Chapter 5 RESULTS

5.1 Effects of Low Level Laser on Cell Counts and Cell Viability

Trypan Blue staining can be used to measure cell viability after exposure to different treatments. The Trypan Blue dye exclusion method is based on the principle that live (viable cells) do not take up certain dyes, whereas dead (non-viable cells) do. Cellular proteins within nonviable cells take up this stain.

This series of experiments was carried out to establish cellular proliferation by direct cell counts as a comparison to the MTT assay and CFSE staining, cell counts were carried out daily using a haemocytometer. The utilisation of Trypan blue also determined the level of cell viability for the different levels of laser irradiation as compared to control groups.

5.1.1 Experimental Design

Cell numbers and cell viability of the osteosarcoma cell population irradiated at the different energy levels were compared with unirradiated cells. Single irradiation and daily irradiation experimental groups were compared.

5.1.2 Expression of Results

Results were expressed as cell number per ml and as percentage viability. Graphs illustrate mean cell number per ml $\pm$ standard deviation of three replicates of quadruplet wells and percentage viability from the same number of wells for the ten days. Counts from irradiated cultures were compared with those from unirradiated controls.

The significance of any difference between cultures was determined using a 2 tailed paired Student's t-test.

5.1.3 Results

Figure 5.1.1 Trypan Blue cell counts of osteosarcoma cells irradiated with a single dose at an energy level of 0.5 Joules compared to unirradiated control cells.

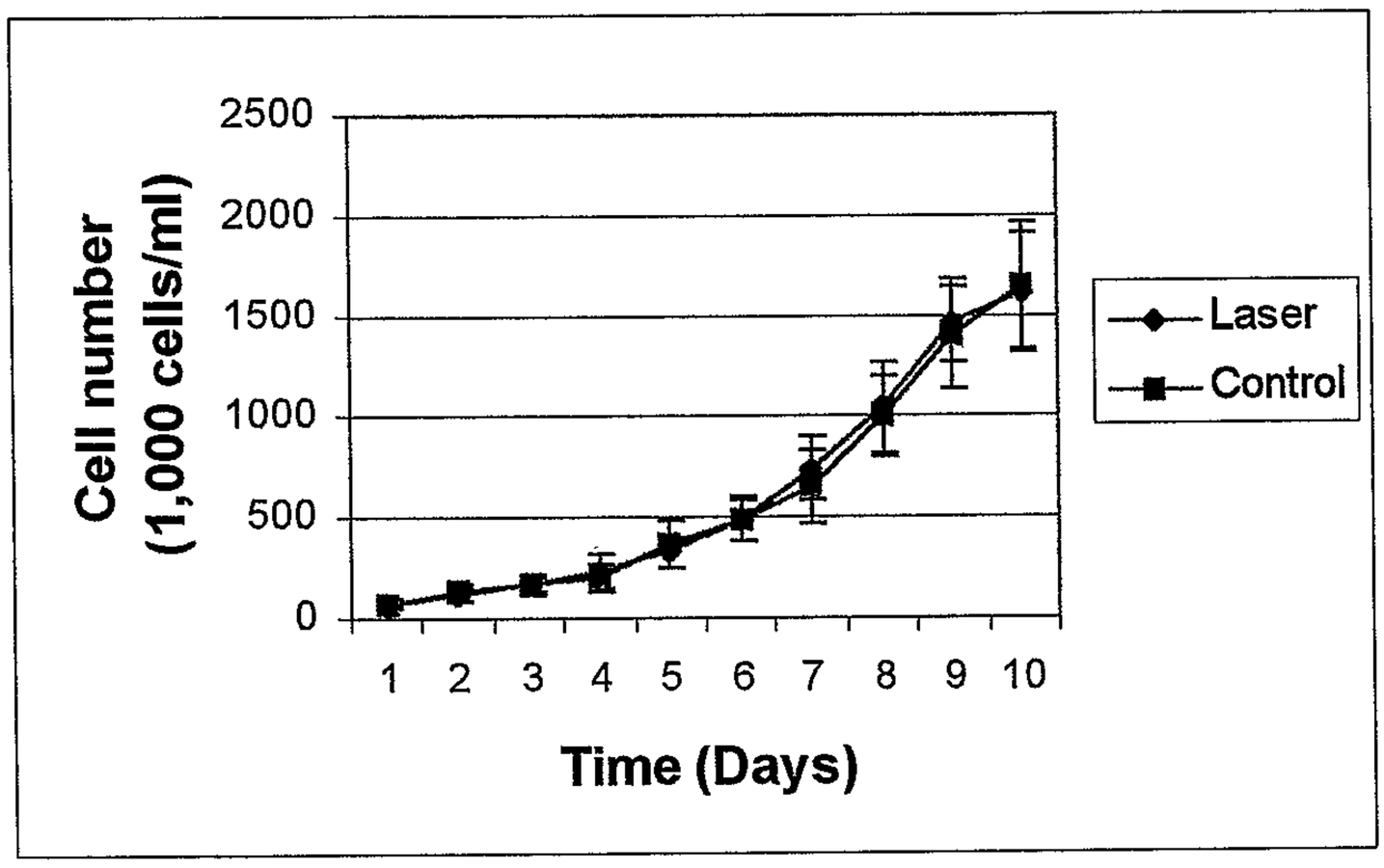


Figure 5.1.2 Percentage viability of cells irradiated at 0.5 Joules as a single dose compared to unirradiated control cells, over the ten day period, utilising Trypan Blue exclusion.

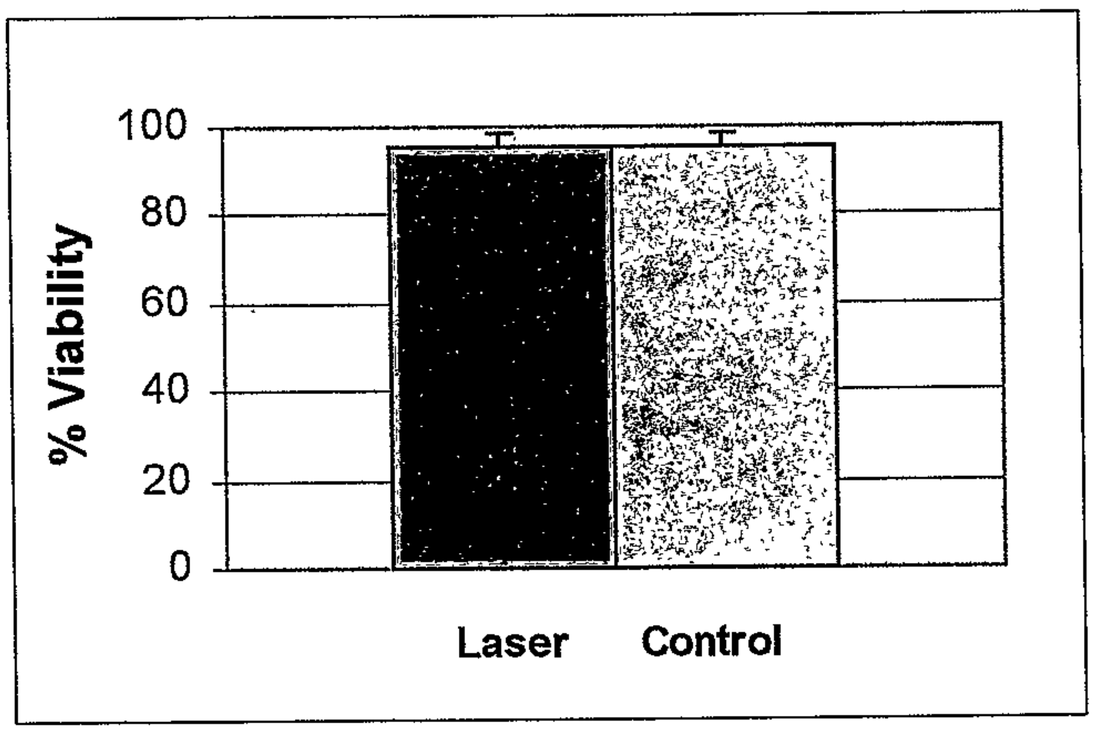


Table 5.1.1 Significance

Day	p value	p<0.05	p< 0.01
1	0.845	-	-
2	0.646	-	-
3	0.980	-	-
4	0.126	-	-
5	0.302	-	-
6	0.915	-	-
7	0.188	-	-
8	0.607	-	-
9	0.571	-	-
10	0.795	-	-
Viability	0.954	-	-

Figure 5.1.3 Trypan Blue cell counts of osteosarcoma cells irradiated with a single dose at an energy level of 1.0 Joules compared to unirradiated control cells.

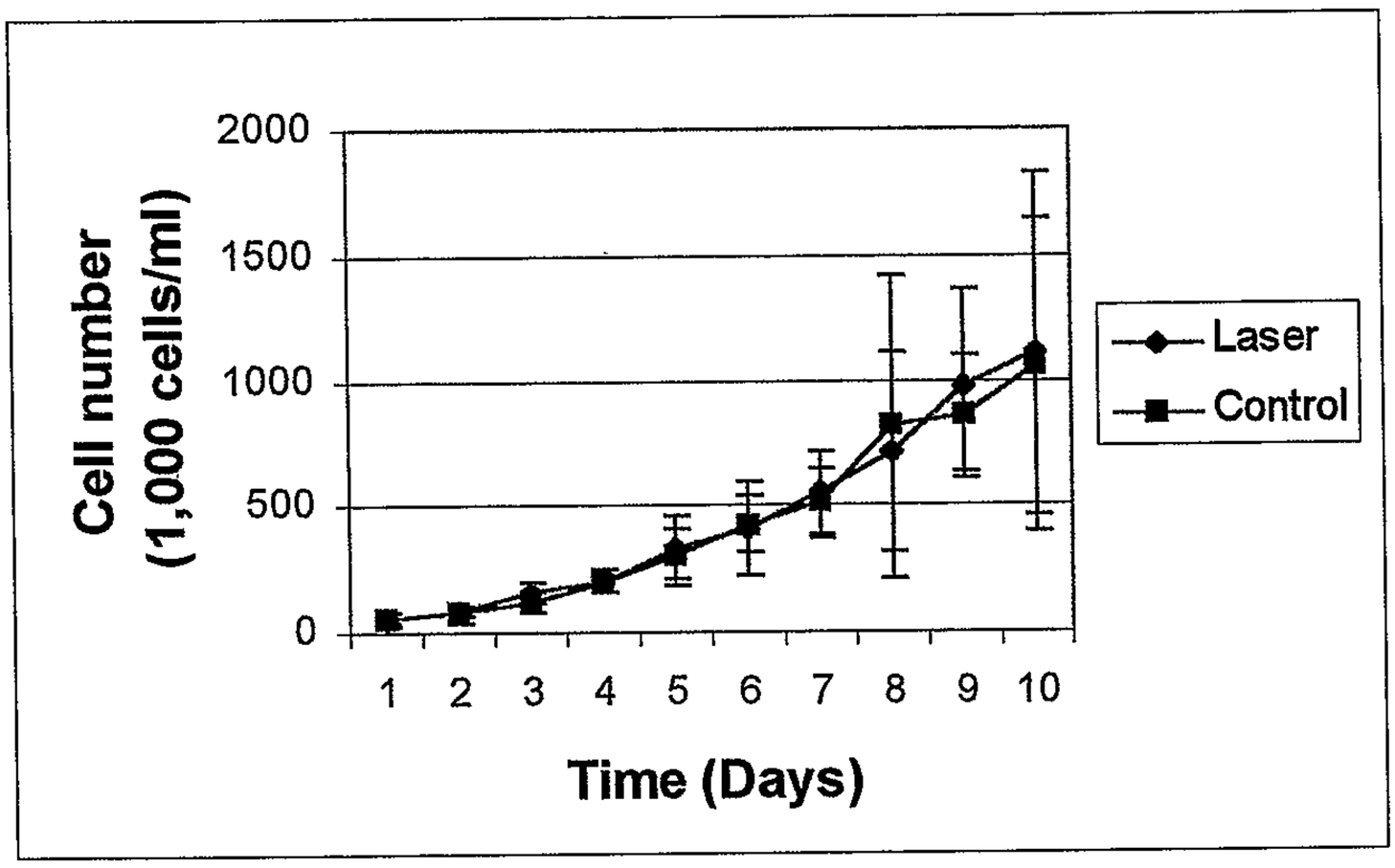


Figure 5.1.4 Percentage viability of cells irradiated at 1.0 Joules as a single dose compared to unirradiated control cells utilising Trypan Blue exclusion.

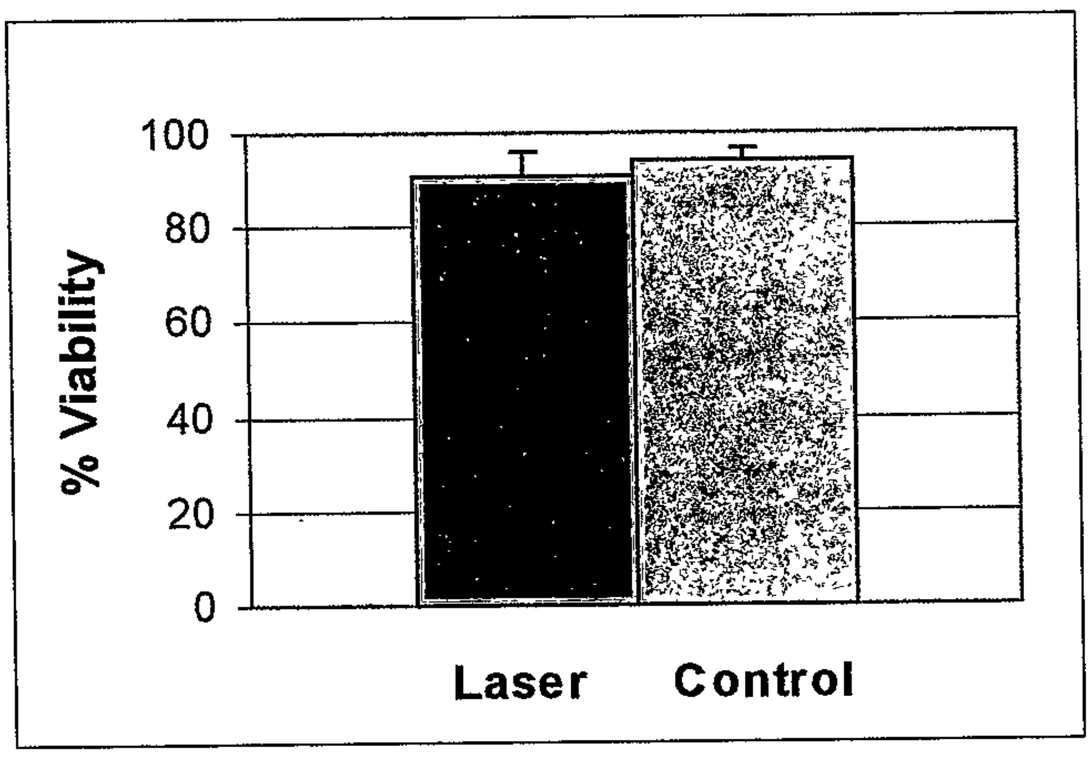


Table 5.1.2 Significance

Day	p value	p<0.05	p< 0.01
1	0.965	-	-
2	0.277	-	-
3	0.078	-	-
4	0.518	-	-
5	0.296	-	-
6	0.719	-	-
7	0.398	-	-
8	0.322	-	-
9	0.306	-	-
10	0.664	-	-
Viability	0.081	-	-

Figure 5.1.5 Trypan Blue cell counts of osteosarcoma cells irradiated with a single dose at an energy level of 2.0 Joules compared to unirradiated control cells.

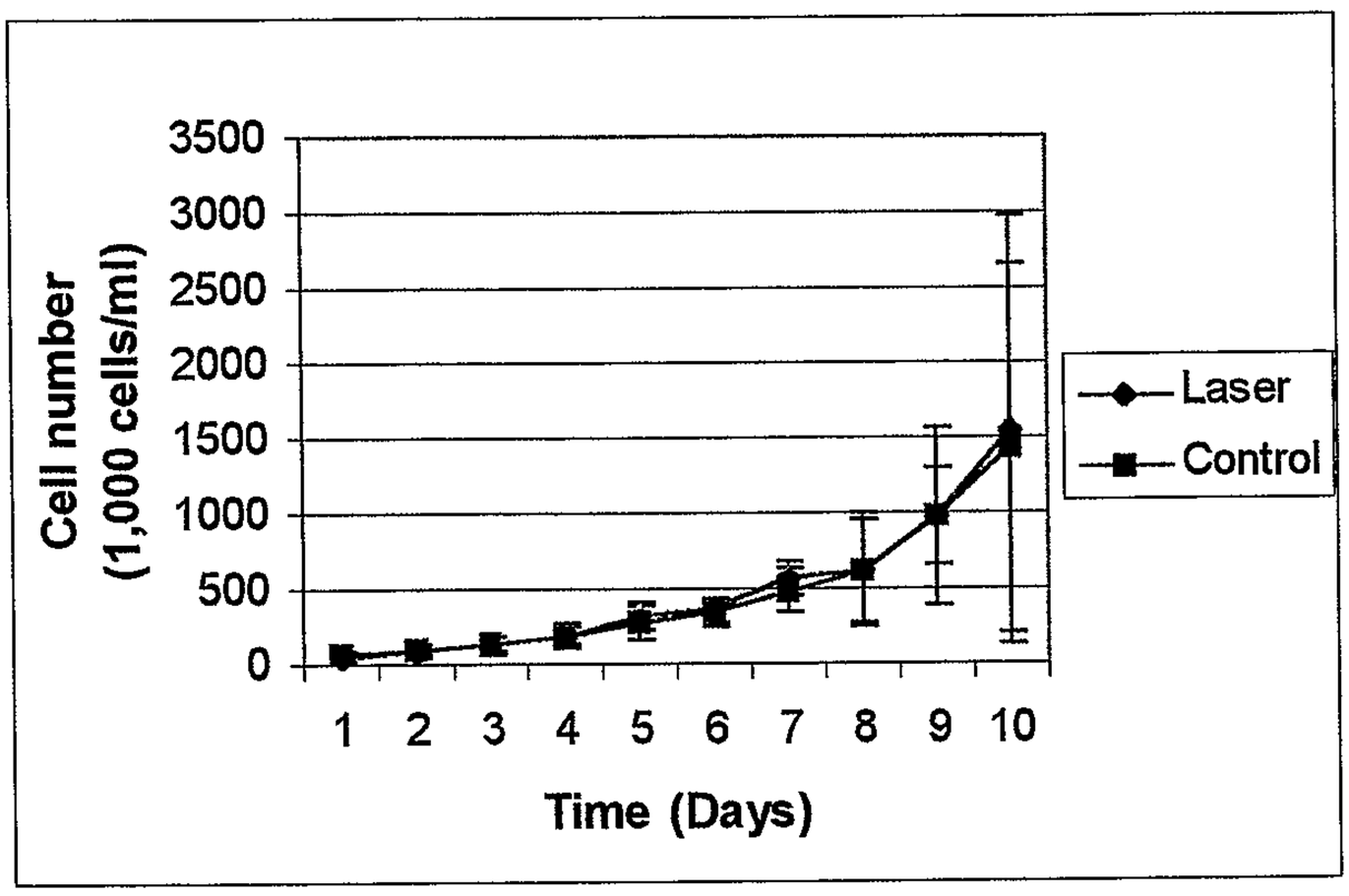


Figure 5.1.6 Percentage viability of cells irradiated at 2.0 Joules as a single dose compared to unirradiated control cells utilising Trypan Blue exclusion.

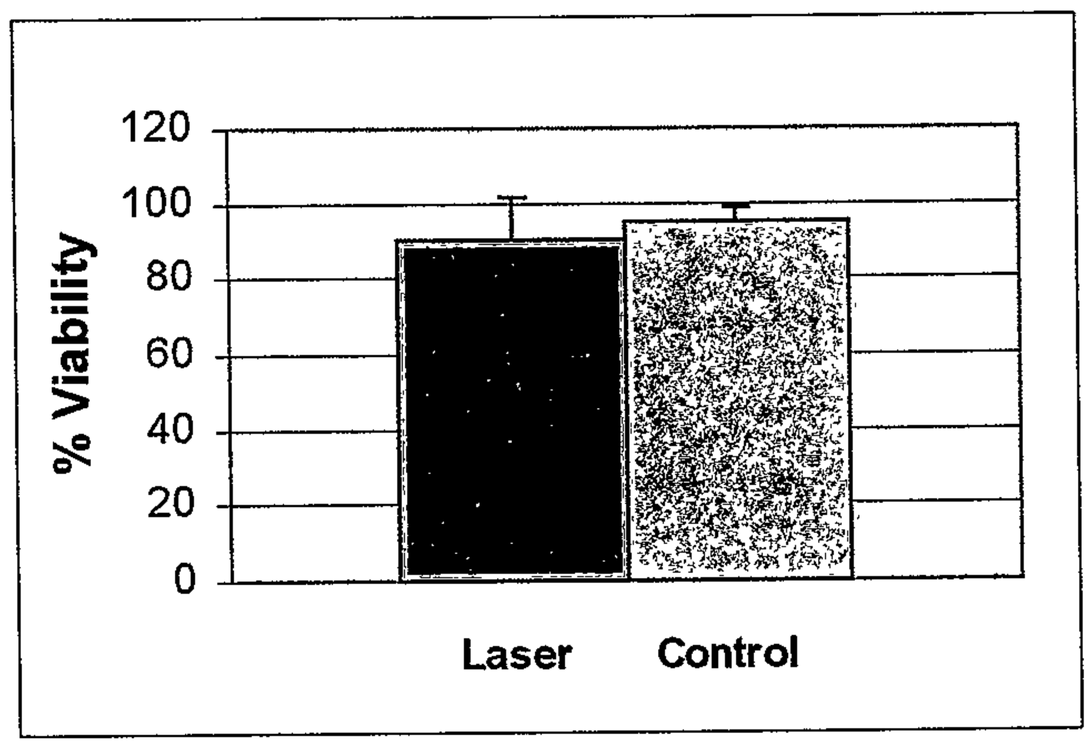


Table 5.1.3 Significance

Day	p value	p<0.05	p< 0.01
1	0.689	-	-
2	0.369	-	-
3	0.839	-	-
4	0.907	-	-
5	0.248	-	-
6	0.536	-	-
7	0.053	*	-
8	0.926	-	-
9	0.979	-	-
10	0.774	-	-
Viability	0.135	-	-

Figure 5.1.7 Trypan Blue cell counts of osteosarcoma cells irradiated with a single dose at an energy level of 4.0 Joules compared to unirradiated control cells.

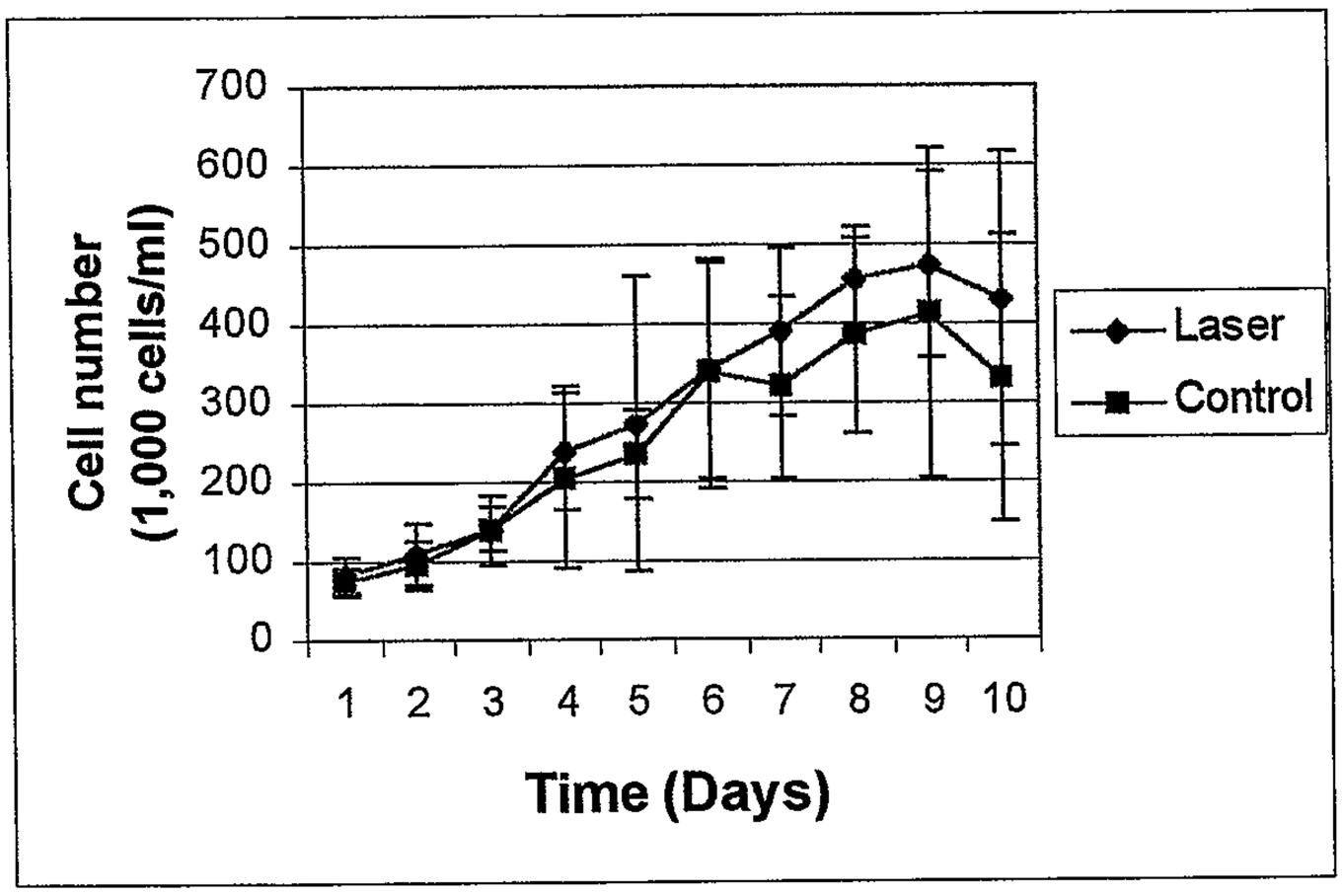


Figure 5.1.8 Percentage viability of cells irradiated at 4.0 Joules as a single dose compared to unirradiated control cells utilising Trypan Blue exclusion.

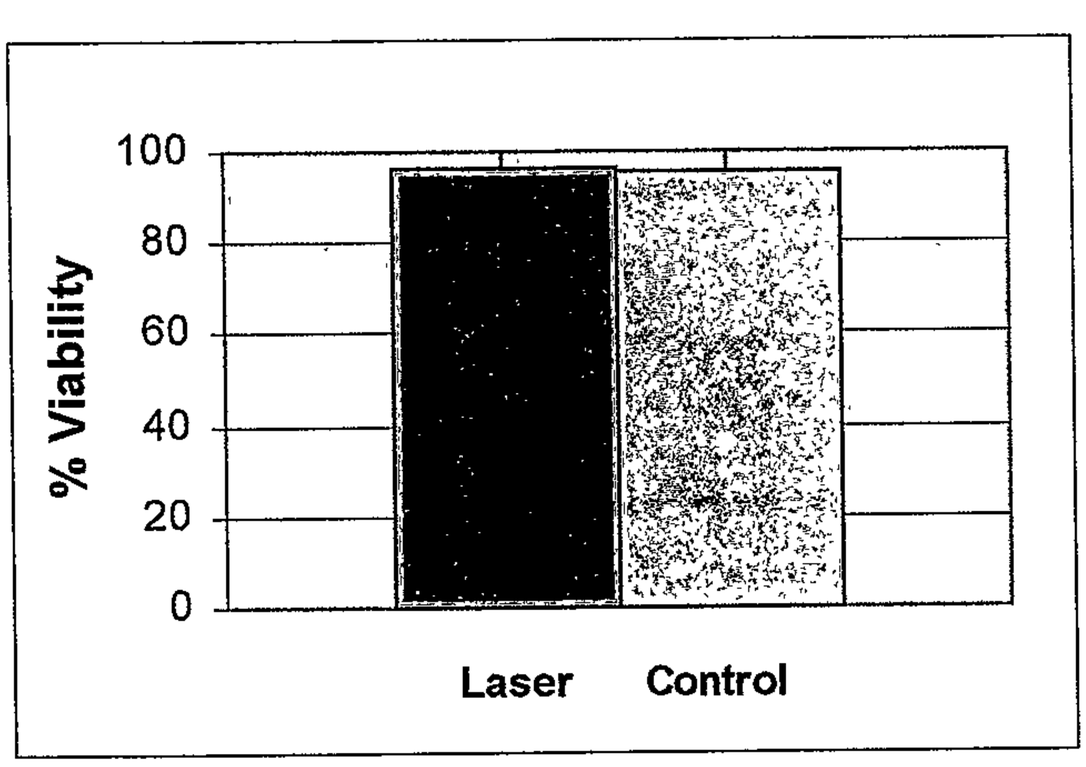


Table 5.1.4 Significance

Day	p value	p<0.05	p< 0.01
1	0.091	-	-
2	0.393	-	-
3	0.910	-	-
4	0.434	-	-
5	0.480	-	-
6	0.863	-	-
7	0.011	*	*
8	0.088	-	-
9	0.220	-	-
10	0.038	*	-
Viability	0.259	-	-

Figure 5.1.9 Trypan Blue cell counts of osteosarcoma cells irradiated daily at an energy level of 0.5 Joules compared to unirradiated control cells.

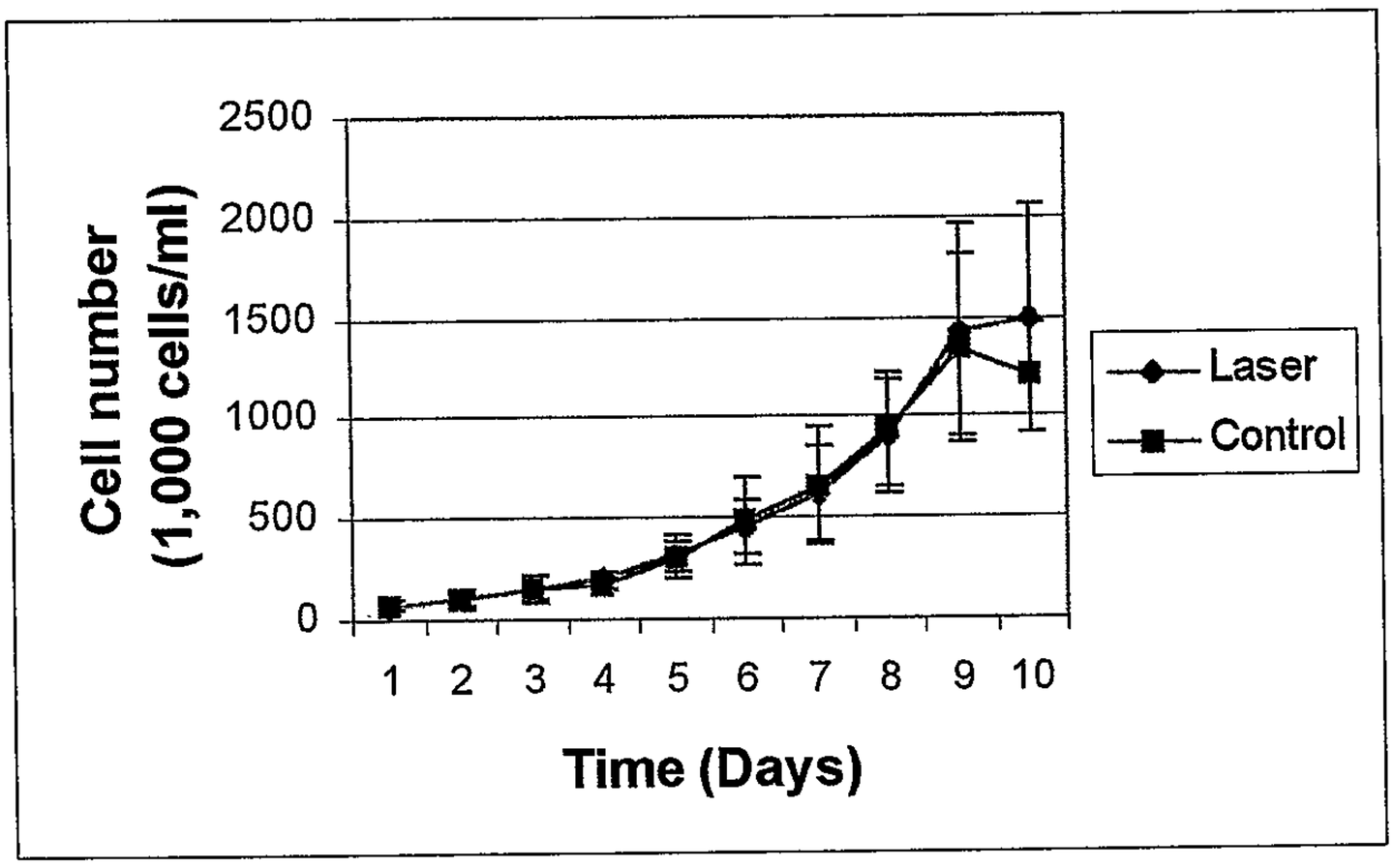


Figure 5.1.10 Percentage viability of cells irradiated at 0.5 Joules daily compared to unirradiated control cells utilising Trypan Blue exclusion.

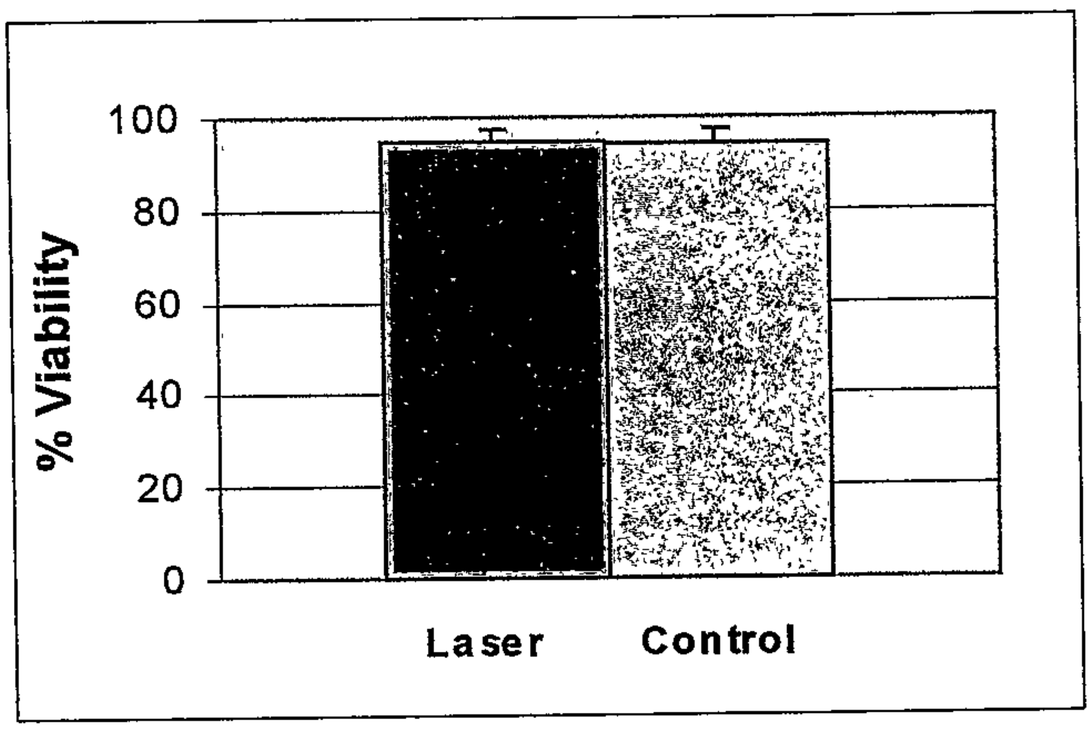


Table 5.1.5 Significance

Day	p value	p<0.05	p< 0.01
1	0.785	-	-
2	0.881	-	-
3	0.558	-	-
4	0.164	-	-
5	0.480	-	-
6	0.429	-	-
7	0.394	-	-
8	0.563	-	-
9	0.305	-	-
10	0.149	-	-
Viability	1.892	-	-

Figure 5.1.11 Trypan Blue cell counts of osteosarcoma cells irradiated daily at an energy level of 1.0 Joules compared to unirradiated control cells.

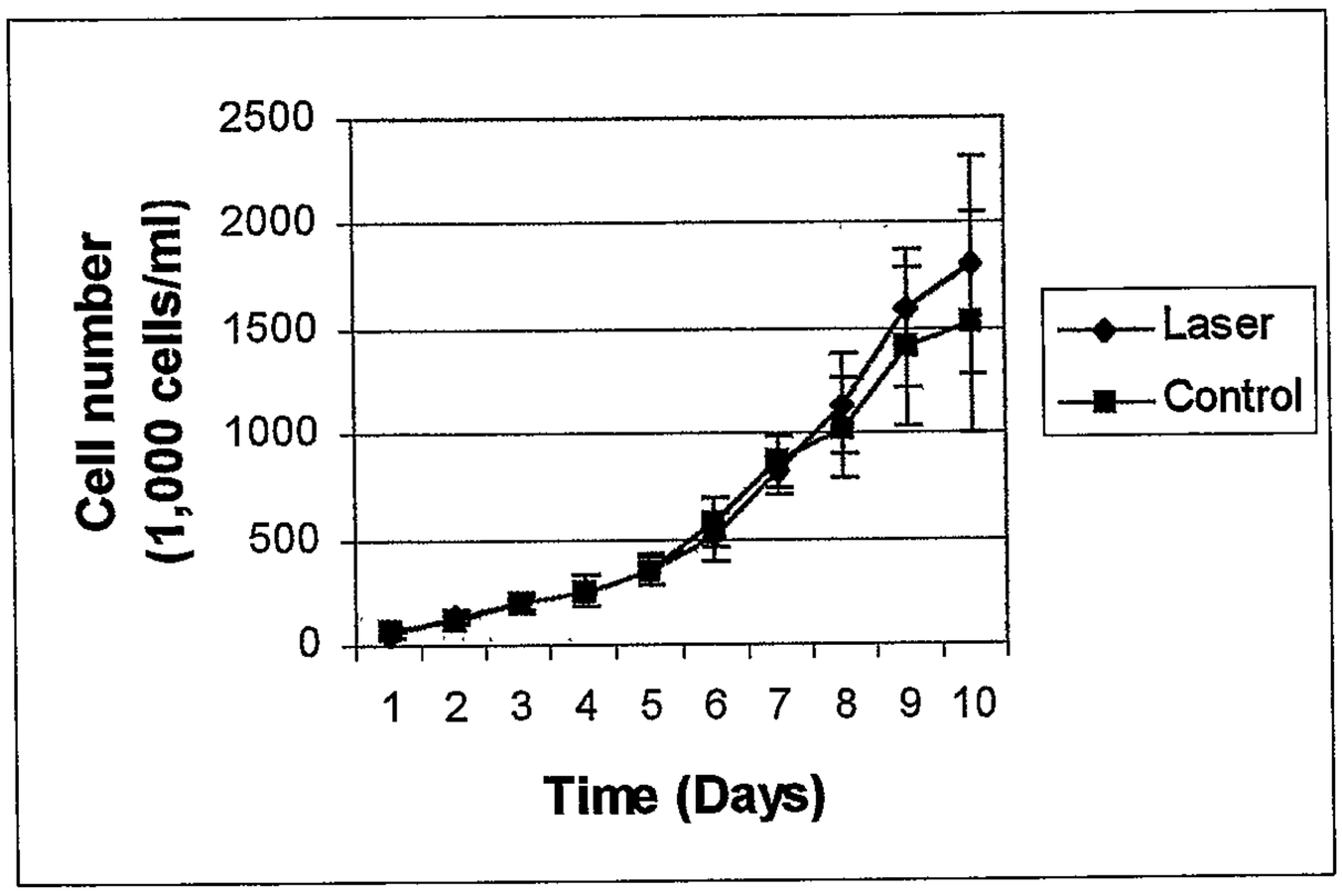


Figure 5.1.12 Percentage viability of cells irradiated at 1.0 Joules daily compared to unirradiated control cells utilising Trypan Blue exclusion.

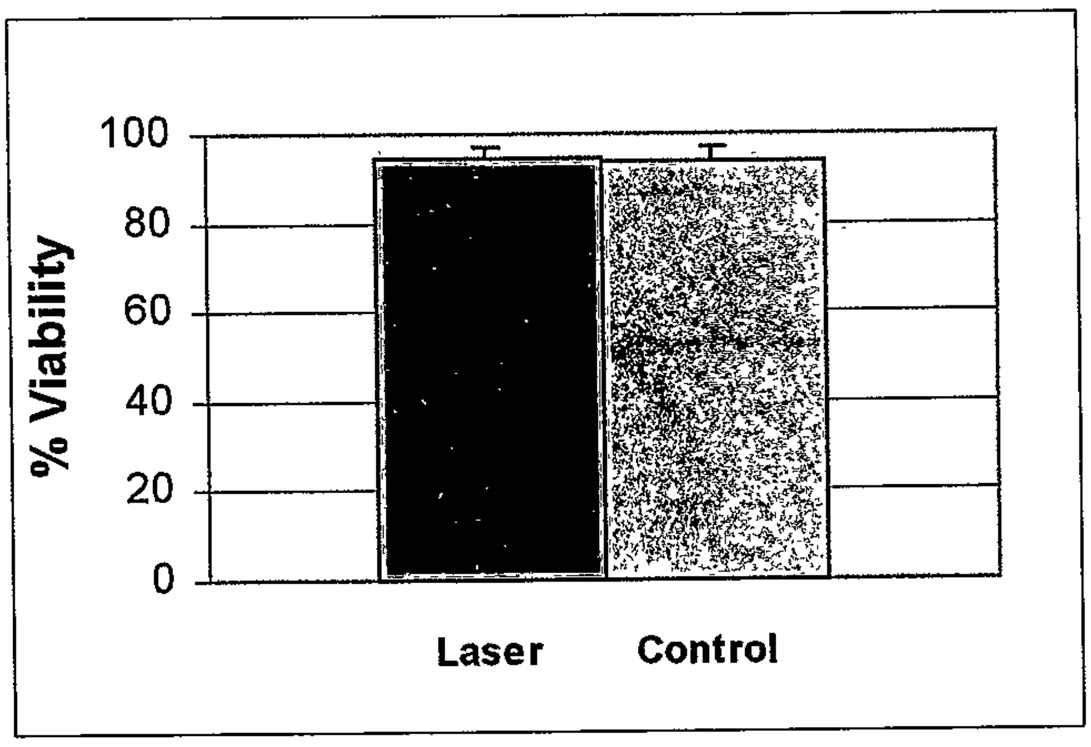


Table 5.1.6 Significance

Day	p value	p<0.05	p< 0.01
1	0.368	-	-
2	0.131	-	-
3	0.843	-	-
4	0.874	-	-
5	0.853	-	-
6	0.018	*	-
7	0.459	-	-
8	0.339	-	-
9	0.207	-	-
10	0.188	-	-
Viability	0.518	-	-

Figure 5.1.13 Trypan Blue cell counts of osteosarcoma cells irradiated daily at an energy level of 2.0 Joules compared to unirradiated control cells.

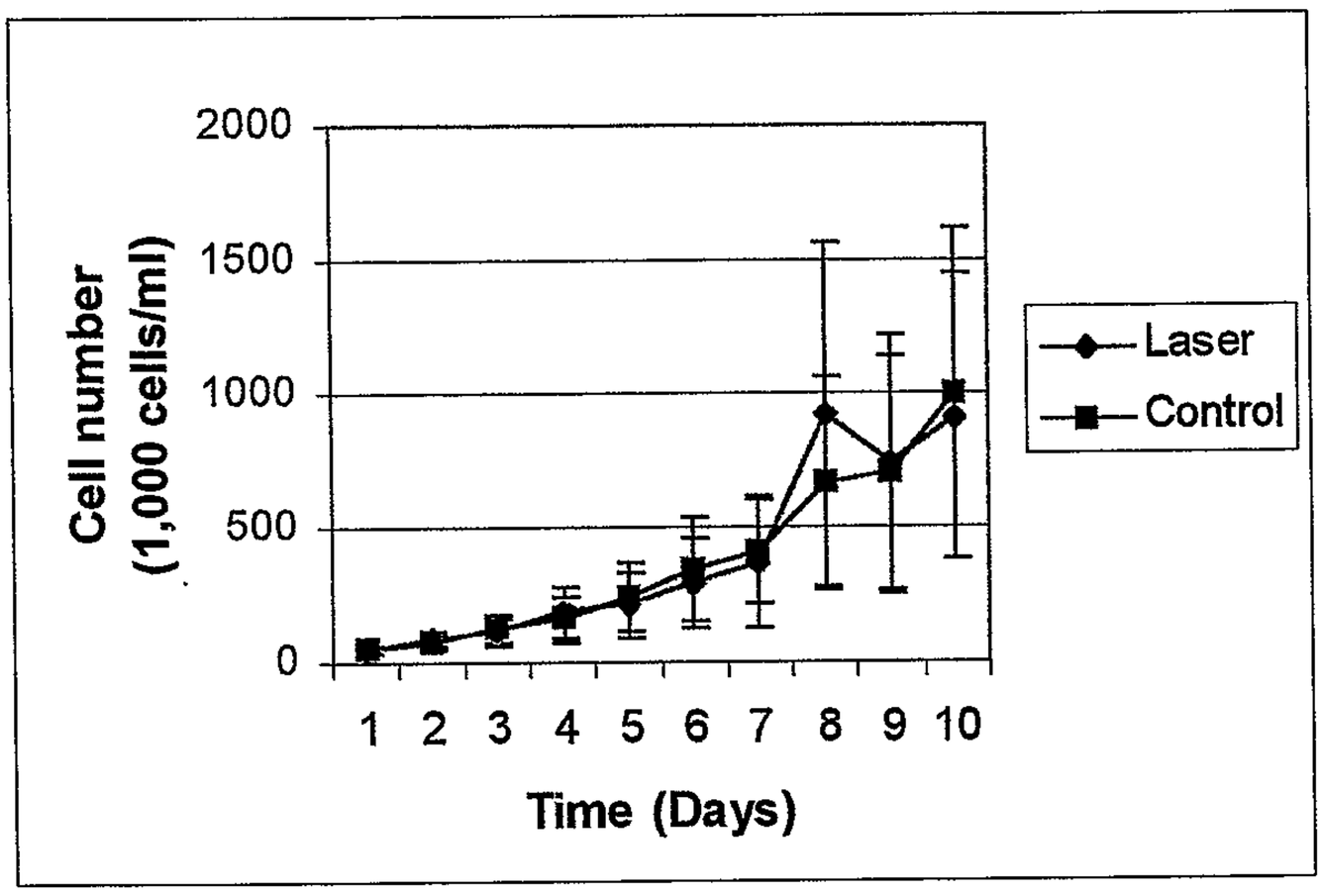


Figure 5.1.14 Percentage viability of cells irradiated at 2.0 Joules daily compared to unirradiated control cells utilising Trypan Blue exclusion.

