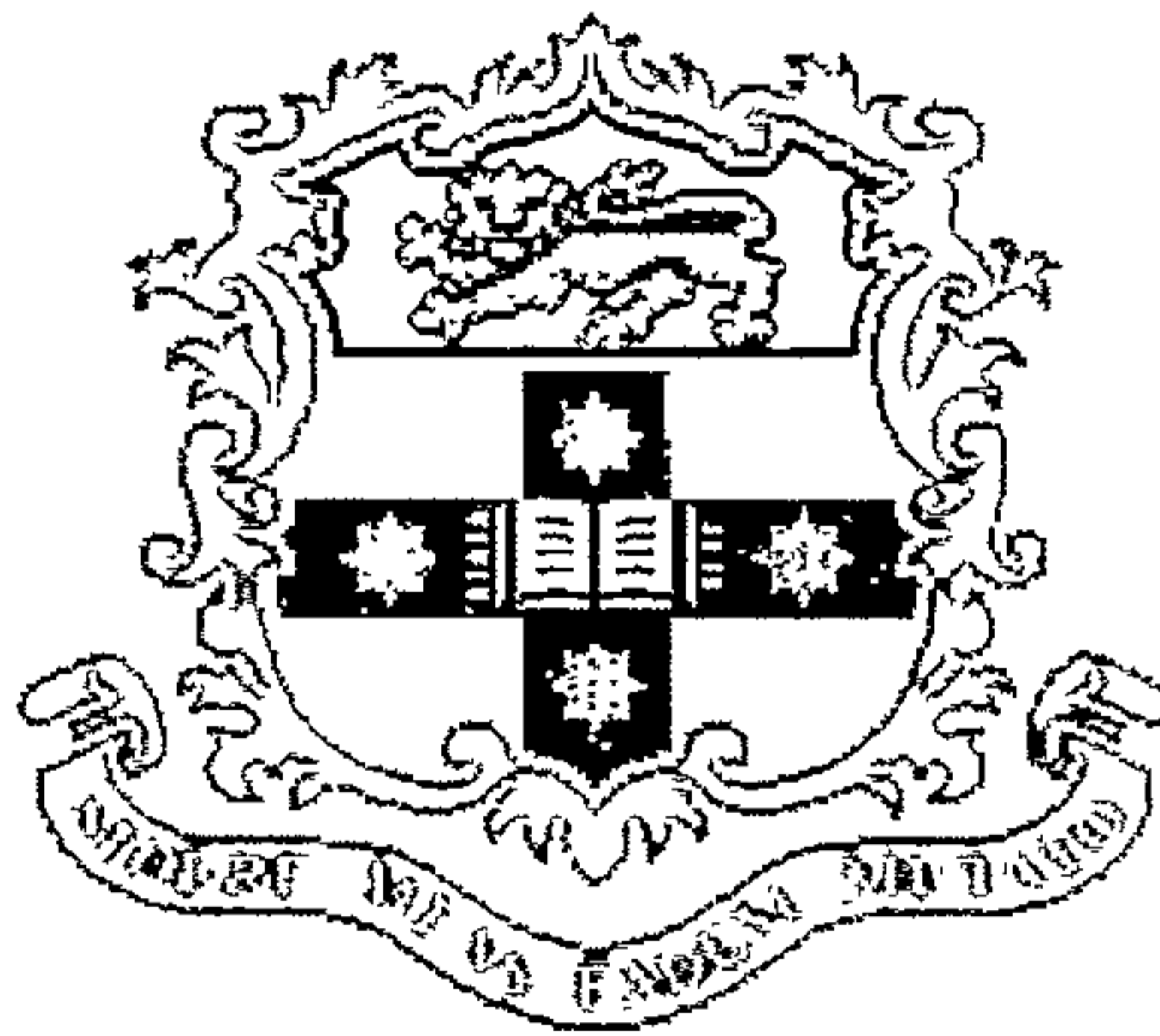

**THE EFFECTS OF LOW LEVEL LASER
ON AN OSTEOGENIC CELL LINE
*IN VITRO.***



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B.D.S. (Otago)

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of the requirements for the degree of
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DEDICATION

To my partner and my parents

My parents for their incredible generosity, support and encouragement in my life, education, and in achieving my goals.

Tony for his love and support, and for sacrificing so much of life to enable me to do this course. A life God given is a life precious enough to hold onto and revere.

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DECLARATION OF AUTHORSHIP

This is to certify that the work presented in this thesis was carried out by the Master of Dental Science candidate, Angela Rose Coombe, who was a postgraduate in the Discipline of Orthodontics, School of Dental Studies, Faculty of Dentistry, University of Sydney, and that the work has not been submitted to any other University or Institution for a higher degree.

31st August 1999

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Angela Rose Coombe

ABBREVIATIONS

ATP	Adenosine Triphosphate
Ca ²⁺	Calcium Ion
cAMP	Cyclic Adenosine Monophosphate
CFSE	5-carboxyfluorescein diacetate-succinimidyl ester
CO ₂	Carbon Dioxide
DMEM	Dulbecco's Modification of Eagles Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diaminetetraacetic Acid
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GaAlAs	Gallium Aluminium Arsenide
He-Ne	Helium-Neon
HSP70	Heat Shock Protein - 70
IgG ₁	Immunoglobulin G ₁
IL-1	Interleukin-1
J	Joules
J/cm ²	Energy Intensity
mJ	Millijoules
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
mW	Milliwatts
mW/cm ²	Power Intensity
µg	Microgram
µl	Microlitres
µm	Microlitres
nm	Nanometres
%	Percent
PBS	Phosphate Buffered Saline
RNA	Ribonucleic Acid
Saos-2	Osteosarcoma cell line utilised

ABSTRACT

Low level lasers have been used in medicine, dentistry, and allied sciences for applications termed low level laser therapy or 'biostimulation'. Cell culture studies and animal models have demonstrated positive effects of low level laser therapy. These biological effects include: acceleration of wound healing (with increased fibroblast proliferation, maturation and locomotion), increased motility of epithelial cells, enhanced neovascularisation, activation and enhanced proliferation of lymphocytes, maturation and regeneration of neural tissue, increased phagocytosis by macrophages, increased proliferation, differentiation, and accelerated calcification of cultured osteoblastic cells. These effects can occur simultaneously.

There is controversy regarding low level laser therapy as a treatment modality. No specific therapeutic window for dosimetry and mechanisms of action has been determined to the level of individual cell types.

Cellular effects on a human osteogenic cell line were examined utilising varying exposure regimes and energy levels with a Gallium/Aluminium/Arsenide (GaAlAs) low level laser (with a specific wavelength [830nm] and energy output [70mW]).

Multiple parameters were observed in conjunction with biochemical assays to determine efficient biostimulation. With the experiments investigating cellular growth changes, cells were analysed over a ten-day period, utilising energy levels of 0.5, 1, 2 and 4 Joules, as single or daily therapeutic doses. Cell counts were carried out and cell viability determined for irradiated cells and non-irradiated controls utilising Trypan Blue exclusion. The MTT assay, specific for mitochondrial succinate dehydrogenase activity was utilised to investigate effects of low level laser on cellular activation and total biomass. The fluorescent labelling dye CFSE more specifically investigated cellular proliferation. The level of physiological stress the osteosarcoma cells were subjected to by laser irradiation was investigated by analysing the expression of heat shock protein-70. Calcium flux was investigated to determine possible effects of low level laser at an intracellular level.

Cell viability was not affected by laser irradiation, with the viability being greater than 90% for all experimental groups. Cellular proliferation or activation was not found to increase significantly in any of the energy levels and varying exposure groups investigated.

Low level laser irradiation did result in a Heat Shock Protein response at an energy level of 2 Joules. Heat shock protein-70 was expressed maximally at 1.5 hours post-irradiation.

Investigation of intracellular calcium concentration revealed a tendency of a transient positive change after irradiation at 1 Joule and at 2 Joules. At the energy level of 1 Joule, effects were greatest after approximately 2 minutes, whereas at 2 Joules more immediate effects were observed. These changes decreased toward or below control levels after approximately 5 minutes. At the energy level of 4 Joules an initial positive change was observed at approximately 30 seconds after irradiation, but calcium levels continued to decrease after this time.

Low level laser irradiation was unable to stimulate the osteosarcoma cells utilised for this research at a gross cell population level. The heat shock response and increased intracellular calcium indicate that the cells do respond to low level laser irradiation. Further research is required, utilising different cell models and animal models, to more specifically determine the effects of low level laser therapy at a cellular level. These effects should be more thoroughly investigated before low level laser therapy can be considered as a viable treatment modality for utilisation in orthodontics.

We are in an era of high-tech devices, with dentists being offered many sophisticated products designed to improve the quality of treatments offered to patients. Already frequently used in medical, physiotherapy and veterinary fields, the laser has begun to impact on dentistry, particularly due to intensive marketing by the manufacturers.

Literature concerning the positive effects of low energy laser irradiation on patho-physiological responses, suggests the possibility of a significant addition to the already available arsenal of physical methods, therapies and rehabilitation procedures.

LASER is an acronym for Light Amplification by Stimulated Emission of Radiation. "Radiation" in terms of laser light is an expression of energy transmission.

Laser instruments cover a broad spectrum of use in medicine, dentistry, and allied sciences for diagnostic and biostimulating applications. For diagnostic purposes there are several types of laser instruments and procedures available, including: Laser Microscopic Masonic Analyzer (LAMMA), Flow cytometry, Doppler effects of laser rays (Laser Doppler velocytometry, Laser Doppler spermokhinezymetry, Laser Doppler spectrometry), Laser fluorescent microscopy, Laser nephelometry, Transillumination by lasers (diaphanography), Laser spectroscopy, Laser holography, Laser retinoscopy, Microirradiation by lasers (Takac *et al.* 1998).

