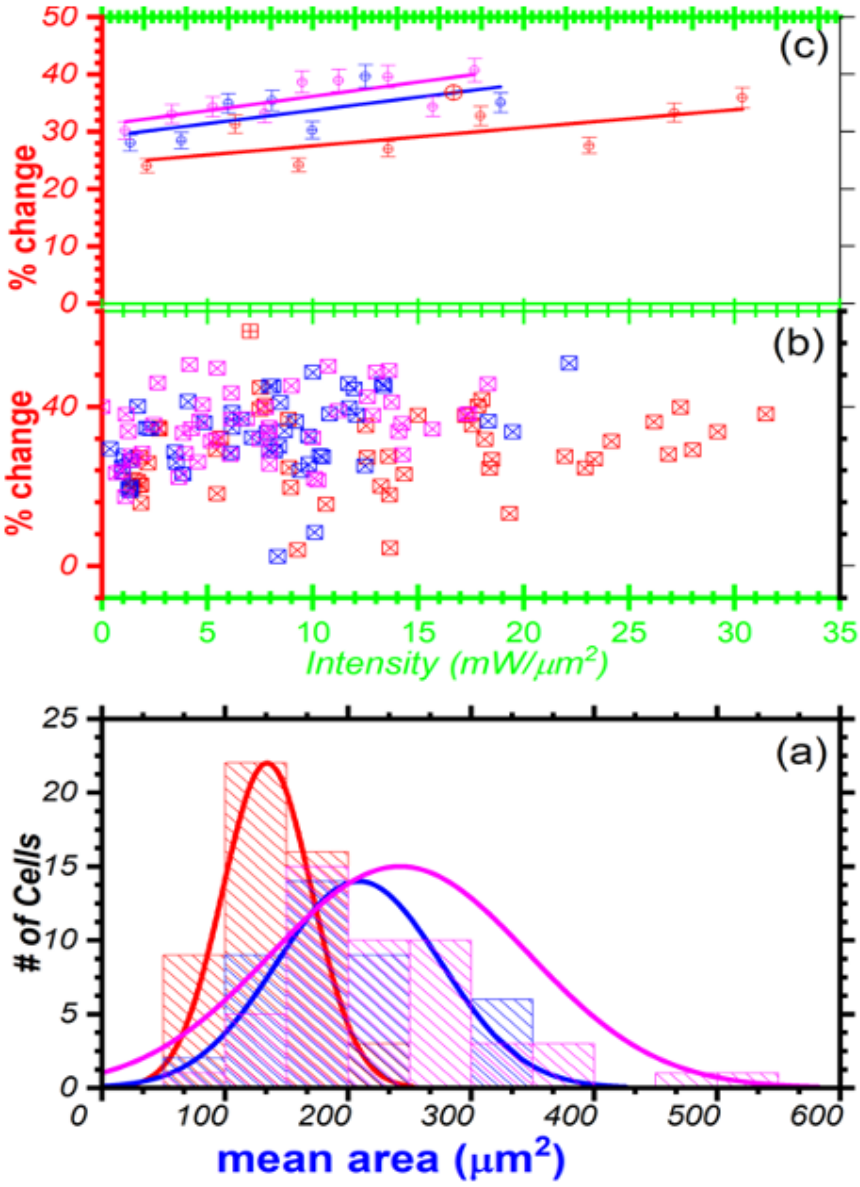
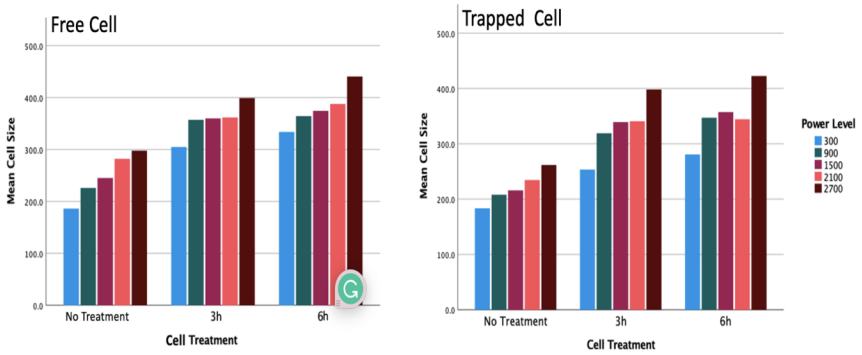