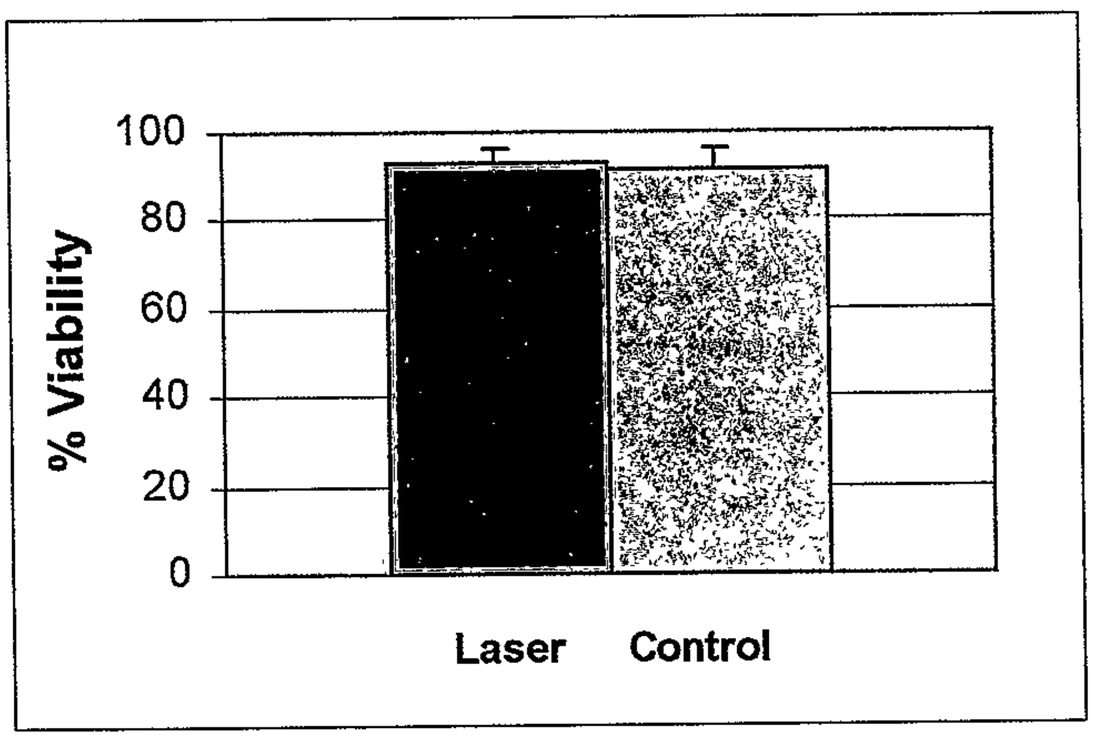


Table 5.1.7 Significance

Day	p value	p<0.05	p< 0.01
1	0.761	-	-
2	0.441	-	-
3	0.285	-	-
4	0.272	-	-
5	0.192	-	-
6	0.183	-	-
7	0.527	-	-
8	0.029	*	-
9	0.655	-	-
10	0.423	-	-
Viability	0.327	-	-

Figure 5.1.15 Trypan Blue cell counts of osteosarcoma cells irradiated daily at an energy level of 4.0 Joules compared to unirradiated control cells.

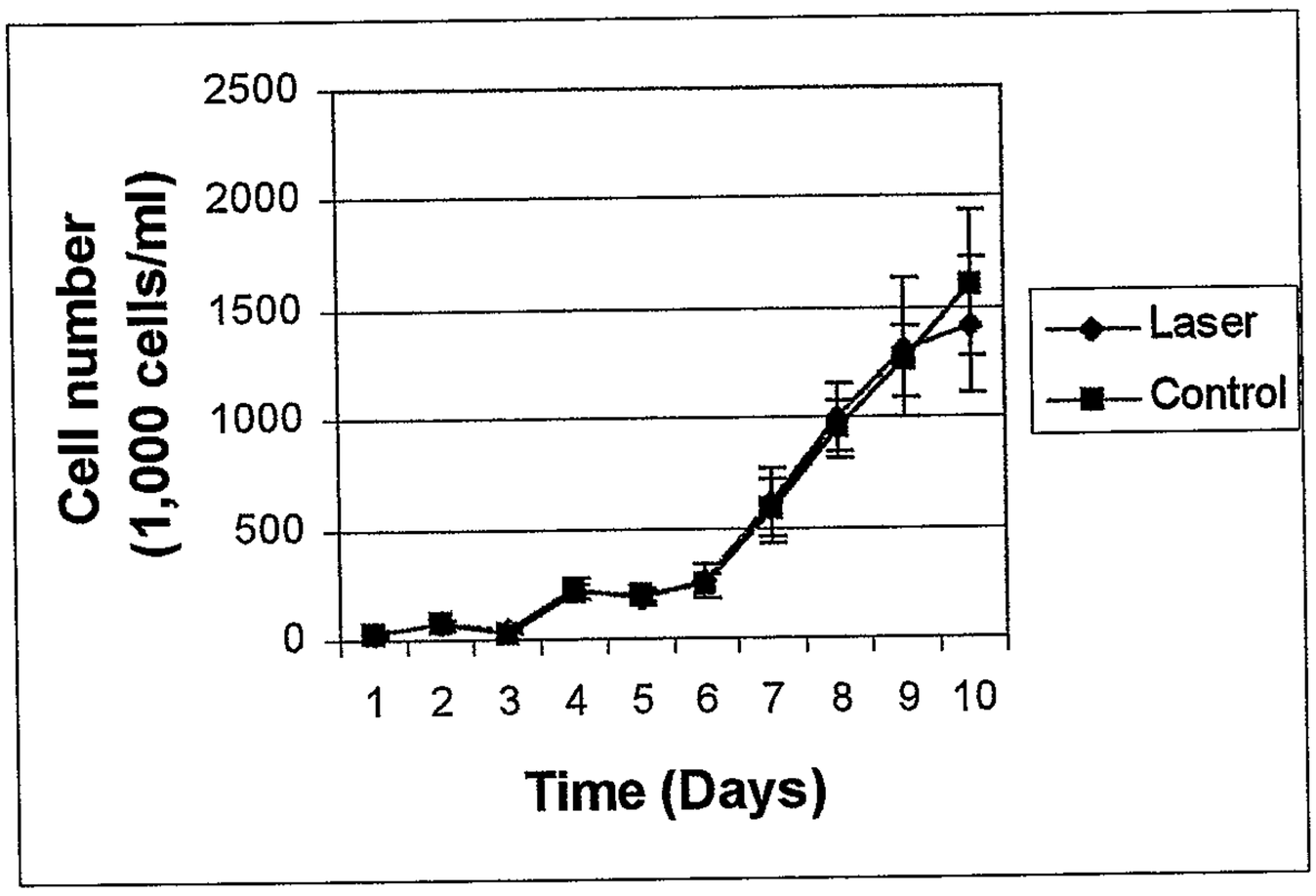


Figure 5.1.16 Percentage viability of cells irradiated at 4.0 Joules daily compared to unirradiated control cells utilising Trypan Blue exclusion.

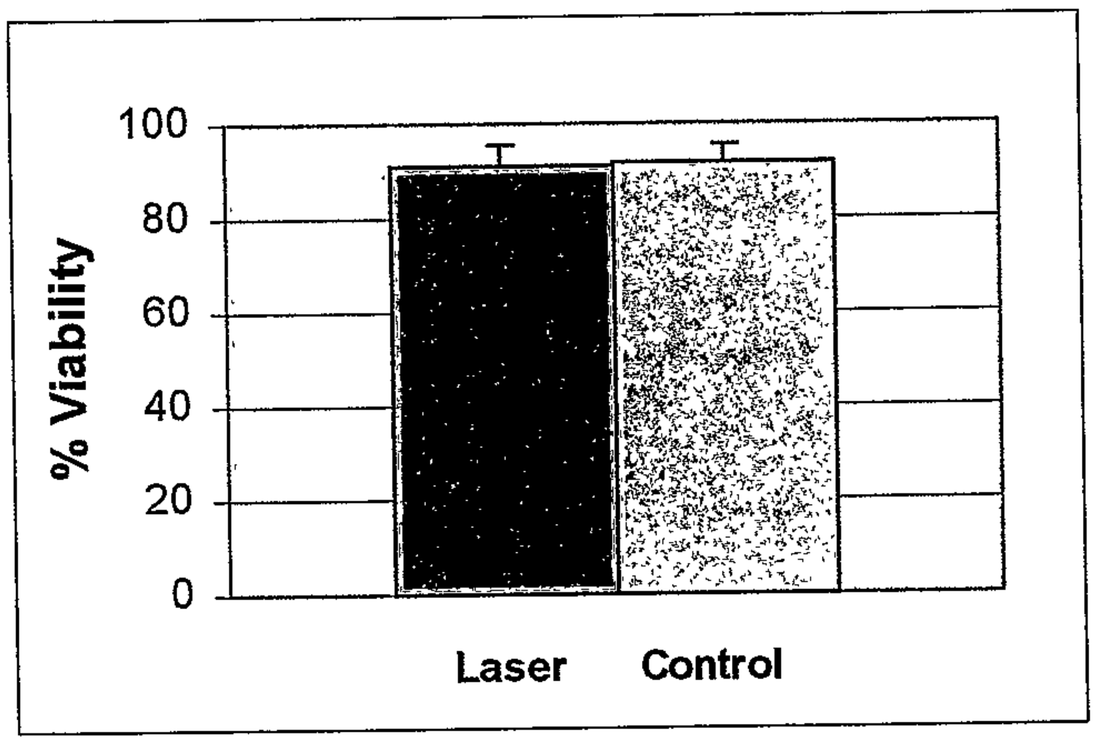


Table 5.1.8 Significance

Day	p value	p<0.05	p< 0.01
1	0.764	-	-
2	0.704	-	-
3	0.195	-	-
4	0.354	-	-
5	0.327	-	-
6	0.417	-	-
7	0.092	-	-
8	0.382	-	-
9	0.599	-	-
10	0.220	-	-
Viability	0.363	-	-

Cell viability was greater than 90% in experimental and control groups. There were no differences in cell numbers when comparing experimental and control groups.

Overall there were no differences in cell numbers over the ten day period when comparing experimental and control groups. Standard deviation of the samples was increased, particularly after day seven. This is to be expected as the cell cultures deteriorate with time.

There was a tendency for an increase in cell numbers in a number of the laser-irradiated groups around day seven. A single irradiation of 2 Joules showed a statistically significant increase on day seven at the 5% level. Single irradiation of 4 Joules on day seven at the 1% level. Day six of daily irradiation of 1 Joule showed a statistically significant increase at the 5% level. Day eight of daily at 2 Joules showed a statistically significant increase at the 5% level.

5.2 Effects of Low Level Laser Irradiation on Cellular Activity and Proliferation

MTT assay (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

The MTT tetrazolium salt colorimetric assay described by Mosmann (1983) is a quantitative assay used as a measure of mammalian cell survival and proliferation. Living but not dead cells reduce the yellow MTT tetrazolium salt to the coloured blue formazan product which is readily detected by its absorbance between 540 and 590nm. The assay reflects the total culture biomass rather than the rate of proliferation (Mosmann 1983).The signal generated is dependent on the degree of activation of the cells (Gerlier et al. 1986), and the method can therefore be used to measure cytotoxicity, proliferation or activation.

Proliferation was more specifically investigated utilising the intracellular fluorescent dye CFSE (see section 5.3).

The amount of formazan generated is directly proportional to the cell number if a homogeneous cell population is used, with activated cells producing more formazan than resting cells (Mosmann 1983).

The dye MTT is cleaved by the mitochondrial enzyme succinate dehydrogenase (an integral part of the inner mitochondrial membrane [Stryer 1988]) to yield an insoluble blue product which can be dissolved in ethanol and the concentration quantified by spectrophotometric analysis (Hansen *et al.* 1989). The results can be read on a multiwell spectrophotometer (Titertek®Plus) and are shown to have a high degree of precision (Mosmann 1983).

Quantitating cell numbers of various cell lines has been found to be highly reproducible using this method (Loveland *et al.*1992).

An almost linear relationship has been found (Hansen *et al.* 1989) between the optical density signals and the number of days cells are in culture until day 5. After this time either cell death or a drop in the optical density curve indicates down-regulation of the cellular metabolism. This has been found to be avoided if the cells are re-fed every third day resulting in a continuous linearity in the development of optical densities.

The aim of this series of experiments was to determine the effects of Low Level Laser irradiation, at different energy levels and exposure regimes, on cellular activity and proliferation.

5.2.1 Expression of results

Results are expressed as changes in optical density (nm) over a ten day period. Control and experimental groups are represented on each graph. Each point in the graphs below represents an average value of three replicate experiments of four samples each.

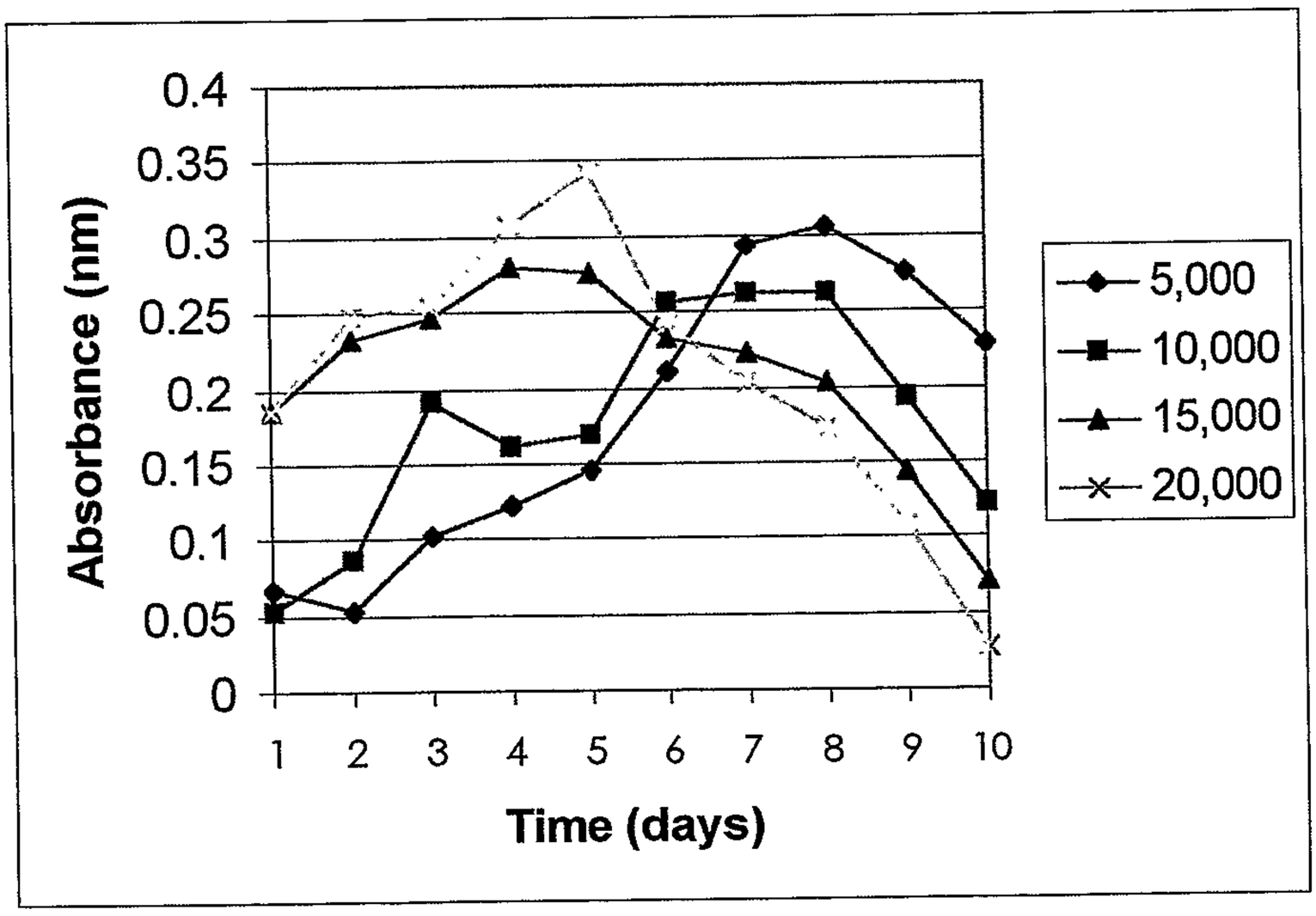
5.2.2 Results

Initial experiments showed excessive variability in the reproducibility. It has been reported that alcohol precipitates serum proteins (Sandberg 1977), giving rise to light scattering phenomena. Experiments carried out with medium containing serum gave inconsistent and inaccurate results, with poor reproducibility. It was found that using serum free medium for the experiment alleviated this problem.

Determination of plating efficiency

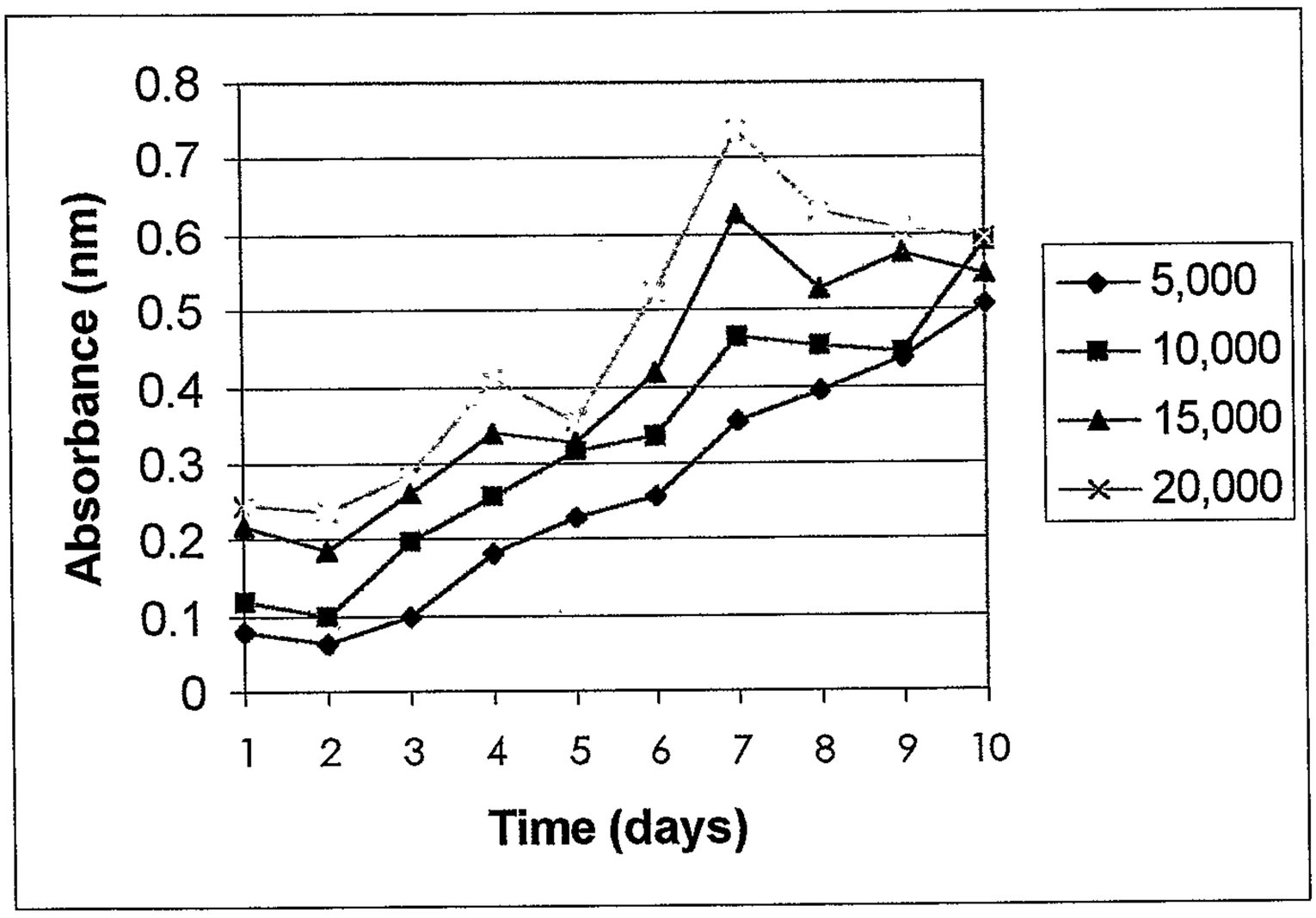
96-well microplates were seeded at densities of 5,000, 10,000, 15,000 and 20,000 cells per well. The plates were analysed daily for a period of ten days with the MTT assay.

Figure 5.2.1 This series was carried out to determine the most efficient seeding density for the ten day time period whilst observing the effects of not changing the medium for the entire experimental period.



For the cells seeded at densities of 15,000 and 20,000 cells per well the optical density was seen to drop off after day five, due to either cell death or down regulation. For the cells seeded at densities of 5,000 and 10,000 cells per well the optical density was observed to drop off after day eight.

Figure 5.2.2 This series was carried out to determine the most efficient seeding density for the ten day time period whilst observing the effects of changing the culture medium every three days for the experimental period.



For the cells seeded at densities of 15,000 and 20,000 cells per well the optical density was seen to drop off after day seven. For the cells seeded at densities of 5,000 and 10,000 cells per well the optical density was not observed to drop off over the ten day period.

The seeding density of 10,000 cells per well was used for all subsequent experiments utilising the MTT assay. This seeding density was also used for the Trypan Blue cell counts and viability determination to be used in conjunction with the MTT assay results. This seeding density was chosen as the optical density was seen to increase for the entirety of the ten day period, with higher readings than those from the wells seeded at 5,000 cells.

Figure 5.2.3 MTT assay for a single irradiation of 0.5 Joules, anlaysed for a period of ten days, comparing experimental and control groups.

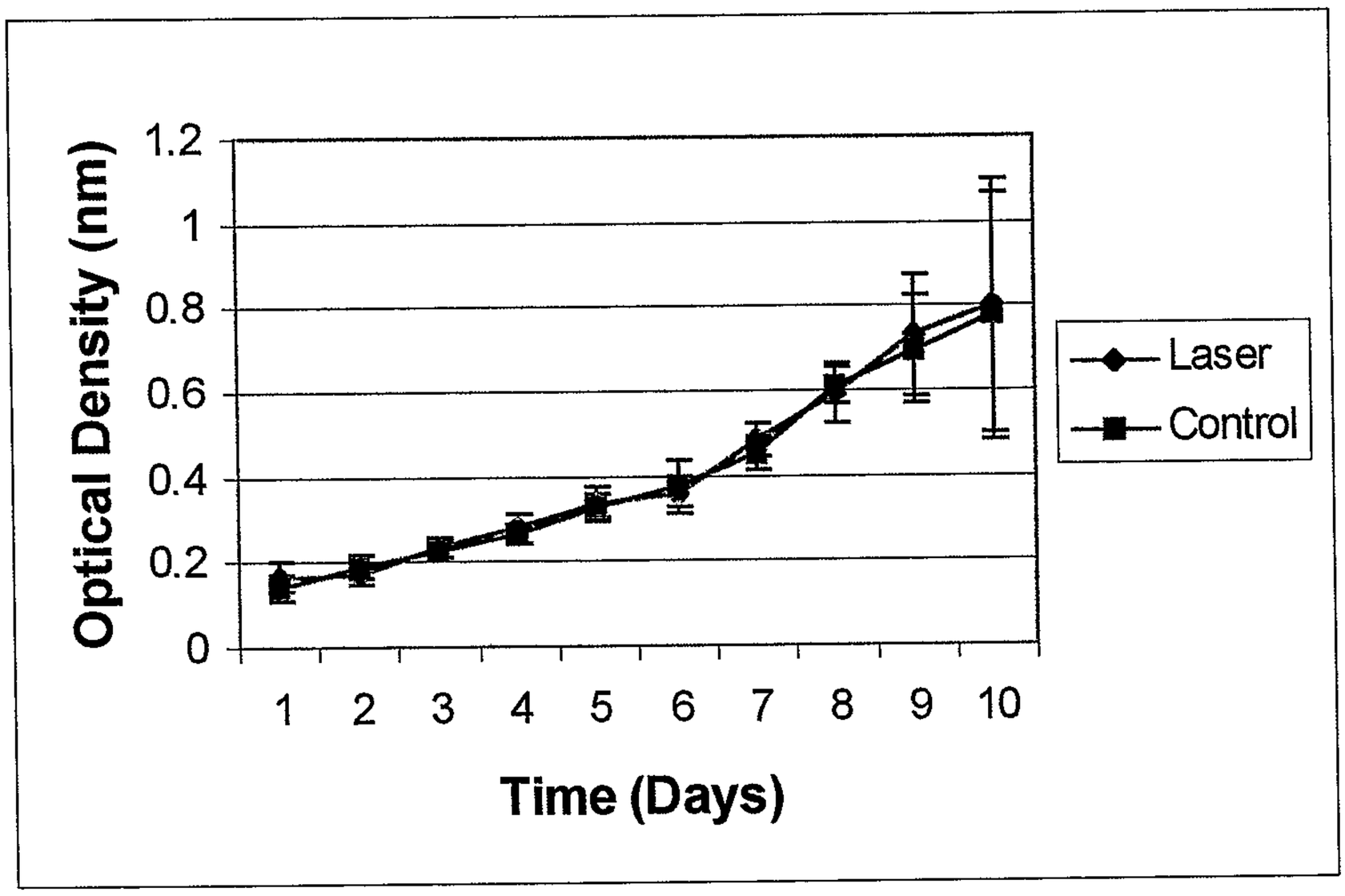


Table 5.2.1 Significance

Day	p value	p<0.05	p< 0.01
1	0.003	*	*
2	0.068	-	-
3	0.580	-	-
4	0.215	-	-
5	0.357	-	-
6	0.337	-	-
7	0.036	*	-
8	0.373	-	-
9	0.107	-	-
10	0.277	-	-

Figure 5.2.4 MTT assay for a single irradiation of 1.0 Joules, anlaysed for a period of ten days, comparing experimental and control groups.

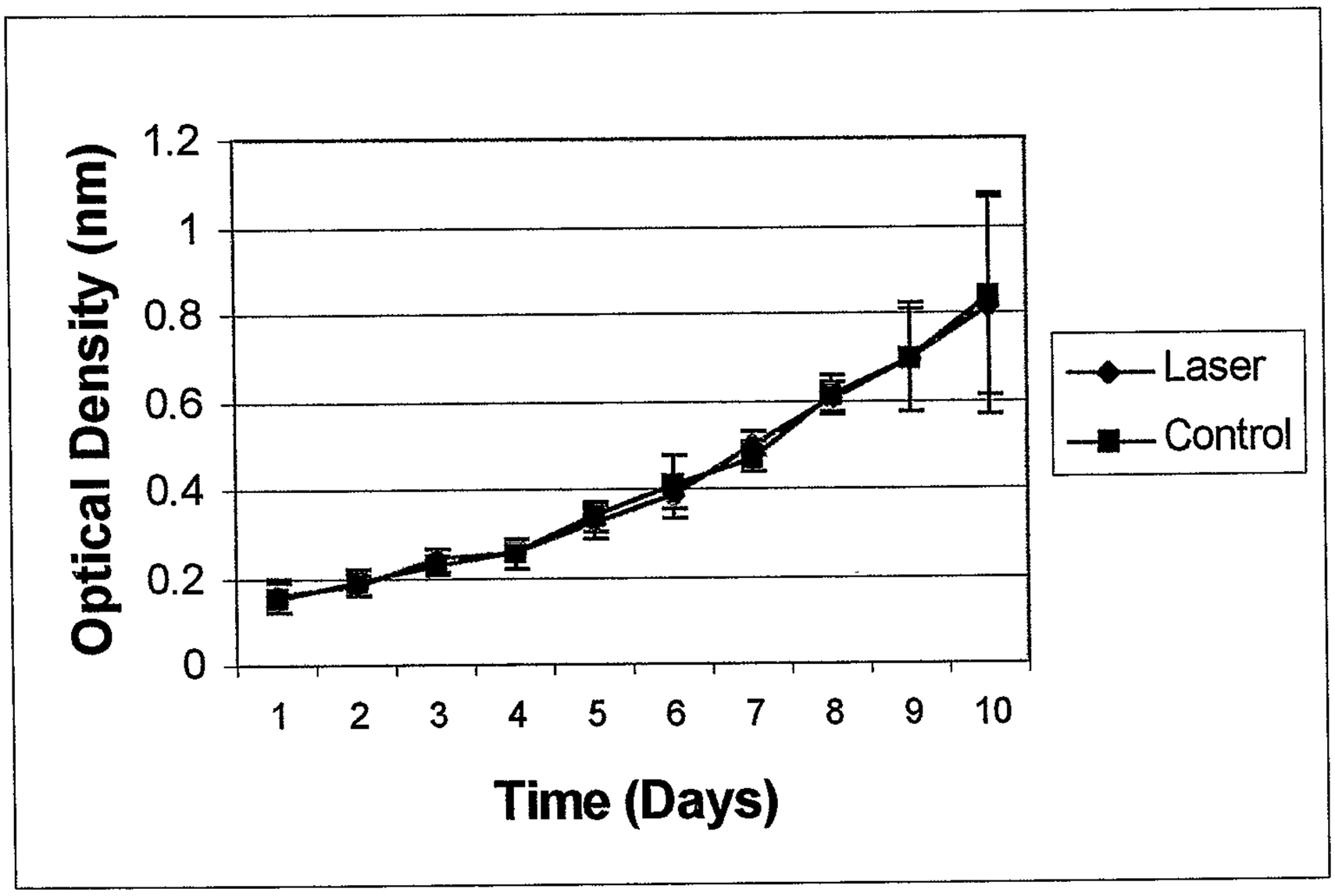


Table 5.2.2 Significance

Day	p value	p<0.05	p< 0.01
1	0.114	-	-
2	0.572	-	-
3	0.266	-	-
4	0.801	-	-
5	0.562	-	-
6	0.470	-	-
7	0.071	-	-
8	0.466	-	-
9	0.914	-	-
10	0.807	-	-

Figure 5.2.5 MTT assay for a single irradiation of 2.0 Joules, anlaysed for a period of ten days, comparing experimental and control groups.

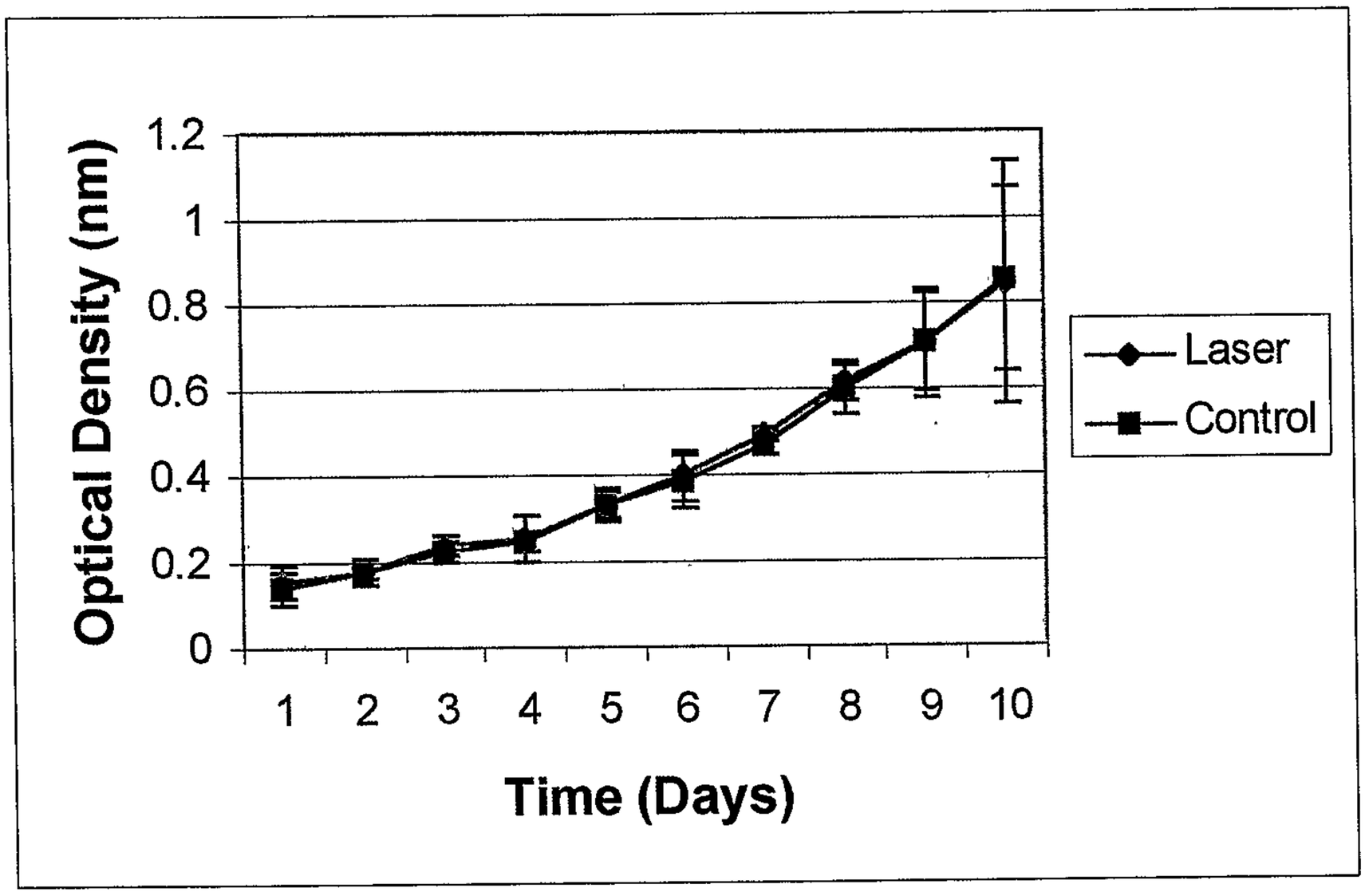


Table 5.2.3 Significance

Day	p value	p<0.05	p< 0.01
1	0.752	-	-
2	0.615	-	-
3	0.307	-	-
4	0.933	-	-
5	0.527	-	-
6	0.309	-	-
7	0.020	*	-
8	0.495	-	-
9	0.862	-	-
10	0.423	-	-

Figure 5.2.6 MTT assay for a single irradiation of 4.0 Joules, anlaysed for a period of ten days, comparing experimental and control groups.

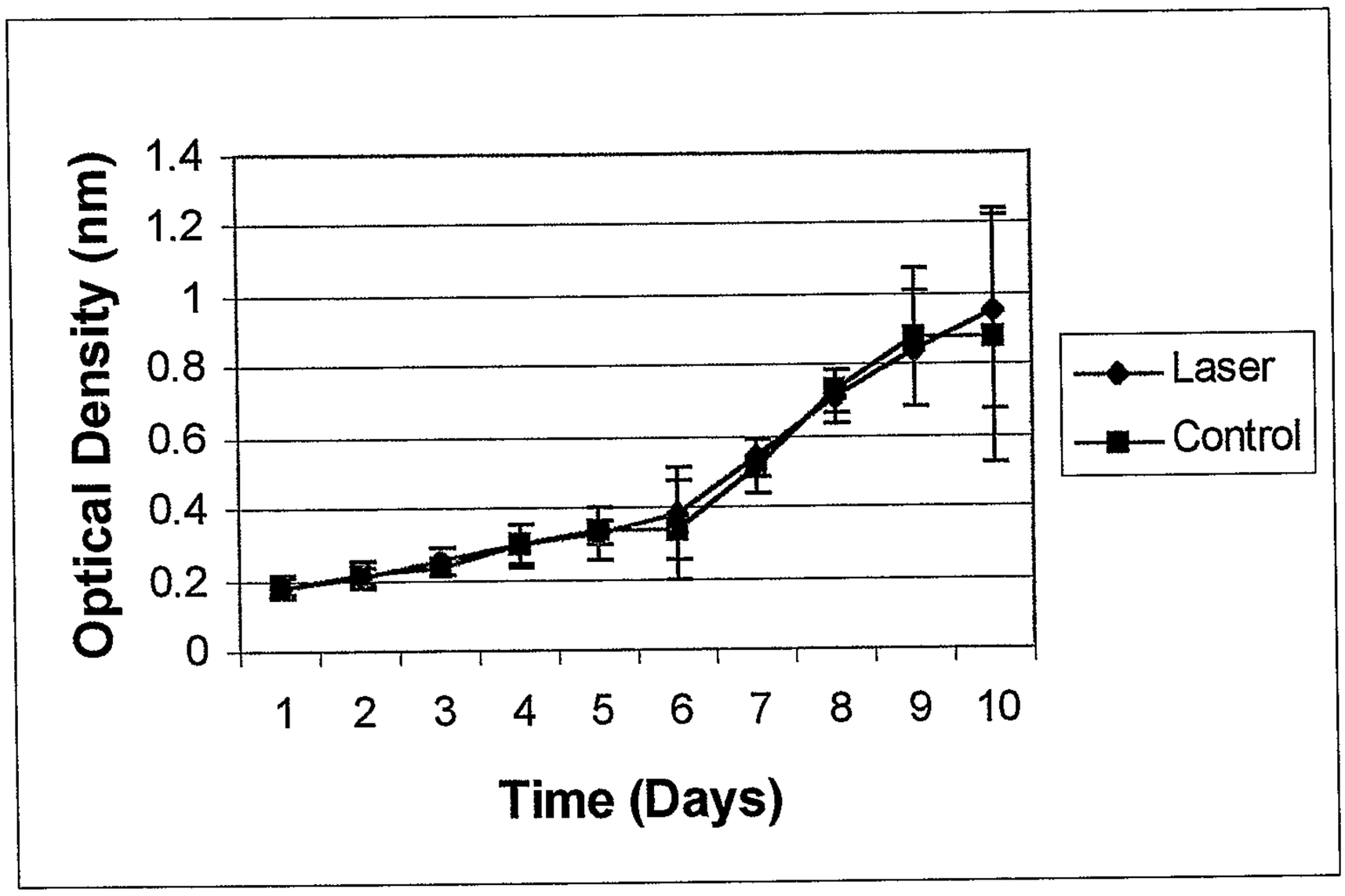


Table 5.2.4 Significance

Day	p value	p<0.05	p< 0.01
1	0.945	-	-
2	0.560	-	-
3	0.071	-	-
4	0.927	-	-
5	0.785	-	-
6	0.168	-	-
7	0.360	-	-
8	0.646	-	-
9	0.593	-	-
10	0.099	-	-

Figure 5.2.7 MTT assay for daily irradiation of 0.5 Joules, anlaysed for a period of ten days, comparing experimental and control groups.

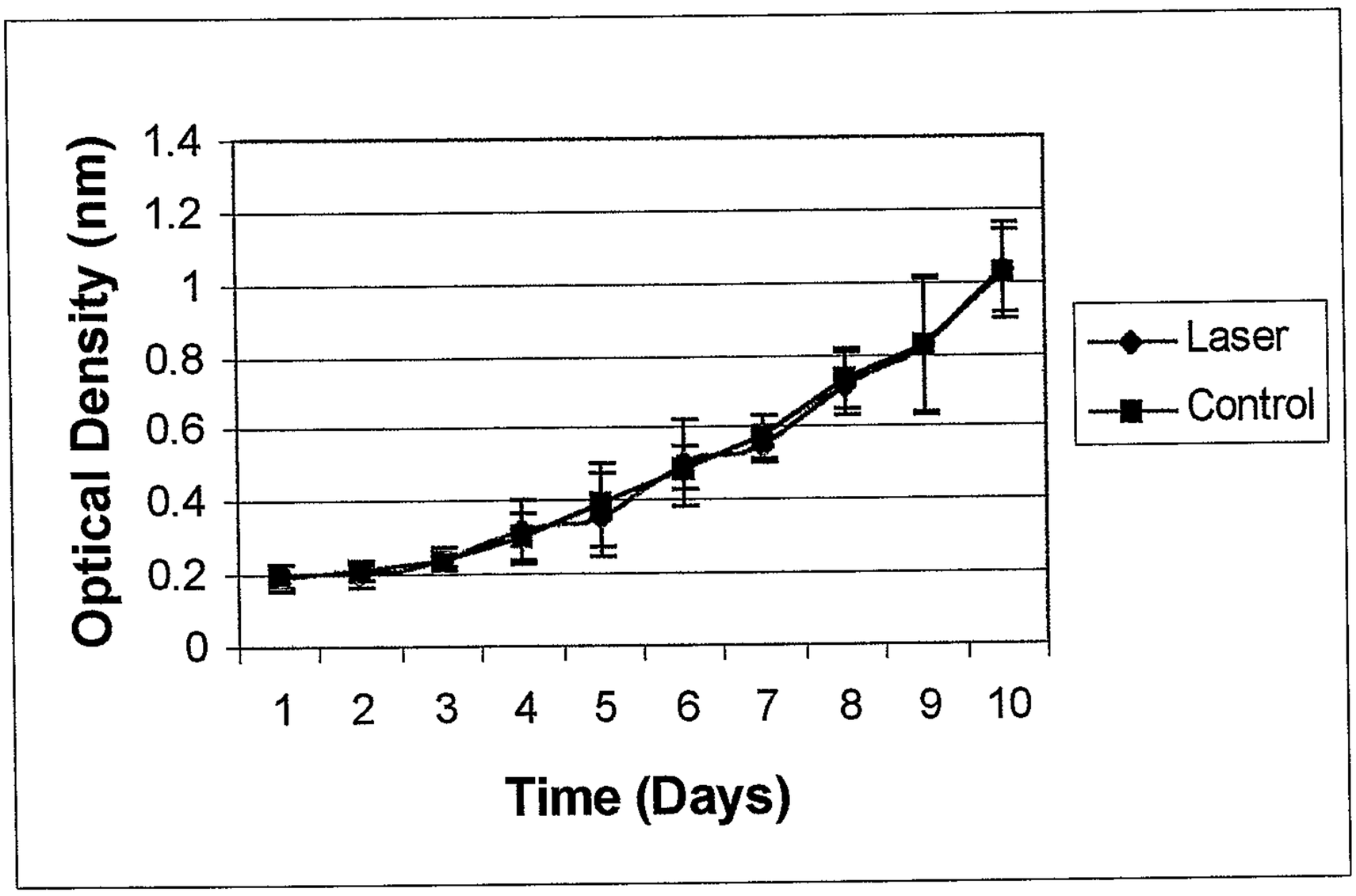


Table 5.2.5 Significance

Day	p value	p<0.05	p< 0.01
1	0.539	-	-
2	0.879	-	-
3	0.585	-	-
4	0.129	-	-
5	0.001	*	*
6	0.515	-	-
7	0.176	-	-
8	0.598	-	-
9	0.935	-	-
10	0.958	-	-

Figure 5.2.8 MTT assay for daily of 1.0 Joules, anlaysed for a period of ten days, comparing experimental and control groups.