The use of laser spectroscopy for quantitative analysis of cations from a single drop of dried blood on a piece of filter paper, and individual analyses of frozen skin biopsies for calcium, arsenic and gold are possible. In Europe, this technique has found its application in forensic medicine (Takac *et al.* 1998).

Laser-based methods have been used to study air pollution with carcinogens in occupational exposures and also for the detection of narcotic drugs. Laser cytofluorimetry utilizes the Argon laser for scanning of single stained cells and has achieved utilization in mass examination programs for Pap-smear determinations. The same technique is used in cell sorting system that is now important in monoclonal antibody production by hybridoma technology (Takac *et al.* 1998).

Other possible diagnostic applications include laser particle size measurement techniques, and laser nephelometry for determination of immunoglobulin classes and autoantibodies such

as rheumatoid factors. Laser Doppler velocimetry is used to measure blood flow by means of a simple probe that rests on the lip (Takac *et al.* 1998).

The Hungarian scientist Endre Mester, from Budapest, is one of the pioneers with the greatest experimental and clinical experience in the use of biostimulating effects of lasers. His former student, Ribari, first used biostimulating effects of Helium-Neon laser (390 mJ power) for the epithelialisation of perforated tympanic membranes and treatment of postoperative fistulas of the neck and of the mastoid (Takac *et al.* 1998).

Physiotherapists are now using low level laser in a wide variety of acute and chronic musculoskeletal pain, as are dentists to treat inflamed oral tissues and to heal diverse ulcerations. Dermatologists treat oedema, indolent ulcers, burns and dermatitis. Rheumatologists use low power laser in the treatment of chronic inflammations, autoimmune diseases and for the relief of pain. Other specialists as well as general practitioners also utilise low power laser. It is also widely used in veterinary medicine (particularly in association with racehorses) and in sports medicine and rehabilitation clinics, particularly for the reduction of swelling and haematoma, pain relief, mobility improvement, and in the treatment of acute soft tissue injuries (Karu 1997).

The clinical effects of light can be classified as direct or indirect, as light either causes an effect to occur within the irradiated tissue, or a nervous or neuroendocrine signal is generated within the irradiated area causing a systemic effect in another part of the body (Karu 1997).

Clinically, low-level laser therapies vary widely in their methodologies, and often lack double-blind tests or even control groups. The laser exposure parameters utilized (dose, wavelength, intensity, continuous wave or pulsed, pulse duration, and repetition rate) vary widely. In many cases no proof has been obtained that laser therapy is better than alternative therapeutic treatments. The operative mechanism at the level of the organism has not yet been elucidated. Low level laser therapy is therefore not a fully developed or accepted method of treatment (Basford 1995).

It has been postulated that low energy laser irradiation may stimulate biological processes (Haina 1982; Mester 1982; Hallman *et al.* 1988). Possible mechanisms for biostimulation of human tissues and organs under low power laser irradiation are many and varied. These

stimulatory and regulatory mechanisms of the cell metabolism appear to be involved in wound epithelialisation, reduction of oedema and inflammation, and re-establishment of arterial, venous and lymph microcirculation and consequently induction of better tissue nutrition. Higher rates of ATP, RNA and DNA synthesis have also been suggested and investigated. Increased absorption of interstitial fluid, better tissue regeneration and stimulation of the analgesic effect are other suggested mechanisms (Takac *et al.* 1998).

The basis for laser therapy is that laser radiation has a wavelength-dependent capability to alter cellular behavior without causing significant heating. Early investigators believed that laser radiation "stimulated" biological processes, as observed with hair growth and accelerated wound healing. "Biostimulation" was the term initially given to this phenomenon. Later evidence was presented indicating that low intensity radiation could inhibit as well as stimulate cellular activity. The early terminology has been replaced with an array of interchangeable terms such as low intensity, low level, and low power, all of which emphasize the nonthermal, low energy characteristics of the approach. This type of laser therapy typically involves the delivery of $\leq 1-4$ J/cm² to treatment sites with lasers having output powers between 10mW and 90mW (Basford, 1995).

Specific types of molecules in tissues absorb specific wavelengths of light (visible and infrared), each biological molecule having its own characteristic absorption spectrum. Absorbed radiation can produce specific biological effects in tissues, depending upon which types of molecules absorb the light.

Tooth and /or jaw movement in orthodontic treatment, tooth extraction and bone fracture, are always accompanied by bone remodelling. Stimulation of bone regeneration by laser treatment may therefore be of great potential benefit in orthodontics. An increased rate of bone healing may result in shorter healing times for orthognathic surgery cases, reduced retention for, and more stable, midpalatal expansion, an increased rate of tooth movement and increased initial stability after orthodontic treatment.

However, clinical laser therapy cannot as yet be applied efficiently, as the mechanisms of action on bone are not fully elucidated. It is thus necessary to investigate comprehensively the effects of low level laser on bone at a cellular level using objective, analytical methods.

Chapter 2 Aims and Objectives

2.1 Specific Aims

1. To determine the effects of low level laser therapy, at different energy levels and exposure regimes, on cell counts and cell viability of an osteogenic cell line *in vitro*.
2. To determine the effects of low level laser therapy, at different energy levels and exposure regimes, on cellular proliferation and activation of an osteogenic cell line *in vitro*.
3. To determine the level of physiological stress experienced by osteosarcoma cells exposed to laser irradiation by quantification of the expression of heat shock protein-70.
4. To determine the effect of low level laser therapy, at different energy levels, on intracellular calcium concentration.

2.2 Hypotheses

1. Low level laser therapy has a dose dependent effect on cell number, with no effect on cell viability.
2. Low level laser therapy has a dose dependent effect on cellular proliferation and activation.
3. Low level laser therapy is not stressful to cells and thus does not induce a heat shock response.
4. Low level laser therapy has a dose dependent effect on intracellular calcium concentration.

Chapter 3 REVIEW OF THE LITERATURE

Over the last few decades many methods have been investigated with respect to their relative merits in biostimulation, with particular reference to the enhancement of wound and bone healing. Included amongst these are vitamin D₃ (Seo *et al* 1996, 1997; Omeroglu *et al* 1997), prostaglandin E₂ (Keller 1996; Talwar *et al* 1996), electromagnetic fields (Otter *et al* 1998; Grace *et al* 1998), synthetic growth factors (Nakamura *et al* 1998; Wang *et al* 1996) as well as low level lasers.

3.1 History of Lasers

Laser therapy has been classed as a light therapy. This type of therapy has been in medical use for centuries. The healing power of the sun was known in the Indian Veda. Hippocrates and Galenus used sunlight as therapy. In the Middle Ages H. de Mondeville embraced the Arabic and Chinese traditions of red light therapy. Finsen, in the 1800's, experimented with light for the first time, and confirmed the findings and experiences of his predecessors. In the 1930's cold 633nm neon light was used in experiments to stimulate tissue responses (Kert and Rose, 1989).

Einstein in his "Zur Quantum Theorie Der Stralung", initially published in 1916, was the first to allude to the principle of laser. He is credited with the development of the theory of spontaneous and stimulated emission of radiation (Miserendino 1995). Einstein stated that absorption occurs at the atomic level when an atom incorporates a quantum of energy called a photon (Einstein 1917). Spontaneous emission is the process by which a photon is spontaneously released from a high-energy atom. Photon energy release allows the high-energy atom to return to a lower and more stable energy level. Stimulated emission of energy occurs when a free photon of energy interacts with a high-energy atom, causing the release of a second quantum of energy. The atom regains a stable energy state, while the two released photons travel as a wave of electromagnetic radiation (laser radiation) in the same spatial relationship (Fuller 1980).

The photon that is released by the energy input speeds on with the incoming photon. These two photons, striking two further molecules, cause the production of another two photons. A cascade reaction thus takes place, with all photons moving in the same direction. The resultant effect is 'microwave amplification by stimulated emission of radiation' and the equipment designed to generate this effect is termed a MASER (Midda and Harper 1991).

Charles Hard Townes, an American physicist pointed out, in 1958, the possibility that the MASER principle can be applied to electromagnetic waves of any wavelength, including light. This process, 'light amplification by stimulated emission of radiation', is the LASER (Midda and Harper 1991).

Theodore Harold Maiman, another American, constructed the first laser in 1960. His work was based on the principles described by Schawlow and Townes (Midda and Harper 1991).

Maiman reported the first light amplification by stimulated emission of radiation effect when he was able to observe stimulated emissions of visible light from a ruby crystal rod (Colvard and Pick 1993). This pulsed ruby laser emitted light of 0.694 μ m wavelength (Miserendino 1995).

Other researchers duplicated Maiman's work, resulting in the rapid emergence of new lasers. A year later (1961) Snitzer developed the second laser, the neodymium laser, (Miserendino 1995). The first gas and first continuously operating laser was described by Javan *et al.* in 1961.

Patel *et al.* (1964) developed the first carbon dioxide laser, and Geusic *et al.* (1964) the first neodymium-yttrium aluminium garnet (Nd:YAG) laser.

Clinical use and research in low level lasers today owe much of their popularity to work begun in Hungary and the Eastern Bloc countries in the mid-1960's (Mester 1981; Mester 1985).

Dentistry was the first profession to conduct research into the diagnostic and therapeutic applications of medical lasers. Goldman *et al.* (1964) reported the first application of laser energy on human tissues when they irradiated human teeth with early engineering lasers.

Stern and Sognaes and Stern *et al.* from 1964 through to 1972 accomplished pioneering dental studies on the effects of laser energy on enamel and dentine. Exploration of a wide range of applications in medicine, especially surgery, also began (Colvard and Pick 1993).

Low-powered laser utilisation was first reported in the late 1960's. Mester, Gynes and Tota in 1969 were one of the earliest to report on the use of low-powered laser, with research on accelerated healing. Various groups, mostly of Eastern European origin have since released numerous reports.