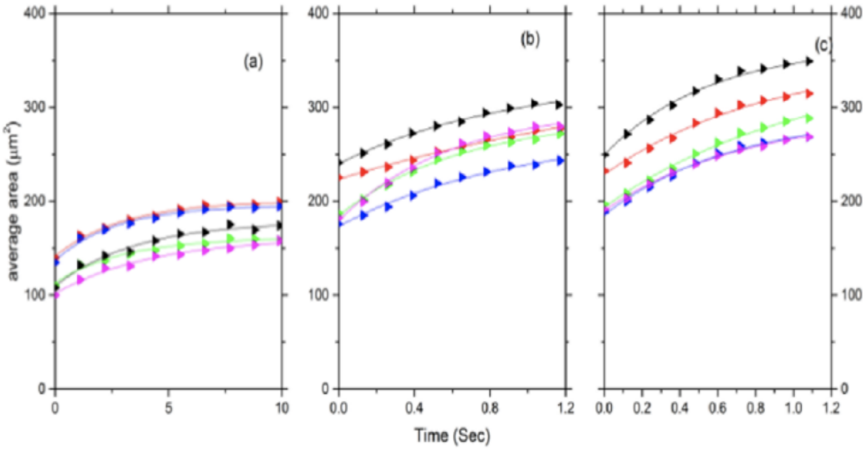


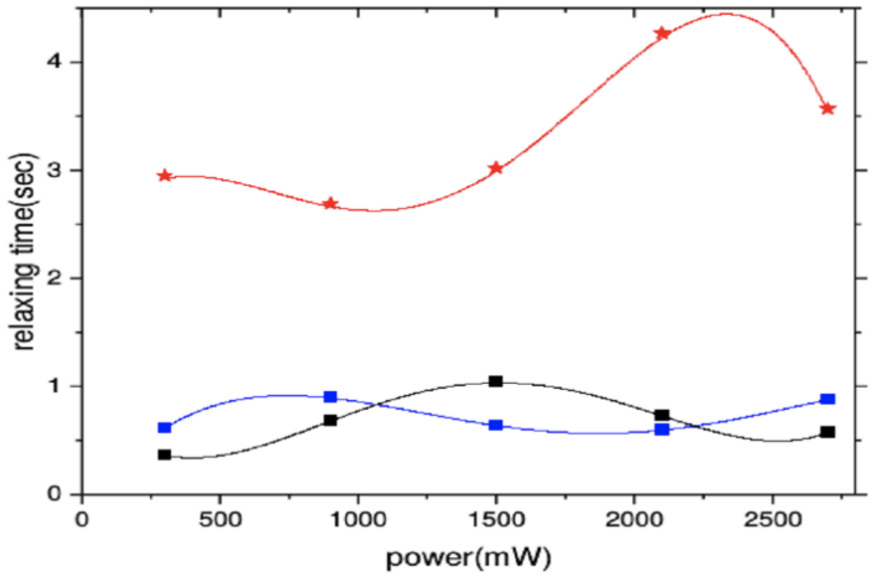


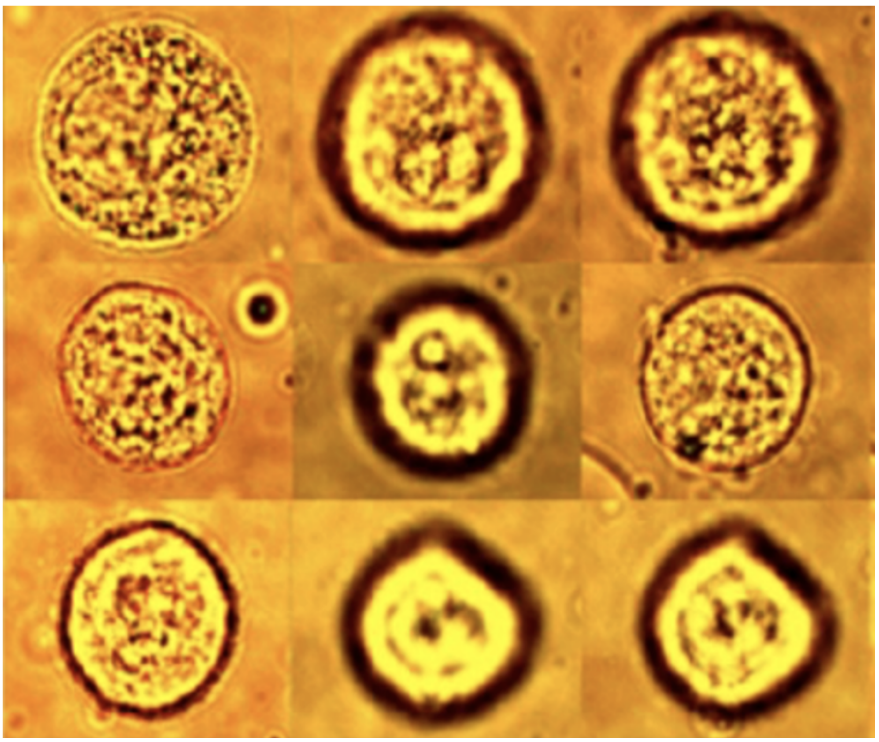


Cell size as a function of treatment length and power









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