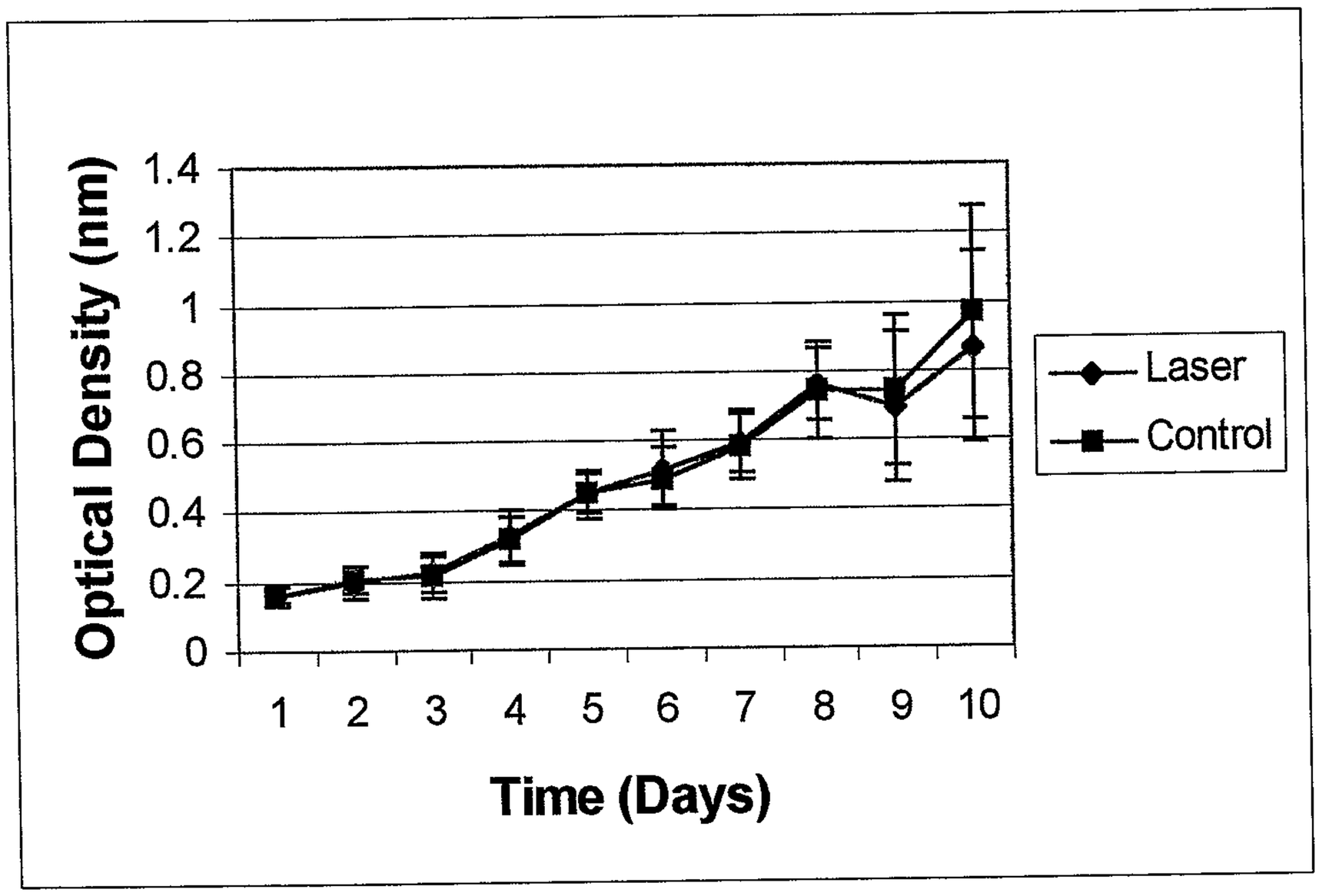


Table 5.2.6 Significance

Day	p value	p<0.05	p< 0.01
1	0.691	-	-
2	0.339	-	-
3	0.509	-	-
4	0.609	-	-
5	0.768	-	-
6	0.278	-	-
7	0.715	-	-
8	0.720	-	-
9	0.333	-	-
10	0.226	-	-

Figure 5.2.9 MTT assay for daily irradiation of 2.0 Joules, anlaysed for a period of ten days, comparing experimental and control groups.

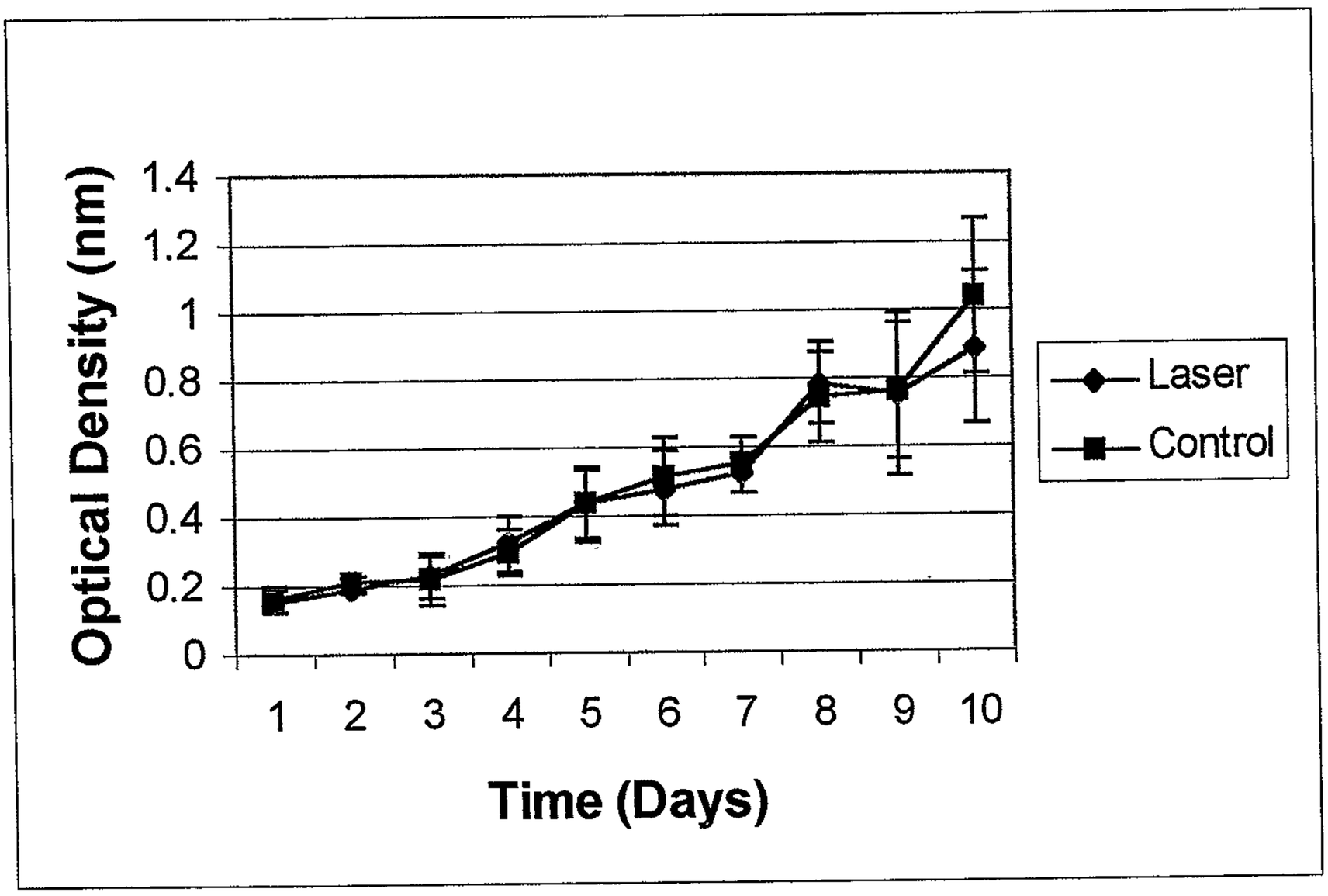


Table 5.2.7 Significance

Day	p value	p<0.05	p< 0.01
1	0.864	-	-
2	0.095	-	-
3	0.975	-	-
4	0.024	*	-
5	0.821	-	-
6	0.923	-	-
7	0.233	-	-
8	0.501	-	-
9	0.972	-	-
10	0.879	-	-

Figure 5.2.10 MTT assay for daily irradiation of 4.0 Joules, anlaysed for a period of ten days, comparing experimental and control groups.

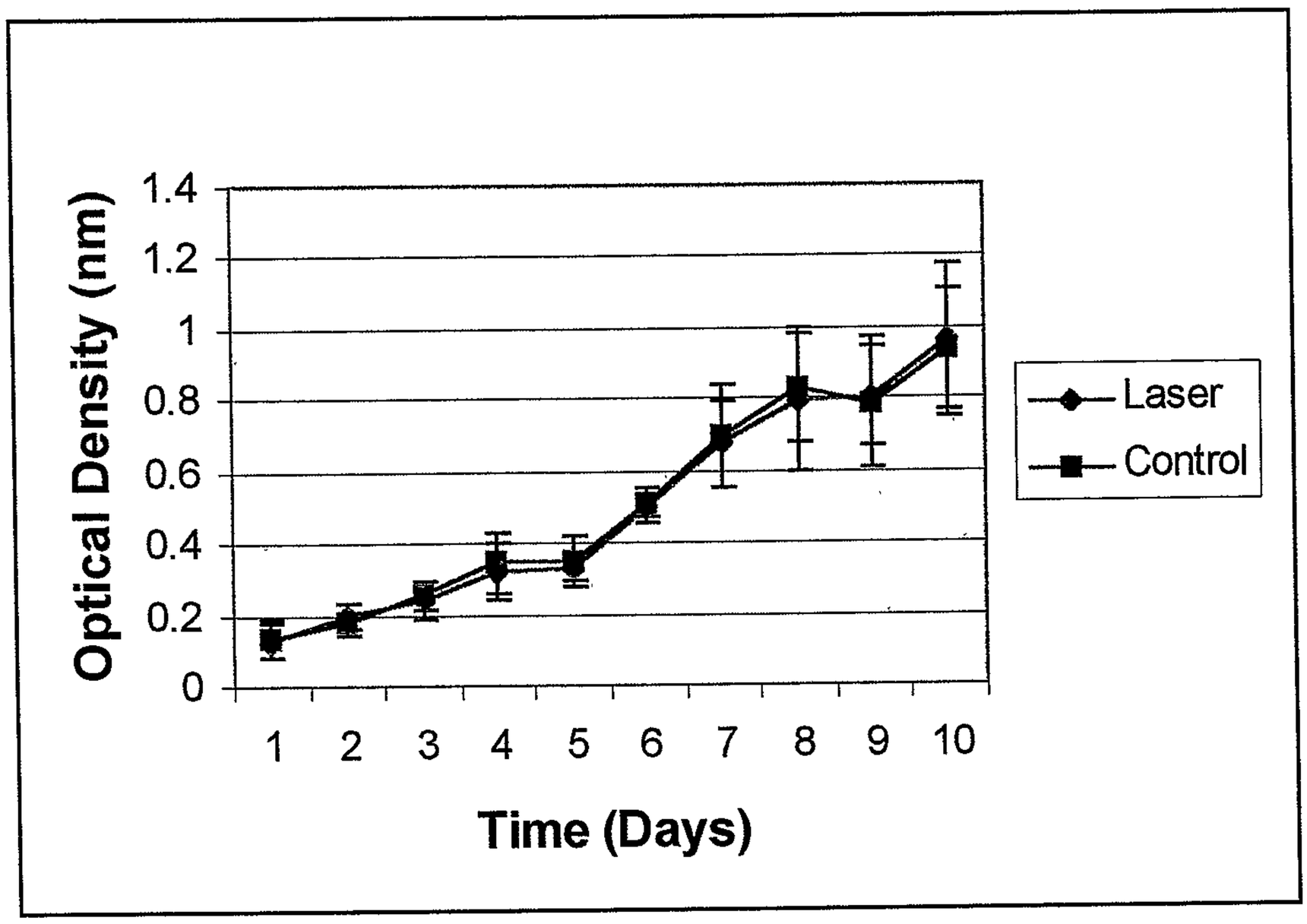


Table 5.2.8 Significance

Day	p value	p<0.05	p< 0.01
1	0.509	-	-
2	0.019	*	-
3	0.356	-	-
4	0.136	-	-
5	0.366	-	-
6	0.618	-	-
7	0.646	-	-
8	0.435	-	-
9	0.850	-	-
10	0.762	-	-

No overall increase in cellular activation or proliferation was observed with low level laser irradiation.

Localised effects on day seven, with a single irradiation of 0.5 and 2 joules, were found to be statistically significant. It is unknown as to whether these are artifactual statistics or not. If these effects are real they are only transient and were not found to be consistent and reproducible throughout the experiments.

Standard deviation increased, particularly after day seven, for the different experimental groups.

5.3 Effects of Low Level Laser Irradiation on Cellular Proliferation Determined by CFSE Labelling

The fluorescent dye 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE) was used to monitor the division of osteosarcoma cells after single dose and daily irradiation of predetermined doses of low level laser irradiation *in vitro*.

The MTT reduction assay (Mosmann 1983) is able to quantify proliferation at a gross level, but is also sensitive to the activation state of cells. The fluorescent dye CFSE is able to specifically resolve multiple successive generations of cells using flow cytometry.

The method is based on the sequential halving of an intracellular fluorescent stain upon cell division (Lyons *et al*. 1994). If the cells are undergoing division, the amount of CFSE present in each daughter cell after a division is half that of the parent.

CFSE is a fluorochrome which can covalently couple with intracellular macromolecules, and can be detected by flow cytometry up to 8 weeks post labelling. There is no evidence that CFSE is toxic for cells (Weston *et al*. 1990; Graziano *et al*. 1998), it is not harmful to cell function, and it has no significant effect on the proliferative potential of cells (Weston *et al*. 1990).

CFSE has been found to be an intense and long-lived marker. Lymphocytes labeled with the succinimidyl ester have been detected up to eight weeks after injection into mice in lymphocyte migration studies, and viable hepatocytes similarly labeled were easily located by fluorescence microscopy even 20 days after intrahepatic transplantation.

This dye can passively diffuse into cells. Once inside the cells, the colourless and nonfluorescent substrates are converted by intracellular esterases into highly fluorescent products (fluorophores) that are retained by cells with intact plasma membranes for days to months. In contrast, both the substrates and their products rapidly leak from dead or damaged cells with compromised membranes, even when the cells retain some residual esterase activity. These fluorophores are reportedly well retained throughout development, meiosis and *in vivo* tracing and can survive subsequent fixation with formaldehyde.

5.3.1 Experimental Design

Ten wells were seeded for each experimental group from which sufficient cells could be obtained for analysis. Control samples were seeded in the same plates utilizing the same number of wells and seeding densities. Irradiation was carried in the laminar flow cabinet using predetermined energy levels. These were 0.5, 1.0, 2.0 and 4.0 Joules as a single dose or daily. Plates were analysed by flow cytometry on consecutive days for ten days.

5.3.2 Expression of results

Dot plots of log FL-2 versus log FL-1 were generated. From this data, histograms and statistical data could be generated. Results are expressed as changes in the peak channel emission. Graphs are used to illustrate the comparison of experimental with control groups over a ten day period for the different energy levels and exposure regimes.

5.3.3 Results

Figure 5.3.1 Initial control experiment to establish flow cytometer parameters for the osteosarcoma cell line utilised. The Peak Channel change was observed to follow an exponential curve.

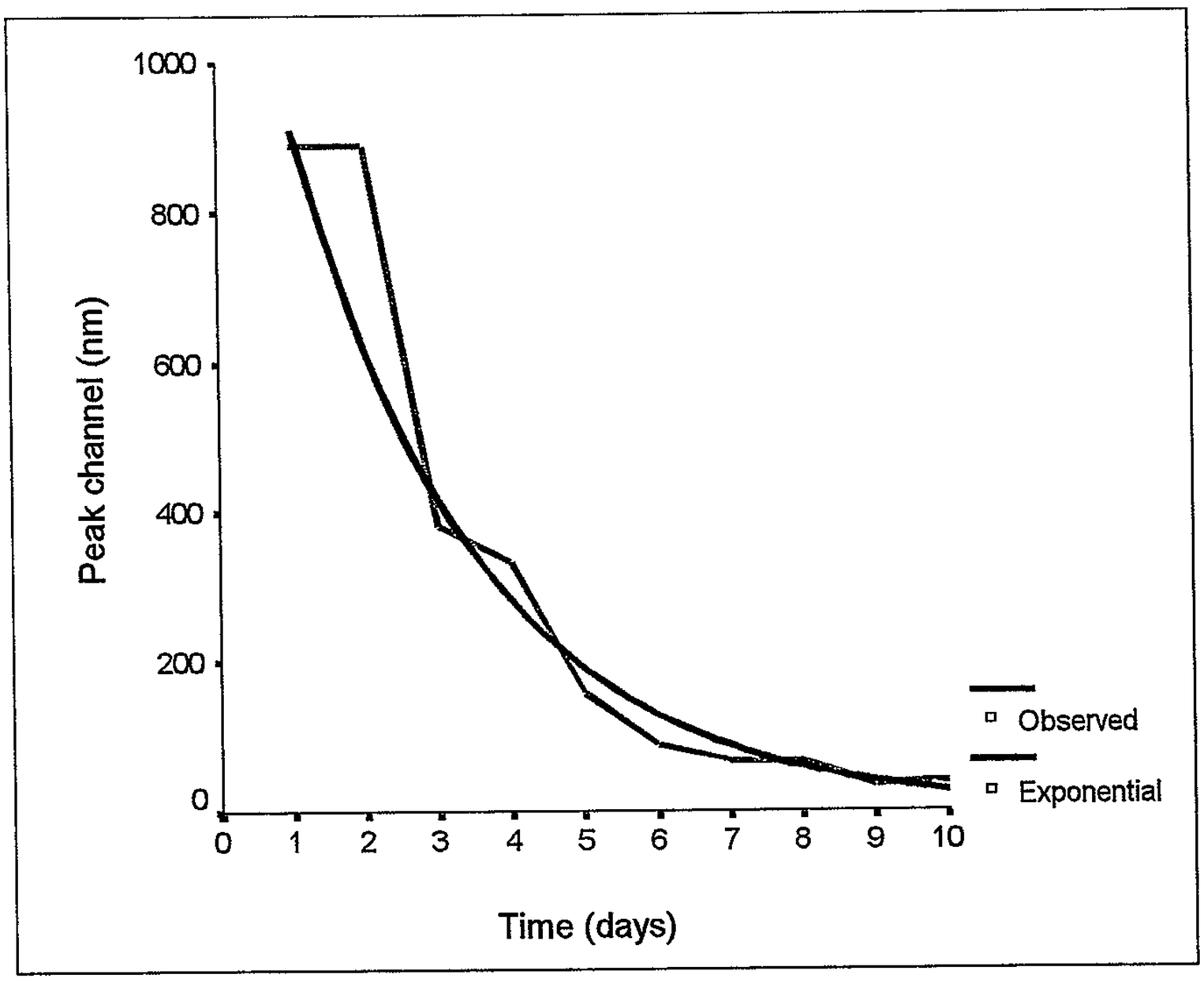


Figure 5.3.2 Comparison of osteosarcoma cells labelled with CFSE, irradiated with a single treatment at 0.5 Joules and unirradiated control cells.

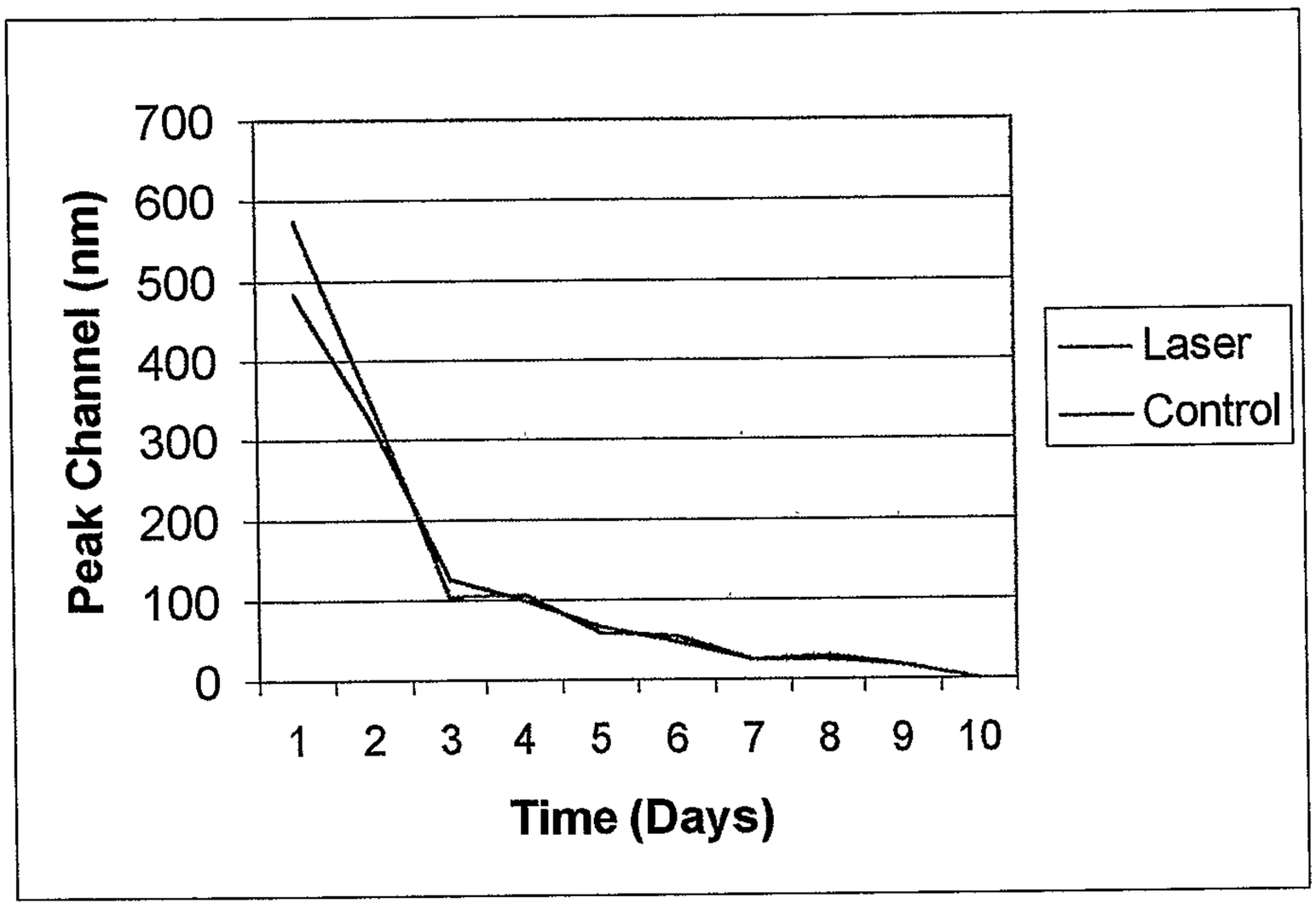


Figure 5.3.3 Comparison of osteosarcoma cells labelled with CFSE, irradiated with a single treatment at 1.0 Joules and unirradiated control cells.

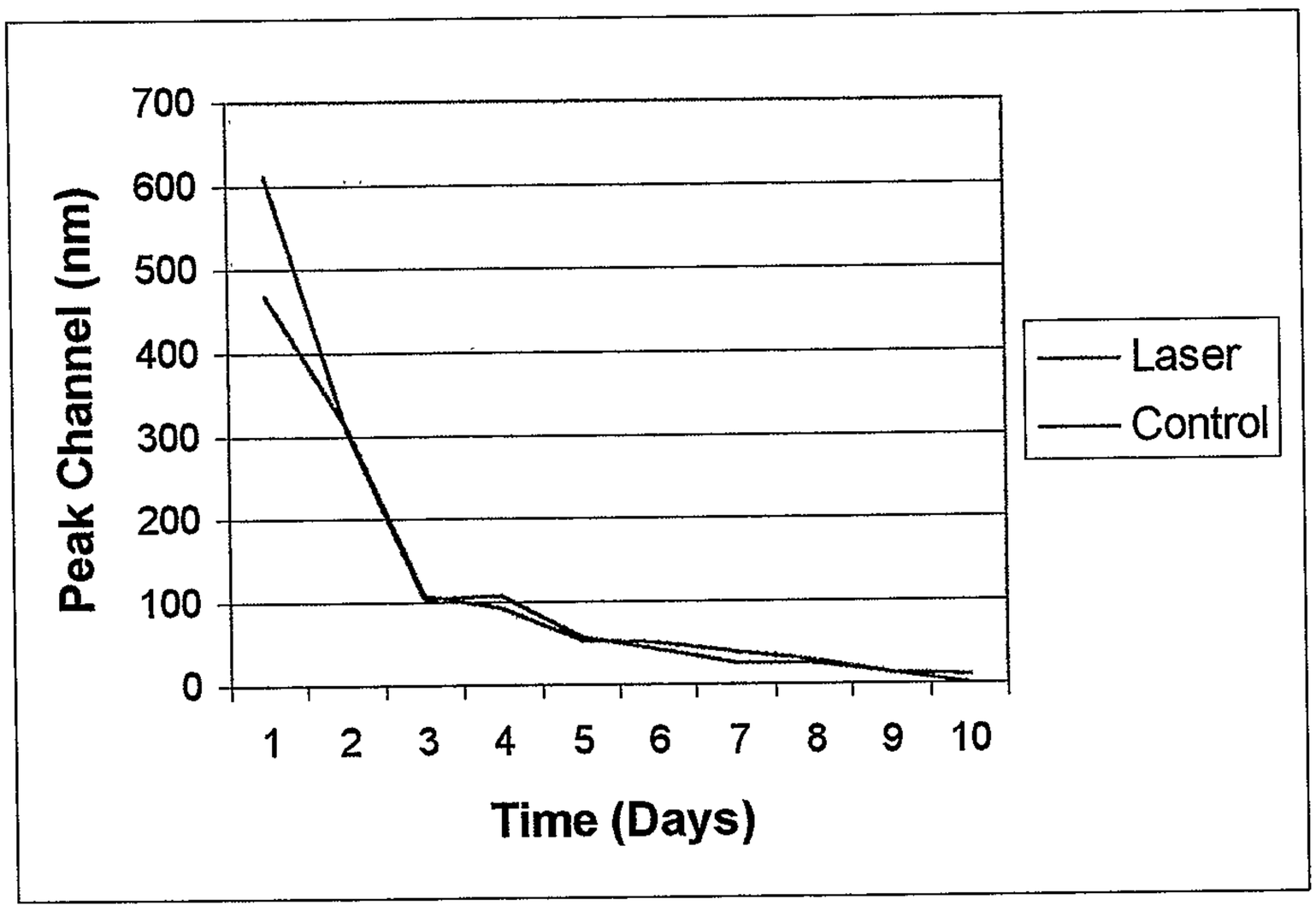


Figure 5.3.4 Comparison of osteosarcoma cells labelled with CFSE, irradiated with a single treatment at 2.0 Joules and unirradiated control cells.

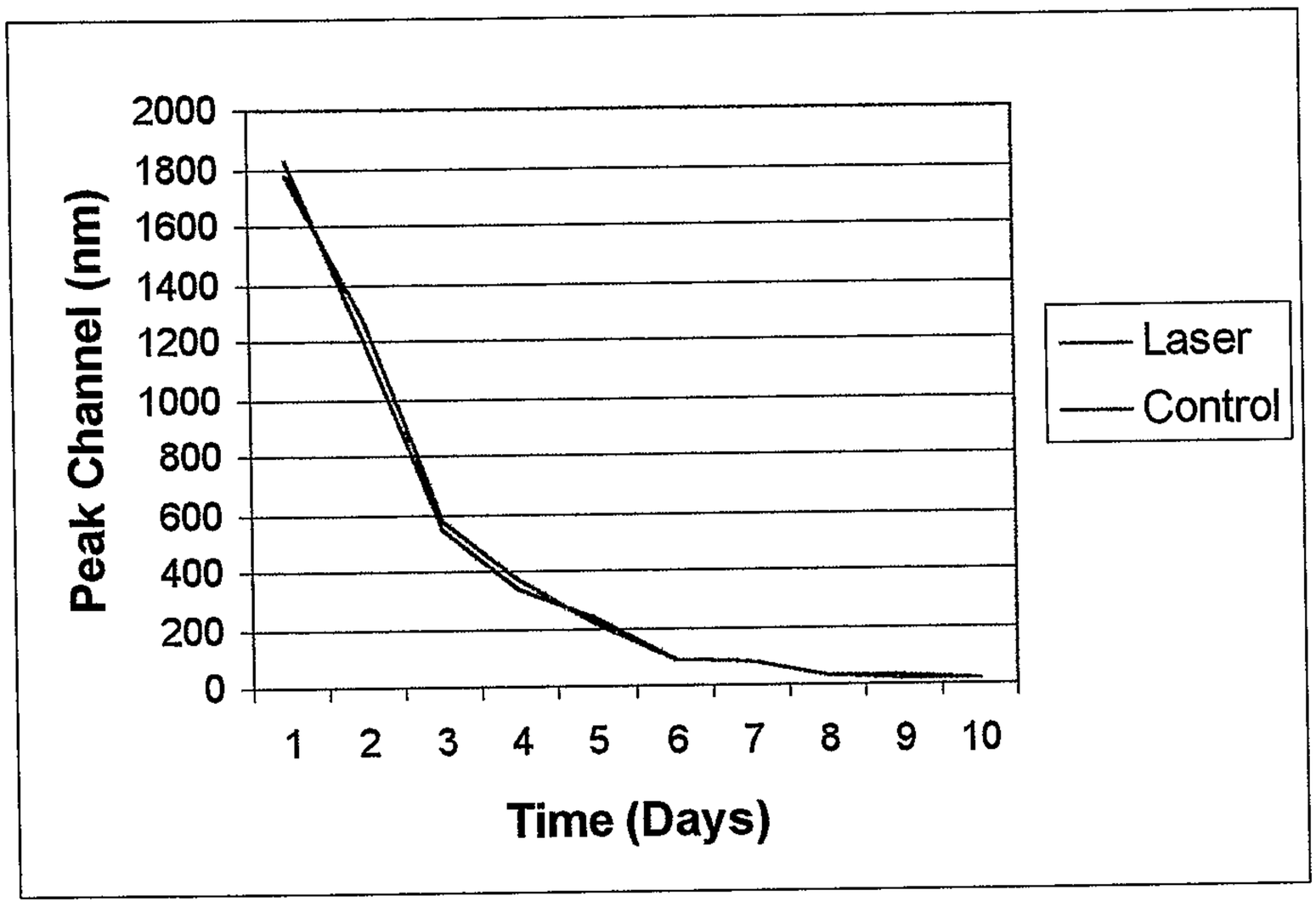


Figure 5.3.5 Comparison of osteosarcoma cells labelled with CFSE, irradiated with a single treatment at 4.0 Joules and unirradiated control cells.

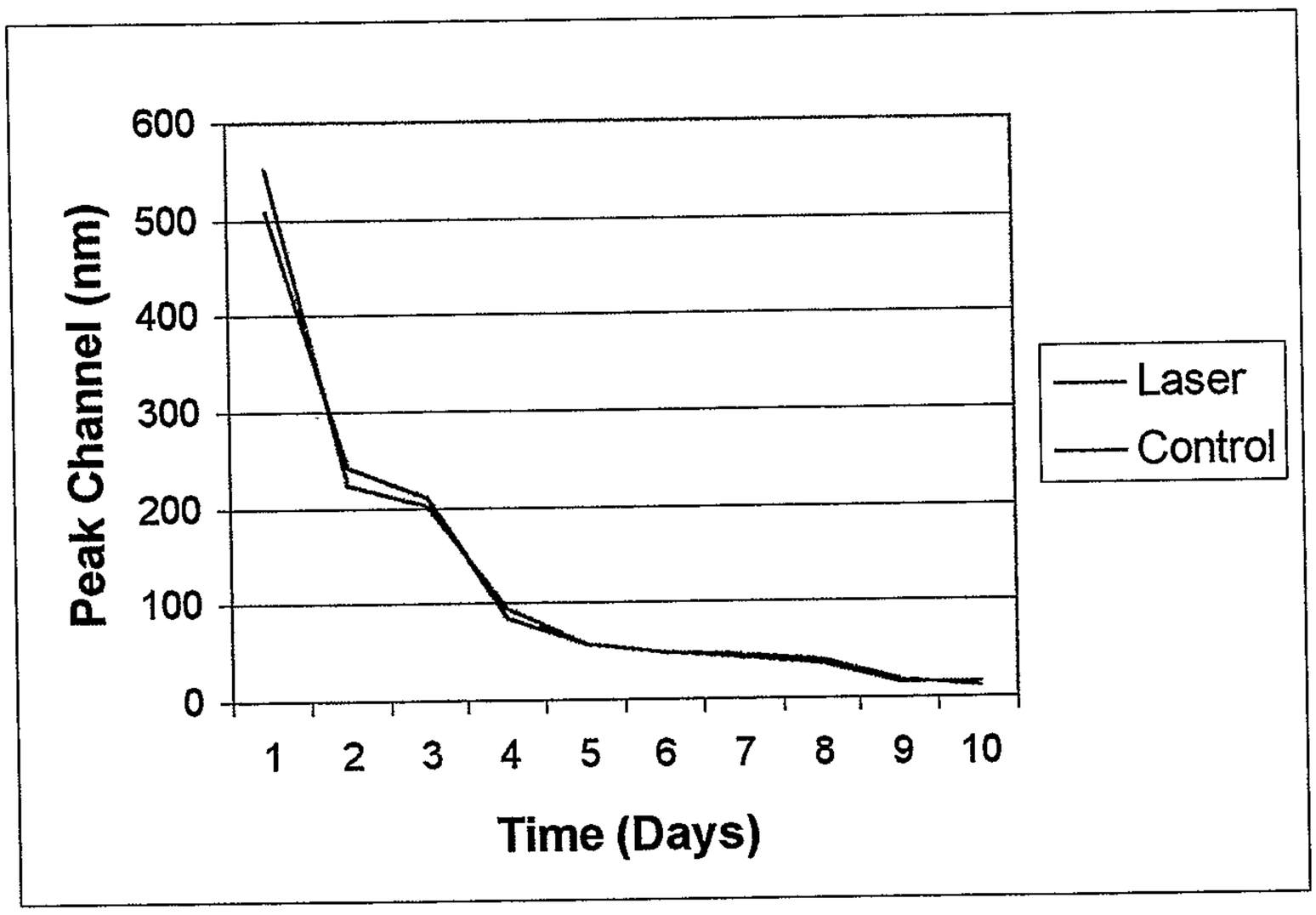


Figure 5.3.6 CFSE labelled osteosarcoma cells irradiated daily at an energy level of 0.5 Joules compared to unirradiated control cells.

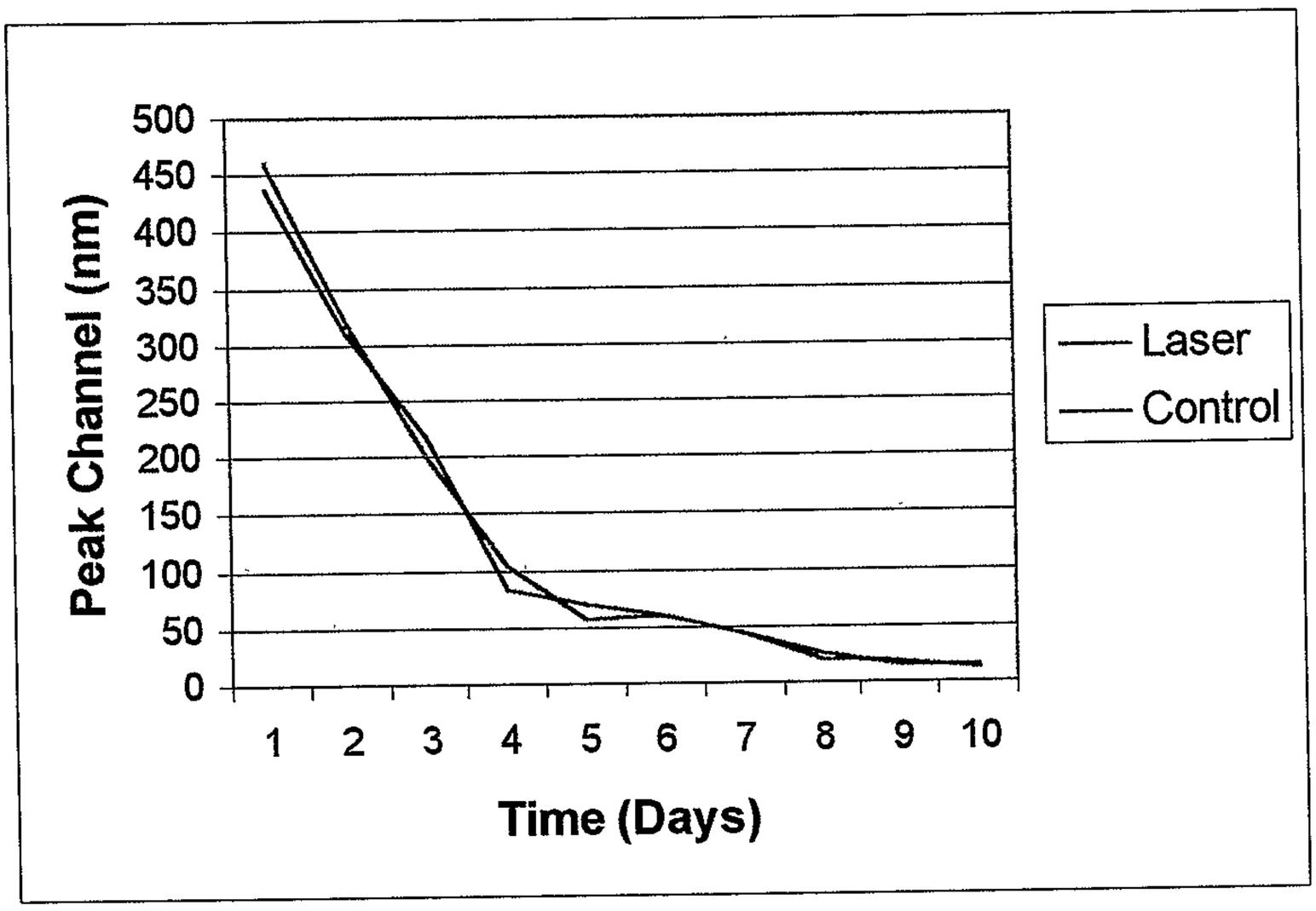


Figure 5.3.7 CFSE labelled osteosarcoma cells irradiated daily at an energy level of 1.0 Joules compared to unirradiated control cells.

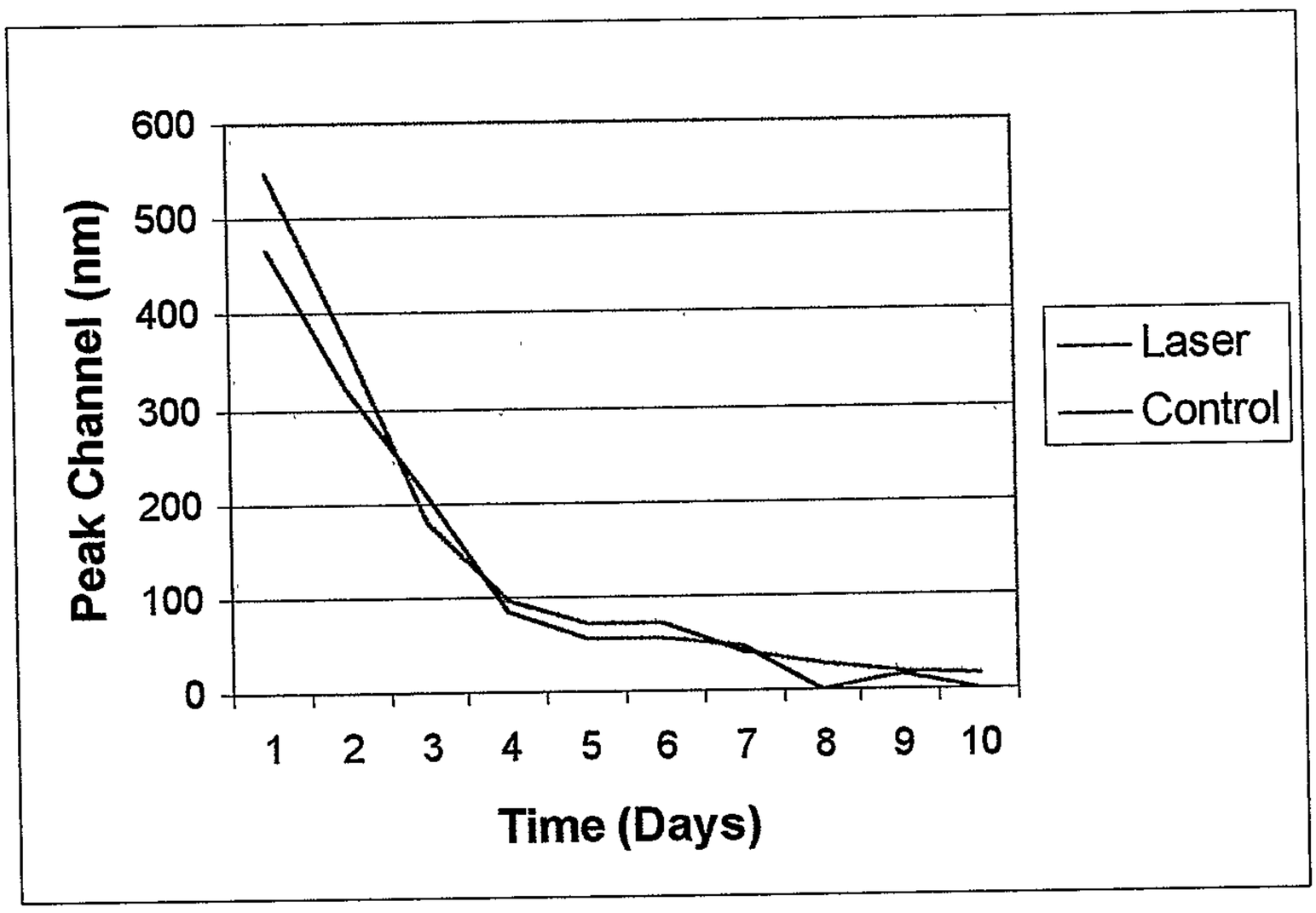


Figure 5.3.8 CFSE labelled osteosarcoma cells irradiated daily at an energy level of 2.0 Joules compared to unirradiated control cells.