The first laser to be used in clinical and research applications was the He-Ne laser (632.8nm), its use extending from the sixties to the mid-eighties. Semiconductor lasers and Light Emitting Diodes became available in the mid-eighties. Gallium-Arsenide or Gallium-Aluminium-Arsenide diodes are now by far the most popular units routinely used in therapeutic as well as in laboratory applications.

Laser therapy is now used therapeutically worldwide, particularly in the treatment of musculoskeletal injury, pain, and inflammation. Surveys show routine use in over 40% of physical therapy clinics in Great Britain and perhaps 30% of dental clinics in Scandinavia. Acceptance in the United States, however, is minimal and the Food and Drug Administration (FDA) has not granted approval for any indication (Basford, 1995).

At present there is much anecdotal and little scientific evidence to support the value of low level laser as a viable treatment modality.

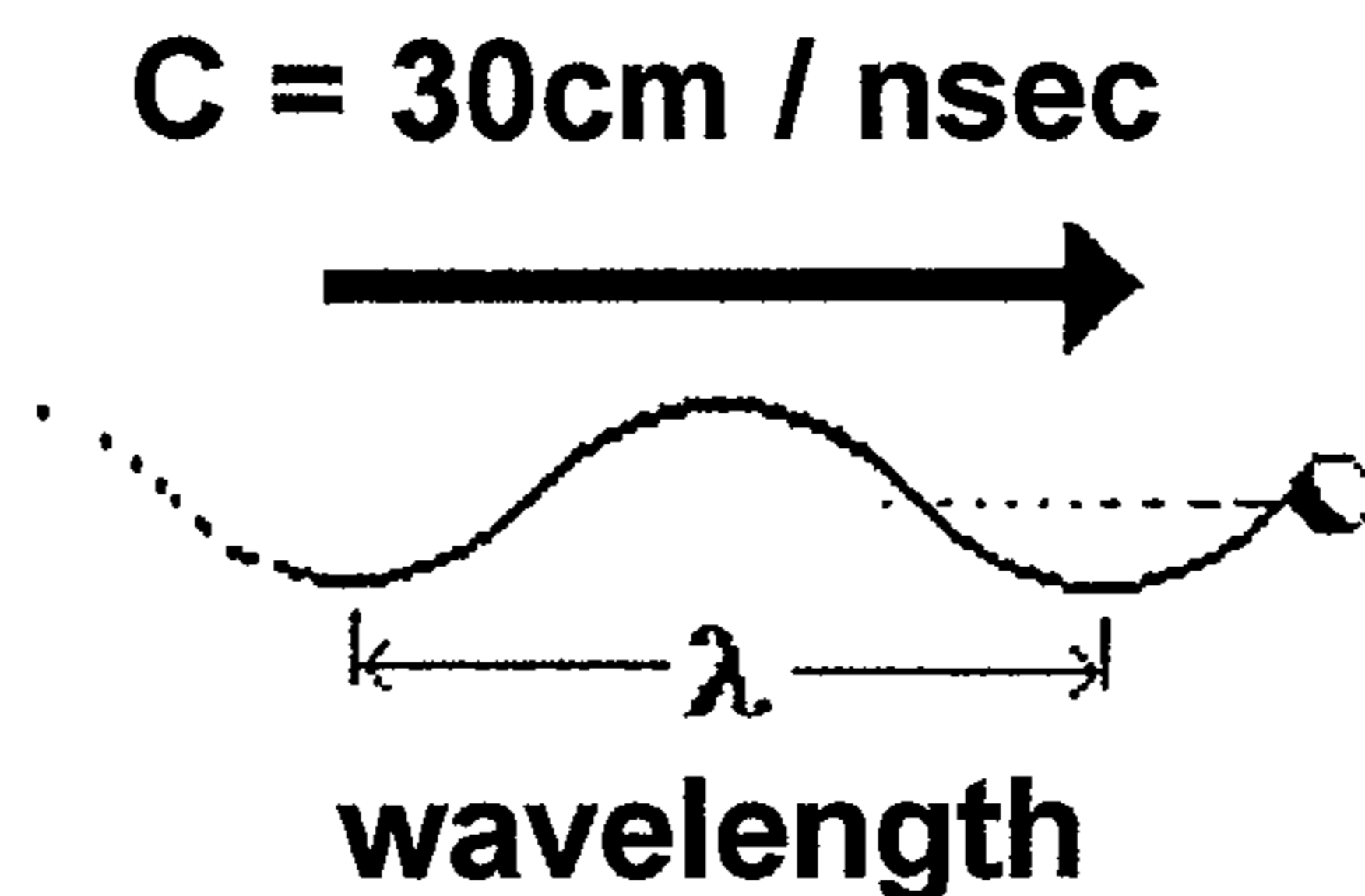
3.2 Laser Physics

Lasers are unique and versatile instruments because of their physical characteristics. The properties of collimation, coherence, and monochromaticity are generated in the high-energy processes occurring inside the optical cavity (Maiman, 1960). The laser beam projected from this optical cavity and into tissues retains these characteristics. Precise control of laser output allows the tuning of the space, time, and wavelength to optimise the outcomes of specific clinical and laboratory procedures (Harris and Pick 1995).

3.2.1 Photons

The basic units, or quanta, of light are called photons. They behave like tiny wavelets similar to sound wave pulses. A quantum of light can be depicted as an electromagnetic wave with an electric field oscillating up and down in the plane of the page, with the photon moving across the page from left to right at 30cm/1billionth of a second (1billionth = 1 nanosecond (ns)). The distance a photon travels through one complete oscillation is its wavelength (Figure 3.1). A magnetic field is also associated with the photon that moves in and out of the page. Photons thus represent quanta of electromagnetic energy (Harris and Pick 1995).

Figure 3.1 Schematic photon showing the approximate speed of light and the definition of wavelength.



Classification of electromagnetic energy is determined by the wavelength. Quanta of electromagnetic radiation are classified as cosmic rays (short wavelengths 10^{-12}m), gamma rays, x-rays, light, microwaves, or radiowaves (long wavelengths 10^3m). Laser light covers the range from about 0.1 to $10\mu\text{m}$ (Harris and Pick 1995).

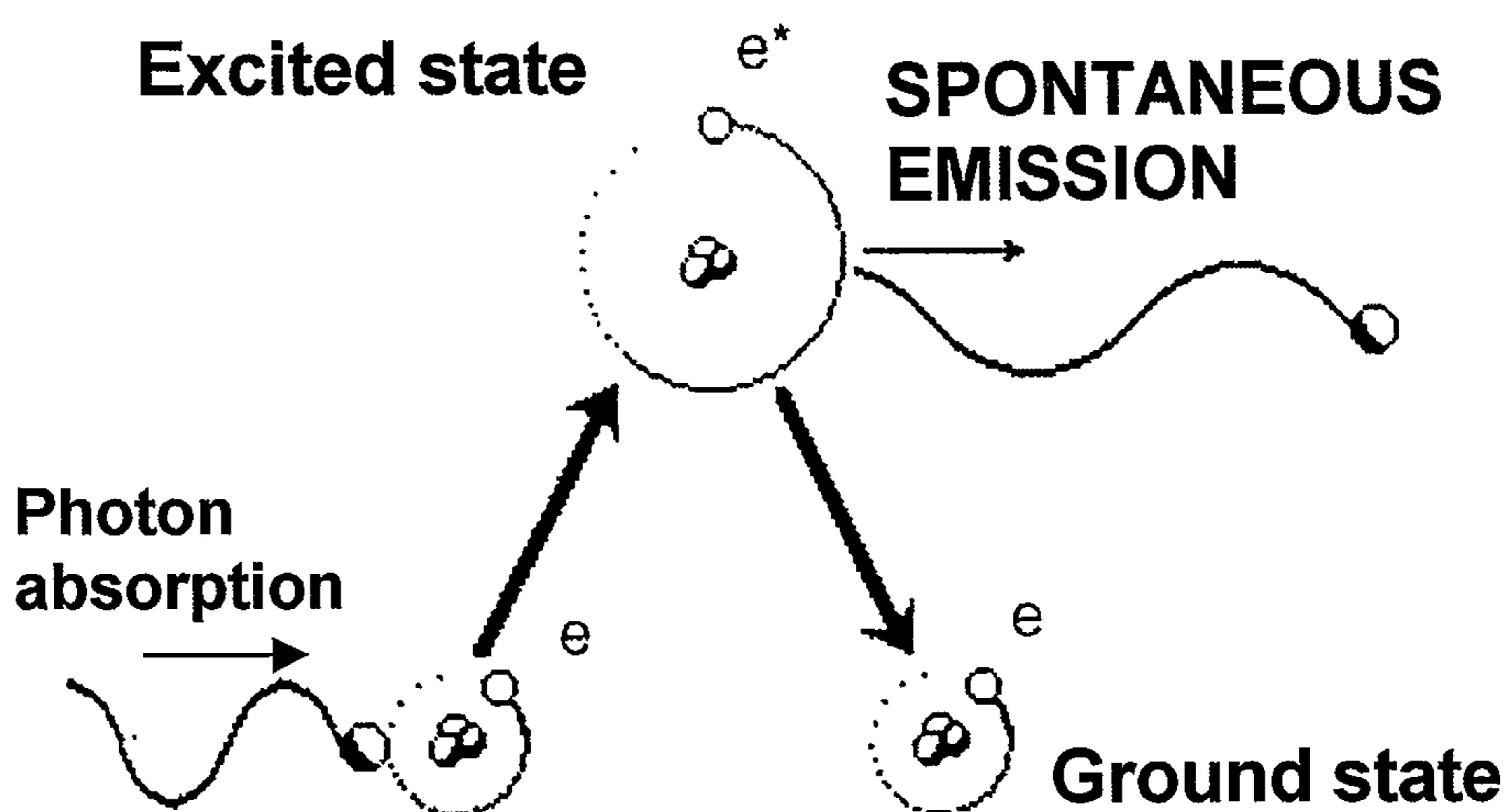
3.2.2 Light Absorption and Emission

When light encounters matter, it can be deflected (reflected or scattered) or absorbed. If a photon is absorbed, its energy is used to increase the energy level of the absorbing atom or molecule (Harris and Pick 1995).

An atom can absorb a photon, in which case the photon ceases to exist, and an electron (e) within the atom jumps to a higher energy level (e^*). The atom is pumped up to an excited state from the resting ground state. In the excited state, the atom is unstable and spontaneously decays back to the ground state, releasing the stored energy in the form of an emitted photon. This entire process is termed spontaneous emission (Figure 3.2). The spontaneously emitted photon has less energy (a longer wavelength) than the absorbed photon. The energy difference usually turns into heat (Harris and Pick 1995).

Only certain orbits (energy levels) in a given atom are allowed. When a photon is absorbed, the atom jumps to one of the allowable energy levels. Thus, each type of atom or molecule can absorb only photons of exactly the right energy (or wavelength). Each species of atom or molecule therefore has a unique absorption spectrum (Harris and Pick, 1995).

Figure 3.2 Spontaneous Emission.



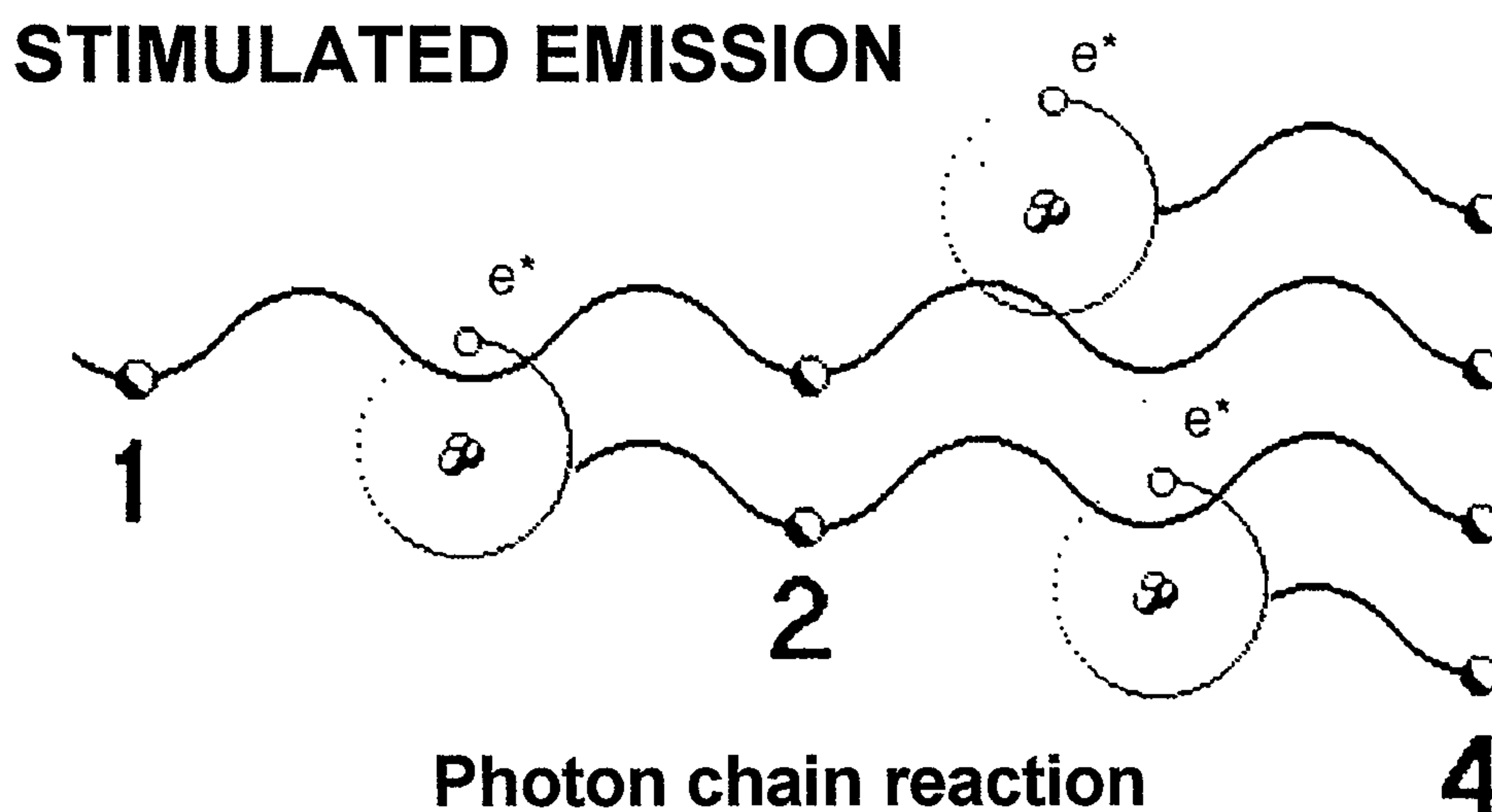
3.2.3 Light Amplification by Stimulated Emission of Radiation

When an excited atom can be stimulated to emit a photon before the process occurs spontaneously, the process of lasing occurs (Figure 3.3). When a photon of exactly the right energy (wavelength) enters the electromagnetic field of an excited atom, the incident photon triggers the decay of the excited electron to the lower energy state. Release of the stored energy in the form of a second photon accompanies this. The first photon is not absorbed but continues on to encounter another excited atom (Harris and Pick 1995).

Stimulated emission occurs only when the incident photon has exactly the same energy as the released photon. The resultant stimulated emission is two photons of identical wavelength travelling in the same direction. The release of the second photon is timed to be the same as the oscillations of the first photon, the two photons oscillating together in phase (Harris and Pick 1995).

If a collection of atoms includes more in the excited state than remain in the resting state, a population inversion exists. This is a necessary situation for lasing. The spontaneous emission of a photon by one atom will stimulate the release of a second photon in a second atom, and these two photons will trigger the release of two more photons, and so on. In a small space at the speed of light, this photon chain reaction produces a brief, intense flash of monochromatic (same wavelength) and coherent (same phase) light (Harris and Pick, 1995).

Figure 3.3 Stimulated emission, an excited atom is stimulated to release stored energy.



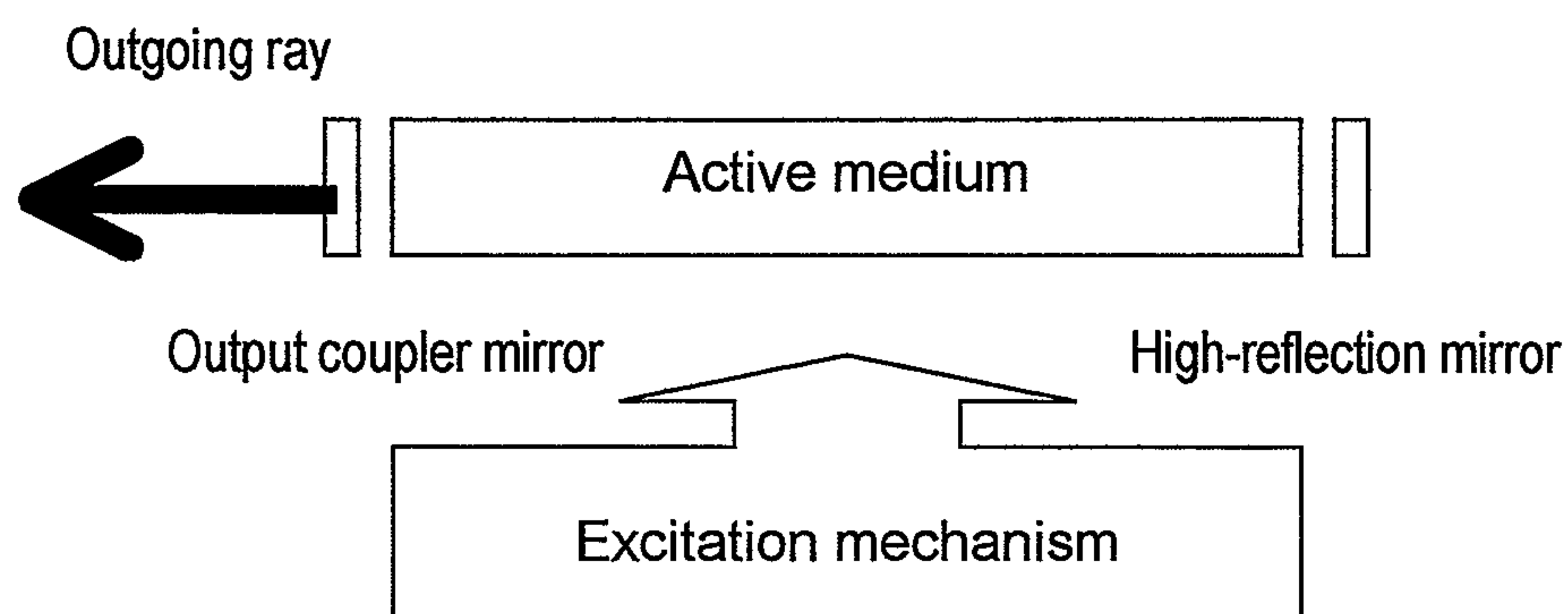
3.2.4 Laser Components and Beam Generation

The basic components of a laser include a lasing medium placed within an optical cavity, a pump energy source, and a cooling system. An optical cavity is required to contain and amplify the photon chain reaction resulting from stimulated emission. An optical cavity consists of two parallel mirrors placed on either side of the laser medium. This configuration allows for the photons to bounce off the mirrors and re-enter the medium to stimulate the release of more photons. If a form of energy is provided, to continuously pump atoms up to the excited state, the population inversion can be maintained and high-intensity light circulating back and forth between the two mirrors can be generated. The energy supplied by the excitation mechanism can be either in the form of intense light or electricity (Kert and Rose, 1989). The mirrors collimate the light, which means that photons exactly perpendicular to the mirrors re-enter the active medium, while those off axis leave the lasing process. The process is not entirely efficient and some energy is converted into heat, therefore making it necessary to provide some form of cooling.