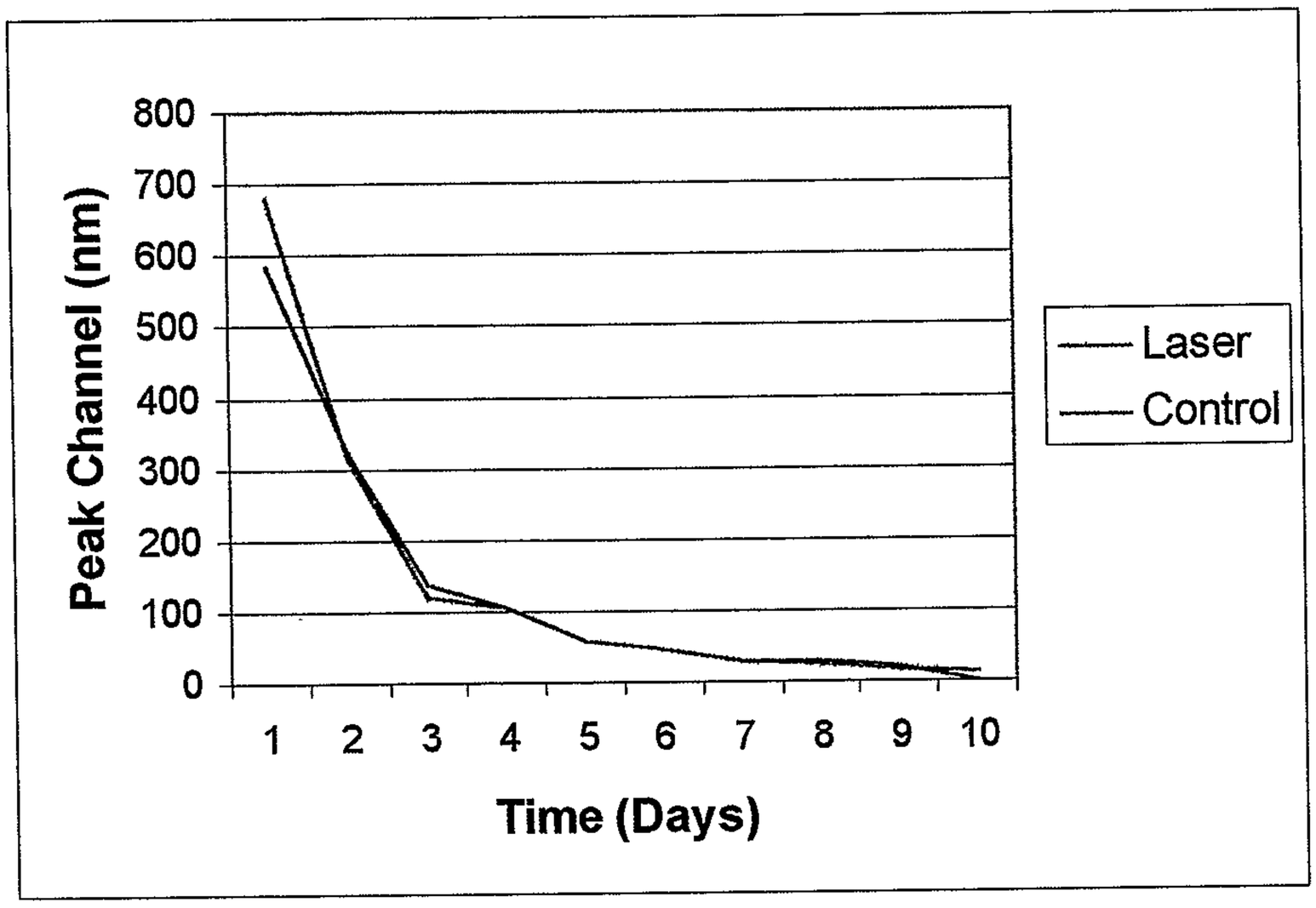
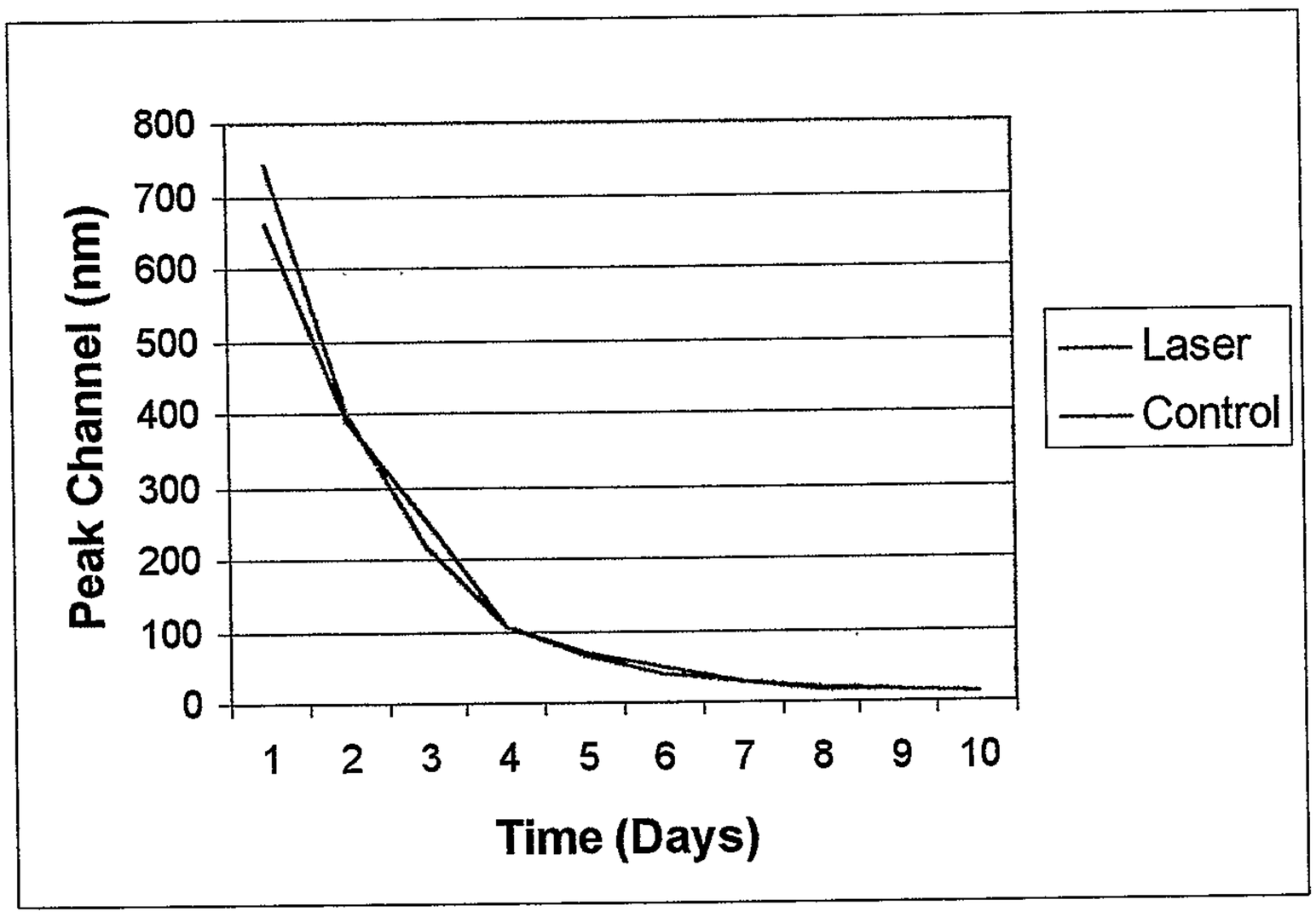
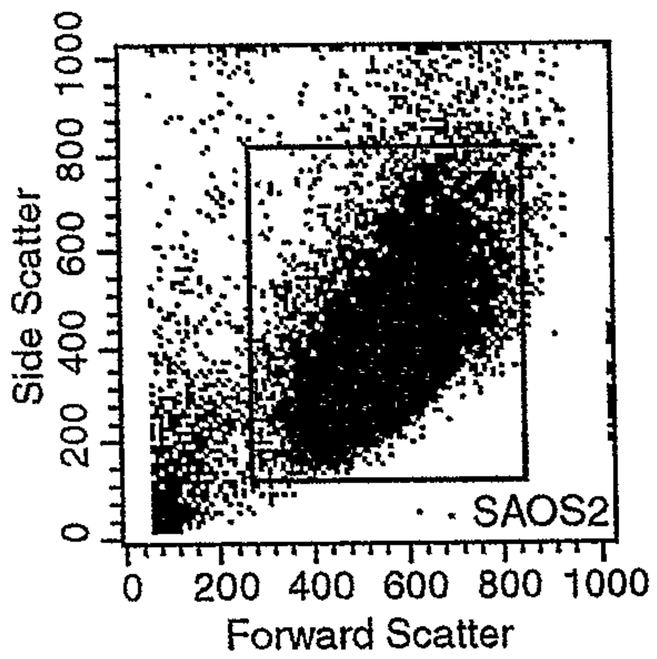


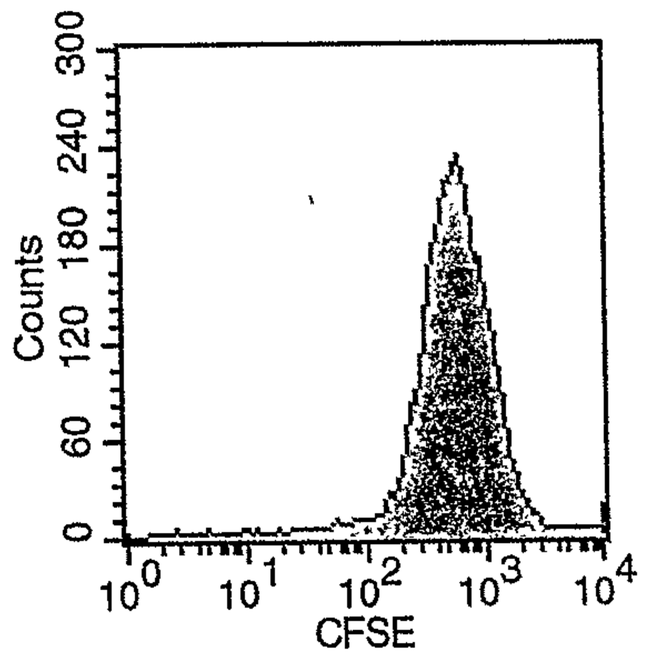
Figure 5.3.9 CFSE labelled osteosarcoma cells irradiated daily at an energy level of 4.0 Joules compared to unirradiated control cells.



The cells followed the expected exponential pattern of growth. No differences were observed between experimental and control groups for any of the energy levels or exposure regimes investigated. Cellular division and proliferation were not effected by low level laser irradiation. These results are consistent with and further support the MTT assay and cell count findings.

Representative Flow Cytometry Plots for CFSE labelling of osteosarcoma cells irradiated daily at 1 Joule.