One mirror is totally reflective and the other partially transmissive (the output coupler), reflecting less than 100%, letting the rest of the light pass through. The light escaping through the partially transmissive mirror becomes the laser beam. The active medium contains the homogenous population of atoms or molecules in the excited state and is stimulated to lase. The exact species of atom or molecules determines the wavelength of the output beam. The active medium is suspended in the optical cavity as a gas, liquid, semiconductor (Kert and Rose 1989), or distributed in a solid state (e.g. a crystal).

A collimated, coherent, and monochromatic beam of light is, thus, generated within the optical cavity. The contents and state of suspension, of the active medium (Harris and Pick 1995) determine the name of the laser.

Figure 3.4. Principal components in a laser.



3.2.5 Laser Energy

Laser energy is expressed in Joules. Energy is a product of mean output and beam duration. Laser power is expressed as output measured in Watts. Mean output is an expression of how many photons per second are released from the laser:

$$\text{Energy (Joules)} = \text{Mean power (Watts)} \times \text{Time (seconds)}.$$

$$1\text{J} = 1\text{W} \times 1\text{sec}.$$

The intensity of the laser beam being used can be expressed as either power intensity (mW/cm^2) or energy intensity (J/cm^2) (Harris and Pick 1995).

3.2.6 Characteristics of Laser Light

The principles governing the production of laser light provides certain unique characteristics not found in ordinary light. These are:

Coherence –all wavelengths are in phase (the peaks and valleys are lined up), even across large distances.

Monochromaticity –it consists of just one wavelength (the beam cannot be split into more than one colour by a prism).

Unidirectionality –it is possible to send a laser beam across large distances and still maintain a concentrated beam, with output intensity being maintained. The laser beam will increase by one millimetre in diameter across a distance of one metre (Basford 1995).

3.3 Laser Biophysics

The maintenance of coherency and collimation does not appear to be essential, as the effect of passage through tissue is to scatter and degrade these parameters of the beam. The fact that both laser diodes and coherent light can alter biological processes supports the relative lack of importance of these properties. Both coherent and noncoherent red light have been found to be equally effective clinically (Sazonov *et al.* 1985; Karu 1986). However, monochromaticity appears vital as investigators have shown that effects present with a narrow bandwidth stimulation are absent with broad spectrum light (Karu 1987, 1989).

There is an optical window in the visible and infrared portion of the spectrum, which extends from approximately 600nm to 1,300nm, and includes most of the lasers currently used in laser therapy. Although specific absorption characteristics are important for some lasers (e.g.

Haemoglobin at 488nm, 515nm, for the Argon ion laser) Helium-Neon, Gallium-Aluminium-Arsenide, and Gallium-Arsenide devices fall in the region of the spectrum without strongly absorbing tissue chromophores and have better than expected tissue penetration capabilities (Basford 1995).

It remains however, that the laser beam experiences an exponential decrease induced by the scattering and absorption occurring as light passes through tissue. Longer wavelengths are more resistant to scattering than shorter wavelengths (Basford 1995).

The interrelationship between reflection, refraction, absorption, spread and transmission determines the distribution of laser light in the tissue. The surface reflects the light, the degree of reflection therefore being dependent on the character of the surface and the angle of incidence. The light is absorbed by particles in the medium, haemoglobin, melanin, and water in practice absorbing most of the light. The penetration of laser light depends on the mean power output and the wavelength. The longer the wavelength, the deeper the penetration, and the firmer the tissue, the less penetration is achieved (Kert and Rose 1989).

The effects of laser are said to penetrate bone tissue to a depth of several millimetres. Approximate soft tissue penetration is suggested to be 2mm at the energy level of 1 Joule and 5mm at the energy level of 6 Joules. Bone penetration is 2mm at 1.5 Joules and 4mm at 6 Joules. The laser beam is always parallel and the diameter increase on $1/1000^{\text{th}}$ of the distance i.e. 1mm of increase across a distance of one metre (Kert and Rose 1989).

Helium-Neon laser light (red) is reported to penetrate 0.5-1mm before losing $1/e$ (37%) of its intensity, and the longer wavelength of infra-red irradiation will penetrate more than 2mm before losing the same fraction of its energy (Anderson *et al.* 1981; Kolari 1985). A penetration depth of 0.5-2.5cm can reportedly be reached at energy densities of $4\text{J}/\text{cm}^2$, thus therapeutic amounts of energy can be expected to reach the superficial nerves, joints and tissues typically treated with laser therapy (Basford 1995).

Low Level Laser treatments typically consist of exposures of about sixty seconds and powers of 1 to 90mW. These energies do not produce appreciable changes in the temperature of the tissues being treated, with temperature changes reported to be in the range of 0.3 to 0.62°C (Boussignac 1985). Thus, any unique effects must be derived from a nonthermal mechanism based on interaction with, or absorption in, specific tissue substructures rather than the generalised heating of a tissue as a whole (Basford 1986).

Biological factors that influence laser tissue interactions include the optical properties of various tissue elements that govern how specific molecular and chemical components in tissue react with light energy. Manipulation of power output, the total energy delivered over a given surface area (energy density), the rate and duration of the exposure and the mode of delivery of the energy to the target tissue, enables the operator to have precise control over the laser in the clinical setting to achieve the desired tissue effect when coupled with the ability to select the appropriate wavelength for the particular tissue target. The optical properties of tissue elements determine the nature and extent of the tissue response through the processes of absorption, transmission, reflection and scattering of the laser beam (Dederich 1991).

3.4 Lasers in Dentistry

Since the development of the first Ruby laser in 1960, multiple uses of lasers in dentistry have been investigated. "Hard" lasers are the lasers used most extensively in dentistry, and a brief summary of these uses will be given. Stern and Sognnaes (1964) were the first to examine the possible uses of the ruby laser in dentistry. Laser studies initially investigated the reduction of subsurface demineralisation (Stern *et al.* 1969; 1972). However, the Ruby laser (Adrian *et al.* 1971) also produced significant heat causing pulpal damage.

Dental laser users employ lasers for the advantages they provide. With the laser, there is little or no bleeding, providing a dry operative field and excellent visibility, as well as reducing the risk of bloodborne contamination. The follow on from this is a reduction in operating time (Pick 1993). Pain during the procedure as well as postoperative pain is said to be reduced. Lasers cause minimal scarring and the requirement for sutures is reduced. Bacterial counts are also reduced (Pick 1993) as is postsurgical swelling (Miller *et al.* 1993). However, surgical incisions heal faster than those made by lasers (Miller *et al.* 1993). There is a high degree of patient acceptance.

Soft tissue procedures have been restricted mostly to incising and excising masses from the gingiva and mucosa in the oral cavity. They can be used for removal or biopsy of tumours and lesions such as fibromas, papillomas and epulides, as well as white lesions, or vapourisation of excess tissues, as in gingivectomies, maxillary or lingual frenectomies, and operculectomies. Hyperplastic tissues can be removed or reduced and haemorrhaging of vascular lesions such as haemangiomas can be controlled (Miller *et al.* 1993). Lasers may also be used in periodontal surgery to impede the postoperative growth of oral epithelium. The laser is used in the second stage of implant recovery, and for crown and bridge soft tissue procedures, for which it can replace retraction cord while providing a high degree of haemostasis.

For patients who suffer from aphthous ulcers, laser treatment can eliminate the painful symptoms.

Many procedures can be carried out without local anaesthesia (Miller *et al.* 1993).

Hard tissue applications include hard tissue ablation, laser dental decay prevention, and laser decay detection, desensitisation of exposed root surfaces, scaling, curettage, and root planing. Dental composite curing, and enamel etching are procedures being commonly carried

out (Pick *et al.* 1993; Wigdor *et al.* 1995). Ceramic orthodontic brackets can be debonded with lasers. In endodontics lasers can be used to vapourise organic tissue, glaze canal wall surfaces and to fuse an apical plug to reduce fluid leakage (Pick *et al.* 1993).

The American Food and Drug Administration had given clearance to "dental" lasers for soft tissue surgery only, by 1995. The only non-soft tissue application, for the oral cavity, cleared by the FDA was for composite curing with the Argon laser (Wigdor *et al.* 1995).

A 1999 FDA fact sheet contained the following information: "...since low level laser therapy is an unproven medical treatment, these devices are also investigational medical devices which may only be distributed to investigators for the purpose of conducting studies of this modality of treatment."

The devices are not to be used commercially. The lasers are clearly labelled that they are for "study" purposes only.

3.4.1 Low level lasers in Dentistry

Low level lasers in dentistry are essentially used as systems to aid in tissue regeneration, primarily being employed with the intent of relieving pain, reducing inflammation and oedema, and accelerating healing.

Clinical dental applications of low level laser have included desensitisation of dentine hypersensitivity, to aid in the healing of acute localised osteitis (dry socket) and to reduce the pain associated with aphthous ulcers and promote their healing (Midda *et al.* 1991). These are all difficult areas to evaluate objectively; thus much of the evidence in support of laser therapy has been somewhat anecdotal. More clinical trials are being conducted to evaluate the use of lasers in a range of maxillofacial disorders. However, some studies lack adequate controls and consist of small sample sizes only.