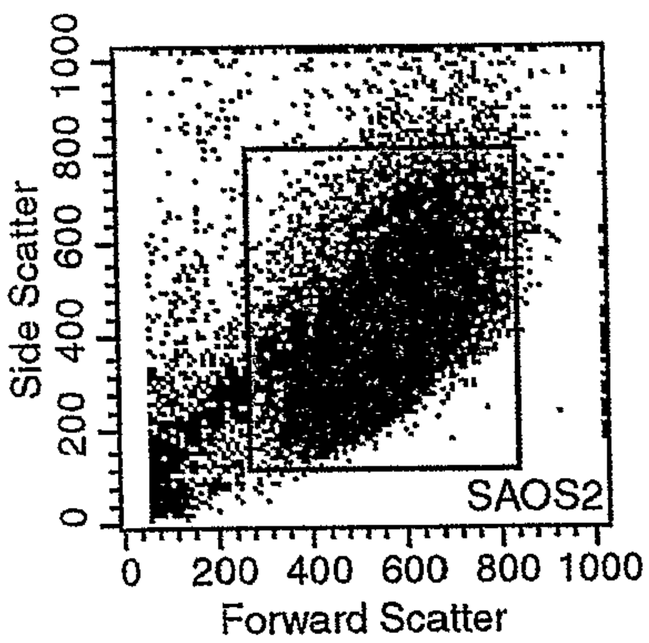


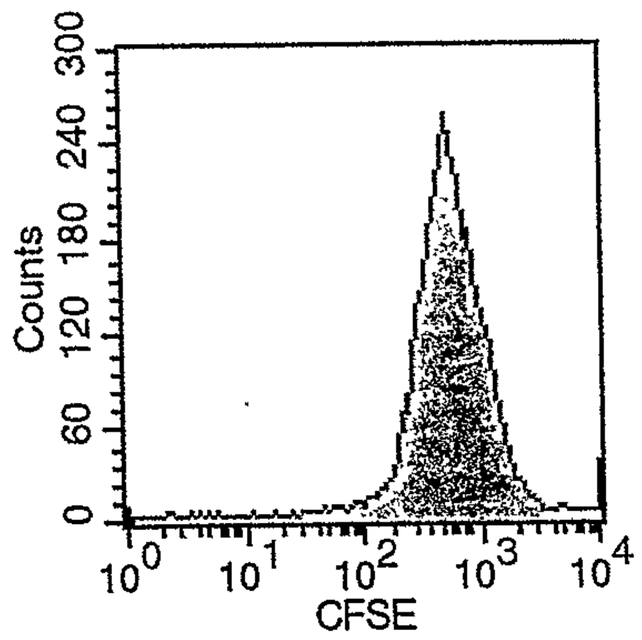


Day 1 irradiated cells

Total Events: 32239

Mean	CV	Peak	Peak Ch
651.79	95.62	230	547

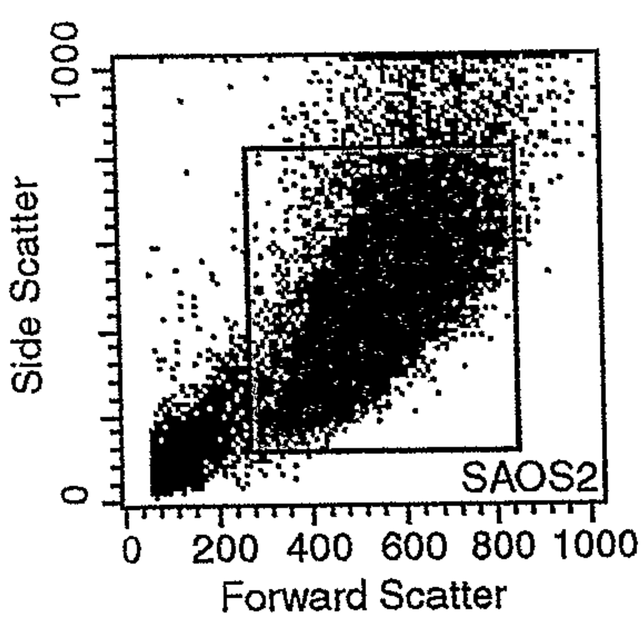


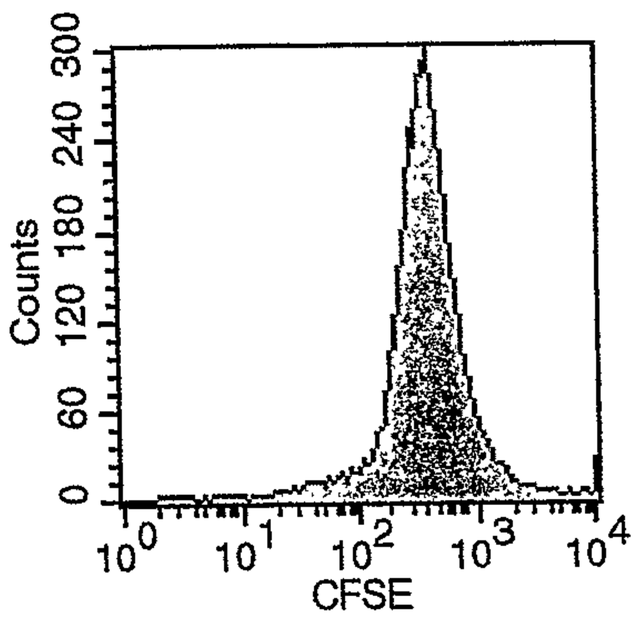


Day 1 control

Total Events: 32680

Mean	CV	Peak	Peak Ch
643.93	112.17	255	465

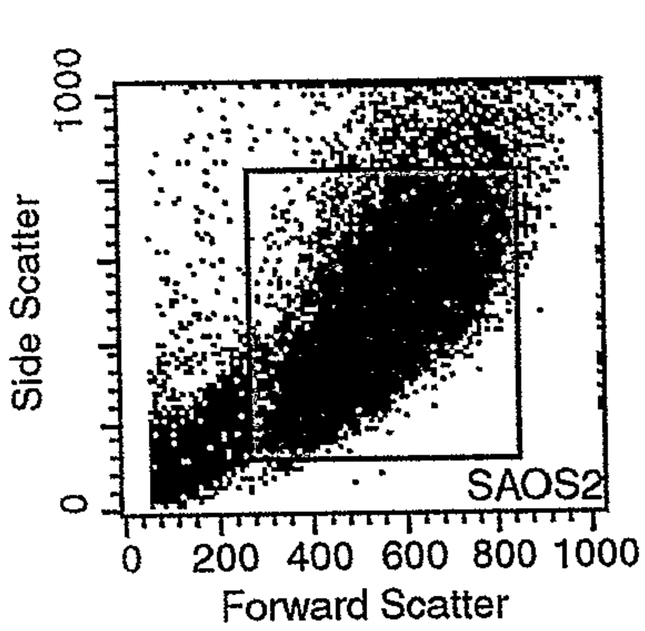


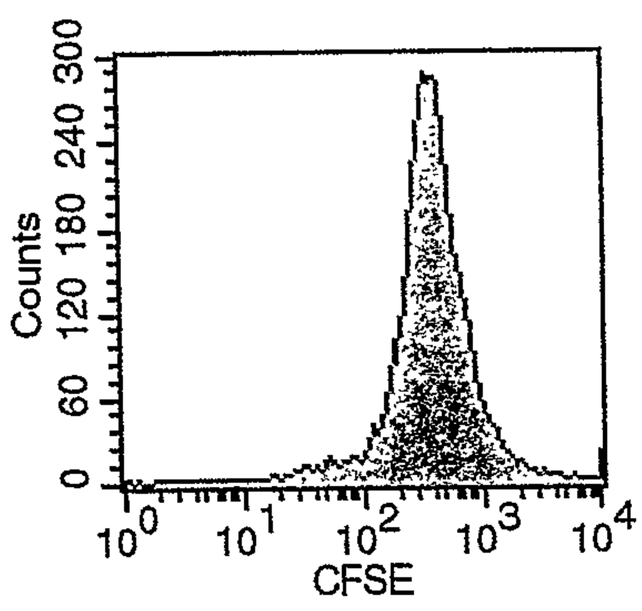


Day 2 irradiated cells

Total Events: 33316

Mean	CV	Peak	Peak Ch
448.30	133.16	295	371

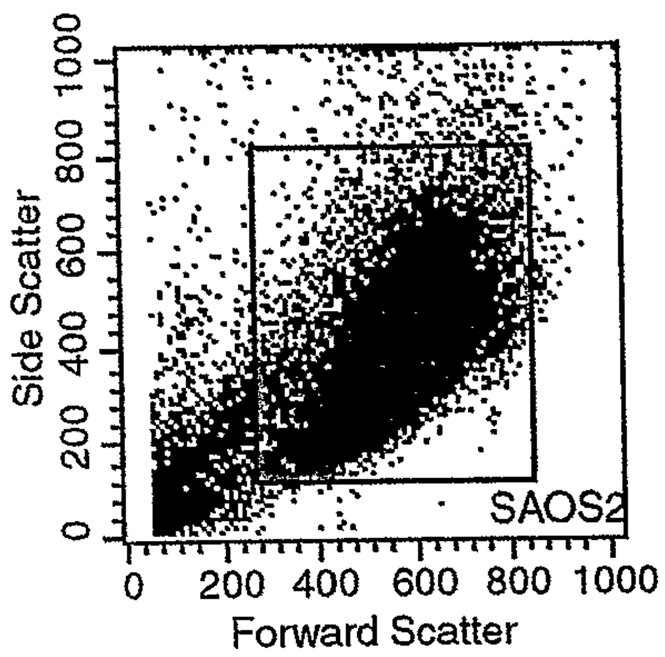


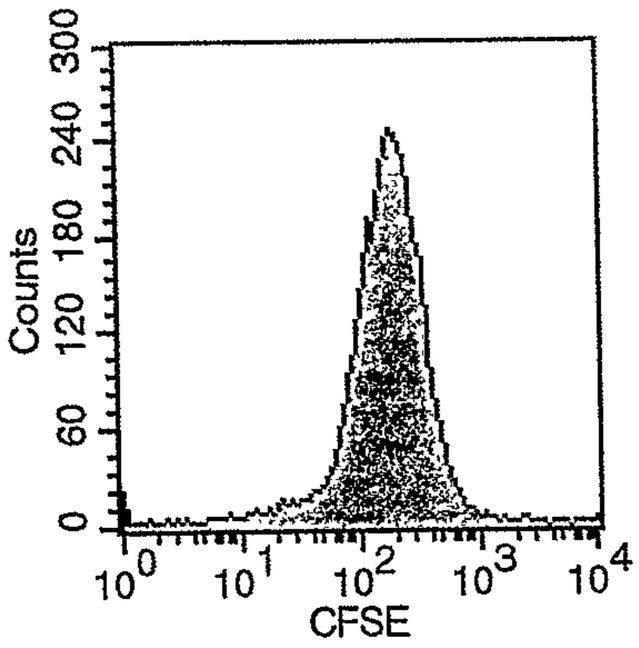


Day 2 control

Total Events: 34349

Mean	CV	Peak	Peak Ch
457.05	128.60	282	321

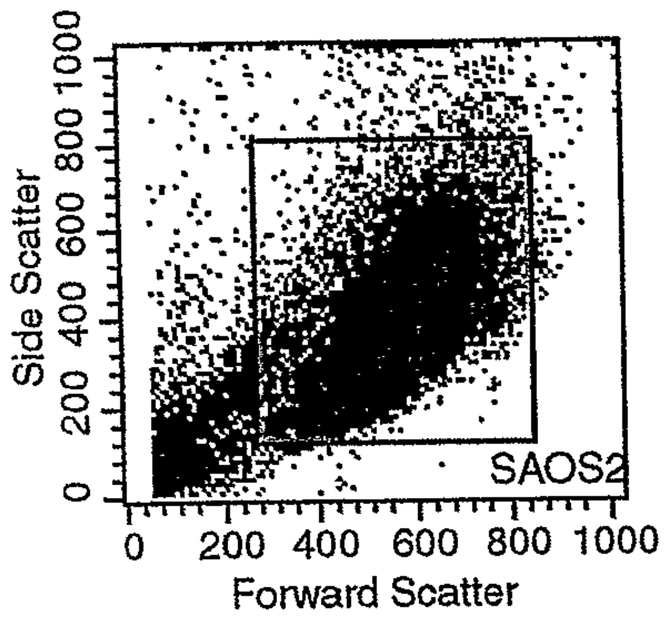


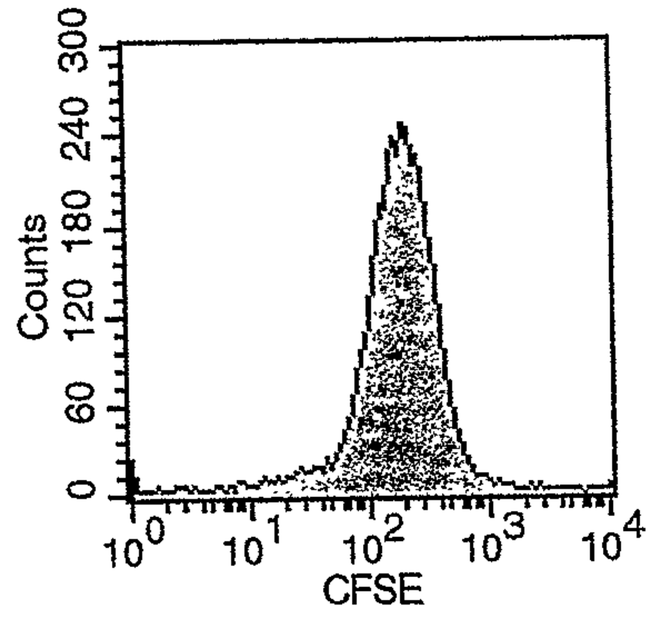


Day 3 irradiated cells

Total Events: 33761

Mean	CV	Peak	Peak Ch
203.00	135.74	244	181

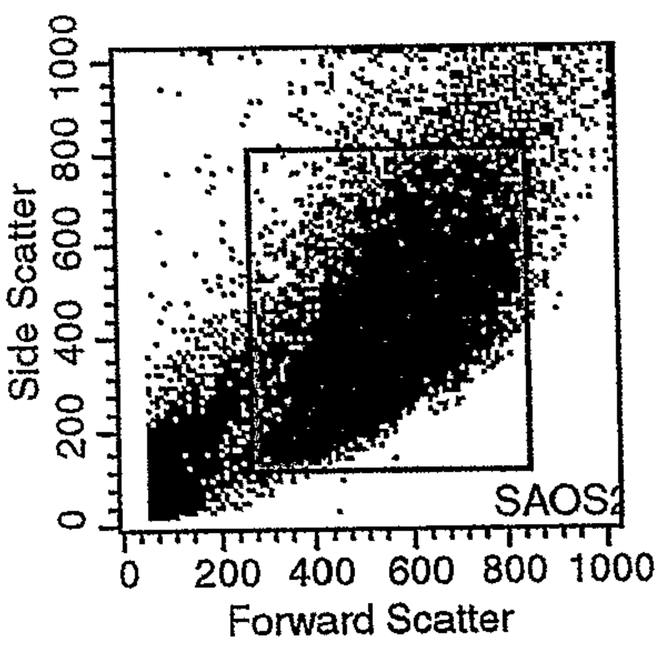


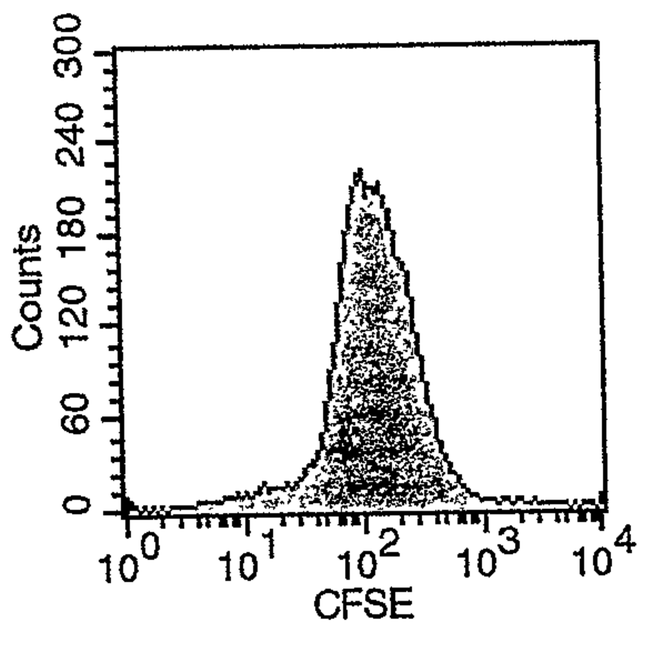


Day 3 control

Total Events: 33761

Mean	CV	Peak	Peak Ch
203.00	135.74	244	181

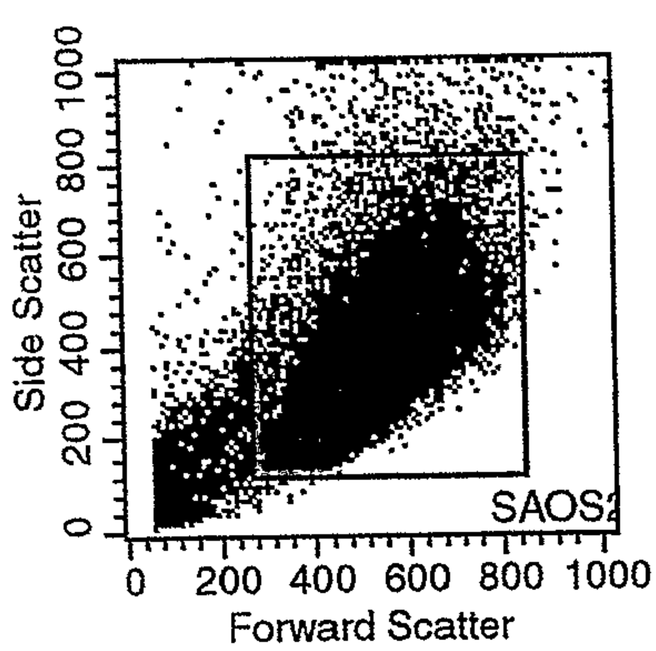


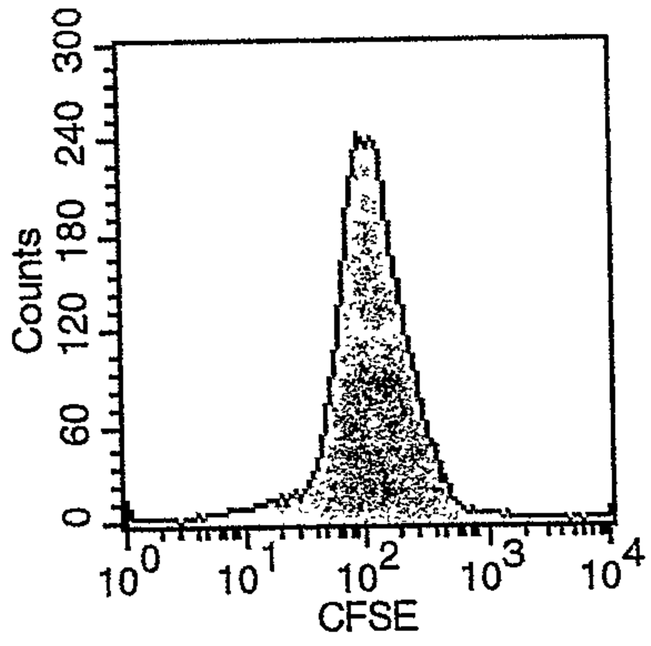


Day 4 irradiated cells

Total Events: 34876

Mean	CV	Peak	Peak Ch
155.33	176.67	217	94

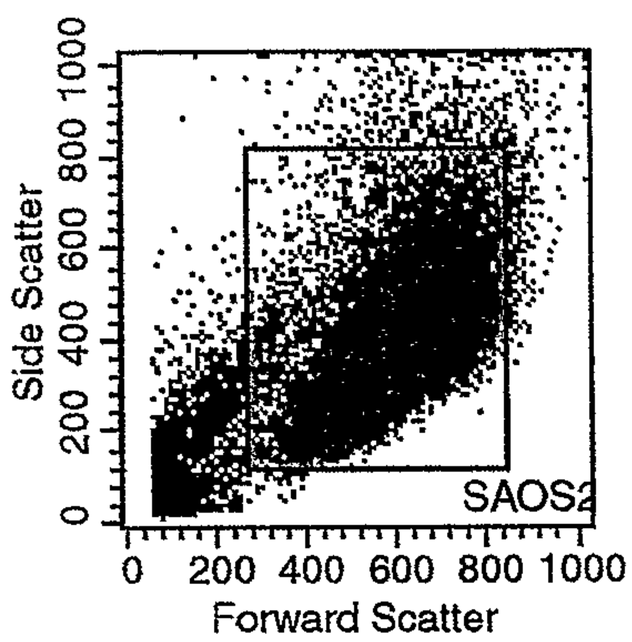


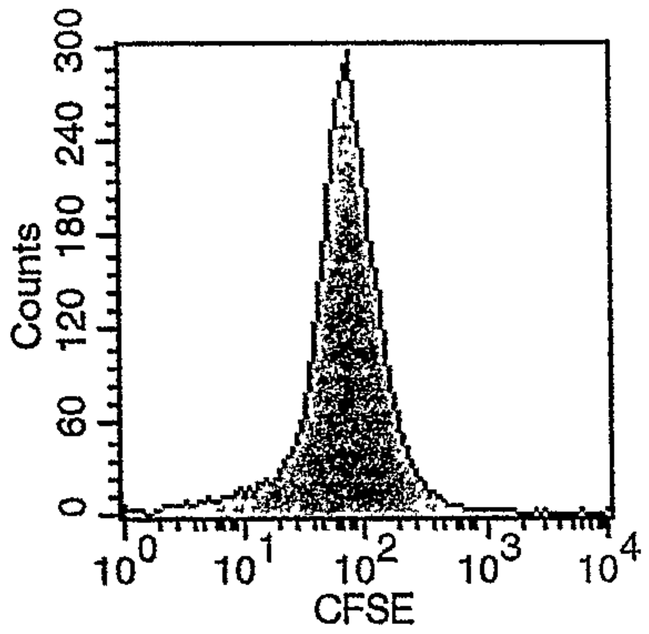


Day 4 control

Total Events: 33745

Mean	CV	Peak	Peak Ch
131.34	211.71	239	84

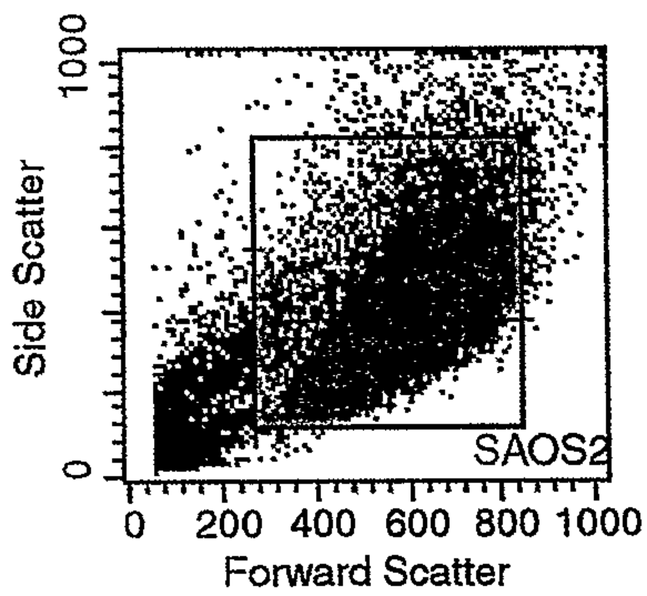


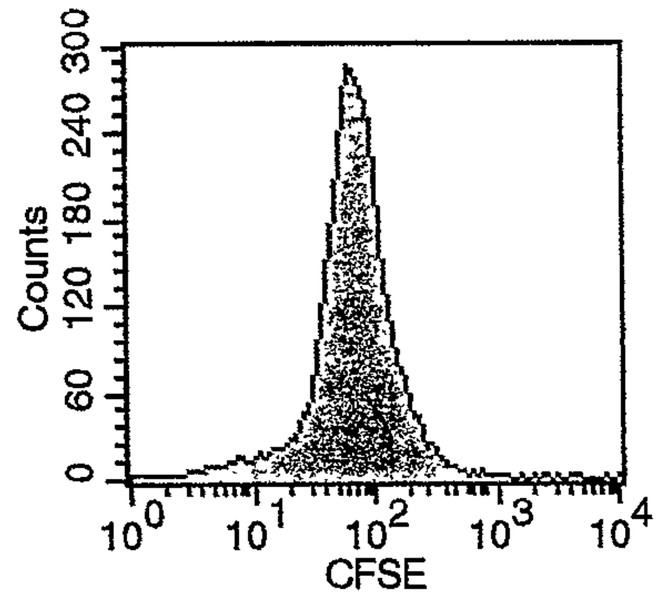


Day 5 irradiated cells

Total Events: 34568

Mean	CV	Peak	Peak Ch
79.57	103.14	293	71

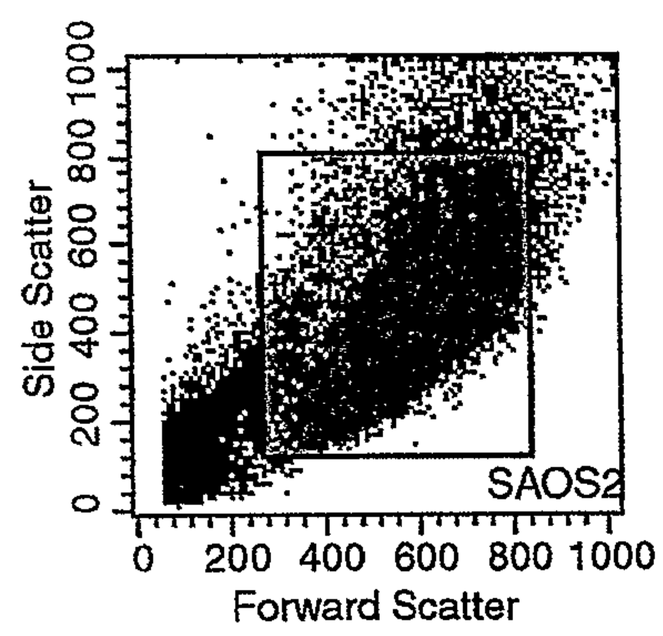


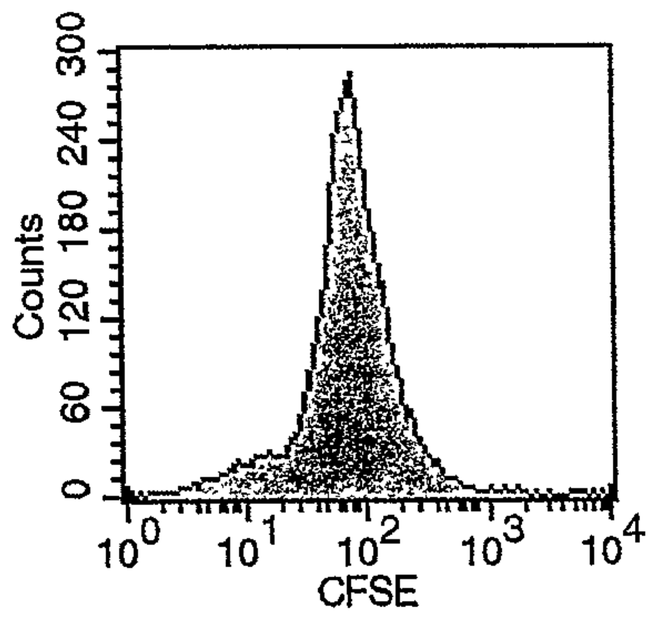


Day 5 control

Total Events: 34833

Mean	CV	Peak	Peak Ch
79.18	178.65	284	57

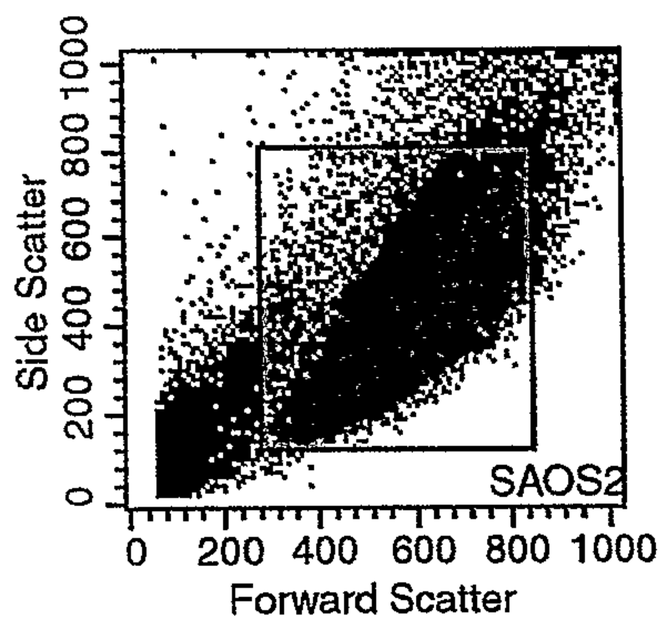


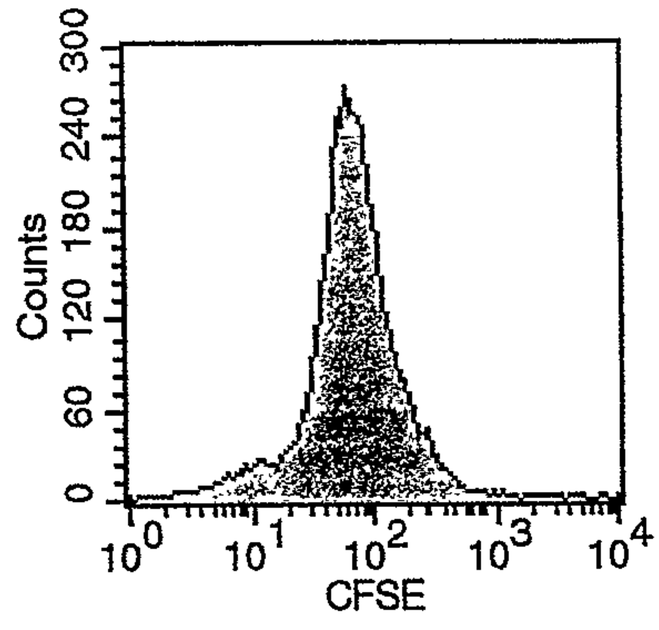


Day 6 irradiated cells

Total Events: 36325

Mean	CV	Peak	Peak Ch
87.59	220.25	281	71

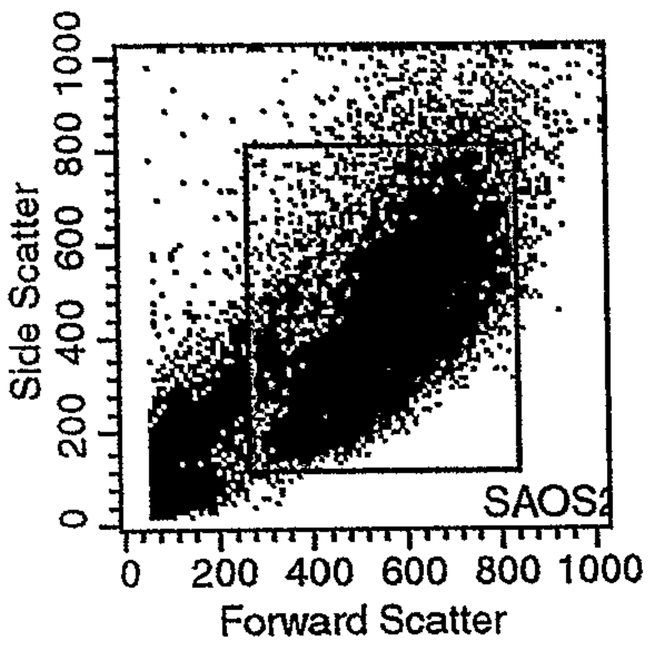


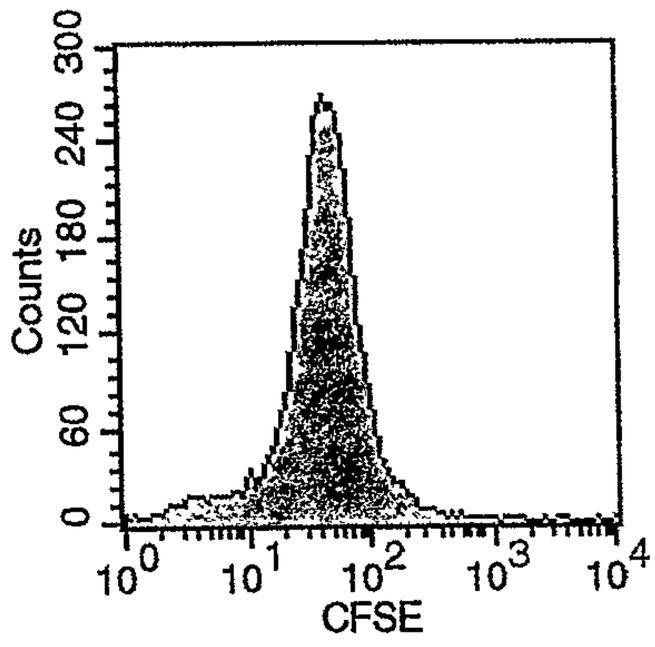


Day 6 control

Total Events: 36721

Mean	CV	Peak	Peak Ch
84.00	201.29	268	57

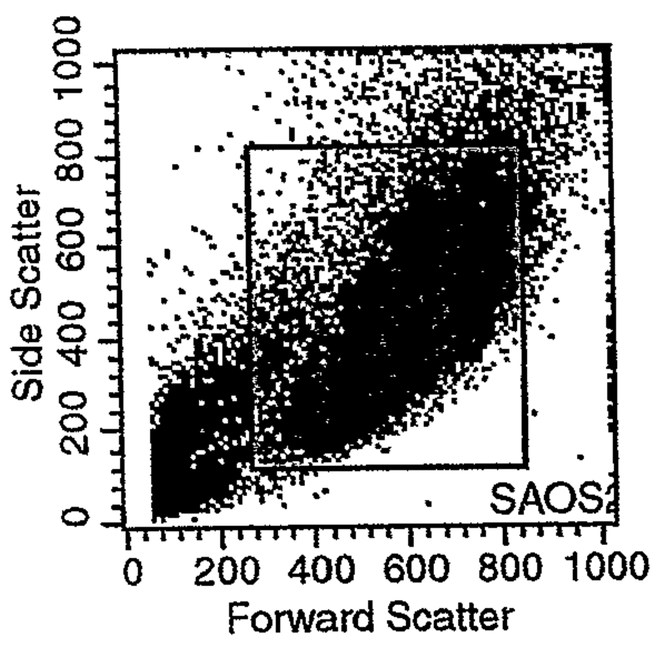


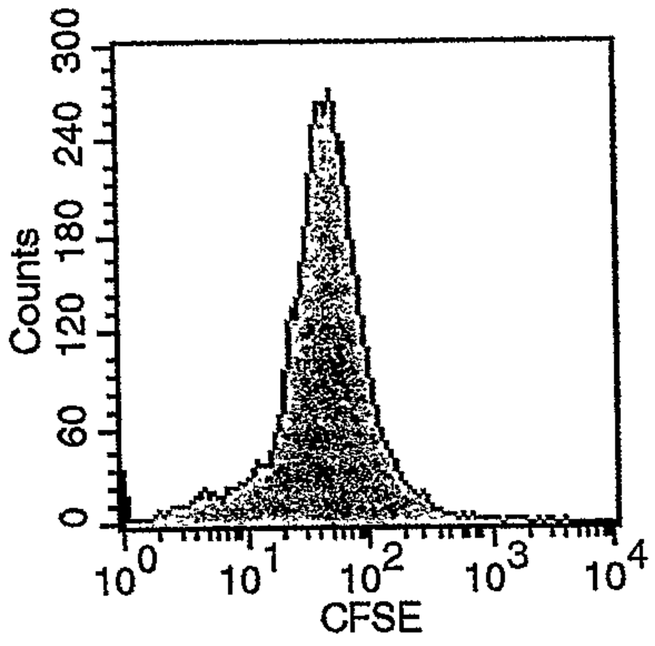


Day 7 irradiated cells

Total Events: 35413

Mean	CV	Peak	Peak Ch
50.53	168.24	265	39

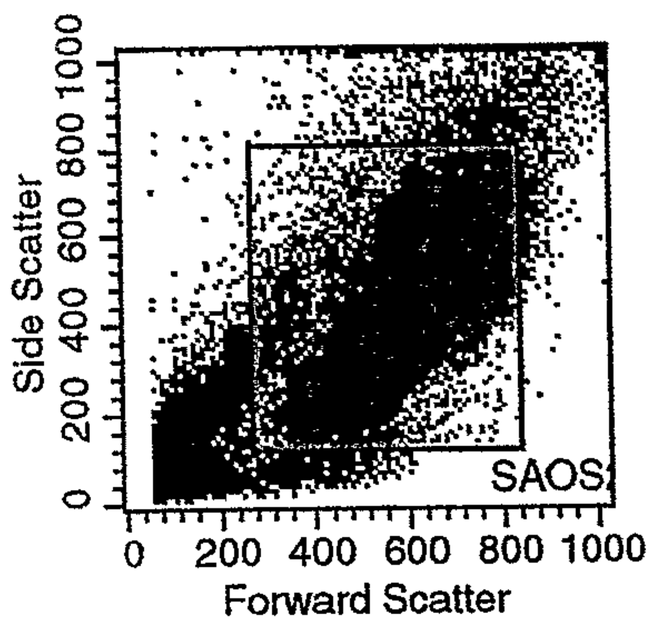


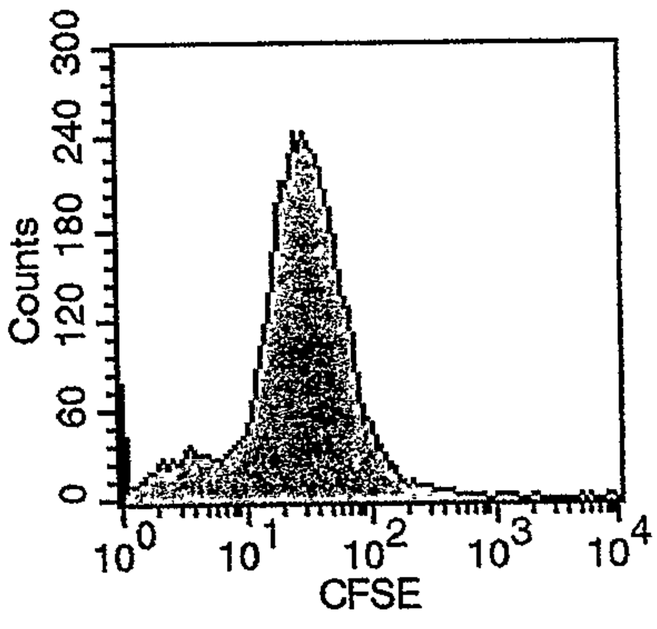


Day 7 control

Total Events: 36983

Mean	CV	Peak	Peak Ch
54.96	187.97	268	46

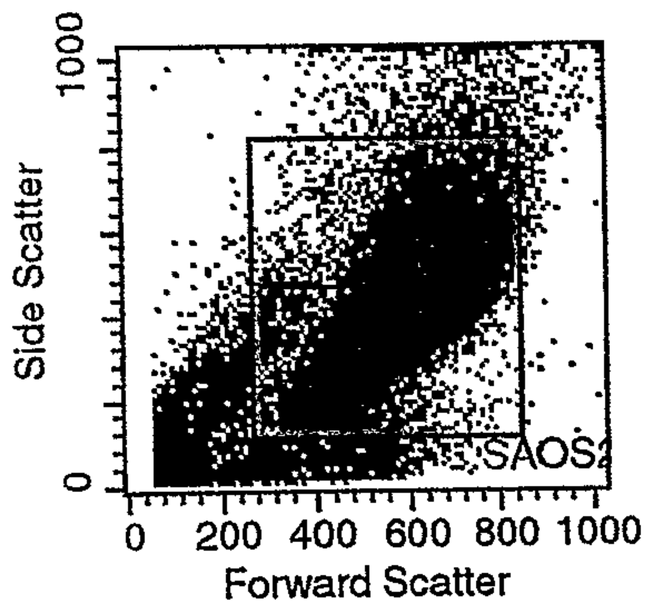


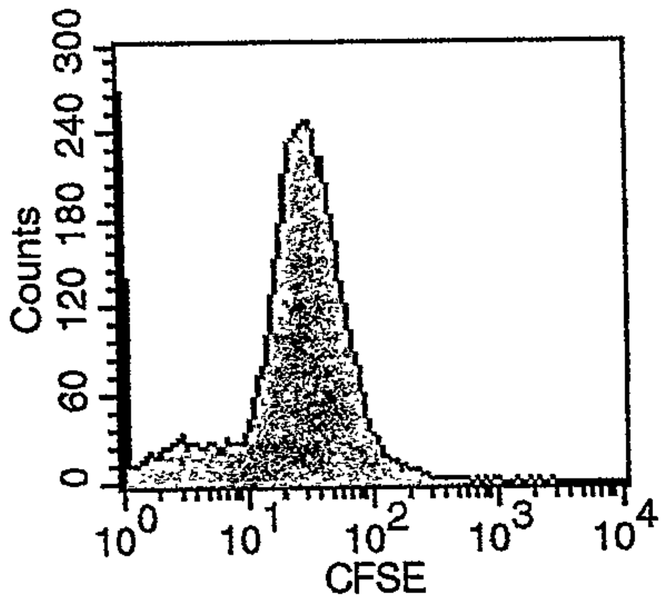


Day 8 irradiated cells

Total Events: 39245

Mean	CV	Peak	Peak Ch
36.36	287.41	241	27

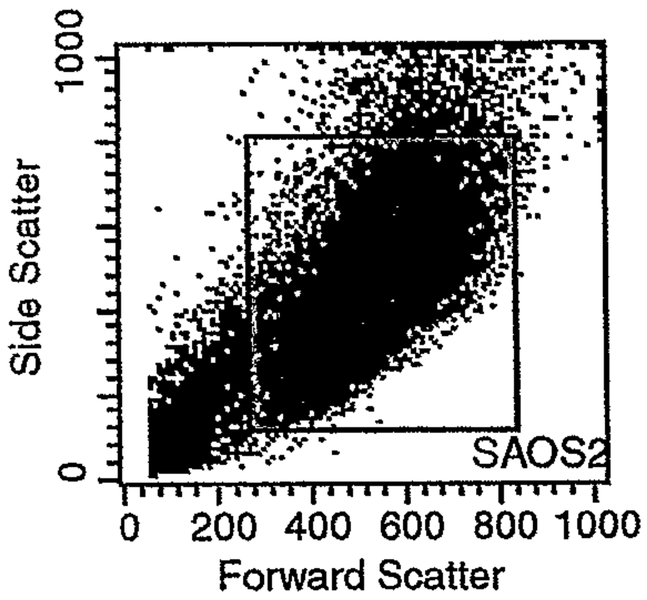


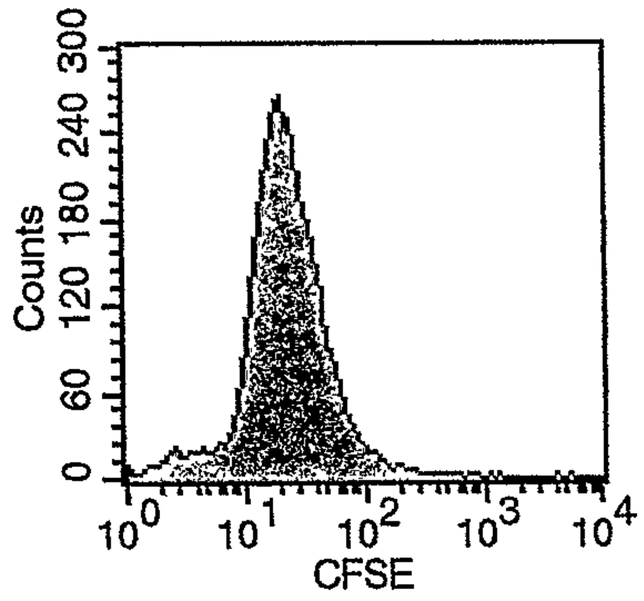


Day 8 control

Total Events: 37293

Mean	CV	Peak	Peak Ch
33.26	280.47	264	1

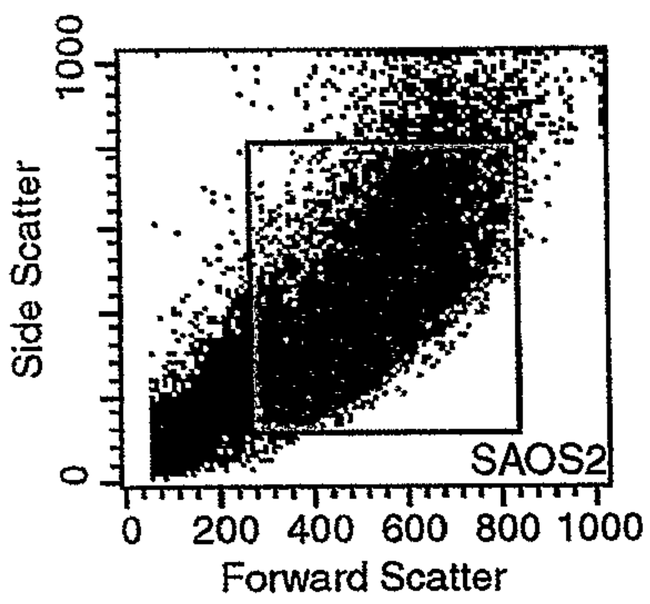


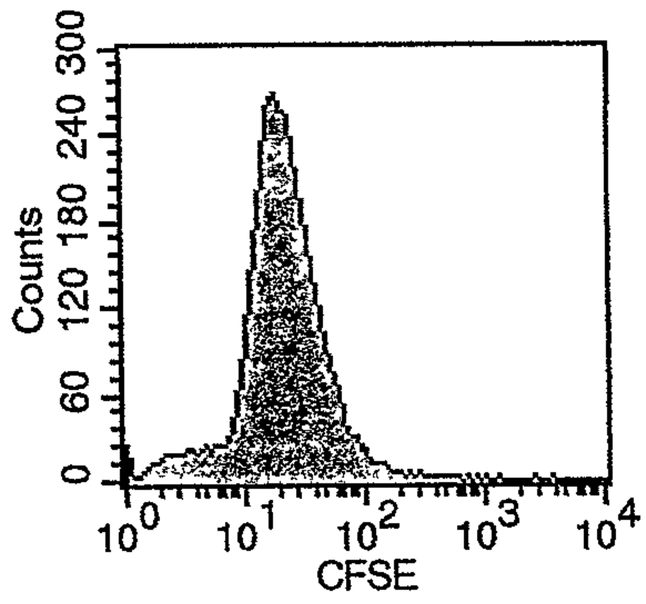


Day 9 irradiated cells

Total Events: 35474

Mean	CV	Peak	Peak Ch
26.69	151.48	261	18

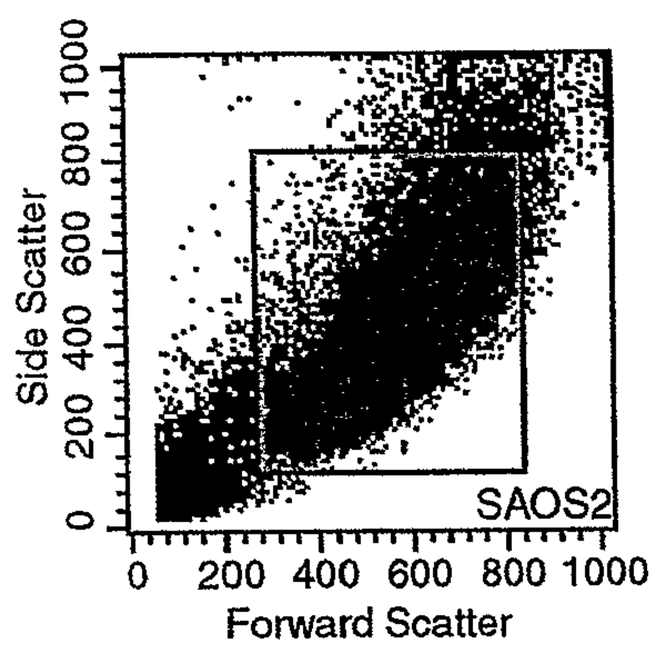


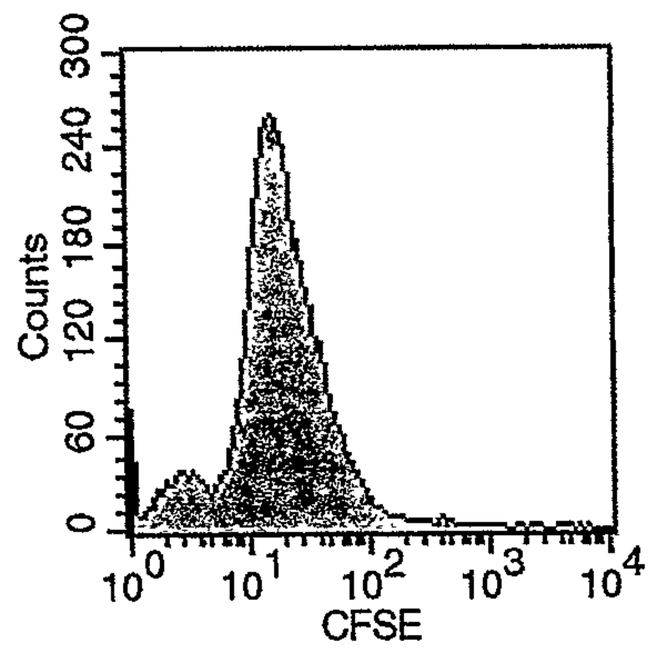


Day 9 control

Total Events: 36067

Mean	CV	Peak	Peak Ch
24.61	126.44	266	17

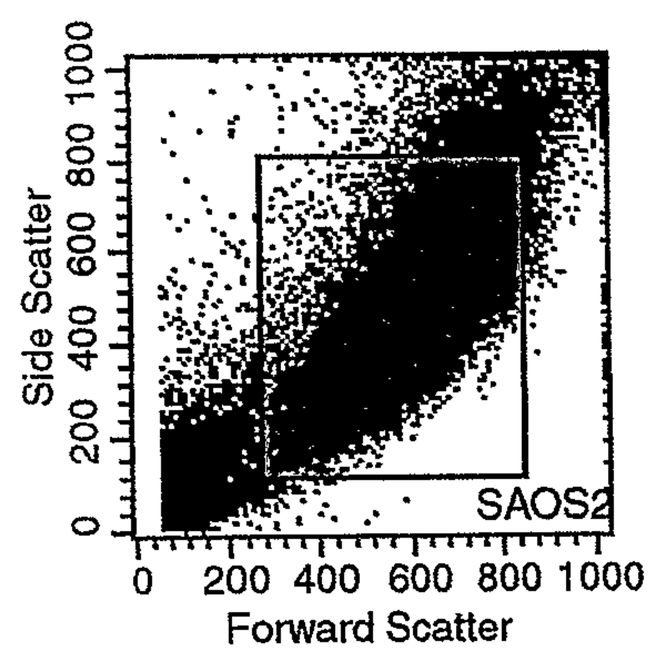


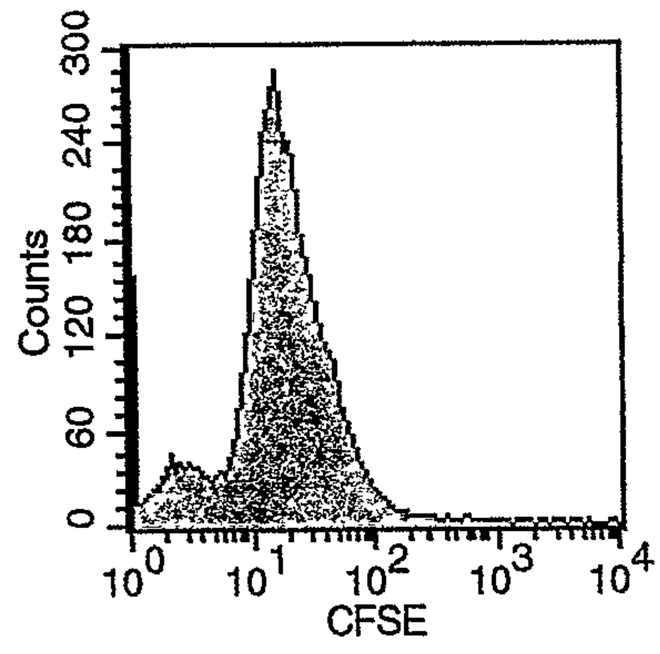


Day 10 irradiated cells

Total Events: 37439

Mean	CV	Peak	Peak Ch
24.37	332.17	256	15



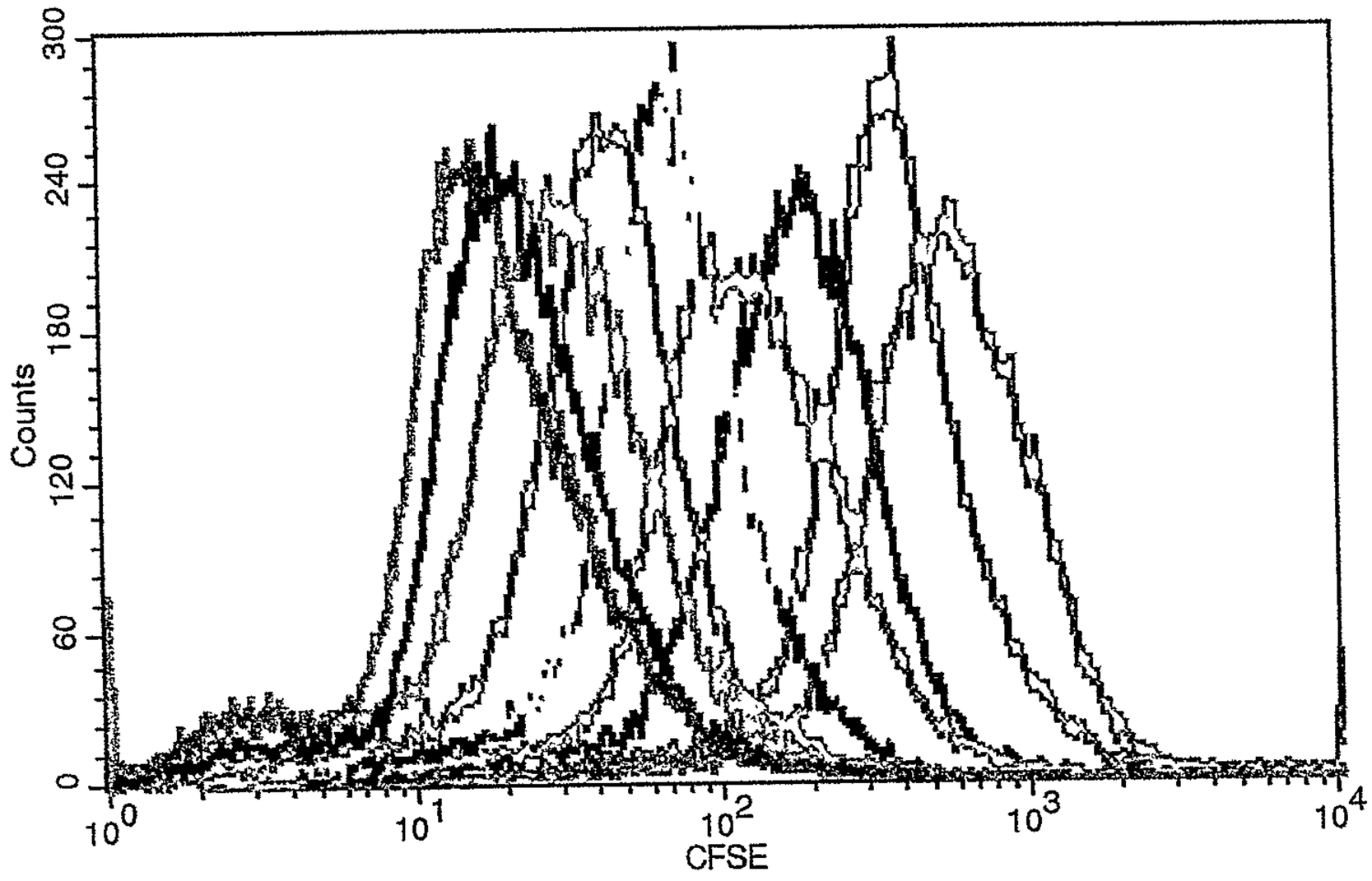


Day 10 control

Total Events: 40356

Mean	CV	Peak	Peak Ch
24.60	343.09	356	1

Overlay flow cytometry plot showing the shift, from day 1 on the right through to day 10 on the left.



5.4 Heat Shock Response of Irradiated Cells

Cells have evolved mechanisms by which to survive extreme environmental insults. A mechanism shared by all cells is the heat-shock response (Alberts *et al*. 1994). A variety of environmental disruptions, such as a sudden temperature increase, induce cells to rapidly synthesize a group of polypeptides known as heat shock (stress) proteins (HSP) that enables them to adapt to this challenge (Stryer, 1988). Studies have shown that various stressors transiently increase production of heat shock proteins as protection against harmful insults. The degree of induction is dependent on the level and duration of exposure to stress. Cell signal transducers, such as changes in intracellular pH, cyclic AMP, Ca^{2+}, N^{+}, inositol triphosphate, protein kinase C, and protein phosphatases (Kiang *et al*. 1998) can modulate their expression.

Heat shock protein 70 is a molecular chaperone whose function it is to help partially denatured proteins fold and assemble into stable, active structures (Alberts *et al*. 1994). Heat shock proteins are expressed in the nucleus of cells of all species, the functions of which are essential in every living cell. They are required for repairing the damage resulting from stress.

Table 5.4.1 Conditions that Induce Expression of Heat Shock Proteins

Physiological	Pathological	Environmental
Cycle of cell division	Viral infection	Heat shock
Growth factors	Bacterial infection	Heavy metals
Cell differentiation	Parasitic infection	Metabolic inhibitors
		Amino acid analogs
Tissue development	Fever	Ethanol
Hormonal stimulation	Inflammation	Antibiotics
	Ischemia	Radiation
	Hypertrophy	
	Oxidant injury	
	Malignancy	
	Autoimmunity	

To gain some insight into the level of physiological stress the osteosarcoma cells are subjected to by laser irradiation, by examination of the expression of heat shock protein-70.

Experiment 5.4.1 Untreated cells were seeded to a confluent monolayer in small flasks ($25cm^2$, Starstedt) and cultured for 24 hours. The cells were heated for a period of one hour in a water bath set to a temperature of 42°C to establish a response profile against which the irradiated cells were compared. After removal from the water bath the cells are returned to the incubator (37°C, 5% CO_2), and removed at pre-determined time intervals for analysis. The

time intervals were 0, 1, 2, 4, and 6 hours. This time series approximated the time at which a maximum response occurs for these cells.

Experiment 5.4.2 Untreated cells were seeded to form a confluent monolayer in small flasks and cultured for 24 hours. The cells were heated for a period of one hour in a water bath set at 42°C. The cells were then returned to the incubator (37°C, 5% CO_2), and removed at pre-determined time intervals for analysis. The time intervals were 0, ½, 1, 1½, and 2 hours. This time series established a more specific time at which the maximum response occurs for this cell line.

Experiment 5.4.3 Untreated cells were densely seeded in 96-well microplates and cultured for 24 hours. The cells were irradiated at 2 Joules and returned to the incubator. Control cells were in the same plates as the experimental group, and were thus exposed to similar environmental conditions for the same period of time. The cells were removed from the incubator at pre-determined time intervals for analysis. The time intervals were 0, ½, 1, 1½, and 2 hours as established in Experiments 5.1 and 5.2.

Experiment 5.4.4 The above series was carried once again to determine that the results were repeatable. Two control groups were compared. The first control was left in the incubator, unexposed to the outside environment. The second control was exposed to the same conditions as the experimental groups.

5.4.1 Experimental design

Response patterns were established for pre-determined time intervals by heating cultured cells at 42°C for one hour, followed by sequential measurement of the HSP70 profile. Responses to laser irradiation of 2 Joules were assessed against the heating profile. Irradiated cells and unirradiated control groups were compared.

Cell suspensions were treated as follows:

Tube	Unstained	Isotype control	HSP-70	FITC IgG_1
1	*	-	-	-
2	-	*	-	*
3	-	-	*	*
4	-	-	-	*

5.4.2 Expression of results

Dot plots of log Fl-2 versus log FL-1 were generated. From this data, histograms and statistical data could be generated. Results were expressed as the percentage changes of the Isotype control and heat shock protein-70 compared to their relative controls.

5.4.3 Results

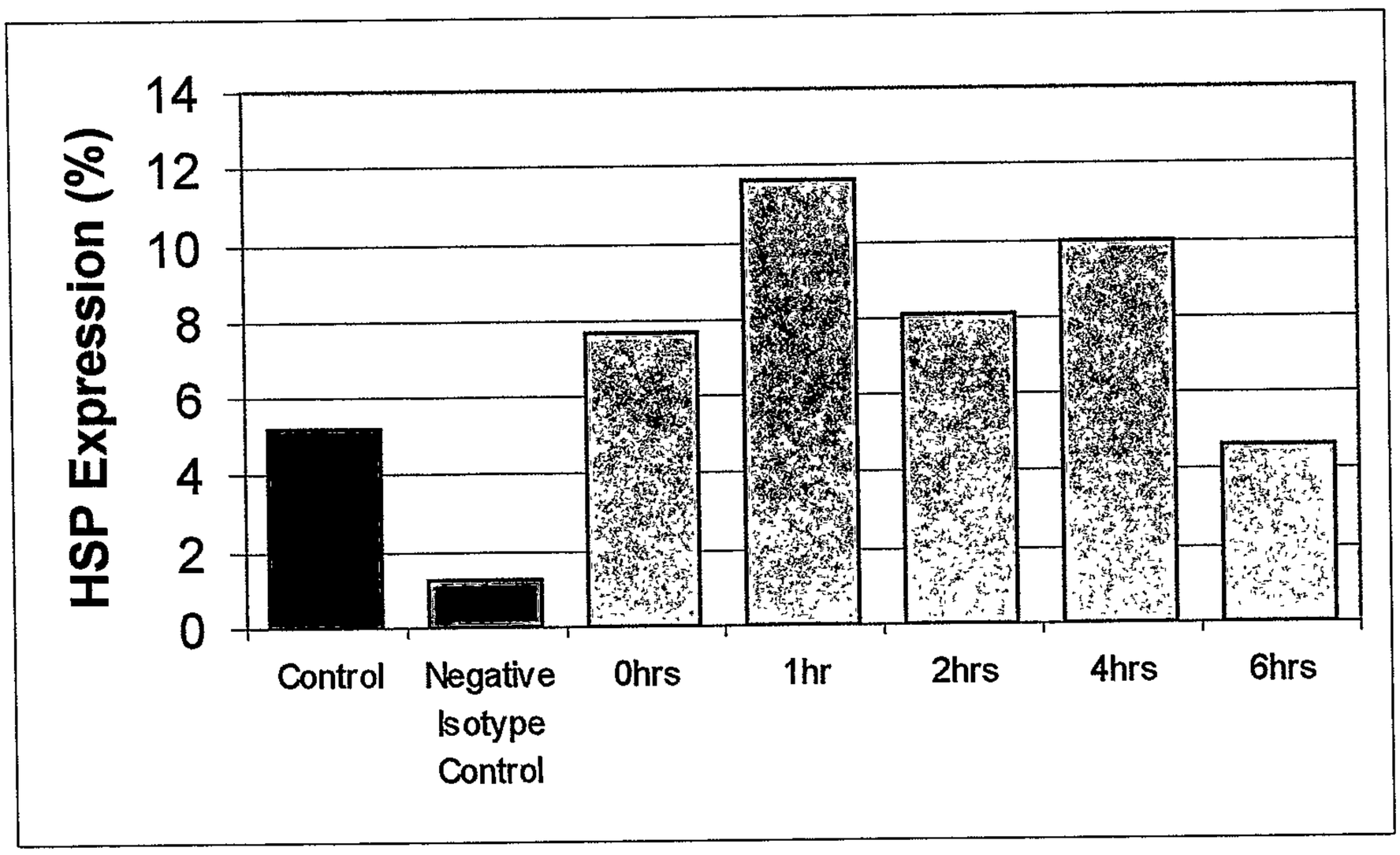
Experiment 5.4.1

Heating profile of osteosarcoma cells from 0 to 6 hours after heating.

Table 5.4.2

Condition Tested in Experiment 5.4.1	% HSP 70
Isotype Control in flasks	0.26
HSP 70 Control in flasks	5.20
0 Hours after Heating	7.64
1 Hour after Heating	10.50
2 Hours after Heating	8.08
4 Hours after Heating	9.98
6 Hours after Heating	4.59

Figure 5.4.1
Percentage HSP70 expression from heating and negative Isotype control relative to the HSP control group.



Heat shock protein-70 was observed to be maximally expressed 1 hour after heating.

Experiment 5.4.2

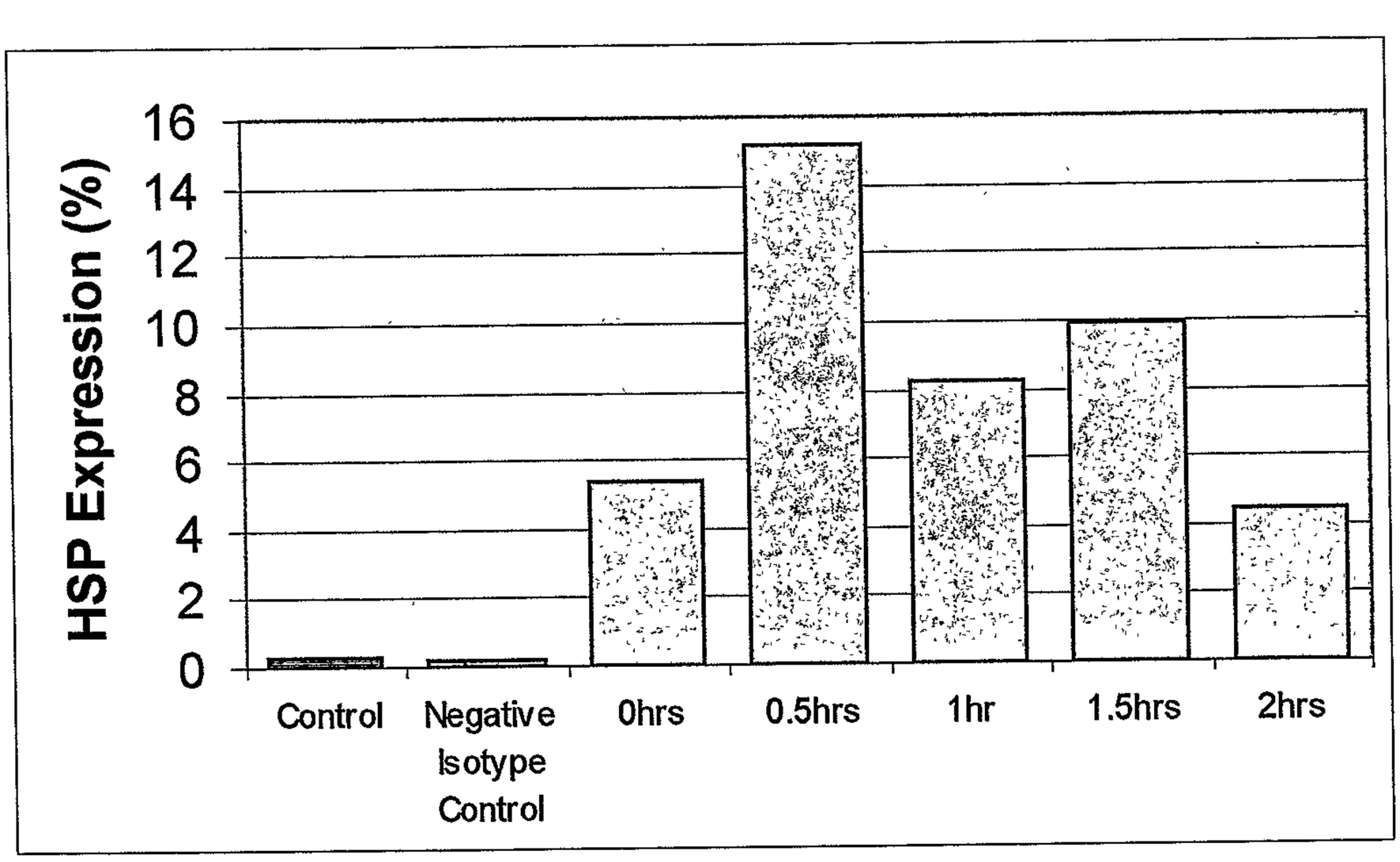
More specific heating profile of osteosarcoma cells from 0 to 2 hours.

Table 5.4.3

Condition Tested in Experiment 5.4.2	% HSP 70
Isotype Control in flasks	0.15
HSP 70 Control in flasks	0.31
0 Hours after Heating	5.40
1/2 Hour after Heating	15.13
1 Hour after Heating	8.27
1 1/2 Hours after Heating	9.86
2 Hours after Heating	4.41

Figure 5.4.2

Percentage HSP70 expression from heating and Negative Isotype control relative to the HSP control group.



Heat shock protein-70 was found to be expressed most from 0.5 to 1.5 hours after heating.

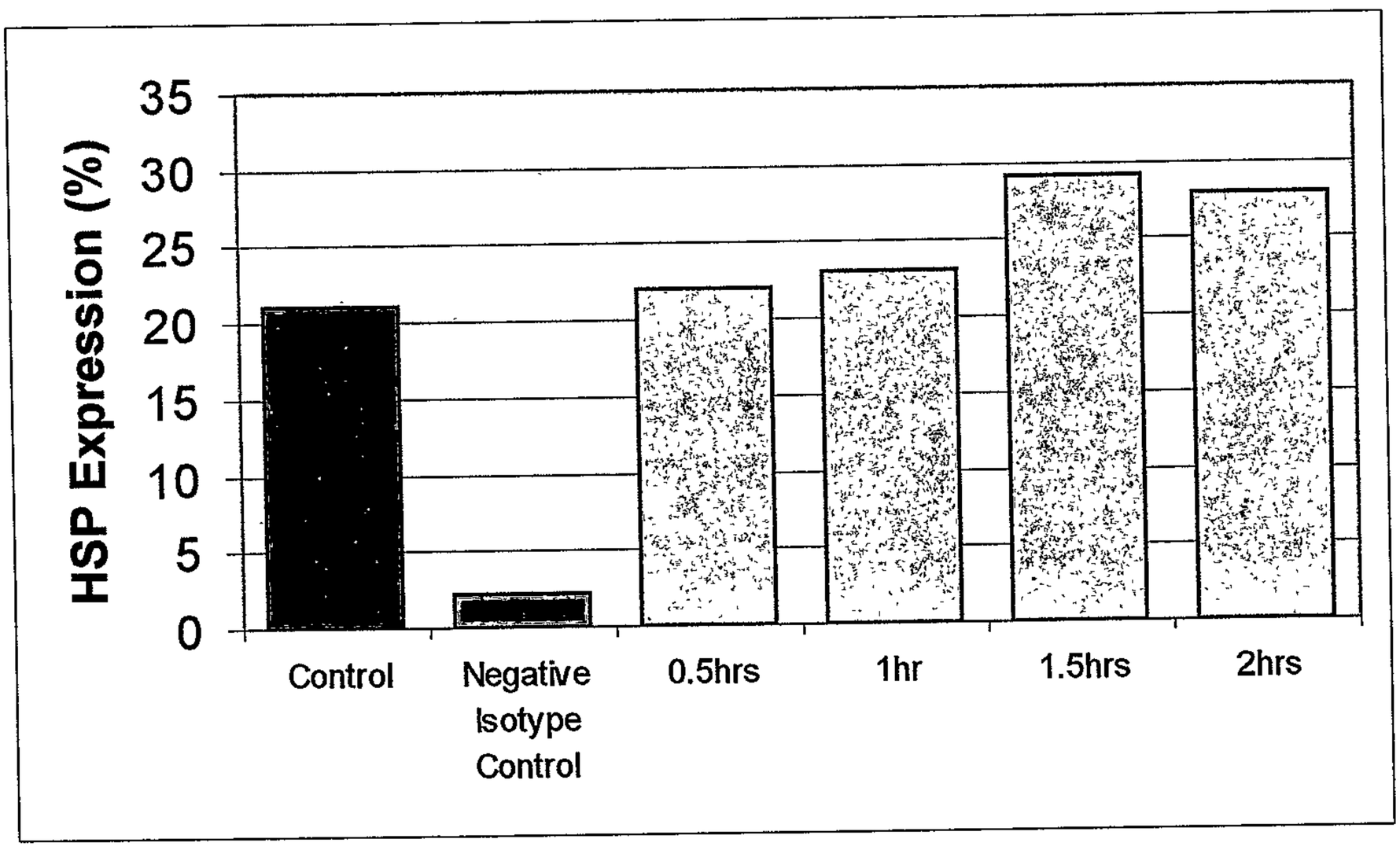
Experiment 5.4.3

Heat shock protein-70 expression after laser irradiation and matched control over the 2 hours following irradiation.

Table 5.4.4

Condition Tested in Experiment 5.4.3	% HSP 70
Isotype Control in 96 well plates	1.73
HSP 70 Control in 96 well plates	21.04
$^1/_2$ Hour after laser	22.15
1 Hour after laser	23.08
$1^1/_2$ Hours after laser	29.23
2 Hours after laser	27.82

Figure 5.4.3
Percentage HSP70 expression and negative Isotype control for irradiated cells.



A heat shock response was observed, with the maximum heat shock protein-70 expression occurring at 1.5 hours post-irradiation.

Experiment 5.4.4
Repeat of Experiment 5.4.3 with additional control left in the incubator.

Table 5.4.5

Condition Tested in Experiment 5.4.4	% HSP 70
Isotype Control in 96 well plates in incubator	2.17
HSP 70 Control in 96 well plates	17.79
Isotype Control in 96 well plates in air	3.27
HSP 70 Control in 96 well plates	6.93
1/2 Hour after laser	12.04
1 Hour after laser	10.37
1 1/2 Hours after laser	36.81
2 Hours after laser	16.84

Figure 5.4.5
Percentage HSP70 expression and negative Isotype control for irradiated cells relative to environment exposed control.

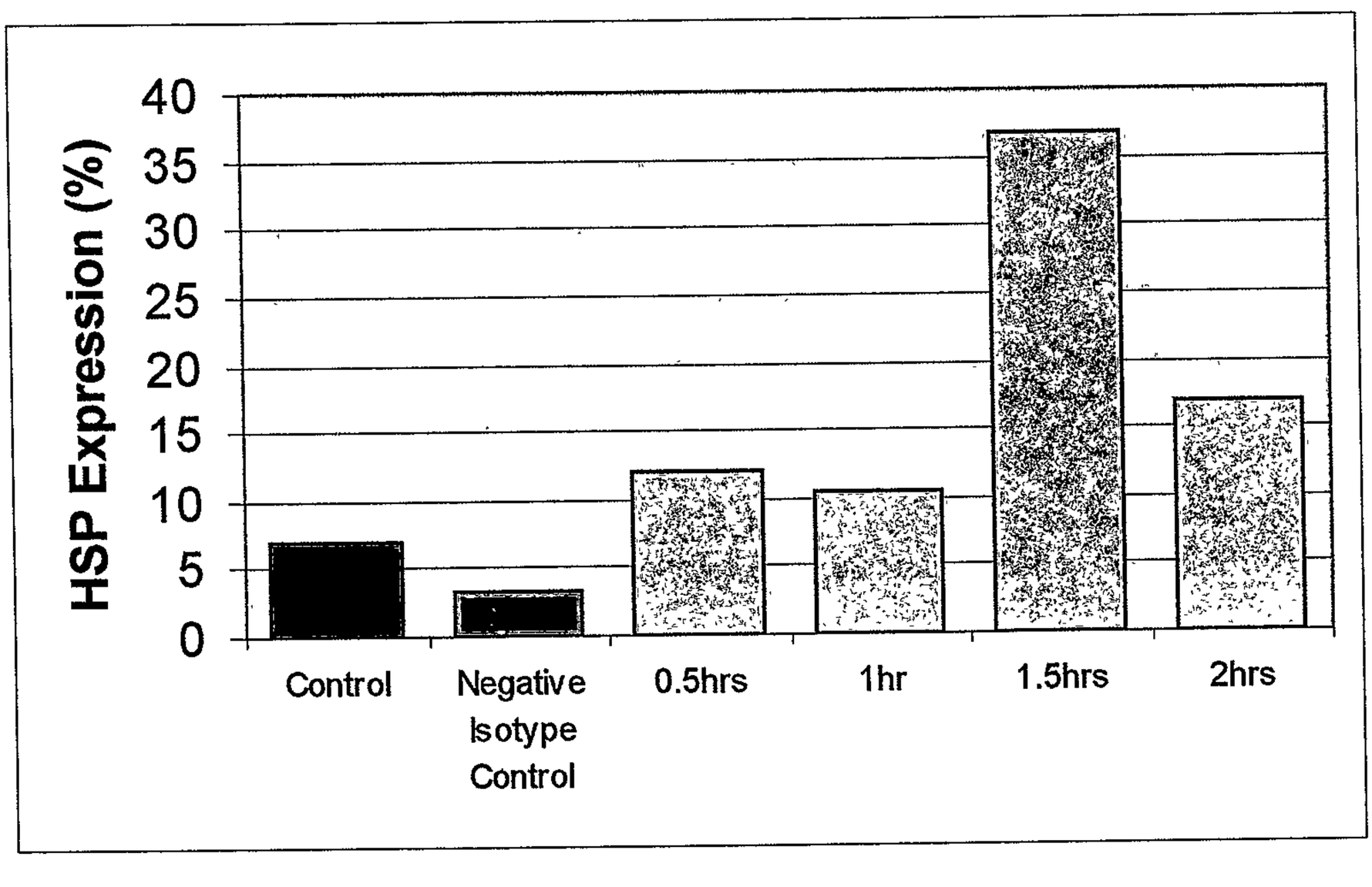
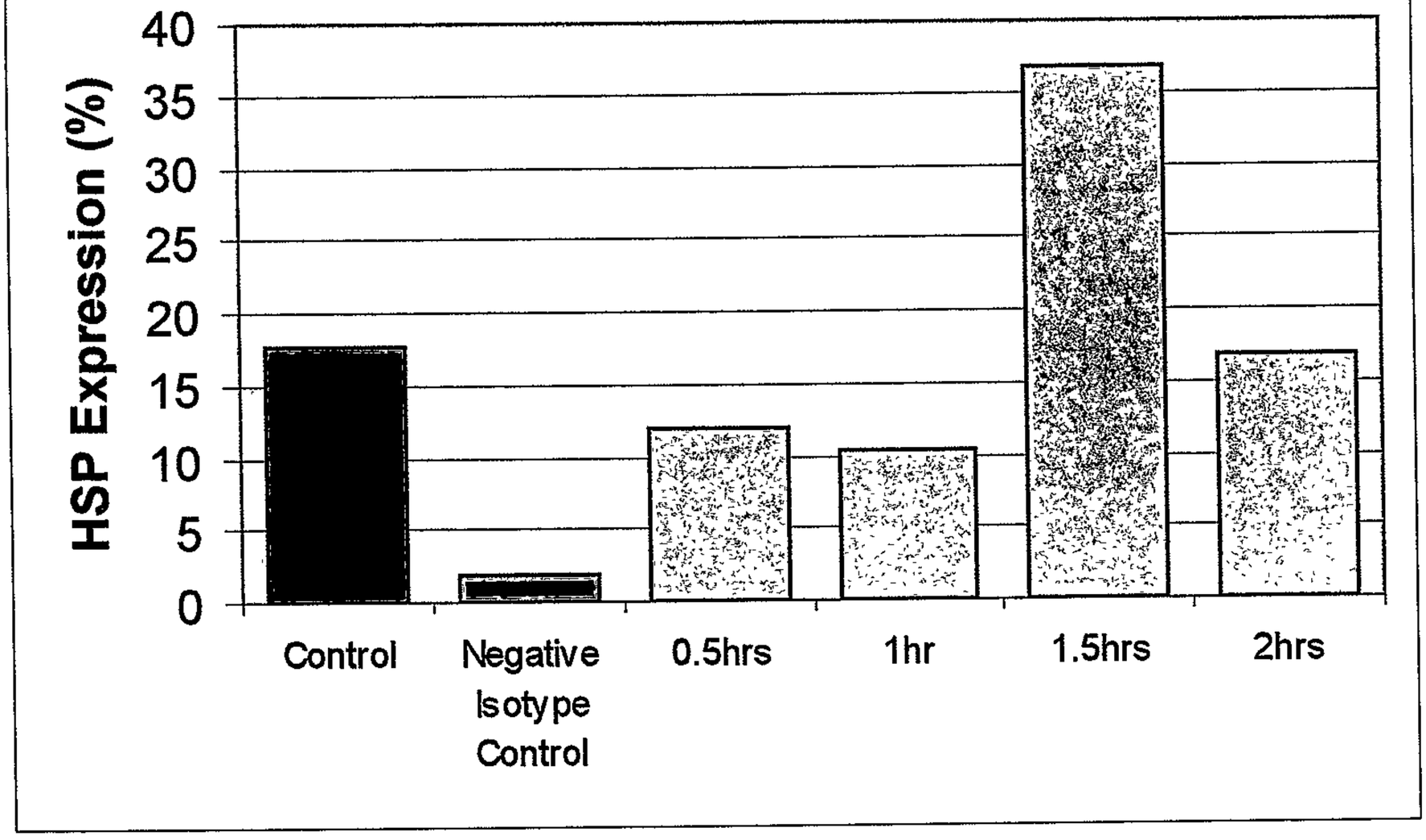
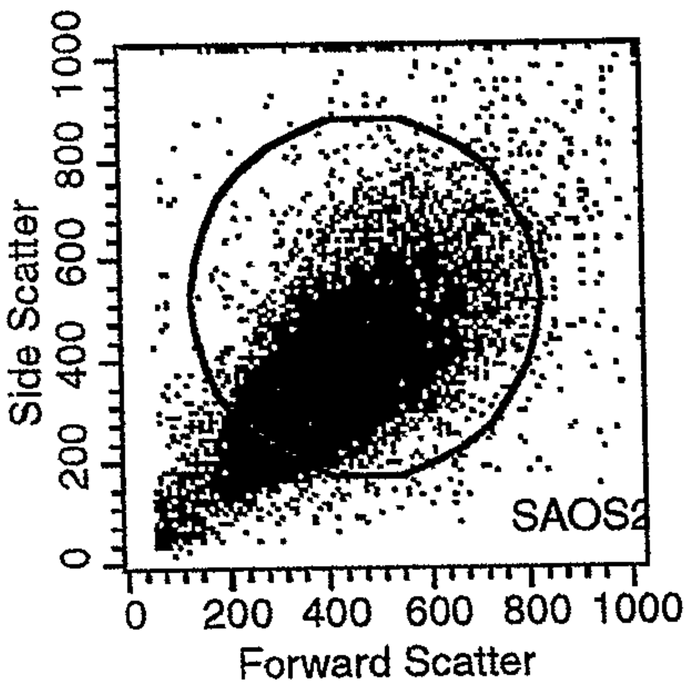


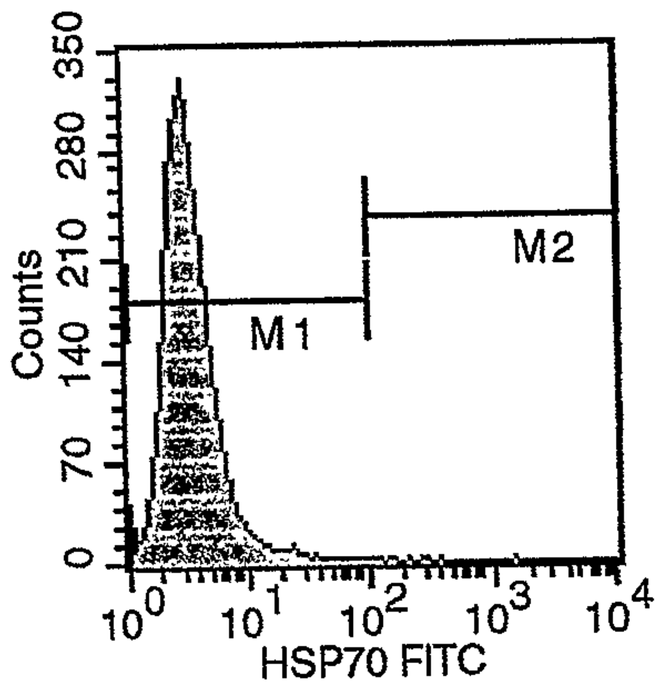
Figure 5.4.6
Percentage HSP70 expression and negative Isotype control for irradiated cells relative to incubated control.



An increase in heat shock protein-70 expression was observed, with peak expression at 1.5 hours post-irradiation. The control exposed to the environment at room temperature expressed less heat shock protein 70 than the control maintained in the incubator. This may be a result of the fact that the temperature in the incubator was higher than room temperature. This finding eliminates the possibility that changes in temperature or air quality / composition due to the time of irradiation may effect heat shock protein-70 expression in a positive manner.

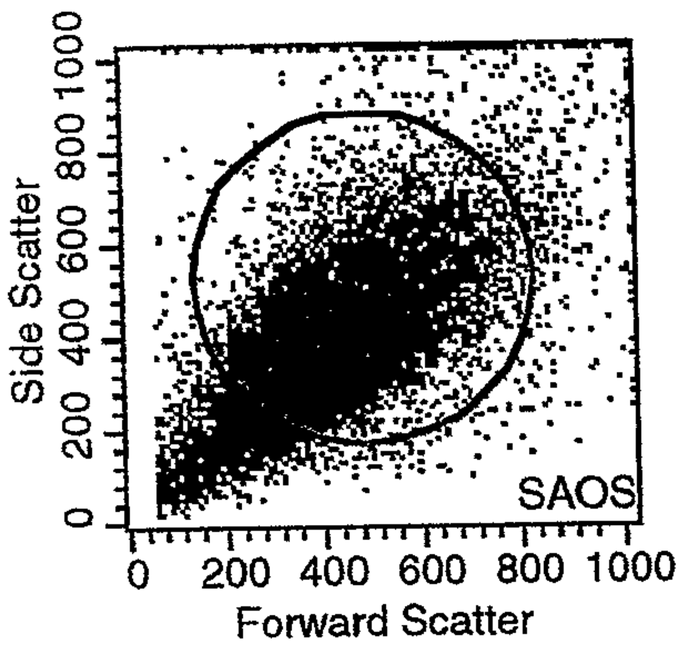
Representative Flow Cytometry plots for Heat Shock Protein-70 experiments.

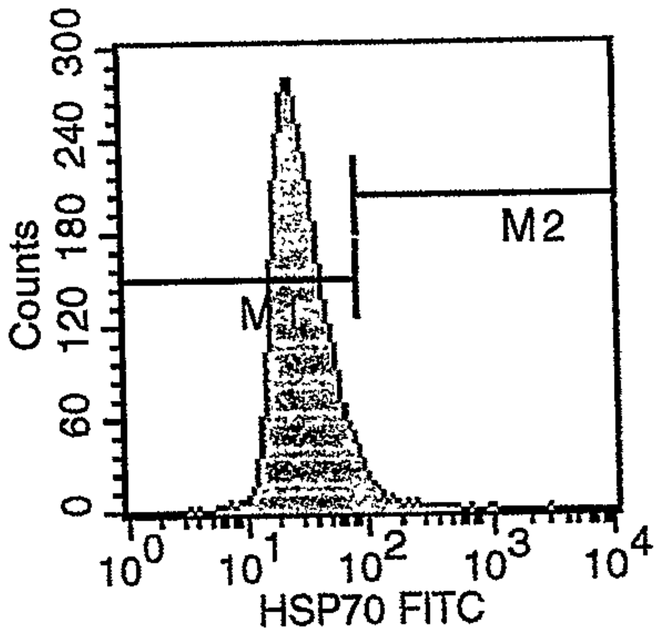




Unstained control

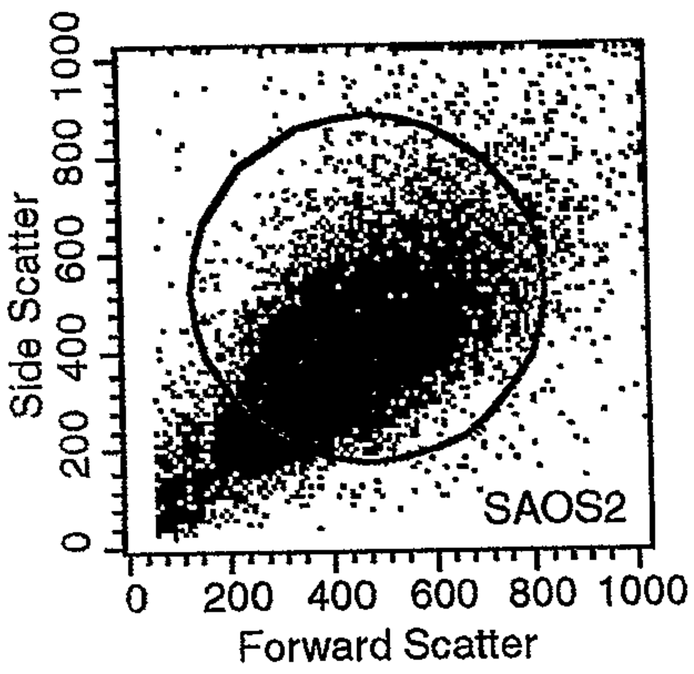
Marker	Events	% Gated	Peak Ch
All	28716	100.00	2
M1	28703	99.95	2
M2	13	0.05	98

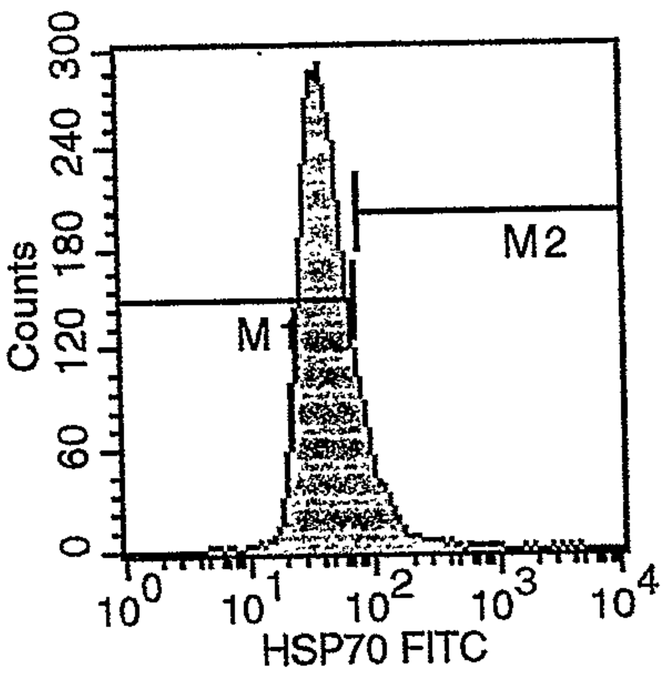




Negative Isotype control

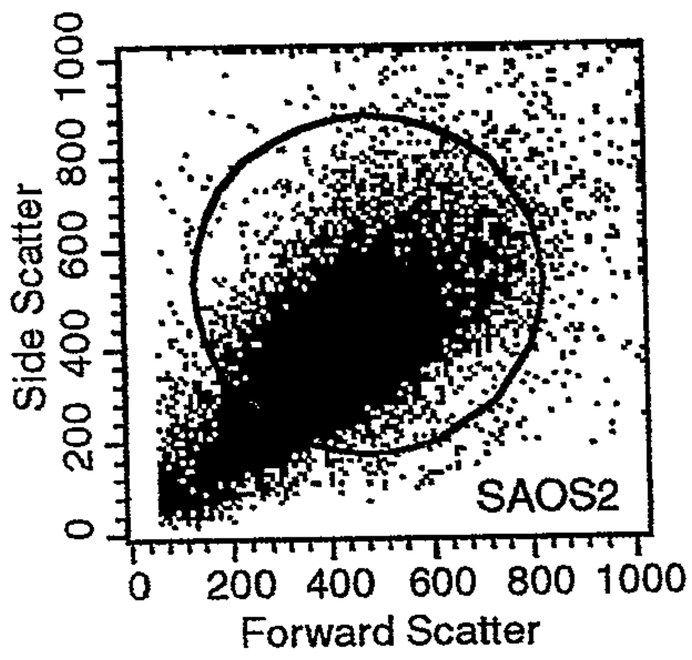
Marker	Events	% Gated	Peak Ch
All	28834	100.00	21
M1	28191	97.77	21
M2	625	2.17	82

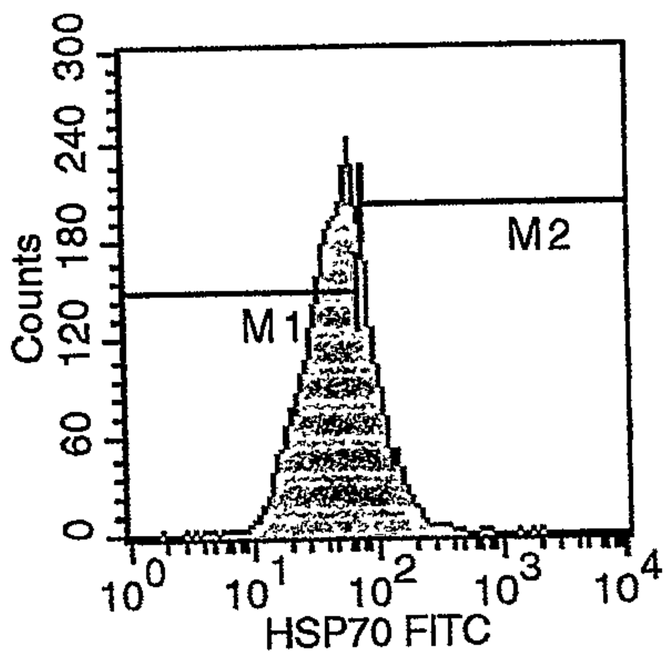




Fitc control

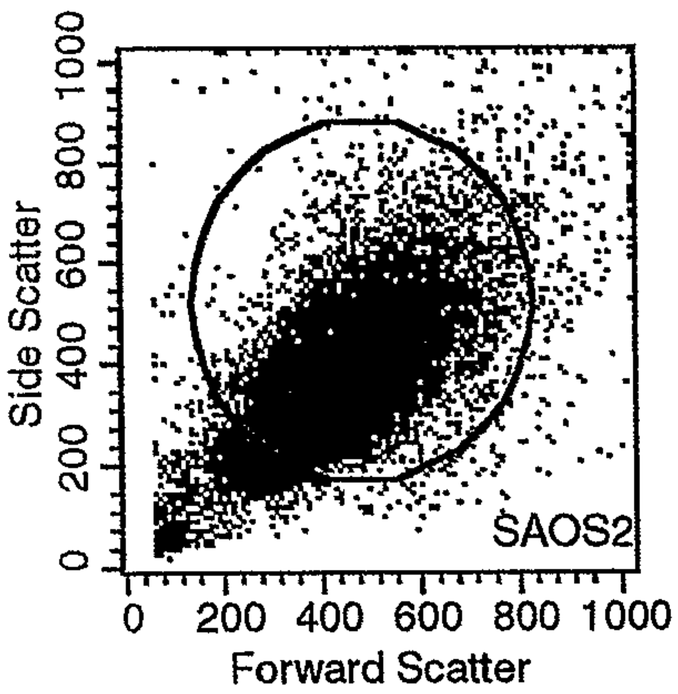
Marker	Events	% Gated	Peak Ch
All	29034	100.00	37
M1	26381	90.86	37
M2	2591	8.92	79

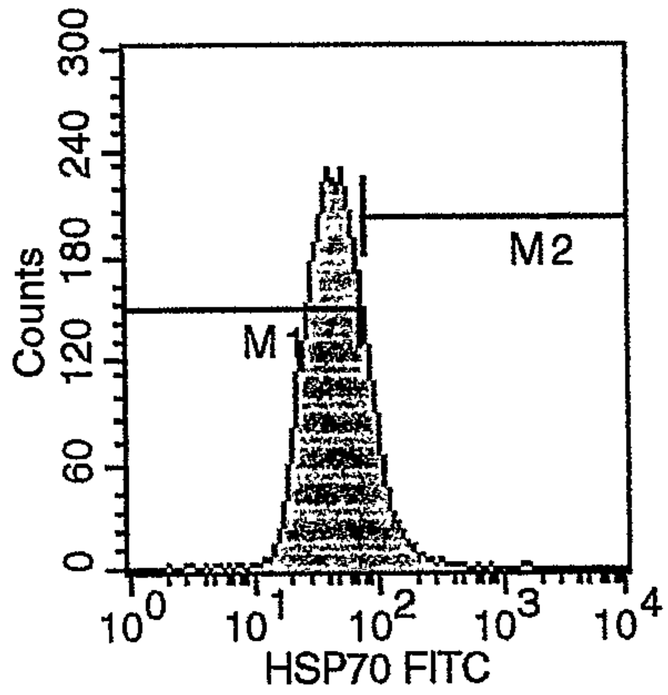




HSP-70 control

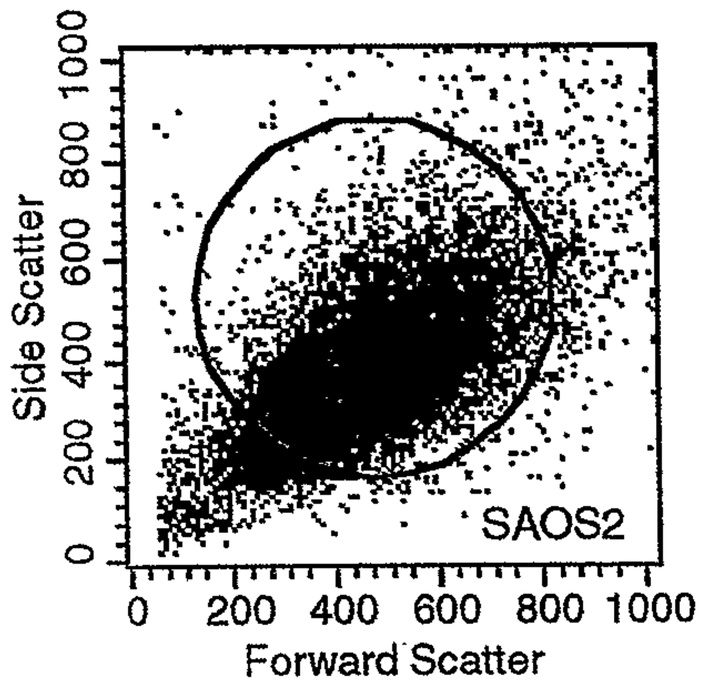
Marker	Events	% Gated	Peak Ch
All	28752	100.00	56
M1	23522	81.81	56
M2	5114	17.79	83

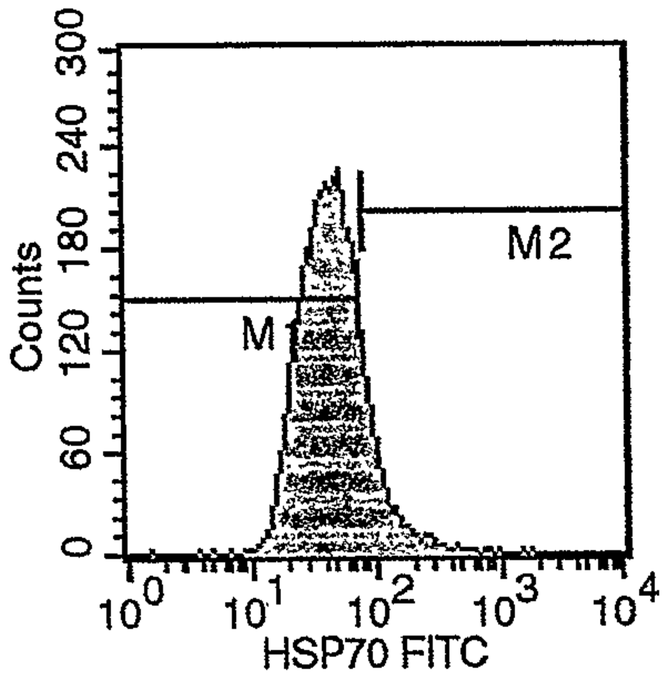




HSP-70 0.5 hours after irradiation

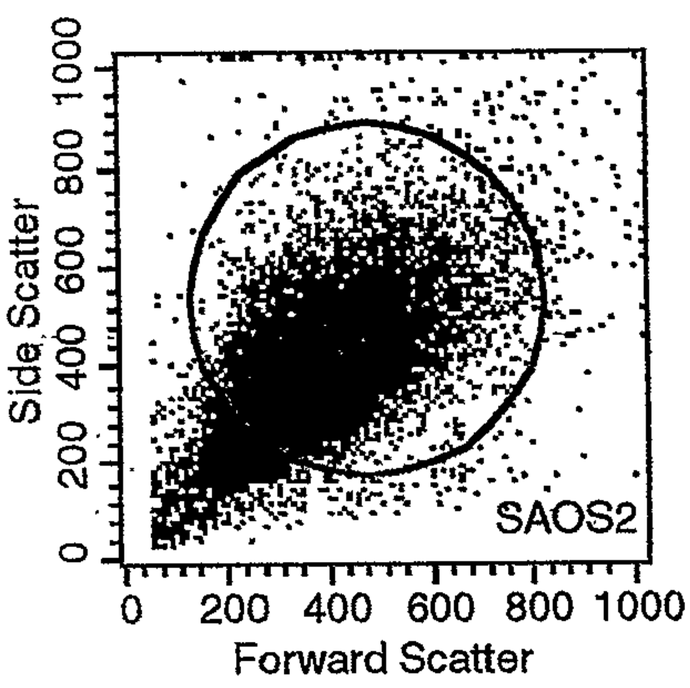
Marker	Events	% Gated	Peak Ch
All	28764	100.00	38
M1	25182	87.55	38
M2	3464	12.04	78

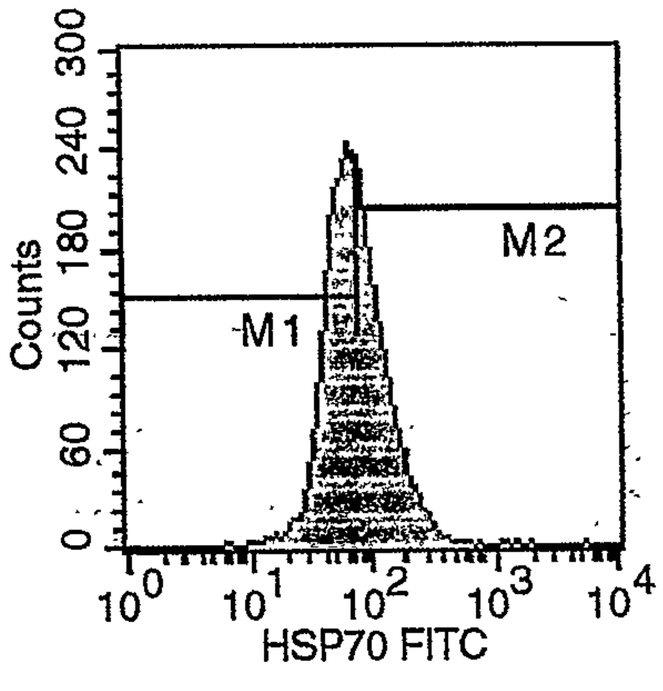




HSP-70 1.0 hour after irradiation

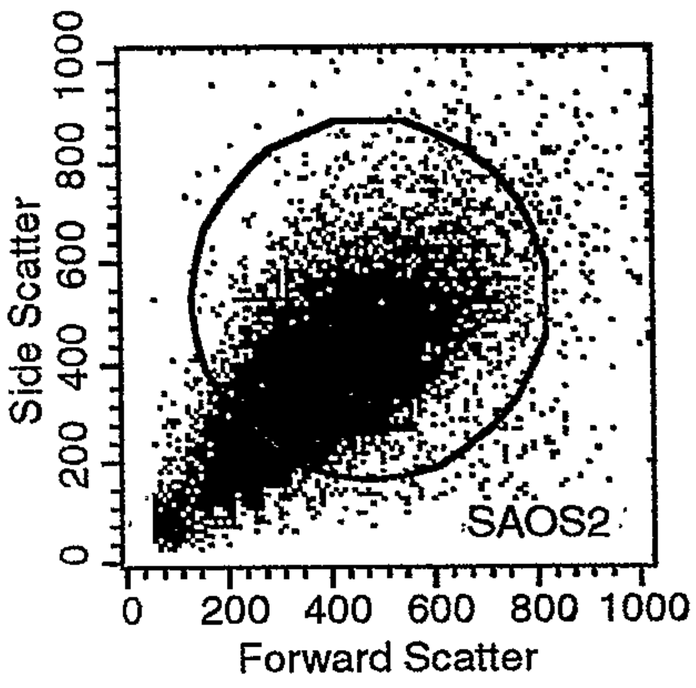
Marker	Events	% Gated	Peak Ch
All	28927	100.00	49
M1	25837	89.32	49
M2	2999	10.37	79

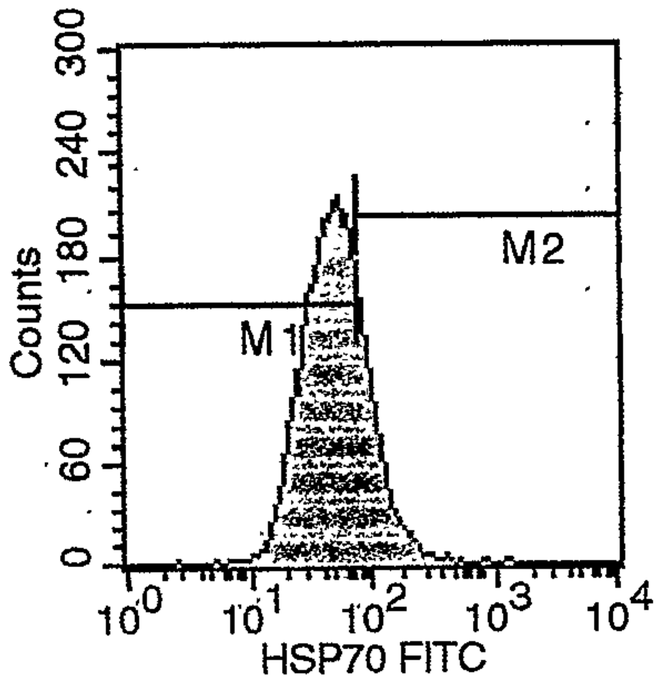




HSP-70 1.5 hours after irradiation

Marker	Events	% Gated	Peak Ch
All	28462	100.00	57
M1	17812	62.58	57
M2	10478	36.81	78

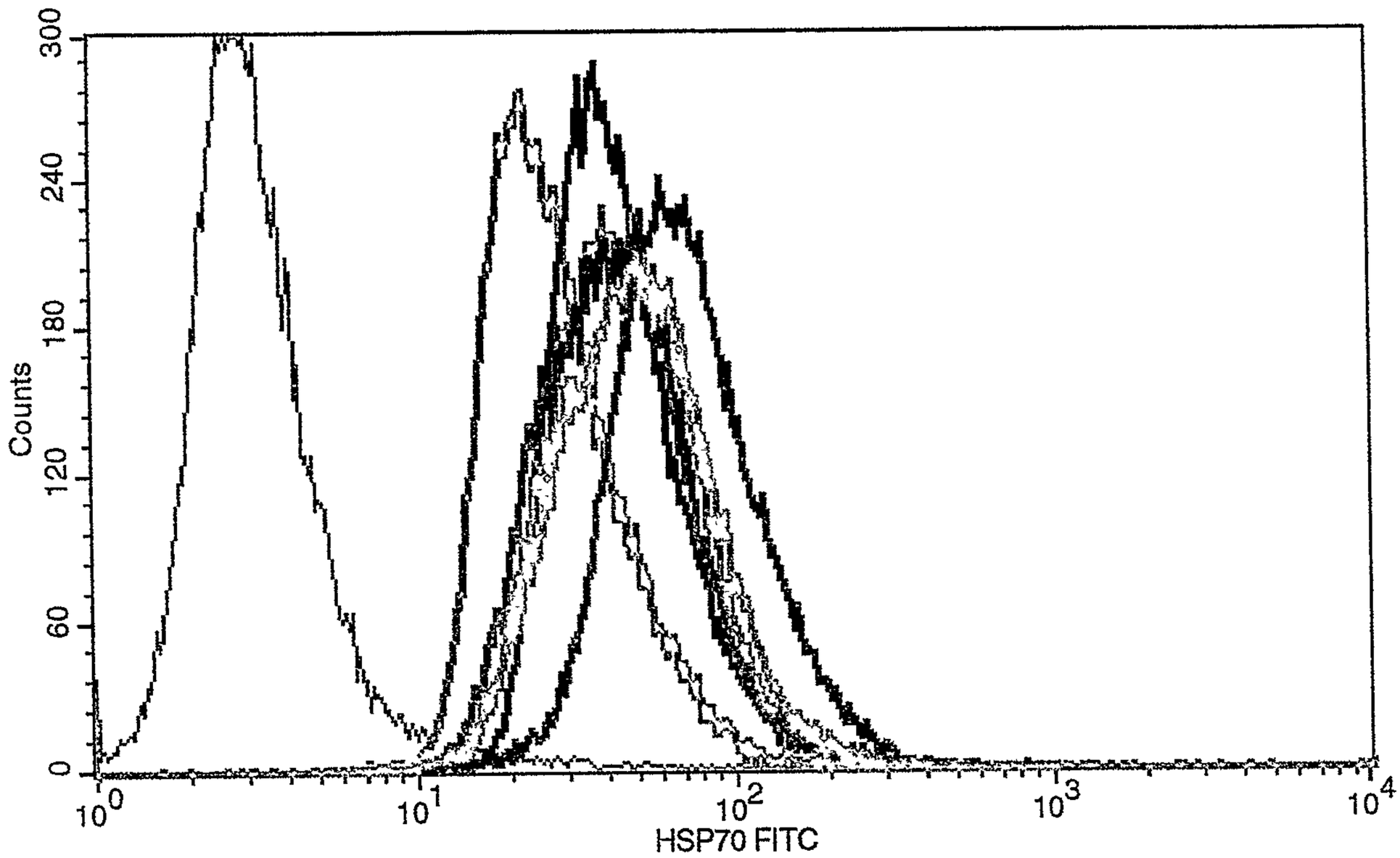




HSP-70 2.0 hours after irradiation

Marker	Events	% Gated	Peak Ch
All	28447	100.00	49
M1	23524	82.69	49
M2	4791	16.84	79

Overlay flow cytometry plot showing HSP-70 response.



Key

- Unstained
- Negative Isotype control
- Fitc
- HSP-70 control
- HSP-70 0.5 hours after irradiation
- HSP-70 1.0 hours after irradiation
- HSP-70 1.5 hours after irradiation
- HSP-70 2.0 hours after irradiation

Heat shock protein-70 is seen to move to the right at 1.5 hours after irradiation.

5.5 Effects of Low Level Laser on Intracellular Calcium Flux

Because of the inability to show an effect with low level laser irradiation at a gross cell culture level, but a heat shock response was observed, it was decided to investigate the effects of low level laser at an intracellular level by determining changes in intracellular calcium concentration.

Changes in cytoplasmic calcium concentrations occur in response to a variety of external signals. This intracellular calcium flux enables a cell to adapt its activities in response to changes in its immediate environment. It is proposed that low level laser irradiation may affect cellular behaviour, in part, via its effect on the permeability of the cell membrane to calcium ions (Young *et al.* 1990).

The cytosolic level of Ca^{2+} in unexcited cells is typically 0.1μM, several orders of magnitude less than in the extracellular environment. This steep gradient allows the opportunity for the cellular cytosolic Ca^{2+} concentration to be abruptly raised for signalling purposes by transiently opening calcium channels in the plasma membrane or in an intracellular membrane (Stryer 1988; Thomas 1994).

The alteration in intracellular calcium levels in response to low level laser therapy may have considerable biological and clinical significance, since calcium ions act as second messengers in many cellular activities. Ca^{2+} is the most common signal transduction element in cells, ranging from bacteria to neurons, and is involved in such diverse cellular processes as fertilisation, muscle contraction and cell growth, synthesis, secretion, intracellular communication, cellular locomotion, endocrine and exocrine secretion, neurotransmitter release and apoptosis (Thomas 1994, Clapham 1995). Cytosolic calcium concentrations are susceptible to rapid and localised increase, which occurs by allowing the calcium ions to enter by gradient density across the plasma membrane or from the endoplasmic reticulum through specialised channels (Clapham, 1995).

Cytoplasmic calcium concentration has been found to change in irradiated cells where accelerated Ca^{2+} transport has been observed (Lubart *et al.* 1992). Transient changes in cytoplasmic Ca^{2+} concentrations can trigger cell mitosis (Lubart *et al.* 1993).

Confocal Microscopy

Confocal laser scanning microscopy is extensively used for studying cellular activities in living cells at a molecular level, through monitoring the temporal and spatial (compartmentalisation) changes of biologically active molecules such as Ca^{2+} which have been rendered visible by fluorescent labels. An image is built up by scanning the confocal pinholes in two dimensions over the sample.

The main disadvantages of confocal microscopes are the relatively low temporal resolution and the high level of excitation light required (Thomas and Delaville 1991). The possibility of spontaneous and sustained increases in fluorescence signal intensity (Lui *et al.* 1997) after being exposed to laser scanning, without added stimuli, is another potential problem. Cells may be pre-stimulated by the argon laser at 488nm. Effects at this wavelength include increased DNA synthesis and therapeutic actions (Cosic 1994).

Cellular calcium homeostasis can be studied with the use of fluorescent indicators of free Ca^{2+} ion concentration, which can be loaded into cells in a non-disruptive manner. Fluorescent calcium indicators offer the most straightforward and accessible of available techniques for measuring cytosolic-free Ca^{2+} (Thomas and Delaville, 1991).

Indicators as membrane permeant esters can be loaded into the cell without any disruption of the cell membrane. This is achieved by incubating intact cells with an esterified form of the indicator. Once in the cytosol, endogenous esterases activate the calcium ion indicators releasing the free acid forms, resulting in the accumulation of trapped indicator in the cell cytosol. Once extracellular dye has been removed, fluorescence signals from the cytosolic dye can be recorded.

Fluorescent probes enable researchers to detect particular components of complex biomolecular assemblies, including live cells, with great sensitivity and selectivity (Johnson 1996). Fluorescence is a molecular process in which emission of electromagnetic radiation results from the absorption of radiation from an extra-molecular source (Thomas and Delaville, 1991). It is the result of a three-stage process occurring in certain molecules called fluorophores or fluorescent dyes. A fluorescent probe is a fluorophore designed to localise within a specific region of a biological specimen or to respond to a specific stimulus (Johnson 1996).

Fluorescent Ca^{2+} indicators with high fluorescence yields have made it possible to measure intracellular calcium concentration at the single cell level and at the subcellular level, using imaging methods (Thomas and Delaville 1991).

5.5.1 Experimental Design

A positive control was established initially and the degree of photobleaching was investigated. Images of cells irradiated at varying energy levels (1.0, 2.0 and 4.0 Joules) captured over a 5 minute period were compared to the pre-irradiated control images.

5.5.2 Expression of Results

Results are expressed as graphs illustrating the percentage change of the average brightness value relative to the pre-treatment control. Standard errors of the mean are included as error bars. Examples of 2 dimensional confocal images are presented.

Figure 5.5.1 Example of 2 Dimensional Confocal images of osteosarcoma cells treated with the positive control Calcium Ionophore.

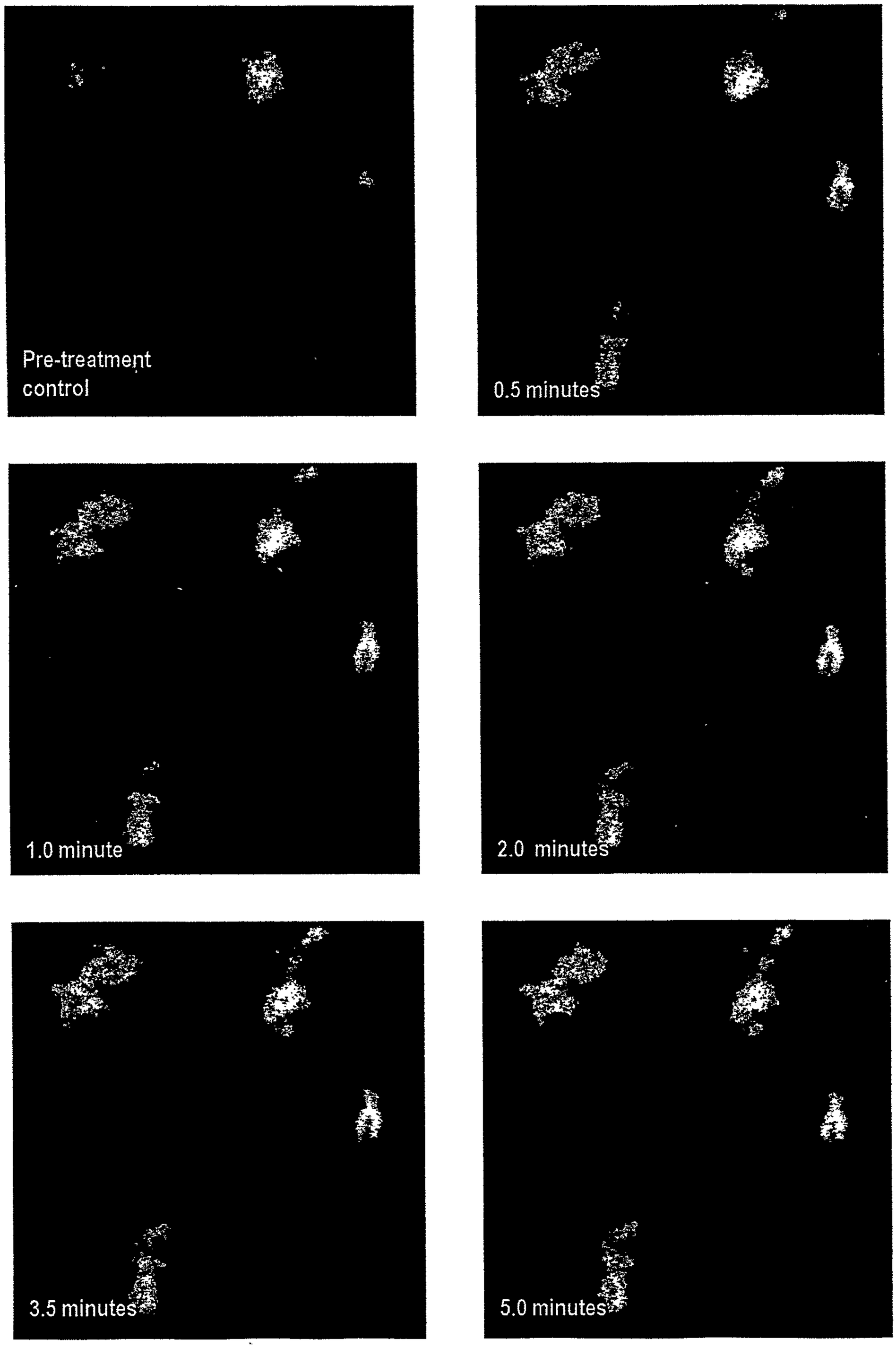
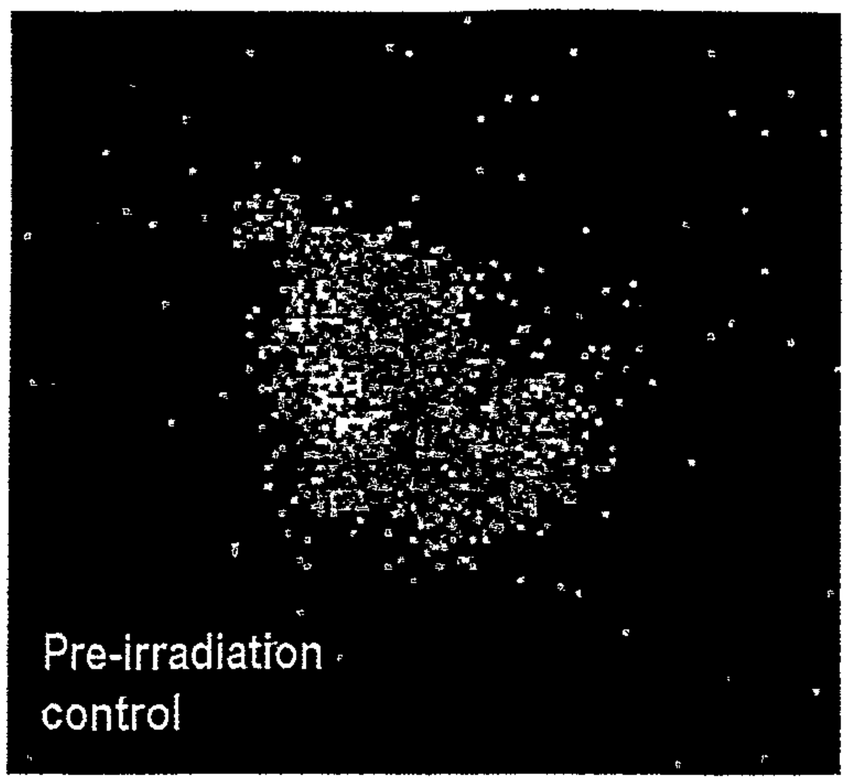
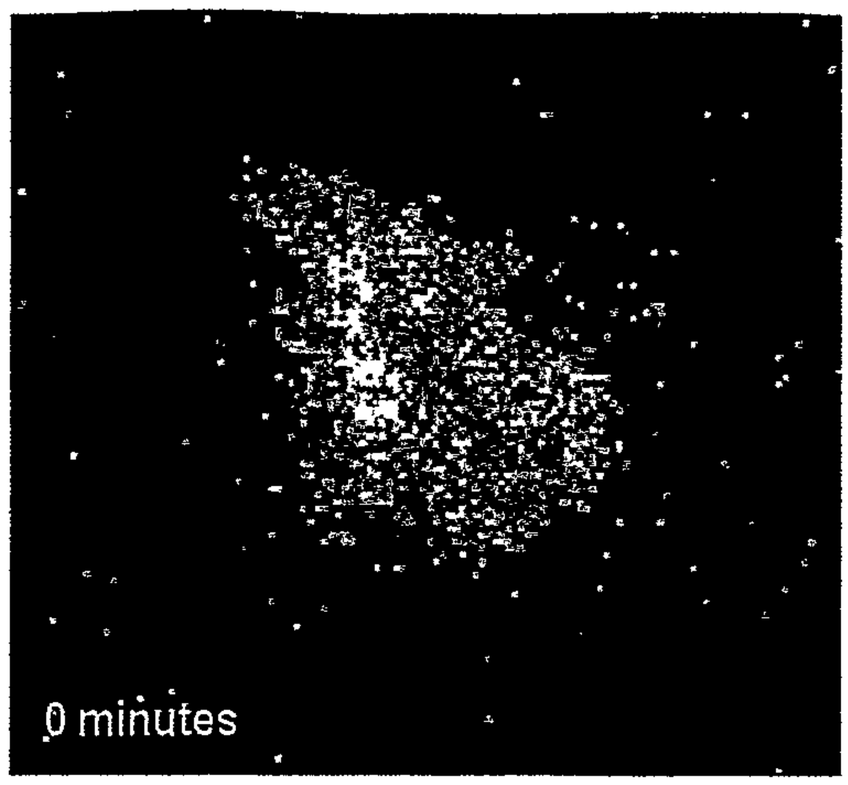
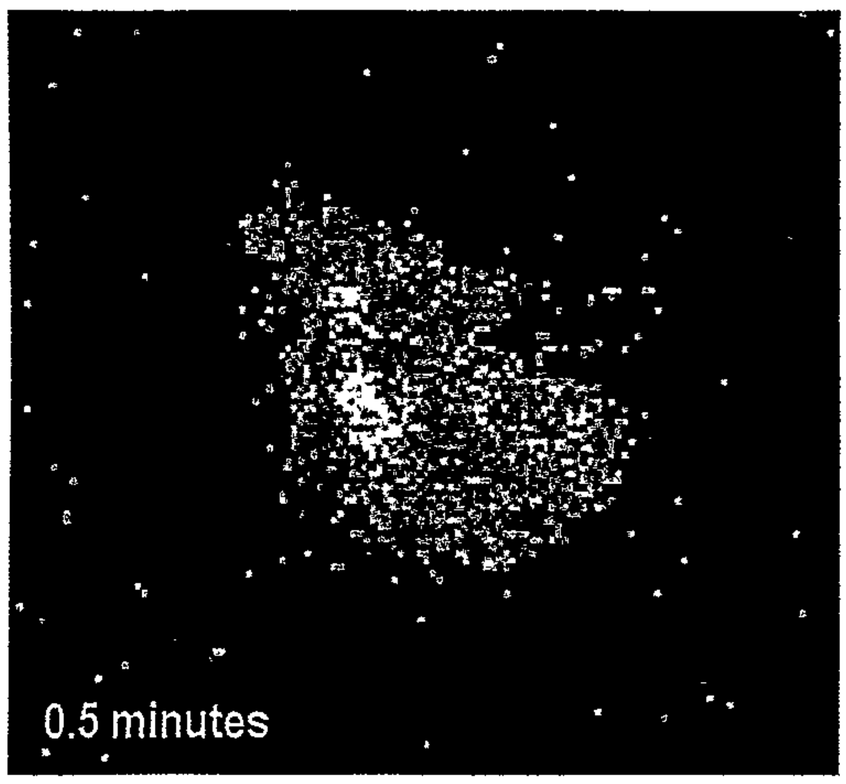
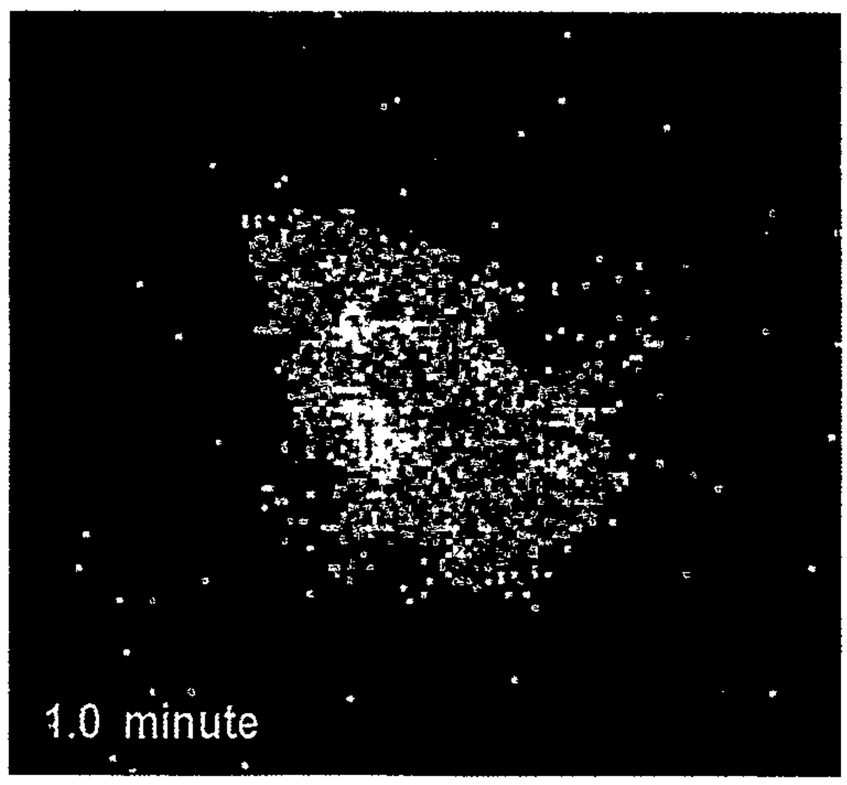


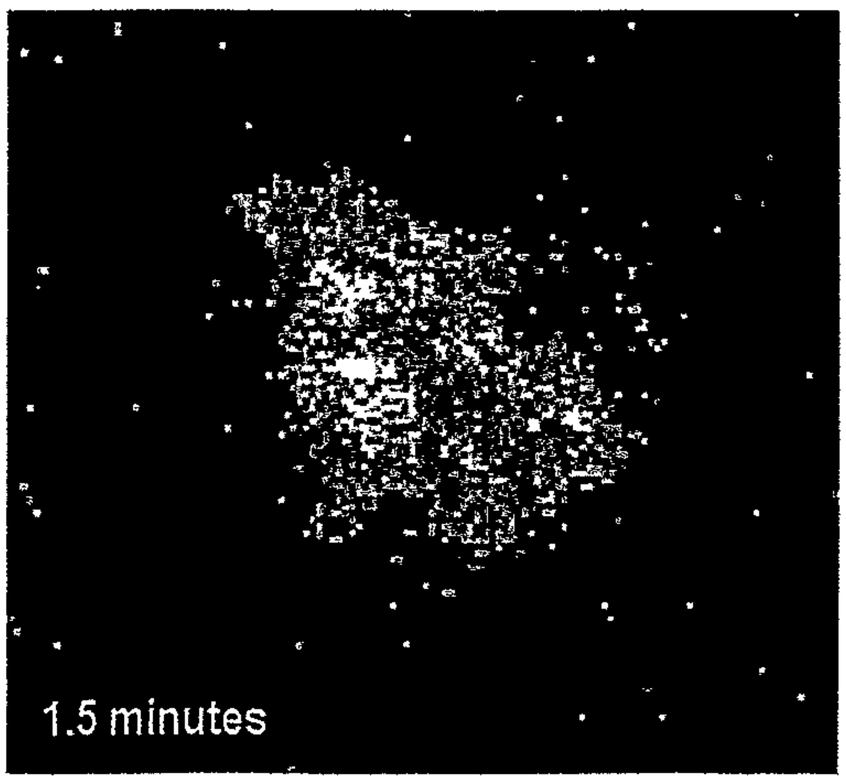
Figure 5.5.3 Example of 2 Dimensional Confocal images of an osteosarcoma cell irradiated at 2 Joules.