3.4.2 Temporomandibular Joint Dysfunction

A comparative study of four physiotherapy treatment methods for the treatment of temporomandibular joint disorders has been carried out (Gray *et al.* 1994). Low level laser use for temporomandibular joint dysfunction had not been investigated scientifically prior to this research. A total of 176 patients were involved in this double-blind trial. The methods investigated were short-wave diathermy, megapulse, ultrasound and soft laser. A Gallium

Arsenide laser (904nm) using an energy level of 4 J/cm² for 3 minutes was applied. They found no statistically significant difference in success rate between any of the methods tested, however, each was individually significantly better than placebo treatment and timing of improvement varied for each treatment. Pinheiro *et al.* (1998) carried out a study of 124 patients with temporomandibular joint dysfunction. The patients were treated with 632.8, 670, and 830nm diode lasers, with an average dose of 1.8J/cm², twice a week for a total of 12 applications. A significant reduction in symptoms was observed, however, there was no control group and low level laser has demonstrated a strong placebo effect.

3.4.3 Third Molar Extractions

Postoperative pain and swelling after surgical removal of impacted third molars has been investigated. A double blind crossover study of 25 patients, with bilaterally identical impacted third molars was carried out by Roynesdal *et al.* (1993). A Gallium Aluminium Arsenide laser (830nm, 40mW), with an energy level of 6 Joules, was used pre- and post-operatively. Swelling, trismus, and subjective registration of pain were statistically analysed. No significant differences were observed for the experimental side compared to the placebo side.

A similarly designed study with a sample size of 64 patients (Fernando *et al.* 1993) also found no evidence of a difference in pain, swelling or healing between laser and placebo sides.

3.4.4 Restorative Dentistry

Low level laser therapy has been reported to reduce fixed prosthodontic pain after tooth preparation to receive a crown (Wafa *et al.* 1990).

A comparative double blind study (Gerschman *et al.* 1994) testing low level laser therapy (Gallium Aluminium Arsenide laser, 830nm, 30mW) compared to placebo was carried out in the management of dentinal hypersensitivity in 71 subjects. Low level laser therapy was applied for one minute (1.8 Joules) to both the apex and cervical area of the tooth, and reapplied at one week, two week and eight week intervals. Differences between the experimental and placebo groups were found to be significant from the first week and increased further in the second and eighth weeks.

3.4.5 Periodontal Treatment

Low level laser therapy has been considered as potentially useful as a healing adjunct for periodontal treatment. Masse *et al.* (1993) carried out a double blind crossover study of periodontal post-surgical patients performed in 28 subjects, to determine the analgesic, anti-inflammatory and healing effects. A Gallium Arsenide laser and Helium-Neon laser were utilised. One treatment was given immediately after surgery. They found no significant differences in the reduction of oedema, pain, inflammation, or healing when compared to the placebo surgical sites.

Treatment of early gingivitis with low level laser therapy has been reported by Kim *et al.* (1987) in a clinical, microbiological, and histological study. They demonstrated that following low level laser therapy, there was a decrease in the proportion of motile bacteria and spirochetes considered to contribute to periodontal diseases, and a concomitant increase in nonmotiles. A decrease in the gingival infiltration by inflammatory cells was also observed. It was indicated that low level laser irradiation had a favourable influence on gingival inflammation, with a change in oral flora composition, but it was not shown whether the decrease in gingival inflammation was due to biostimulation or a direct effect on the composition of the flora. It has since been investigated and proposed (Kim *et al.* 1992; Lee *et al.* 1993) that cellular activity due to the biostimulation effect of low level laser therapy in the surrounding normal tissue predominates over the tissue irritation due to the bacterial growth in an infected lesion.

3.4.6 Neurosensory deficits

A number of studies have looked at the effects of low level laser therapy on inferior alveolar, mental and lingual nerves after traumatic injury (mainly from third molar removal). Low level laser therapy has been shown to promote neurone maturation and regeneration following injury, and to reduce the production of inflammatory mediators of the arachadonic acid family by injured nerves (Mester *et al.* 1991; Soloman *et al.* 1991). It has also been shown to be effective for promoting axonal growth in injured nerves in animal models (Anders *et al.* 1993).

Midamba *et al.* (1993) observed considerable improvement of sensation in a group of 15 patients with short and long-term neurosensory impairment. Low level laser therapy was

applied using a GaAlAs laser (830nm, 70mW), with a dose of 4.9J/cm² at each point three times per week for two to eight weeks. However, there was no control group and some of the patients were examined at only one week post-surgery. The same authors carried out another study of 40 patients, using the same laser system at a dose of 6J/cm² for all patients. Effective improvement in sensation was determined. Again, no control group was included.

A study by Khullar *et al.* (1996), applying a double blind design, investigated the effect of low level laser treatment on neurosensory deficits subsequent to bilateral sagittal split osteotomy. The aim was to determine whether low level laser treatment resulted in an objectively verified improvement in sensory function and whether this correlated with the patient's subjective evaluation subsequent to treatment. Thirteen patients, two years post-surgery, were randomly divided into two groups. The laser treatment group received 4 x 6 Joules per treatment along the distribution of the inferior alveolar nerve for a total of twenty treatments using a GaAlAs laser (820nm, 70mW). The other group received an equivalent placebo treatment. The patients in the laser treatment group experienced a statistically significant subjective improvement in both lip and chin sensory function after completion of the course of treatment. This group also showed a significant reduction in the area of mechanoperception neurosensory deficit compared with no difference in the placebo group. The laser treatment group exhibited a strong tendency toward improvement in mechanoreceptor neurosensory deficit in the areas of most damage for the lip and chin (the lip region showing the greater tendency, $p=0.06$). This tendency was not illustrated in the placebo group. However, given the sample size and previously demonstrated placebo effect of this treatment, a "tendency" should be viewed with some skepticism. Neither group showed any significant change or improvement in thermoception.

3.4.7 Analgesia

The ability of low level laser therapy to exert analgesic effects has been a dominant clinical application of the technique. Studies *in vivo* of nerves supplying the oral cavity have demonstrated that low level laser therapy decreases the frequency of firing of the nociceptors, with a dosage dependent threshold effect to exert maximal suppression (Mezawa *et al.* 1988). Low level laser therapy is found to selectively inhibit a range of nociceptive signals arising from peripheral nerves, including neuronal discharges evoked by pinch, cold, heat and chemical irritation (Sato *et al.* 1994; Tsuchiya *et al.* 1994). It is also suggested that laser

irradiation may selectively target slow conducting fibres, particularly afferent axons from nociceptors (Tsuchiya *et al.* 1994; Baxter *et al.* 1994).

An alternative approach to direct irradiation is to irradiate target skin points used in acupuncture or acupressure. Few controlled trials are available investigating the effectiveness of this technique. A large case study (Zhou 1984) involving 562 patients who had undergone surgical exodontia and 48 patients who had minor oral surgery has been reported. Low level laser acupuncture was able to provide adequate postoperative analgesia in all cases. No sedatives or analgesics were administered before or during any of the procedures.

3.5 Lasers in Orthodontics

Use of lasers in orthodontics is limited. In clinical orthodontics there have been studies on laser enamel etching (Cooper *et al.* 1988; Walsh 1991, 1996; Walsh *et al.* 1994; Obata 1999) and laser bracket removal (Strobl *et al.* 1992; Mimura *et al.* 1995; Obata 1995, 1999) utilising hard lasers, and more recently post-adjustment pain reduction utilising low level laser treatment (Harazaki *et al.*, 1997, 1998; Lim *et al.* 1995).

Lim *et al.* (1995) carried out a double-blind study with placebo control on thirty-nine volunteers. Elastomeric separators were placed in each quadrant to induce orthodontic pain. A 30mW GaAlAs laser (830nm) was utilised, with exposure times of 15, 30, 60 seconds, and a placebo group, over a five-day period. The differences between treatments and placebo within each subject were not found to be statistically significant.

An initial study by Harazaki *et al.* (1997) observed the effects of low level laser irradiation on the reduction of pain in patients undergoing orthodontic treatment. It involved 84 patients divided into three groups (non-treated control, blind irradiation group, and laser-irradiated group). Irradiation was carried out on the day of appliance placement. A Helium-Neon laser (632.8nm, 6mW) was utilised, with a total irradiation time of 12 to 24 minutes for each patient. Pain reduction was evaluated by means of a questionnaire. The start of pain was found to be delayed in the laser irradiation group. However, the starting day of those with the most severe pain was not different between the groups. The authors also claimed that the laser group had fewer patients in the high pain categories. Statistical analysis of the data was not carried out, however.

A further study by Harazaki *et al.* (1998) evaluated 34 patients undergoing orthodontic treatment. Again pain reduction was evaluated using a questionnaire, with the addition of pulp sensitisation measured by pulp electrodiagnosis. Irradiation (He-Ne laser, 632.8nm, 6mW) was carried out on the day following appliance placement. Each painful tooth was irradiated for three minutes. Pain reduction was evaluated at 30-second intervals, and irradiation discontinued when effects were observed. In the control group painful teeth were irradiated with a halogen lamp used for resin polymerisation. Laser irradiation was found to be statistically significantly effective in reducing pain compared to the control group. The mean time required for pain reduction in the young group (<18 years) was 2 minutes and 13

seconds and in the older group (≥ 18 years) 3 minutes. Pulp electrodiagnosis showed no statistically significant differences between the laser and control groups.

Low level laser therapy as it relates to orthodontics *in vitro* has also been minimally investigated. Some researchers have attempted to elucidate in part, the mechanism of orthodontic pain relief. High levels of prostaglandin E₂ and interleukin-1 beta are found in the periodontal ligament during tooth movement, and both factors are involved in pain induction (Shimizu *et al.* 1995). The effects of low level laser irradiation on prostaglandin E₂ and interleukin-1 beta production in stretched periodontal ligament cells have been studied *in vitro* (Shimizu *et al.* 1995). The stretched cells were irradiated with a GaAlAs laser (60mW) once a day for 3, 6, or 10 minutes (10.8 to 36.0 Joules) for 1, 3, or 5 days. Increase in prostaglandin E₂ was found to be significantly inhibited by laser irradiation in a dose-dependent manner. The increase in interleukin-1 beta production was also significantly inhibited, although the inhibition was only partial.