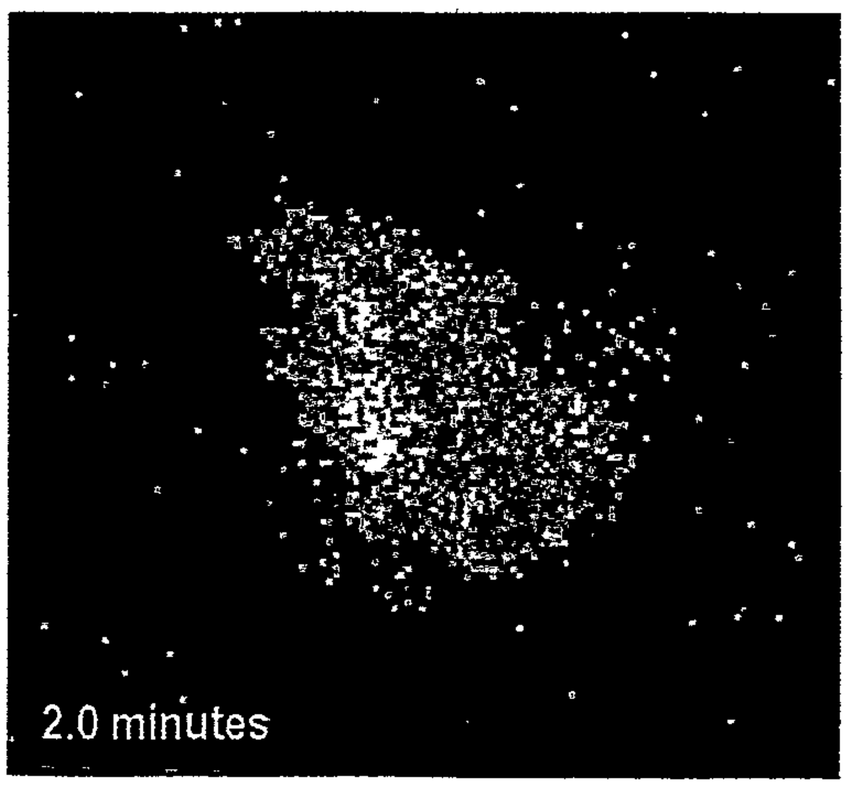


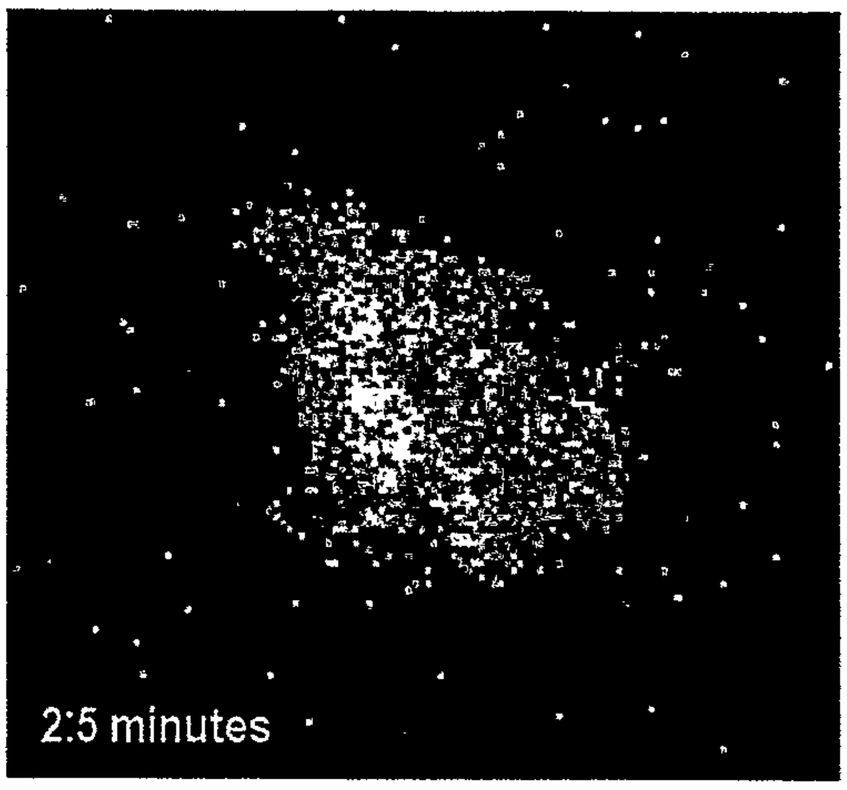


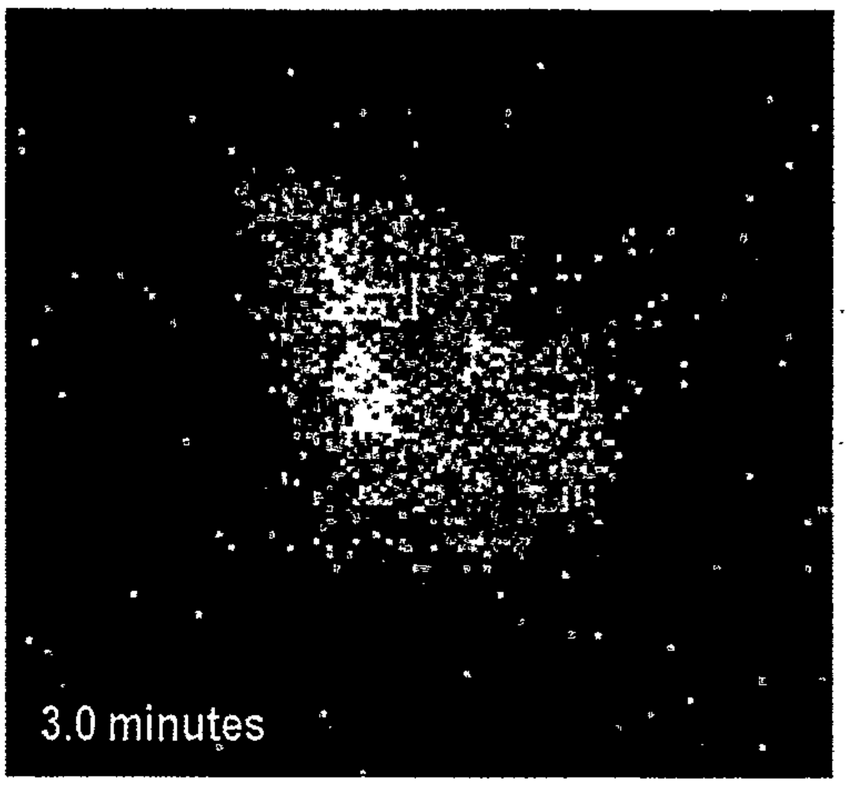


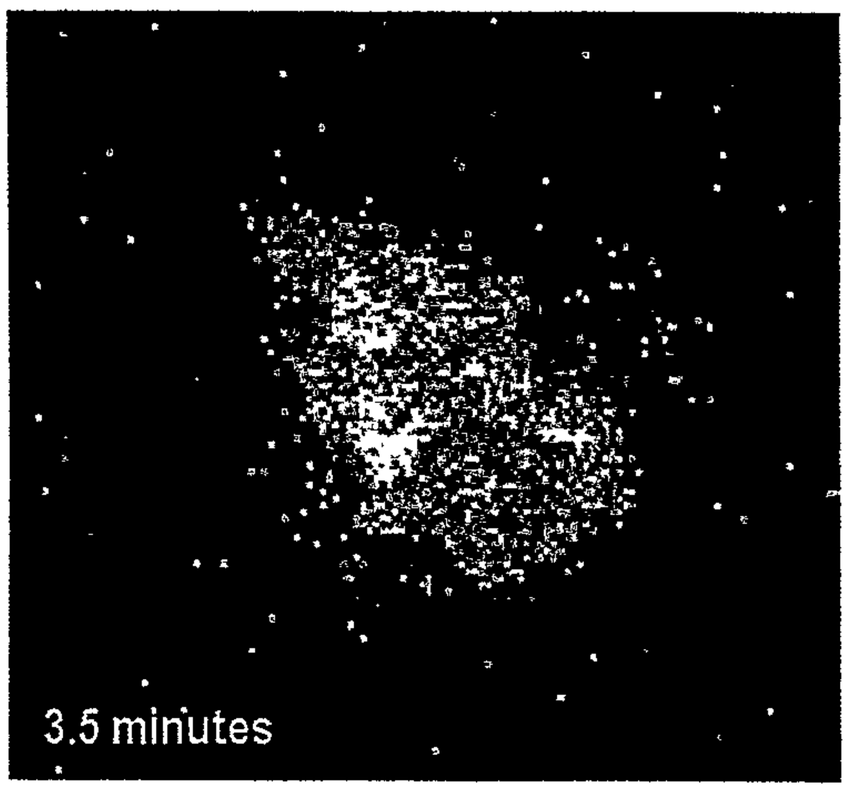


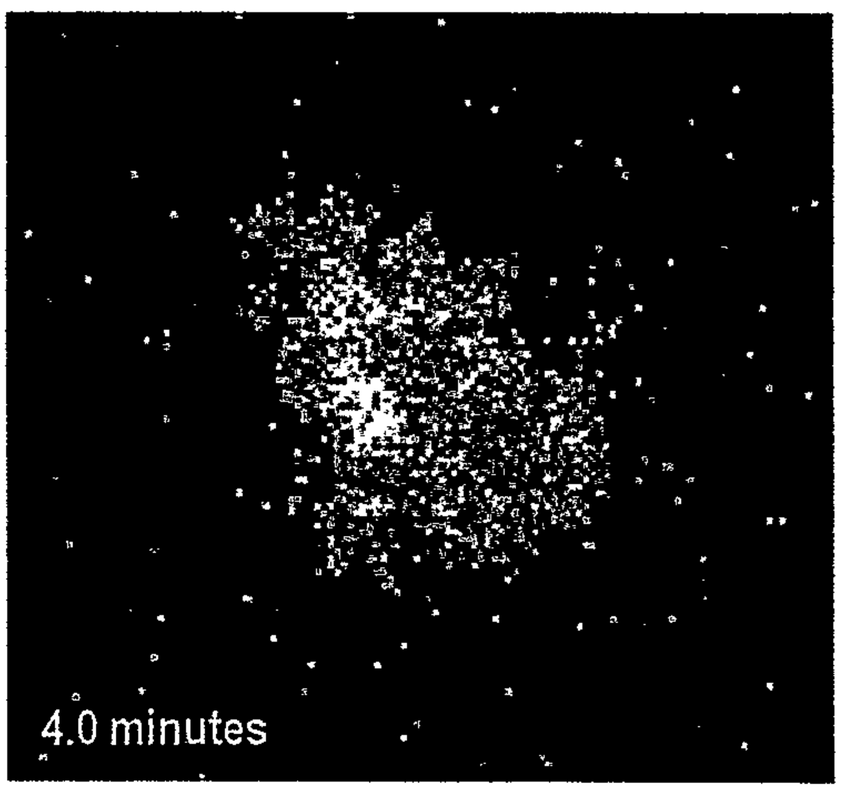


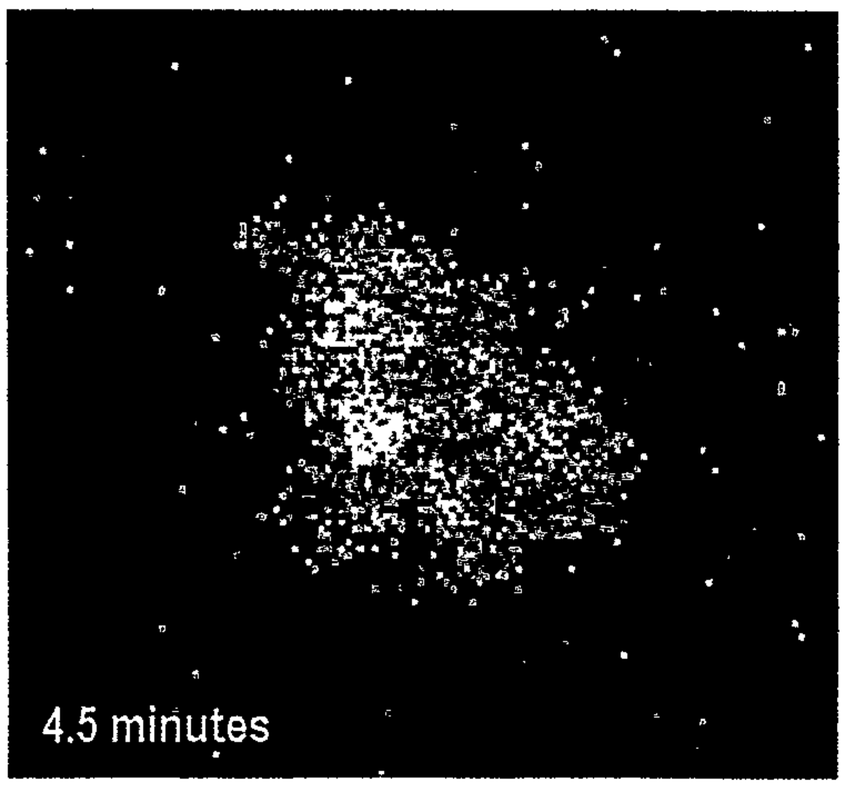


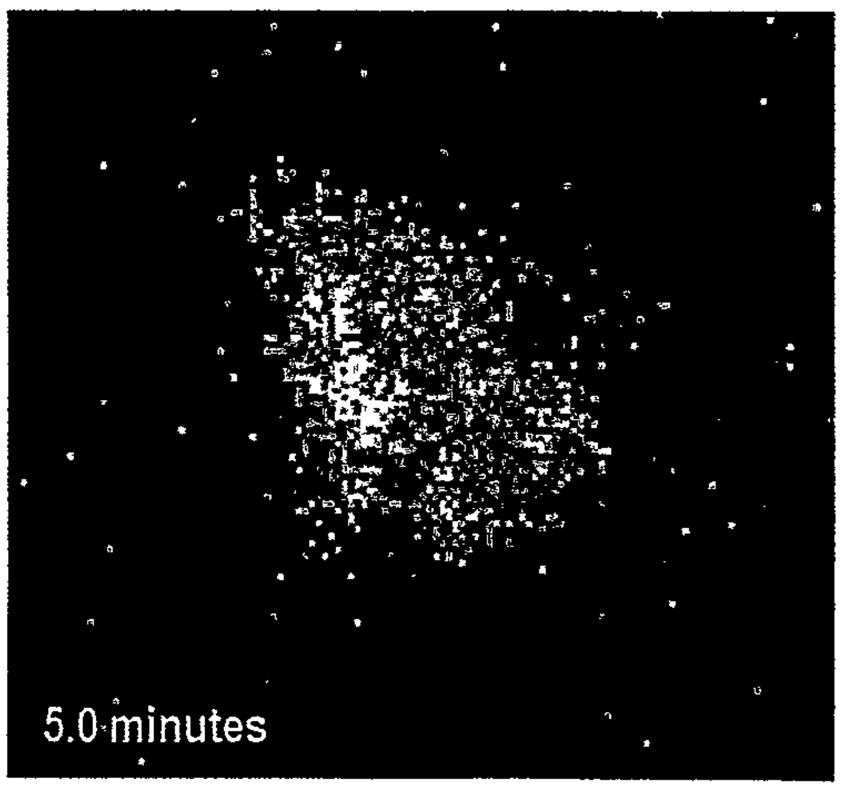

2:5 minutes


3.0 minutes


3.5 minutes


4.0 minutes


4.5 minutes


5.0 minutes

5.5.3 Results

Experiment 5.5.1 Effects of photobleaching on confocal images of osteosarcoma cells after ten scans, expressed as percentage change from the initial image.

Figure 5.5.4 Effects of photobleaching observed on entire field acquired.

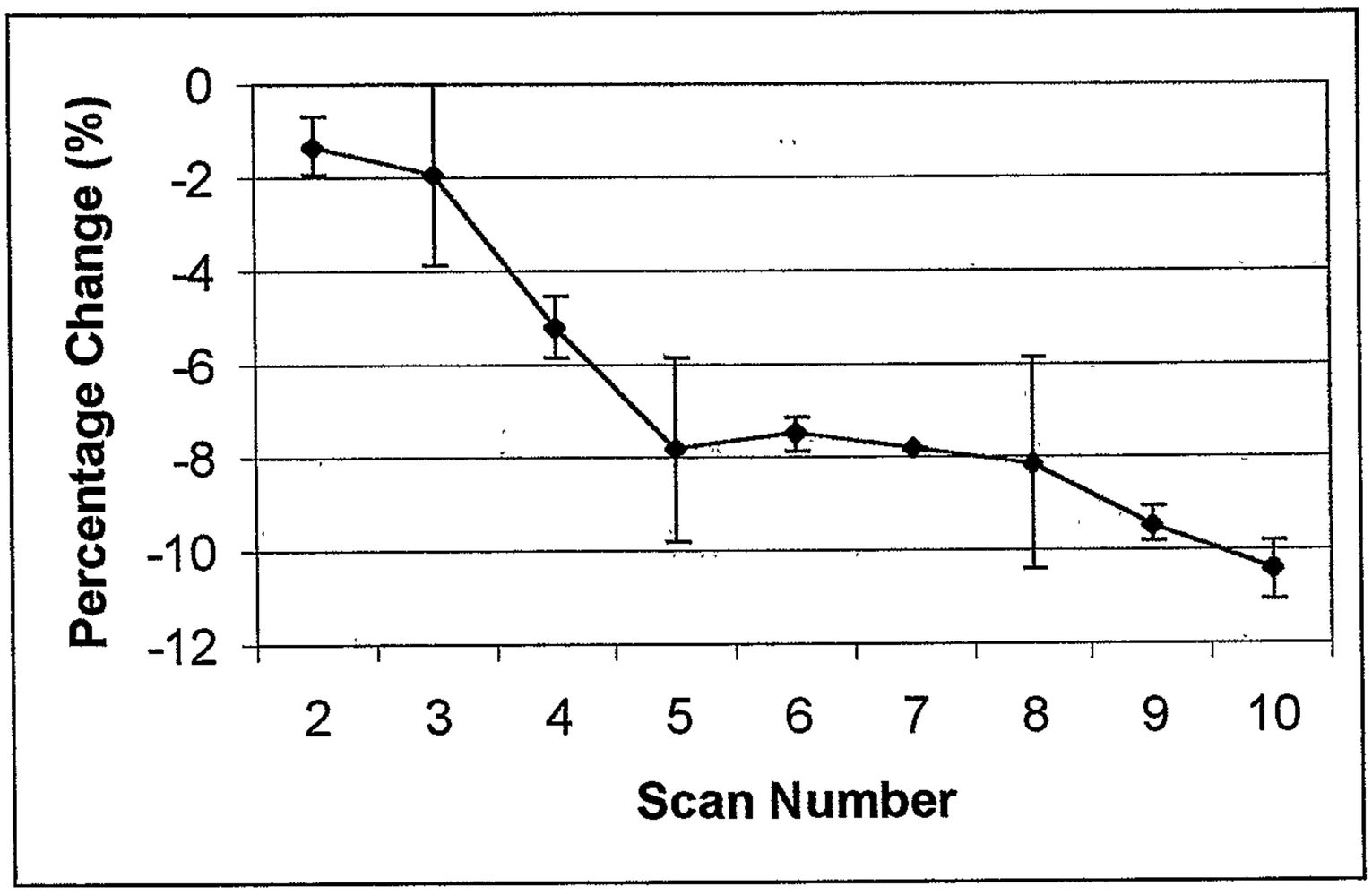
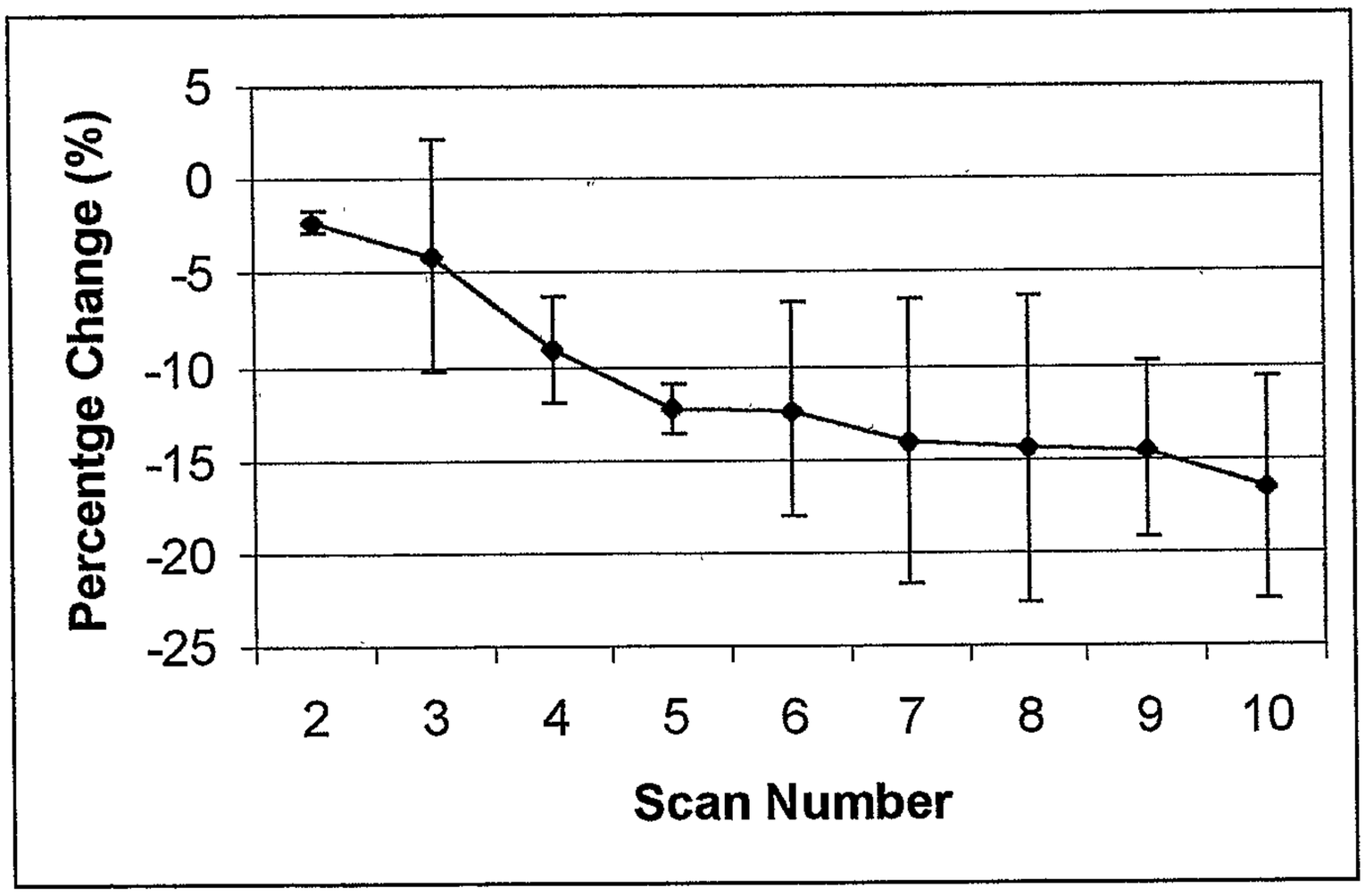


Figure 5.5.5 Effects of photobleaching at the level of individual cells in the field.



Repeat scanning of the cells to acquire images results in a reduction of luminosity of the image relative to the initial image scanned.

Experiment 5.5.2 Positive control utilising Calcium Ionophore A23187, with results expressed as a percentage change relative to the pre-treatment image.

Figure 5.5.6 Effects of the addition of the Ionophore to the osteosarcoma cells determined from the entire field acquired.

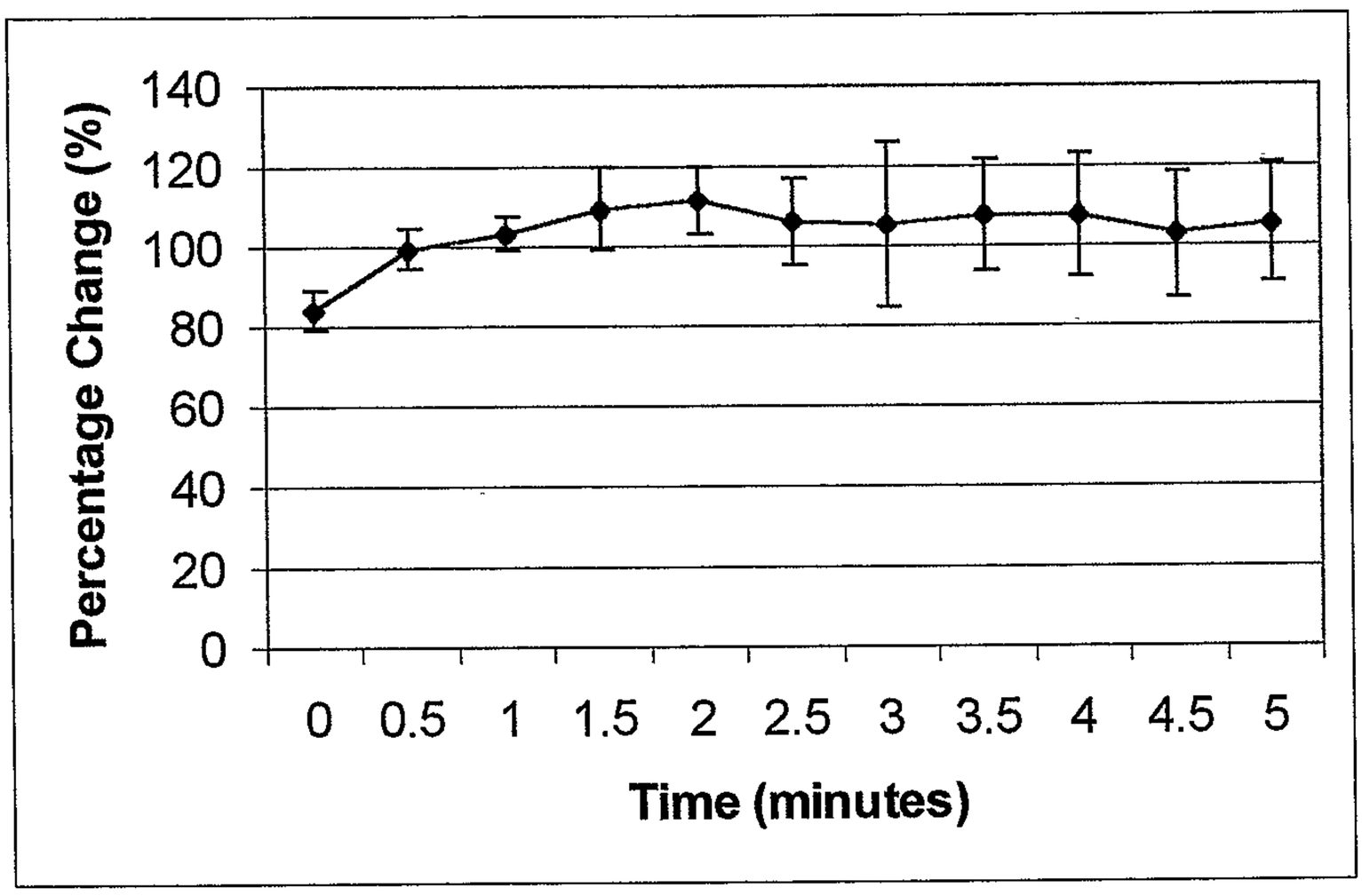
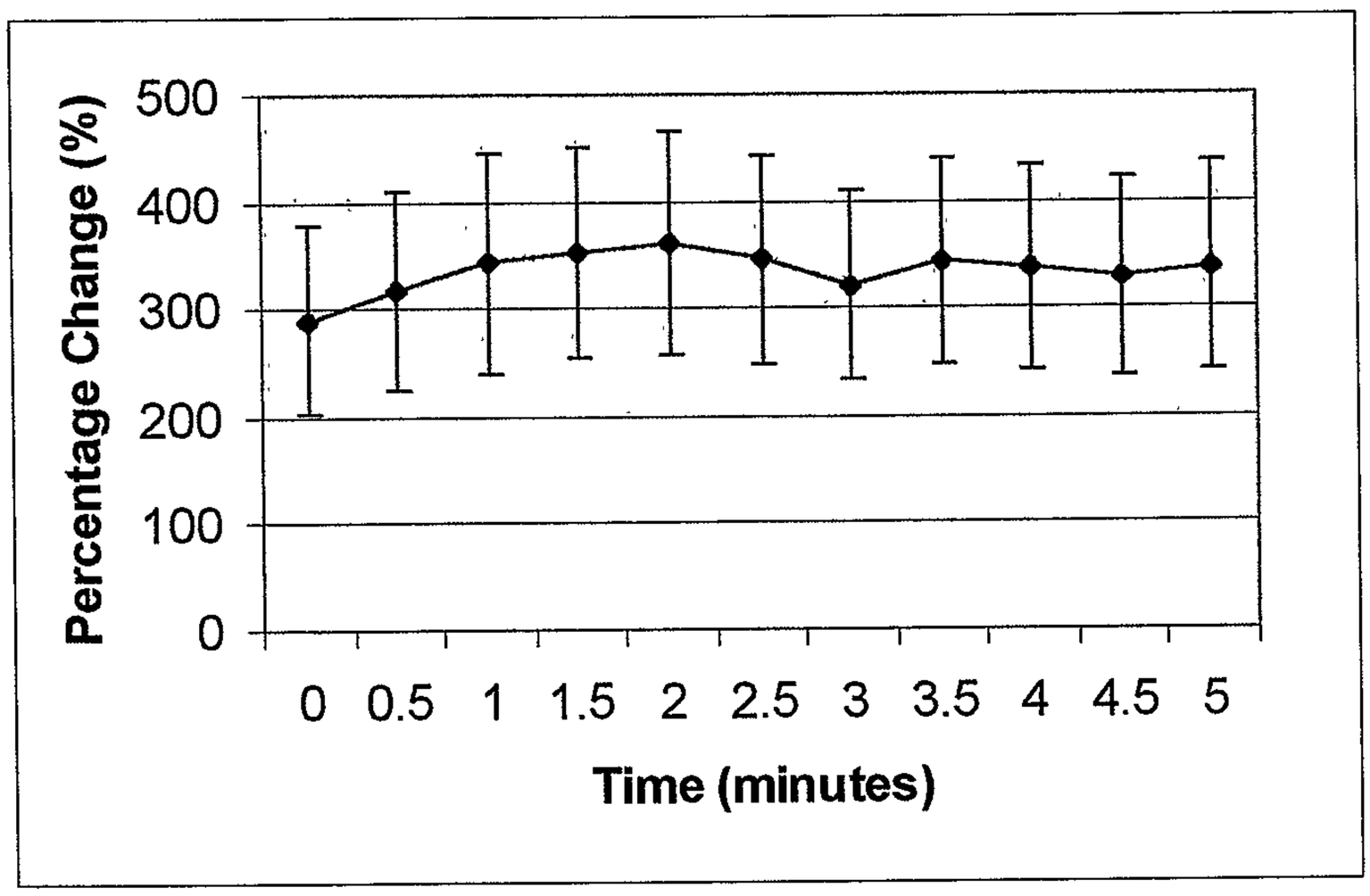


Figure 5.5.7 Effects of the addition of the Ionophore to the osteosarcoma cells determined at an individual cellular level.



A large positive percentage change was observed with addition of the Ionophore. The results of the photobleaching assessment and positive control suggest that the method is satisfactory for determining intracellular calcium change.

Experiment 5.5.3 Effects of low level laser irradiation at an energy level of 1.0 Joule, with results expressed as percentage change relative to the pre-irradiation control.

Figure 5.5.8 Effects of laser irradiation of osteosarcoma cells at 1.0 Joule determined from the entire field acquired.

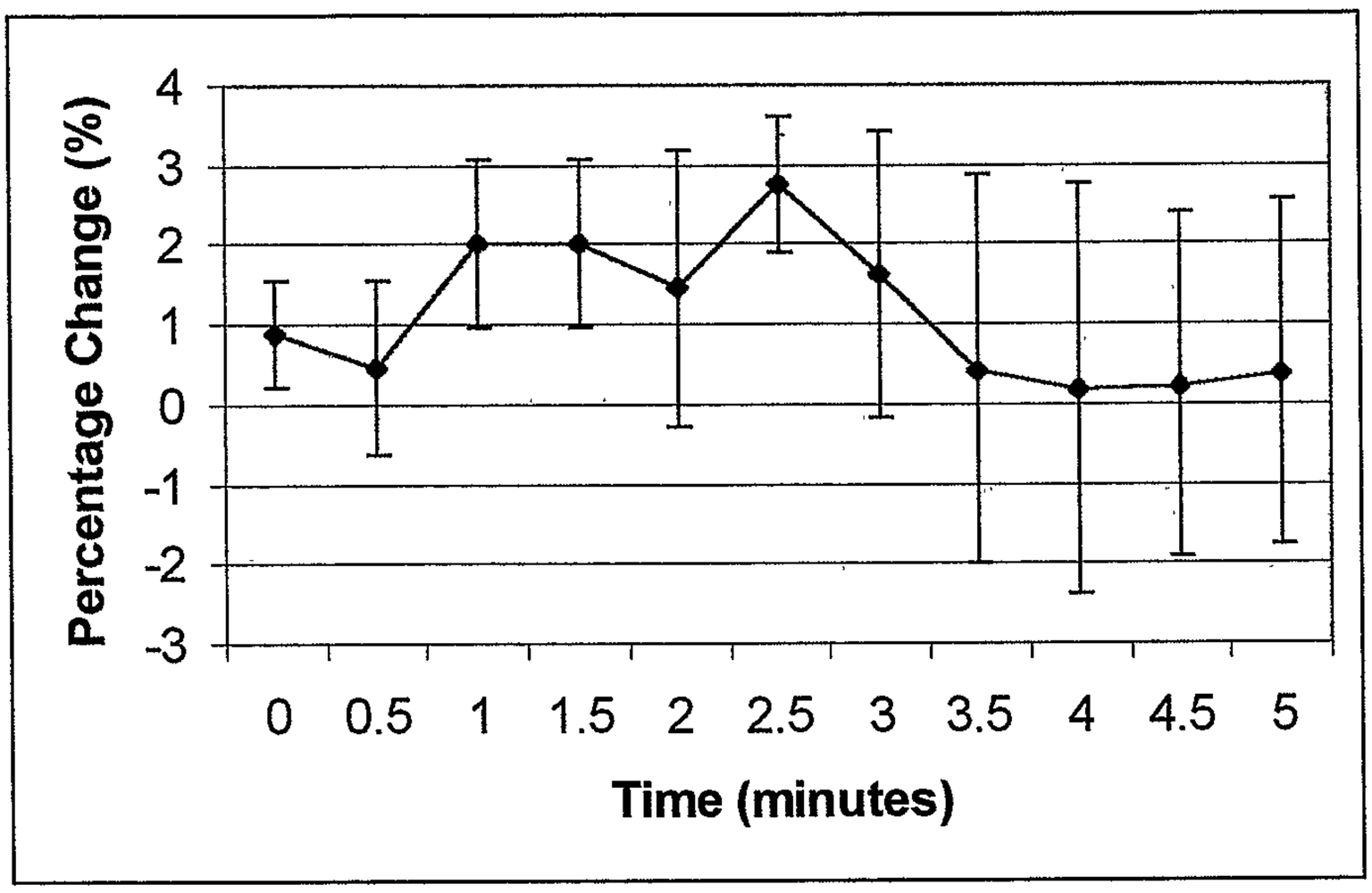
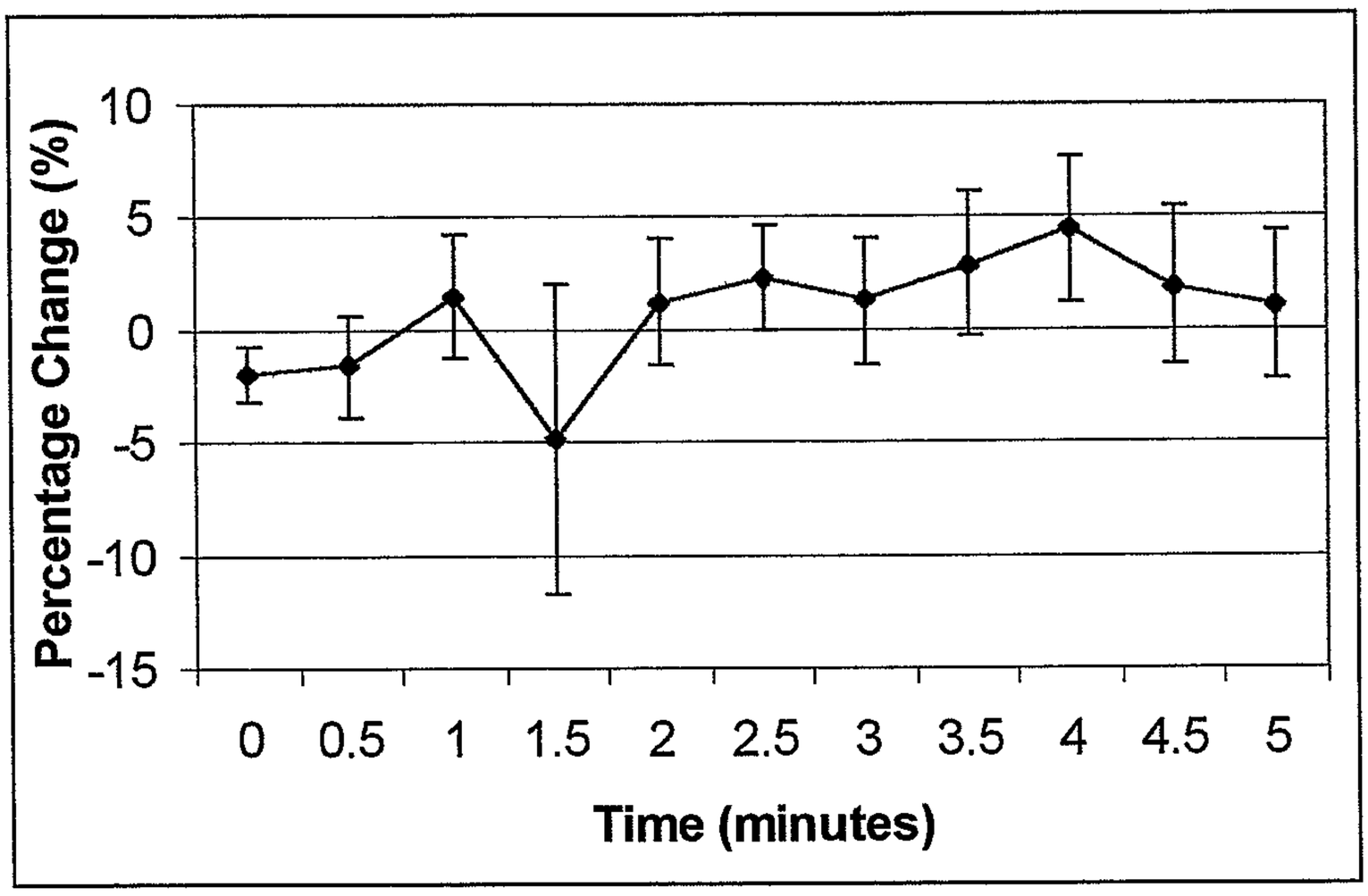


Figure 5.5.9 Effects of laser irradiation of osteosarcoma cells at 1.0 Joule determined at the level of individual cells.



A positive change was observed in intracellular calcium levels after irradiation of 1.0 Joule, particularly after 2 minutes.

Experiment 5.5.4 Effects of low level laser irradiation at an energy level of 2.0 Joules, with results expressed as percentage change relative to the pre-irradiation control.

Figure 5.5.10 Effects of laser irradiation of osteosarcoma cells at 2.0 Joules determined from the entire field acquired.

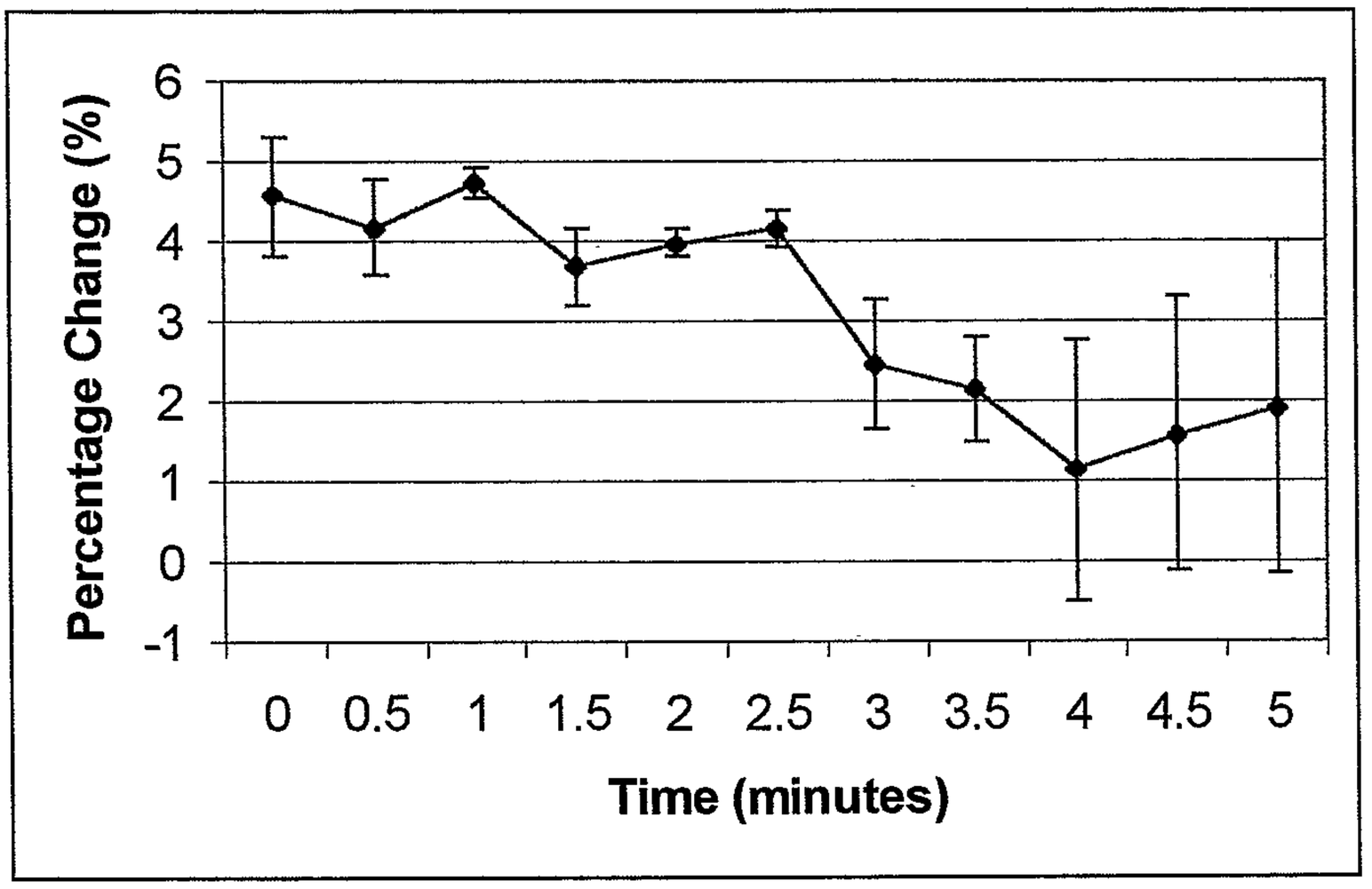
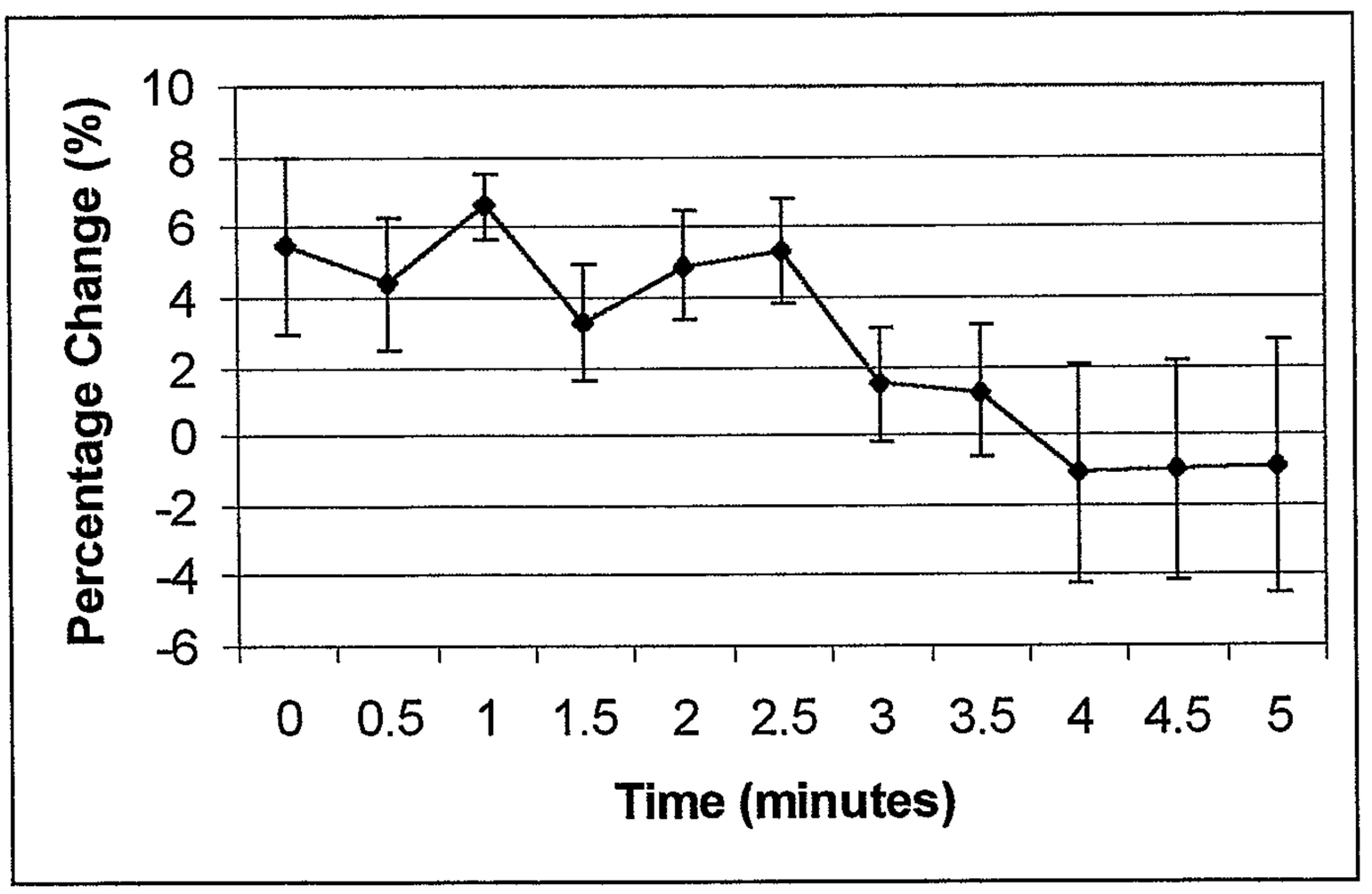


Figure 5.5.11 Effects of laser irradiation of osteosarcoma cells at 2.0 Joules determined at the level of individual cells.



A positive change was observed in intracellular calcium levels after irradiation at 2.0 Joules.

Experiment 5.5.5 Effects of low level laser irradiation at an energy level of 4.0 Joules, with results expressed as percentage change relative to the pre-irradiation control.

Figure 5.5.12 Effects of laser irradiation of osteosarcoma cells at 4.0 Joules determined from the entire field acquired.

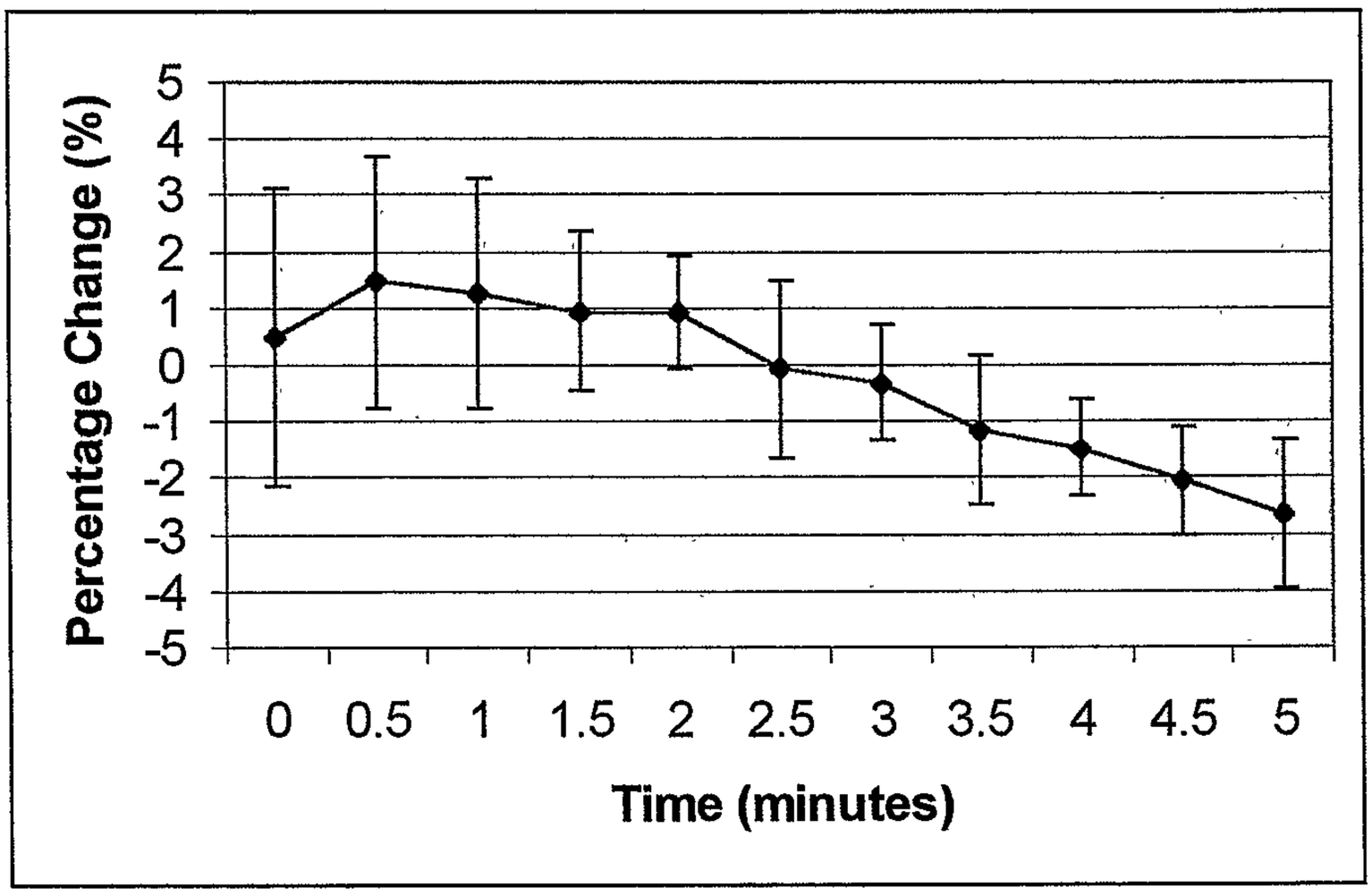
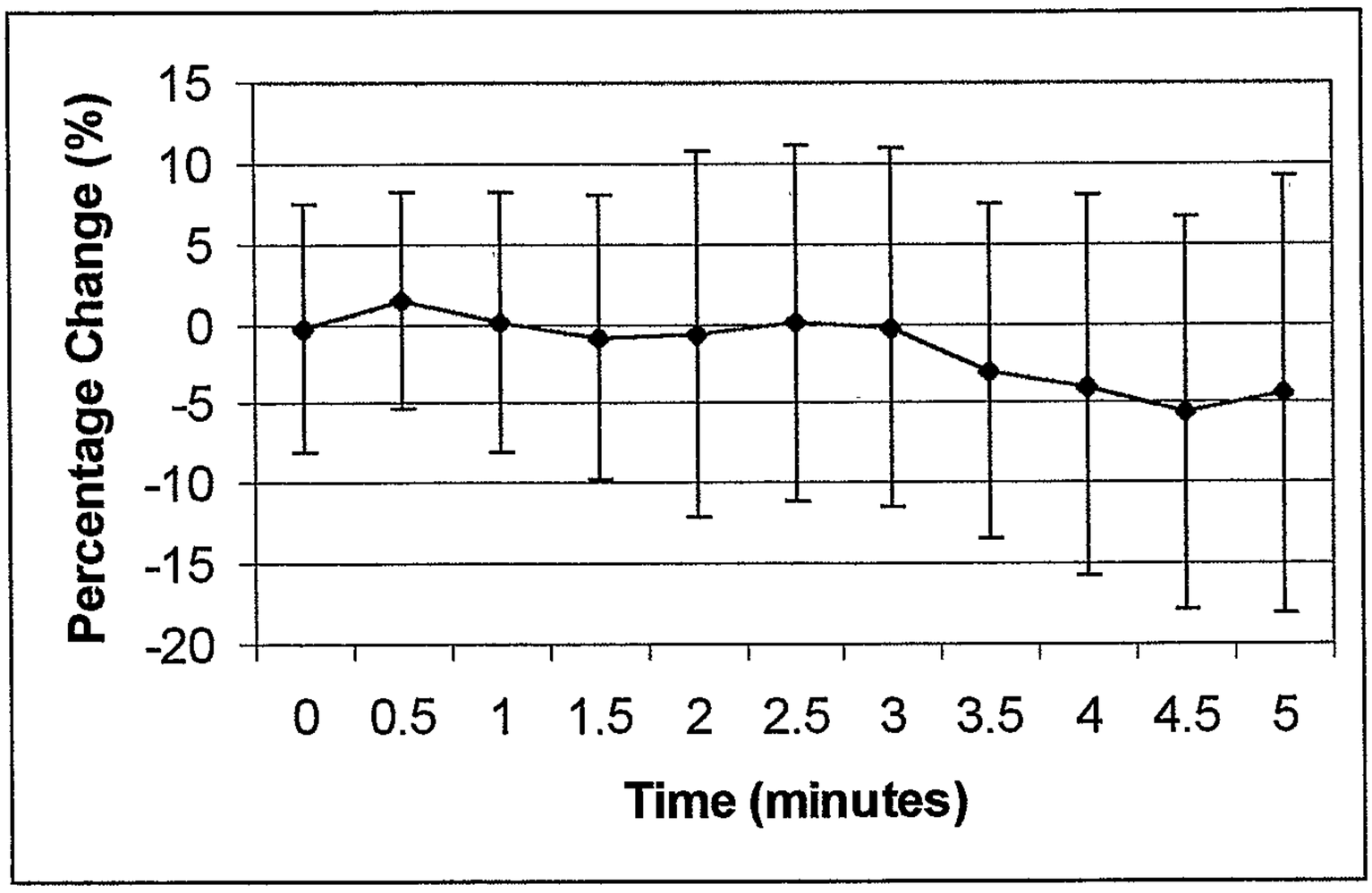


Figure 5.5.13 Effects of laser irradiation of osteosarcoma cells at 4.0 Joules determined at the level of individual cells.



An initial positive change was observed at approximately 30 seconds after irradiation, but calcium levels continued to decrease after this time. Large variations were observed in this sample group as indicated by the standard error of the mean.

Although the sample size was small for these experiments, a trend of increased intracellular calcium concentration was observed at the different energy levels utilised for this experiment.

Changes induced by the Ionophore positive control were clearly evident. Observations of images of laser irradiated cells did not reveal obvious effects. However, analysis of average brightness values of the images indicates a tendency toward increased calcium concentrations.

Repeat scanning of the cells to acquire images resulted in a reduction of luminosity of the image relative to the initial image scanned. A large positive percentage change was observed with addition of the Ionophore. The method was therefore shown to be sensitive by the Ionophore positive control.

A transient positive change was observed in intracellular calcium levels after irradiation at 1.0 Joule, particularly after 2 minutes, and at 2.0 Joules. These changes decreased toward or below control levels after approximately 5 minutes.

At the energy level of 4.0 Joules an initial positive change was observed at approximately 30 seconds after irradiation, but calcium levels continued to decrease after this time. Large variations were observed in this sample group as indicated by the standard error of the mean.

Chapter 6 DISCUSSION

Overall there were no differences in cell numbers, as determined by Trypan Blue exclusion and cell counts, over the ten day period when comparing experimental and control groups. Standard deviation of the samples was increased, particularly after day seven. This is to be expected as the cell cultures deteriorate with time.

Cell viability was greater than 90% in experimental and control groups. There were no differences in cell numbers when comparing experimental and control groups.

There was a tendency for an increase in cell numbers in a number of the laser-irradiated groups around day seven. A single irradiation of 2 Joules showed a statistically significant increase on day seven at the 5% level. Single irradiation of 4 Joules on day seven at the 1% level. Day six of daily irradiation of 1 Joule showed a statistically significant increase at the 5% level. Day eight of daily at 2 Joules showed a statistically significant increase at the 5% level.

Any potential effects of low level laser as observed from these experiments are minimal and transient if present. Irradiation did not have a consistent, reproducible, lasting effect on the osteosarcoma cell line utilised.

Daily counting of the number of cells has some stated limitations including possible increased attachment of irradiated cells to the flask (Karu 1997). Some authors irradiate a monolayer of cell culture and others irradiate a cell suspension. Differences may also be due to handling of the cells e.g. in the dark or light before irradiation, or different laboratory techniques (the nutrient media chosen), and the wide spectrum of normal and malignant cells used.

The accuracy of the cell counts performed using a haemocytometer depended on a series of factors. Errors arose either because of the random distribution of cells through the chamber or because of technical problems. The random streaming of cells across the chamber due to fluid currents gave rise to a random distribution of cells through the chamber (Berg 1945). This caused an uncontrollable error inherent in the method. This was reduced by counting sufficiently large cell numbers and by performing counts on multiple samples. Avoidable technical errors may have arisen from inaccurately measuring volumes and poorly mixing

suspensions so that the cell distribution was uneven; using inferior or poorly cleaned equipment; employing poor technique; or inaccurately counting the cells in the field (Sanford *et al.* 1951).

No overall increase in cellular activation or proliferation was observed with low level laser irradiation, as determined by the MTT assay and CFSE labelling.

Localised effects on day seven, with a single irradiation of 0.5 and 2.0 Joules, as measured by MTT, were found to be satistically significant at the 5% level.

Standard deviation increased, particularly after day seven, for the different experimental groups. Formazan production per cell at higher cell densities is found to be diminshed (Sieuwerts *et al.* 1995), which may account for some of the variabilities in cell numbers observed after day seven. A relatively large sample size was utilised to counteract some inherent errors in the methodology, as previously described

CFSE labelling, as a more specific determinant of cellular proliferation, did not detect any differences between experimental and control groups.

A physiological stress response was observed to occur, with peak expression of heat shock protein-70 at 1.5 hours post-irradiation, at an energy level of 2 Joules, suggesting that the cells experienced an environmental insult. The reason for the increased expression of Heat shock protein-70 at 1.5 hours remains unclear.

Investigation of intracellular calcium concentration revealed a tendency of a transient positive change after irradiation at 1.0 Joule and at 2.0 Joules. At the energy level of 1.0 Joule, effects were greatest after approximately 2 minutes, whereas at 2.0 Joules more immediate effects were observed. These changes decreased toward or below control levels after approximately 5 minutes.

At the energy level of 4.0 Joules an initial positive change was observed at approximately 30 seconds after irradiation, but calcium levels continued to decrease after this time. Large variations were observed in this sample group as indicated by the standard error of the mean.

Calcium uptake has previously been found to increase at two minutes after irradiation, a two- to threefold increase of intracellular Ca^{2+} concentration being observed 2 minutes after irradiation of lymphocytes with a He-Ne laser (Karu *et al.* 1991). Studies of neutrophils (Loevschall *et al.* 1994) as well as bovine sperm cells (Lubart *et al.* 1994) and rat Schwann cells (van Breugel *et al.* 1993) have reported similar results.

Although the constraints of the methodology meant a small sample size for these experiments, a definite trend of increased intracellular calcium concentration was observed. Further investigation is therefore indicated to obtain a more definitive result utilising a method that would enable analysis of greater cell numbers, such as fluorimetry.

Over the last decade dentists in different parts of the world, particularly in Scandinavia, have been subjected to forceful marketing of low level laser equipment by various medical firms. Although the cost of equipment is high, clinicians have bought, and use the equipment uncritically for a wide variety of oral diseases, with many anecdotal reports being published on the beneficial effects of laser treatment (Roynesdal *et al.* 1993).

Potential benefits of pain alleviation, increased bone turnover and healing, by low level laser therapy, to the practice of clinical orthodontics was the motivation behind this research. This study aimed to investigate some of the reported effects of low level laser therapy utilising an osteogenic cell line.

This research was limited the use of a laser with a wavelength of 830nm and initial power output of 70mW. There are wide ranging variations in the laser parameters used in published reports on low level laser therapy. The most frequently used low-power lasers are the infrared (830nm) Gallium/ Aluminium/ Arsenide and the visible (630nm) Helium-Neon lasers. A combination of ease of use, broader experimental background, low cost, and availability are cited reasons for this preference (Basford 1986). A survey of the literature tends to suggest that the 633nm irradiation of the Helium-Neon laser may be more effective for wound healing and analgesia than other wavelengths. The Gallium/Aluminium/Arsenide laser appears to be commonly used for relief of pain, particularly in conditions such as rheumatoid arthritis, and

nerve healing. Clinically, lasers have also been found to have a strong placebo effect (Heussler *et al.* 1993; Bulow *et al.* 1994; Lim *et al.* 1995).

Most research is centered around the clinical setting, with limited research having been carried out on individual cell lines. Most laboratory research has been carried out utilising fibroblasts, with low level laser being found to have a stimulatory effect (Loevschall *et al.* 1994; Hrnjak *et al.* 1995; Skinner *et al.* 1996). The laser used in this research has never been used in association with the osteogenic sarcoma cell line, Saos-2.

Results of this research, utilising the Saos-2 osteosarcoma cell line, have not confirmed the findings of previous research utilising fibroblasts or other osteogenic tissue cultures. An immortalised tumour cell line may not react to low level laser therapy in the same way as primary cultured tissues.

As previously discussed, the analyses employed for determining the effects of different energy levels and exposure regimes detected no overall differences in cellular proliferation, activity or viability of experimental cells compared to unirradiated controls. Laser irradiation on the day of seeding significantly stimulated cellular proliferation on or around day 7 compared with control samples. Although it is yet unclear as to whether these are artifactual statistical results, these findings are consistent with those of Ozawa *et al.* (1998) who found statistically significantly stimulated proliferation of osteogenic calvariae cells on day 6 and on day 9 after pulsed laser irradiation (GaAlAs 830nm laser, 500mW, energy density 3.82J/cm^2) on day 1. Their day 6 was equivalent to day 7 of this research. They found that irradiation at any later time had no effect on cellular proliferation.

It has been reported that the effect of a single dose of irradiation at an early stage of culture is similar to that following a daily multiple dose of irradiation (Osawa *et al.* 1995). Similarly, the stimulatory effect of bone regeneration by laser treatment in the midpalatal suture during rapid maxillary expansion in rats, has shown bone regeneration by laser to be most effective during the early period after expansion (Saito *et al.* 1997). It is thus proposed that the extent of the stimulatory effect of bone formation is dependent on not only the total dosage of laser irradiation, but also the timing of irradiation.

The finding that the stimulatory action of laser irradiation occurs during the proliferative and earlier stages of differentiation of immature precursors but does not occur during later stages (Ozawa *et al.* 1998) is suggestive of the fact that precursors rather than mature well differentiated cells are required to be present. More highly differentiated cells become unresponsive to laser treatment the differentiated cells around mineralised nodules are unaffected by laser irradiation.

Other studies have also suggested that laser effects may be transient (Takeda 1988; Yamada 1991).

Not all mammalian cell lines cultivated *in vitro* respond to irradiation in a similar manner; some do not respond at all (Marchesini *et al.* 1989). One of the principal differences between primary cell cultures and established cell lines is the capability of the latter for unlimited and vigorous growth (Richter and Issinger 1986).

Failure to show general gross growth changes in cellular proliferation may be due to the independent, rapidly proliferative nature of the cell line used. The vigorous growing nature of this cell line may mask any effects that the laser induces.

It has been found that irradiation does not influence the proliferation of cellular subpopulations to an equal degree (Karu 1989). Cultured cells are heterogeneous with regard to proliferative activity of subpopulations (Nias 1968; Azzarone *et al.* 1982; Hassel *et al.* 1983). Subpopulations and even cells at an individual level do not respond in exactly the same way to irradiation (Karu 1997). Light stimulation of a faster growing culture is weaker than that of a slower growing one, the stimulative effect of irradiation is found to be most noticeable in the proliferation activity of slowly growing subpopulations (Karu 1989). It has been clearly shown that there is a limit in the specific growth rate of all populations, which is said to be independent of growth conditions. It is not possible to stimulate populations that are already growing at this rate (Tiphlova and Karu, 1991). It is also not possible to activate a process that is activated already or occurring at speed near maximal. Laser stimulation is not a general phenomenon, but occurs only in certain circumstances (Karu 1997).

Interactions *in vitro* may not represent those occurring *in vivo*. The conditions *in vitro* are optimised and many factors are not represented. For example the complexities of cell migration and interaction within the framework of the PDL during tooth movement, or within a healing wound or fracture and the cellular microenvironment can not be simulated *in vitro*. Factors such as immunological mediators, the vasculature and growth factors are not present. Inquiry into the action of different radiation wavelengths on cellular cultures stemmed from the knowledge that disorders which respond to laser therapy, such as indolent wounds and trophic ulcers (Mester 1981) may be associated with increased proliferation of the cells surrounding the injuries. However, wound healing may also be due to changes in cellular migratory, metabolic or secretory functions.

Like many tumour cell lines, osteosarcoma cells are immortal and undifferentiated. Unlike fibroblasts, and other freshly explanted cell cultures, they have no critical requirements for external growth factors. Cellular proliferation as a result of low level laser irradiation may be dependent on the autocrine production of these growth factors. Growth factors are a group of hormone-like polypeptides that have been shown to play a central role in different phases of wound healing. Yu (1994) has provided evidence that fibroblast proliferation in cell culture as a result of low level laser irradiation may be associated with the autocrine production of bFGF from fibroblasts.

It is proposed that the stimulatory mechanism of laser irradiation on bone formation may also be mediated by some growth factors, cytokines, or prostaglandin's with differentiation inducing properties produced from the cells of osteoblast lineage. Their production may be affected by laser irradiation, and act as autocrine or paracrine stimulators to these cells.

It is proposed that the sensitivity of cells is not an all-or-nothing phenomenon, but depends to various degrees on the physiological state of the cell before irradiation. In mammalian cell culture studies, after irradiation with a He-Ne laser, the growth rate of slow-growing subpopulations was increased, and was not increased in fast-growing subpopulations. In experiments with microorganisms, seasonal variations of growth stimulation were established. In the summer there was almost no photostimulation growth, and in the winter the effect was observed to be maximal. It was thus proposed that the seasonal variation in the magnitude of the effect depends on the growth rate of the culture before irradiation (Karu 1987).

It has also been indicated that laser irradiation is effective when applied at healing sites, such as those of bone fracture, but ineffective when applied at inactive or normal tissue. In areas of injuries such as trophic ulcers and indolent wounds conditions are created (low oxygen concentration and pH, lack of necessary nutrients) which prevent proliferation, so that the cells enter the G_0 phase or remain in the G_1 phase of the cell cycle. Light may serve as a signal to increase proliferation for such cells. The effect of irradiation when irradiating fresh wounds can be minimal, or not present particularly when proliferation is active and the regeneration of tissue integrity occurs at a normal rate (Karu 1989).

Cellular sensitivity seems to be increased in injured or otherwise stressed systems. Under normal physiologic conditions mammalian cells maintain a precise intracellular pH, between 7.0 and 7.2 (Roos and Boron 1981). The normal pH of arterial blood is 7.4 and healthy tissues between 7.0 and 7.4 (Vaupel 1977). Growth factors, neurotransmitters, and direct cell-cell interactions can modify the regulation of intracellular pH in receptive cells (Roos and Boron 1981).

Wound healing and chronic inflammation are the two important areas of low level laser therapy and where the most positive effects of laser therapy are found. Both of these conditions are characterised by decreased oxygen tension (decreased partial pressure of oxygen and hypoxia) and acidosis (decreased pH) (Kittlick 1986).

In hypoxic states the partial pressure of oxygen in tissue drops from 40mm Hg to 0-5 mm Hg (Vaupel 1977; Freitas 1991). Wound hypoxia in normal regeneration is a transient condition. Chronic inflammation on the other hand is characterised by continued aerobic glycolysis and a redox shift towards a reduced state. An example of this is that the partial pressure of oxygen of rheumatoid synovial fluid is found to fall as low as 0 mm Hg, local hypoxia being considered one of the primary causes of rheumatism (Kittlick 1986).

It is proposed that the cellular response is weak or absent when the overall redox potential of a cell is optimal or near optimal for the particular growth conditions, and stronger when the redox potential of the target cell is initially shifted to a more reduced state (intracellular pH is lowered). This may explain why the degree of cellular responses can differ markedly in different experimental studies, and why there may be no effect at all.

In a series of experiments, analysis of the data has shown that when one cellular function was altered by irradiation, the others remained unchanged. For example, there was found to be no effect on keratinocyte proliferation when their motility was changed (Haas *et al.* 1990) and when proliferation of keratinocytes was found to be increased, there were no significant effects on their migration (Loevschall and Arenholt-Bindsev 1996). The production of collagen type I by fibroblasts has been found to be affected by irradiation in an inverse manner to cell proliferation (van Breugel *et al.* 1992). It has been proposed that there is a priority in the utilisation of the pH gradient for ATP synthesis, nutrient transport, taxis, and initiation of genetic processes. This priority is said to enable the metabolic processes utilising the pH gradient to be regulated and coordinated (Tiphlova and Karu, 1991).

The reason for the increased expression of heat shock protein 70 at 1.5 hours remains unclear. This finding was not supported by Hashieh *et al.* (1997) who studied heat shock protein level in fibroblasts by gel electrophoresis and Western blotting using monoclonal antibodies. Their utilisation of a Helium-Neon laser (632.8nm, 60mW) and energy densities of 1.5 J/cm^2 and 3 J/cm^2 did not stimulate HSP70 synthesis. However, due to the different laser parameters and analytical process utilised, a direct comparison with this study cannot be made. Ultraviolet irradiation (280-320nm and 254nm) of organ cultured normal human skin (Muramatsu *et al.* 1992) has, on the other hand, been shown to induce HSP70 synthesis.

Laser irradiation has been found to produce no significant temperature increase. Schneede *et al.* (1988) using a microthermoprobe in a monolayer of cells found the temperature increase to be less than 0.065°C at laser irradiation of 40mW/cm^2. The temperature of tissues being treated with low level laser, have temperature changes reported to be only as much as 0.3 to 0.62°C (Boussignac 1985) or 0.1°C (Gerschman *et al.* 1994). Thus, cellular effects of the mechanisms of laser irradiation are proposed to be due to biomechanical rather than thermal effects.

However, even if no rise in temperature is observed after laser irradiation, the treatment may be stressful, stimulating the synthesis of stress proteins indicating an alteration of the metabolic pathways due to an oxidative stress leading to free radical formation and missfolding an aggregation of proteins (Hashieh *et al.* 1997).

Ultrastructural changes have been observed with prolonged laser irradiation indicating damage of the cells. It was also observed that this damage did not appear to be irreversible (Schneede *et al.* 1988).

Laser irradiation may intensify the formation of a transmembrane electrochemical proton gradient in mitochondria. This may occur by either singlet oxygen formation by endogenous prohyrins which activates the respiratory chain in the mitochondria, or alternatively, by activation of the redox reactions in the respiratory chain by exciting the mitochondrial cytochromes (Lubart *et al.* 1992). Increased efficiency of the proton motive force may results in more calcium being released into the cytoplasm from the mitochondria (Friedman *et al.*, 1991). It is proposed that at low laser doses, mitosis is triggered by the additional Ca^{2+} transported into the cytoplasm (Meininger and Binet, 1984), enhancing cell proliferation, too much Ca^{2+} may be released at higher doses. Hyperactivity of the Ca^{2+}-ATPase calcium pumps results and exhausts the ATP reserves of the cell. The intramolecular osmotic pressure becomes larger than that of the medium. Water influx produces blebs extruding from the cell membranes. At even higher laser doses, more Ca^{2+} influx may completely exhaust the cell energy and the intracellular osmotic pressure may explode the cell (Malik and Lugaci, 1987).

Increases in intracellular calcium concentration, have been shown to promote heat shock protein-70 production in human and rat cells (Kiang *et al.* 1998). Sustained elevation of intracellular Ca^{2+} concentration to micromolar levels leads to cell death. The reduction of the intracellular Ca^{2+} concentration in response to heat shock or hypoxia in cells overexpressing heat shock protein-70 is one of the cellular defense mechanisms that promotes cell survival (Kiang *et al.* 1998).

Investigation at an intracellular level does suggest elevation of calcium concentration in response to laser irradiation, particularly at energy levels of 1 and 2 Joules. These results are not definitive due to the small sample size and require further investigation.

The divergent results found in these studies as compared to others could also be explicable by the differences in the cell line, the dosage and duration of irradiation and the wavelength used in these studies. The possibility also exists that the actual performance of the laser may differ from the theoretical values given by the manufacturer (Masse *et al.* 1993).

Experimental conditions including the fluences and the intensities of the light as well as the incubation periods, culture handling and irradiation methods have been cited as possible reasons by which results of investigators may differ. Some authors irradiate a monolayer of cell culture and others irradiate a cell suspension. Cells may be handled in the dark or light before irradiation, different laboratory techniques (e.g. the nutrient media chosen), and a wide spectrum of normal and malignant cells are used. (Karu 1997). Detection of effects is also dependent on the time elapsed after the irradiation, varying for different cell types and cellular processes (Karu 1997). Methods of measurement may not have been sensitive enough to demonstrate subtle growth changes.

Future Directions

In light of the findings of this research and findings of other authors, future investigations may include the utilisation of fresh tissue cultures of clonal osteoblasts as opposed to an immortal cell line. The effects of the wavelength and output power used in this research, as well as other wavelengths and power outputs should be compared for osteogenic cell lines.

Further research could involve the manipulation of the cellular environment, including media supplements, pH and temperature variations, utilising these results obtained from cells cultured in optimal growth conditions as baseline data.

The investigation of the primary photoacceptors, endogenous porphyrins and mitochondrial cytochromes is another direction for further investigation of intracellular changes in an osteogenic cell line.

Singlet oxygen and intracellular calcium flux should be further investigated as possible reasons for the heat shock protein response. Further investigation of the effects on intracellular calcium concentration is also indicated. Utilisation of a method such as fluorimetry, that would enable the analysis of greater cell numbers, would be beneficial.

A measurable consistent and reproducible effect is required before we can determine specific energy levels or exposure regimes for this particular cell line, or any other osteogenic cell line.

Histological analyses should then be carried out at the level of the animal model. Specific effects and optimal laser parameters should be determined.

At this stage low level laser therapy cannot be considered as a viable adjunct to the clinical practice of orthodontics. Specific effects at the cellular level and in animal models must be more thoroughly investigated before this type of treatment should be initiated in the human model.

In terms of the clinical application of this laser, it may be impractical to carry out low level laser therapy on all patients in fixed appliances. The time it would take to irradiate all teeth included in full fixed appliances would be extremely time consuming for the clinician. This treatment would also be limited by the inherent limitation of a laser, including their relatively short battery life, particularly at energy levels that may be required to have any effect.

Appendix 1 P-Laser System®

1.1 P-Laser System® Technical Data

Laser class:	3B.
Lasing medium (Diode) :	GaAlAs (Gallium/Aluminium/Arsenido).
Wavelength:	830nm (invisible).
Mean output:	70mW
Beam mode/ frequency:	Continuous wave.
Beam divergence:	9º
Polarisation:	Random.
Target light:	Visible red spectrum.
Signal/alarm:	Acoustic bleep for On/Off and failure alarm.
Dimensions:	Length: 205mm Diameter: 22mm Weight: 120grams
Battery duty cycle:	Maximum 40 minutes with fully-charged battery. Recharging time 15 hours. LED indicator for charging and charge error.
Timer:	Choice of 15, 30, 60 or 120 seconds.
Safety lock:	Key-lock beam disablement.
External power supply:	Ordinary mains adapter capable of 6-9 volts AC, 9-12 volts DC.
Fiber optic glass tips:	Available in a range of designs to suit all applications. All tips provide typical effective beam transmission rate of 75-95%. All fiber optic glass tips are autoclavable and can be cleaned easily using a disinfectant.
Power meter:	Separate digital power meter for monitoring actual output.

Appendix 2 Laboratory Procedures

A.2.1 Method of Culturing Osteosarcoma Cells

1. Flat culture flasks were removed from the incubator and placed under the lamina flow hood, a class II biological chamber
2. The growth medium was aspirated from the flask
3. The flask was gently rinsed three times with 5 ml of Phosphate buffered saline, this was aspirated
4. 5 ml of 5 percent trypsin / ethylene diaminetetraacetic acid was placed in the flask and the flask was replaced in the incubator at 37°C and 5 percent carbon dioxide atmosphere for three minutes
5. The side of the flask is tapped to dislodge the cells on the flask base
6. 5 ml of serum free growth medium was added to neutralise the trypsin / ethylene diaminetetraacetic acid. The growth medium and trypsin / ethylene diaminetetraacetic acid mixture containing the cells was transferred by pipette to a 15ml centrifuge tube.
7. The centrifuge was counterbalanced and spun at 100,000 revolutions per minute for ten minutes.
8. The 10 ml of supernatant is removed and the tube flicked to resuspend the cells. Growth medium was then added.
10. A cell count was performed using a haemocytometer at a dilution factor of 5.
11. The fluid volume was adjusted to give a cell concentration per ml as required for either cell passaging or for seeding 96-well microplates.
12. Cells to be passaged were passed into $75cm^2$ flasks and the flask labelled with the date, passage number and cell type.
13. Incubate at 37°C and 5 percent carbon dioxide atmosphere.
14. Change the media and passage cells as required, at least twice a week.

A.2.2 Freezing down cells

1. Make up 1mL, 100µL DMSO (Dimethyl sulphoxide) in 900µL growth medium in a cryotube (1.8mL, Nunc) and place in cold room until needed (DMSO is toxic to cells at room temperature).
2. Trypsinize confluent flask of osteosarcoma cells. Take cryotube from cool room as centrifugation is finishing (DMSO freezes at a relatively high temperature) and resuspend pellet in DMSO/growth medium. Approximately 10^6 cells should be present for each tube.
3. Sandwich cryotube between two foam racks and place in -70°C freezer (this allows cells to freeze slowly, at about 1°C per minute).
4. Cryotube is transferred to liquid nitrogen (N_2) store the following day.

A.2.3 Bringing cells up from liquid N_2

1. Thaw cryotube quickly in 37°C water bath, alternatively run in warm water until cells have thawed. Rub over the lid of the tube with ethanol.
2. Transfer cells to 20mL centrifuge tube containing 10mL of DMEM and 10% FCS and centrifuge at 1100 rpm for 10 minutes. Alternatively use a 5mL syringe to add 2mL of DMEM and FCS into a vial. Resuspend the cells and transfer to the centrifuge.
3. Pour off the supernatant (not disturbing the pellet) and resuspend in 5mL growth medium. Transfer the resuspended cells to a flask.

A.2.4 Cell counting and viable cell number

Cell counts were performed using Trypan Blue dye and Haemocytometers. Trypan Blue is a stain recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualisation of cell morphology.

1. 80µL of 0.4% Trypan Blue solution is transferred to Eppendorfs. 20µL of the cell suspension (dilution factor = 5) is added and mixed thoroughly.

2. With the cover slip in place, a pasteur pipette is used to transfer a small amount of Trypan Blue-cell suspension mixture to both chambers of the Hemocytometer. Each chamber is filled by capillary action.
3. All cells in the 1mm center square and four 1mm corner squares are counted. Non-viable cells stain blue. A separate count is kept of the viable and non-viable cells.
   Cells on the top and left touching the middle line of the perimeter of each square are counted. Cells touching the middle line at bottom and right sides are not counted.
4. This procedure is repeated for both chambers.
5. Cell counts –Each square of the Hemocytometer with cover-slip in place, represents a total volume of $0.1mm^3$ or $10^{-4}cm^3$. Since $1cm^3$ is equivalent to approximately 1 mL, the subsequent cell concentration per mL (and the total number of cells) is determined using the following calculations:

   Cells per mL = the average count per square x dilution factor x 10^4

   Total cells = cells per mL x the original volume of fluid from which cell sample was removed.

   Cell viability (%) = total viable cells (unstained) ÷ total cells (stained and unstained) x 100.

A.2.5 Seeding of 96-well plates

1. Cells are harvested from confluent flasks by trypsinisation, centrifuged, and resuspended in growth medium with 10% foetal bovine serum.
2. Cells are counted using Trypan Blue and a Hemocytometer.
3. The cells are then diluted to the desired concentration.
4. The cells suspended in media with 10% foetal bovine serum are placed in a trough from where they are transferred to the 96-well plates using a multipipetter.
5. Cells are incubated at 37°C and 5% carbon dioxide in air.

A.2.6 MTT Assay

A.2.6.1 Seeding 96-well microplates for MTT assay

- Plates were seeded as per diagram (see 6.1) at an initial density of 10,000 cells per well. The first column of the plate contains no cells.
- Medium is placed in this column the same as with the sample columns. The same procedure is carried out with this column as for the experimental rows (i.e. MTT assay – rinsed with PBS, MTT added, alcohol added).
- The mean column blank is calculated from this row. All calculations of absorbances are made from subtracting this column.
- The next column is left blank.
- The plates are seeded starting with the top row moving down so that each control group is drawn from the same end of the Multipipetter. There are eight sample columns in each plate.

A.2.6.2 MTT assay procedure

1. Tip medium out of 96-well microplate.
2. Gently rinse cells twice with Phosphate buffered saline.
3. Add 100µL serum free medium to each well and to the column mean blank wells.
4. Add 10µL MTT 5mg/mL to each well and column mean blank wells.
5. Incubate 3.5 hours at 37°C (Switch on Titertek , ~45min to warm up).
6. Gently tip out MTT/medium.
7. Add 100µL absolute ethanol to each well.
8. Shake for 30 seconds in Titertek prior to initial reading.
9. Read using Titertek plate reader (Program 4 then program 6) 690nm, 540nm.

A.2.6.3 Titertek® Twinreader Plus

(D & A Laboratory Services, Baulkham Hills, NSW, Australia)

Preprogrammed settings are used for the MTT assay. Program 4 (Filter 8, 690nm) followed by Program 6 (Filter 6, 540nm). The 690nm readings are subtracted from the 540nm readings.

Twinreader PLUS is a single channel vertical light path filter photometer with built-in incubator, shaker and computer control.

It is used with flat-bottomed 96-well microplates, as utilised in our experiments.

A.2.6.4 Principle of operation

The optics of Titertek® Twinreader Plus is based on the concept of vertical photometry in which the light beam always passes through the whole sample and light absorption is proportional to the amount of light absorbing substance in the well.

Absorption is expressed by the following equation:

$$A = a/S \cdot m$$

where A = absorbance

a = molar absorptivity of the substance

m = mass of absorbing substance

s = cross-sectional area perpendicular to the light path

The advantages of this method are:

(1) inaccurate pipetting of nonabsorbing liquids does not affect the measured absorbance values

(2) evaporation of nonabsorbing liquids during the reaction does not affect the measured absorbance values and

(3) a certain degree of inhomogeneity in the solution, for example as a result of layering in turbidity measurements, does not affect the results.

A.2.7 5-Carboxyfluorescein Diacetate-succinimidyl Ester (CFSE) staining for Flow Cytometric Analysis

1. Take 1 x 10^8 or 5 x 10^7 cells, wash twice with serum-free medium (DMEM)
2. Resuspend to 5 x 10^7/ml

3. Warm for 5 minutes at 37°C
4. Dilute CFSE (10mM stock) 1 in 10 in warm DMEM
5. Add 10µl of the 1 in 10 dilution of CFSE for each ml of cell suspension (final concentration 10µM)
6. Incubate for 10 minutes at 37°C, invert every 3 to 4 minutes
7. Stop reaction with several volumes of cold DMEM / foetal bovine serum
8. Wash twice with DMEM / foetal bovine serum
9. Seed as per usual

A.2.8 Intracytoplasmic Immunoglobulin Staining for Flow Cytometric Analysis

From: Current protocols in Immunology (1995) Volume 1 of 3 pages 5.3.1 – 5.3.23 "Preparation of cells and reagents for flow cytometry." Holmes K, Fowlkes BJ, Schmid1, Giorgi JV.

Cell Harvesting

1. Harvest cells, disperse the cells with trypsin / ethylene diaminetetraacetic acid
2. Wash twice with Phosphate buffered saline / 2 percent foetal calf serum / 0.1 percent Sodium Azide
3. Count cells and determine concentration and viability
4. Resuspend to 1 x 10^6/ml
5. Aliquot 1ml of the suspended cells into a "Facs" tube
6. Centrifuge for 5 minutes, 300g, at 4°C, remove the supernatant, resuspend in residual fluid

Fixation

7. Add 875µl cold Phosphate buffered saline to 10^6 stained cells, mix gently
8. Add 125µl cold fixation solution (Paraformaldehyde), mix again
9. Incubate for 1 hour at 4°C (30 minutes for single cell population)
10. Centrifuge 5 minutes, 300g, 4°C
11. Remove supernatant carefully (aspirate or decant rapidly)

Permeabilising

12. Add 1ml permeabilisation solution (0.2 percent Tween 20), mix gently
13. Incubate 15 minutes at 37°C
14. Add 1ml Phosphate buffered saline, centrifuge for 5 minutes, 300g, 4°C
15. Remove supernatant carefully

Staining

16. Add 100µl antibody in permeabilising solution to pellet, mix well (anti-HSP70 mAbs and IgG negative isotype control mAbs [2µl 1:100 HSP or IgG and 98µl Tween 20])
17. Incubate 30 minutes, 4°C
18. Add 1ml washing buffer (0.1 percent Tween 20), centrifuge 5 minutes, 300g, 4°C
19. Remove supernatant, wash pellet again with 1ml washing buffer
20. Centrifuge 5 minutes, 300g, 4°C
21. Add secondary antibody (fluorescein isothiocynate anti-mouse IgG1), 2µl to residual fluid, repeat steps 17-20
22. Resuspend to 10^6 cells/ml in staining buffer (Phosphate buffered saline / 1.0 percent foetal calf serum / 0.1 percent Sodium Azide)
23. Analyse within 2 hours (store on ice)

A.2.9 Flow cytometry

Flow cytometric analysis of cell suspensions was performed using a FACScan system (Becton Dickinson, California, U.S.A.). This automated cell analyser consisted of a FACScan flow cytometer, and a Cell Quest computer software designed for the acquisition, analysis and stroage of data obtained from the flow cytometer. The cells to be analysed were suspended in FACScan tubes in a volume of 0.3-0.5ml. It was essential to allow the FACScan to warm up for at least 5 minutes on "Standby" before switching the machine to "Run" for data acquisition. Initially the cell population to be analysed was gated. Then for each subsequent suspension the same detector and compensation settings were used and a sample of events acquired. Typically 30,000 gated events were acquired from each cell suspension.

FACScan data was analysed in dot plots involving light scatter and fluorescence. From these dot plots statistical data could be obtained about the different fluorescing cell fractions in the sample in question. Histograms could also be generated.

A.2.10 Oregon Green AM Ester Loading technique for Confocal Microscopy

- Stock solution of 0.1mM of the probe in DMSO (*anhydrous* dimethylsulfoxide) is prepared and aliquoted and stored desiccated at –20°C
- Seeding density 400,000 cells/ well for 4-well chamber slides and 10µM concentration of Oregon Green

1. Wash cells twice with serum-free DMEM
2. Warm for 5 minutes at 37°C
3. Dilute Oregon Green (0.1mM stock) in warm serum free DMEM at appropriate pH and Hepes (2µl/ml).
4. Add 10µl of the 1 in 100 dilution of Oregon Green for each 100µl of cell suspension (final concentration 10µM). Incubate for 25 minutes at 37°C.
5. Tip out medium gently and add a large volume of serum free medium gently. Return to the incubator for 15-30 minutes.
6. Gently rinse with several volumes of cold serum free DMEM. Return to the incubator until required.

Appendix 3 Cell Culture Formulae

Dulbecco's Modification of Eagles Medium

Dissolve in water to make 1 litre

- DMEM 1 packet
- Sodium Bicarbonate (7.5%) 20mL
- Penicillin/Streptomycin (5000 IU/mL solution) 10mL
- (final concentration 50IU/mL)
- Adjust pH (HCL or NaOH)
- Filter Sterilise
- Supplement with 10% FCS and L-Glutamine if required (2mL /100mL of a 200mM stock solution)

Antibiotics

Combine in equal amounts

- Penicillin at 5000 IU / ml
- Streptomycin at 5000 IU / ml

Phosphate Buffered Saline

Dissolve Tablets in water to make 1 litre

- 10 PBS Tablets
- Adjust pH to 7.2
- Autoclave
- Store at 2.7°C

L-Glutamine (200mM)

- L-Glutamine 2.922g
- Medium (DMEM) 100mL
  (fully supplemented)
- Store frozen
- L-Glutamine is added to the medium every two weeks.

Trypsin –EDTA (trypsin / ethylene diaminetetraacetic acid)

- Make up 0.2% EDTA/PBS solution

  EDTA 200mg

  PBS 100mL

- Dissolve 50mg Trypsin in 90mL PBS
- Add 10mL 0.2% EDTA/PBS to Trypsin solution
- Filter Sterilise
- Store 2-8°C

MTT (5mg/mL)

(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

- MTT 25mg
- PBS 5mL
- Vortex
- Stand for 30 minutes at room temperature
- Filter Sterilise
- Store at 4°C in foil wrapped bottle
- Absorbance measured at 570nm, background subtraction at 690nm

5-Carboxyfluorescein Diacetate-succinimidyl Ester (CFSE) Stock

- 5-carboxyfluorescein diacetate, succinimidyl ester
- 10mM in DMSO
- Store dessicated at -70°C as 20µl aliquots
- CFSE from Molecular Probes, Oregon, USA
- Catologue Number: C-1157
- Molecular Weight 557.47

Solutions for Intracytoplasmic staining for HSP70 and cell cycle analysis

Phosphate Buffered Saline (PBS)

- NaCl 8g/l
- KCl 0.2g/l

- KH_2PO_4 0.2g/l
- $NaH_2PO_4.2H_20$ 1.15g/l
- Milli Q Water

10x PBS (2L)

- NaCl 160g
- KCl 4g
- KH_2PO_4 4g
- $NaH_2PO_4.2H_20$ 23g
- Milli Q Water 2000ml

Phosphate buffered saline / 0.1 percent Sodium Azide

- NaN_3 100mg
- PBS 100ml
- Store at 2-8°C for up to two months.

Phosphate buffered saline / 2 percent foetal calf serum / 0.1 percent Sodium Azide

- Foetal Calf Serum 2ml
- PBS/ NaN_3 100ml
- Store at 2-8°C for one month.

Fixation Solution

- Paraformaldehyde 2g
- Phosphate buffered saline 100ml
- Heat to not more than 70ºC to dissolve
- Adjust pH to 7.2 with 0.1M HCL or NaOH
- Store protected from light up to 1 month at 4ºC

Permeabilisation solution

- Tween 20 200µl (0.22g, 0.2%)
- Phosphate buffered saline 100ml
- Store up to 1 month in amber container at 4ºC

- Warm to room temperature before use

Washing Buffer

- Tween 20 100µl (0.11g, 0.1%)
- Phosphate buffered saline 100ml
- Store up to 1 month at 4ºC
- Tween 20 d=1.1g/ml

Appendix 4

PRESENTATIONS OF THIS RESEARCH

1998 Faculty of Dentistry Research Day -poster

1998 Biennial Australian Orthodontic Conference, Canberra –oral presentation

1998 Australian Society of Orthodontics Clinical Day -poster

1999 Australian Society of Orthodontics Clinical Day -Best poster presentation by a postgraduate

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