Stimulatory effects of low level laser irradiation on bone regeneration in the mid-palatal suture during expansion in the rat have also been investigated (Saito and Shimizu, 1997). It was proposed that it would be potentially beneficial to accelerate bone formation in the midpalatal suture after expansion to reduce retention time and prevent relapse and of the arch width. During expansion the mid-palatal suture was irradiated with a GaAlAs laser (100mW), of power density 35.3 J/second/cm², over 1, 3 and 7 days. The effects were determined histologically. Irradiation during the early period of expansion (0-2 days) was found to be the most effective, the other time periods had no effect on bone regeneration. Single irradiation had no effect on the newly formed mineralized bone area. This suggests the effects to be transitory as well as time, frequency and dose dependent.

3.6 Low Level Laser and Pain Reduction

As outlined above, low level laser has been investigated with regard to pain reduction in a number of procedures in dentistry, including removal of third molars, temporomandibular joint dysfunction, post-orthodontic adjustment pain, relief of dentinal hypersensitivity, and in periodontal postoperative relief. Due to the limitations and lack of literature on this subject, and considering the relative importance of pain alleviation to the practice of orthodontics, literature concerning the efficacy of low level laser therapy for pain reduction in other areas of medicine may also be considered.

A trial conducted by Moore *et al.* (1992) tested the hypothesis that low level laser therapy reduces the extent and duration of postoperative pain. A double blind trial of twenty consecutive patients undergoing elective cholecystectomy, randomly allocated for laser treatment or as controls, was carried out. Laser treatment patients received 6-8 minutes of irradiation (GaAlAs laser, 830nm, 60mW) to the wound area immediately after skin closure prior to emergence from general anaesthesia. The demands for postoperative analgesia and pain scores were recorded. It was reported that no patient in the laser therapy group required narcotic analgesic after 24 hours. The requirement for oral analgesia was similarly reduced in the low level laser therapy group. Control patients assessed their overall pain as moderate to severe as compared to mild to moderate in the laser treatment group.

Pain attenuation in 63 patients with post-herpetic neuralgia was evaluated (Kemmons *et al.* 1991) after treatment with low level laser (GaAlAs, 830nm, 60mW). The total irradiation time for each session was 10 to 20 minutes, using 10 seconds for each painful spot along the pathway of the affected nerves. Laser therapy was applied 2 to 3 times a week for outpatients and 4 to 6 times a week for inpatients. Decreases in pain scores and increases of the body surface temperature by low level laser therapy were significantly greater than those that occurred in the control group. A placebo effect was also observed.

Rheumatoid arthritis and osteoarthritis are painful conditions to which low level laser therapy has been applied. An uncontrolled study by Asada *et al.* (1989) of 53 patients with rheumatoid arthritis treated with a GaAlAs laser (830nm) observed effective pain reduction. However, a number of double-blind placebo controlled trials (Heussler *et al.* 1993; Bulow *et al.* 1994; Hall *et al.* 1994) utilising the same laser diode (GaAlAs, 830nm) found no significant differences in any of the effect variables between the two groups at any time during or after the trial period.

The treatment did, however, produce analgesia through a powerful placebo effect in each of the trials.

To study the pain relief effects of laser irradiation Jimbo *et al.* (1998) set up a model using the distal portion of cultured murine dorsal root ganglion neurons associated with C-fibers and the GaAlAs laser (830nm). Laser light irradiation was found to reversibly suppress action potentials elicited by bradykinin in the cell body. It was proposed that laser light might block the conduction of nociceptive signals in primary afferent neurons. Quantification of substance P in the rat spinal dorsal root ganglion has illustrated that laser irradiation suppresses the excitation of the unmyelinated C-fibers in the afferent sensory pathway (Ohno 1997).

Low level laser therapy may be valuable as a treatment modality for alleviation of pain because it is a noninvasive, painless and safe method of therapy, and is reported to be well accepted by patients. However, consistent efficacy in pain relief is yet to be demonstrated, with many of the well designed trials showing no significant gain. A powerful placebo effect is always observed.

3.7 Low Level Laser and Bone

The effect of low level laser irradiation on bone growth and repair has been minimally investigated also. Faster callus formation and revascularisation in induced fractures in mice after 12 consecutive Helium-Neon laser applications have been reported (Trelles *et al.* 1990). Promotion of bone formation by low level laser irradiation (780nm) of focal injuries in mandibular bone has been observed Kusakari *et al.* (1992). Barushka *et al.* (1995) reported an approximately two-fold enhancement in bone repair in hole injuries of the rat tibia after two applications of Helium-Neon laser (632nm, 31J/cm²). Calcium, phosphorous and collagen hydroxyproline quantities have been found (Chen *et al.* 1989) to be significantly higher in irradiated bone callus compared to a contralateral unirradiated control in a rabbit model. Bilateral mandibular hole-like osteotomies were irradiated with a CO₂ laser (10600nm, 2W), with a power density of 225mW/cm², every day for seven consecutive days.

Increased vascularisation, faster osseous tissue formation, and more dense trabecular network after Helium-Neon doses of 2.4 Joules in a series of 12 treatments on alternative days, has been observed Trelles *et al.* (1987). Maximal load at failure and the structural stiffness of the rat tibia have been found to be significantly elevated after Helium-Neon irradiation, applied for 30 minutes daily for 14 days, to the area of the fracture (Luger *et al.* 1998). The extension maximal load was reduced; presenting a potentially undesirable consequence.

However, studies that show no and in some instances even negative effects, are just as numerous.

Radiological, biomechanical, and histological effects of Helium-Neon irradiation on fracture healing in a rat model were investigated in a thorough study by David *et al.* (1996). Laser radiation of 0, 2 or 4 Joules on alternate days for 2 to 6 weeks to a fixated leg fracture, with the other leg serving as a control was studied with respect to healing. Radiological and histological examinations of the osteotomy sites failed to show any enhancing effect on the bone healing process. Biomechanically, the irradiated bones of some of the test groups were significantly weaker than the controls.

Research has indicated that laser irradiation may be most effective when applied at healing sites, such as those of tooth extraction (Nagasawa 1991; Takeda 1988), bone fracture (Abe 1990; Trelles 1987), and bone defects (Nagasawa 1991; Tang 1986). In contrast, laser

irradiation has been found to be ineffective when applied at inactive or normal tissue sites (Karu 1997).

Takeda (1988) has suggested that irradiation by low level laser has a beneficial effect on the initial healing of the bony socket. He observed more pronounced proliferation of fibroblasts from remnants of the periodontal ligament in rat sockets irradiated with a Gallium-Arsenide laser. The formation of trabecular osteoid tissue and the deposition of lead in newly formed trabecular bone were observed sooner in the irradiated bone.

3.8 Wound Healing

Wound healing is one of the more notably studied areas of low level laser application. However, the literature on the biostimulatory effects of low level laser on wound healing in controlled animal experiments remains limited and contradictory. The reports by Mester *et al.* (1971; 1985) in the late 1960's and early 1970's that laser irradiation induced healing of chronic nonhealing soft tissue ulcers formed the basis for the advent of clinical laser therapy. Although these studies grew to include over a thousand patients, they remained poorly controlled (Basford *et al.* 1995).

Reports of low level laser therapy applied to soft tissues *in vitro* and *in vivo* suggest stimulation of specific metabolic processes in wound healing. The type of tissues studied and mode of delivery vary widely, however it is commonly implied in these studies that while low doses of low level laser therapy are stimulatory, high doses of laser irradiation are suppressive (Hall *et al.* 1994; Talar *et al.* 1993).

In the rat animal model of the healing of skin wounds by secondary intention, many reparative processes are proposed to be affected by low level laser therapy. These include stimulation of capillary growth and neovascularisation (Bisht *et al.* 1994), granulation tissue formation, altered cytokine production, altered keratinocyte motility and fibroblast movement (Noble *et al.* 1992) following low level laser irradiation. Additional studies have found stimulation of collagen formation (Mester *et al.* 1973; Kana *et al.* 1981; Tolstykh *et al.* 1991) and increased strength of the forming scar (Kovacs *et al.* 1974; Tolstykh *et al.* 1991; Efendiev *et al.* 1992; Lyons *et al.* 1991), enhanced fibroplasia (Abergel *et al.* 1984, 1987), early epithelialisation, increased fibroblast proliferation and matrix synthesis (Bisht *et al.* 1994). More specific wound healing

effects that have been cited include acceleration of the inflammatory phase of wound healing, increasing ATP synthesis, increased protein synthesis due to quickening of DNA and RNA synthesis, and increased fibroplasia (Chukuka *et al.* 1988).

It has been reported that at higher energy levels of laser irradiation these reparative processes are slowed or adversely altered (Tolstykh *et al.* 1991; Efendiev *et al.* 1992). Whilst many authors support the above findings, others have reported contradictory findings (Kana *et al.* 1981; Surinchak *et al.* 1983; Jongsma *et al.* 1983; Basford 1986).

Although no direct evidence is available, a survey of the literature suggests that the Helium-Neon laser (632.8nm) may be the more effective for wound healing than other wavelengths. Other factors suggested (Basford 1986) to be important in the study of wound healing are the species-specific effects. In pig and human skin, healing by wound contraction is much less important than it is in loose-skinned animals such as rodents. Skin thickness and turnover times are also similar between humans and pigs (Hunter *et al.* 1984; Bal 1977; Marcarian *et al.* 1966). Most studies of rabbits and rodents (Kana *et al.* 1981; Lyons *et al.* 1985; Cummings *et al.* 1985; Surinchak *et al.* 1983) have shown considerable improvements in wound healing following low level laser therapy, particularly in the early phases. On the other hand, experiments involving swine with skin wounds have shown no benefit from laser treatment (Hunter *et al.* 1984; Basford *et al.* 1986). Thus, although animal studies offer some basis for human, clinically based treatment, the applicability of these findings to man remains unclear.

In humans, anecdotal clinical observations and small case studies have suggested that low level laser therapy stimulates wound healing. However, controlled clinical trials have produced otherwise conflicting results. Variations of wound size, patient characteristics, and other coincidental activities make it extremely difficult to carry out well-controlled and blind human wound healing studies.

The few blind and well controlled studies available (Santoianni *et al.* 1984; Lundeberg *et al.* 1991), observing the effects of low level laser therapy on the healing of venous ulcers, have reported no significant differences in healing between experimental and placebo groups. However, a similar study (Sugrue *et al.* 1990) conducted elsewhere at the same time showed

positive responses to low level laser therapy over a twelve-week period. The patients had chronic venous leg ulcers that had proven to be unresponsive to conservative measures.

A trial was carried out in the 1980's (Mester *et al.* 1987) involving the treatment of human skin wounds and ulcers that had failed to heal after all other approaches had been tried. A total of 1120 of these wounds were treated with Helium-Neon and Argon lasers. A large percentage (over 93 percent) of the wounds and ulcers had either healed or significantly improved. In a study involving 152 diabetic patients (Kuliev *et al.* 1991) with purulent injuries of skin and underlying soft tissue, daily low level laser therapy resulted in a shortened healing phase of the wounds. In another study involving 512 patients (Chentsova *et al.* 1991) with corneal wounds, burns, or ulcers, treatment with daily low level laser therapy resulted in accelerated healing compared to a reference group of patients receiving conventional treatment.

Growth factors are a group of hormone-like polypeptides shown to play a central role in the different phases of wound healing. Injury repair begins with the release of peptide growth factors from both inflammatory cells and injured cells as soon as tissue damage occurs (Cromack *et al.* 1990). Epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF- β), and basic fibroblast growth factor (bFGF) are found to be expressed and distributed in the same areas of the early skin wound. The area of expression of these growth factors was observed to be associated with the presence of wound inflammatory cells and wound fibroblasts (Yu *et al.* 1994a). In a further study Yu *et al.* (1994b) provided evidence that the proliferation of fibroblasts resulting from low level laser irradiation (660nm, 2.16J/cm²) may be associated with the autocrine production of bFGF from fibroblasts.

Wound healing is a complex process of many interconnected and overlapping factors, all of which require separate investigation with respect to low level laser irradiation. It will be many years before this arduous task is ever completed.

Further to this, potential risks have not been ruled out with respect to low level laser therapy. Experiments *in vitro* have revealed that irradiated cells can show chromosomal damage. This has been reported in association with both pulsed lasers (Rounds *et al.* 1965; Gordon *et al.* 1967; Moskalik *et al.* 1974; Stepanov *et al.* 1977) and low level gas lasers (Rabkin *et al.* 1968; Belcheva *et al.* 1973; Stepanov *et al.* 1977; Nakajima *et al.* 1983). Both chromosomal

aberrations and malformations have been produced with lasers in susceptible experimental developing embryos (Belcheva *et al.* 1973). These changes seen with visible light are said to be similar to those observed with ultraviolet light. Tumour cell proliferation and tumour growth have been shown to be stimulated by low level lasers (Mims 1968; Jamieson *et al.* 1969; Skibenko *et al.* 1976). Long-term studies have not been carried out to exclude this potentially uncontrolled reaction by cells and tissues.

3.9 Laser and Cell Cultures

The need to examine the action of different visible radiation wavelengths, energy densities and exposure regimes on cell cultures stems from the knowledge that disorders such as indolent wounds and trophic ulcers respond to laser therapy and may thus be associated with increased cellular proliferation.

Studies have been conducted with objects of varying complexity levels by a number of authors –bacteria (Karu *et al.* 1983; Tiphlova and Karu 1986, 1987a, 1987b, 1988), yeasts (Fedoseyeva *et al.* 1984, 1986, 1987), and mammalian cells (Karu *et al.* 1982, 1984a, 1984b, 1984c, 1985; Fedoseyeva *et al.* 1988; Yamada 1991; Loevschall 1994; Rigau *et al.* 1991), to clarify the mechanisms of low level visible light action at the cellular level.

3.9.1 Bone Cells

Research at the level of bone cells is even more limited than investigations of animal models. There is only one known study investigating the effects of low level laser irradiation on osteoblastic cells. Yamada (1991) studied the effects of Helium-Neon laser irradiation on cultured clonal osteoblast cells, analysing their rates of proliferation, differentiation, and calcification. A Helium-Neon laser (wavelength 632.8, power output 8.5mW), utilising energy densities of 0.01J/cm² to 1.0J/cm², was used to irradiate cells at different stages in the phase of culture. Laser irradiation was observed to accelerate proliferation of the cells only in the growing phase, when the cells are considered to be undifferentiated osteoprogenitor cells. Cellular growth rate and DNA synthesis were found to be increased. Calcium accumulation was enhanced during long-term culture by irradiation at 1.0J/cm². No significant increase in alkaline phosphatase activity was produced. There was a tendency of enlargement of the Golgi apparatus in irradiated cells.

Low level laser irradiation effects at various cell culture stages has been further investigated with respect to cellular proliferation, bone nodule formation, alkaline phosphatase activity, and osteocalcin gene expression (Ozawa *et al.* 1998). Osteoblast-like cells isolated from foetal rat calvariae were irradiated once with a GaAlAs laser (830nm, 500mW) at various cell culture stages (days 1-16). Laser irradiation at early stages of culture was found to significantly stimulate cellular proliferation, alkaline phosphatase activity, and osteocalcin gene expression. These effects were not observed by irradiation at a later stage. It is suggested that bone-formation-stimulating roles may be exhibited by laser irradiation to immature cells only.

It was proposed (Ozawa *et al.* 1998) that the stimulatory mechanism of laser irradiation on bone formation may be mediated by some growth factors, cytokines or prostaglandins with differentiation-inducing properties produced from the cells of osteoblast lineage. They are presumed to act as autocrine or paracrine stimulators to these cells, and their production may be affected by laser irradiation.

3.9.2 Tumour Cells

This research involves working with a tumour cell line. Published effects of low level laser irradiation on tumour cell lines were therefore investigated. The literature available in this area is limited. Most of the tumour cell-associated research is in the area of photodynamic therapy rather than biostimulation. There was no literature found linking osteosarcoma cells and low level laser therapy.

The growth of tumour cells such as HeLa cells and Erlich's ascites tumour cells is said to be stimulated by laser irradiation, as for undifferentiated osteoprogenitor cells. It is proposed that undifferentiated cells are more photosensitive than differentiated cells (Yamada 1991).

Mester *et al.* (1971) investigated the effect *in vitro* of the Ruby laser on the growth of Erlich's ascites tumours. Growth of the tumour, mitosis-index of the tumour cells, electron microscopy of the tumour cells, and survival of test animals were compared to corresponding features of a control group inoculated with tumour cells from the same donor that were untreated by laser irradiation. In six series of experiments, body weight values of animals inoculated with cells treated with laser irradiation surpassed that of animals in the control group by 10 to 16%, and the number of tumour cells was 19-30% higher in the experimental group when compared to the control group. The differences were highly significant in three series. Survival time in a group of ten mice, following inoculation with irradiated cells, was shorter than in the control group.

3.9.3 Fibroblasts

Fibroblasts are one of the most commonly investigated cell types, and are one of the main models utilised to determine mechanisms of action of low level laser therapy at an intracellular

level. Studies on human fibroblasts have also shown conflicting evidence as to the effects obtained from low level laser therapy.

A study of cultured human fibroblasts (Hallman *et al.* 1988), treated in a controlled, randomised manner, to determine the effects of daily low level laser irradiation (Helium-Neon laser, 0.9mW) on cellular proliferation found no significant stimulatory or inhibitory effect on replication. Fibroblast viability, proliferation and metabolic changes in human fibroblasts have been investigated (Rigau *et al.* 1991) following *in vitro* irradiation (He-Ne laser, 10mW). No increase in cell number was observed, but metabolic rates were significantly increased compared to the unirradiated controls.

Low level laser therapy effects on normal dermal tissue fibroblasts and hypertrophic scar-derived fibroblasts have been investigated (Webb *et al.* 1998). A Helium-Neon laser (660nm, 17mW), using single dosages of 2.4 and 4J/cm², was utilised. Significantly higher cell counts than controls were observed for both cell lines.

Helium-Neon laser irradiation has been found to significantly enhance the replication of human gingival fibroblasts as well as to induce morphological changes at the ultrastructural level (Poureaux-Schneider *et al.* 1989). Collagen production (Lam *et al.* 1986; Abergel *et al.* 1984; Balboni *et al.* 1986) and DNA synthesis (Loevschall *et al.* 1994; Skinner *et al.* 1996) are also shown to be increased with low level laser therapy.

3.9.4 Epithelial Cells

A study was carried out by Schneede *et al.* (1988) investigating the cellular mechanisms of action of the low level Helium-Neon laser (632.8nm, 25mW). Rat kidney epithelial cell cultures were analysed with regard to the effect of laser treatment on DNA-synthesis, ultrastructure, glucose consumption and lactate production as a measure of cell energy metabolism, and the synthesis of prostaglandin E₂ with a view to laser-induced pain relief. Relatively high doses of irradiation were utilised. Significant ultrastructural changes in the cells were seen after He-Ne irradiation at 40mW/cm² for 4 hours (569J/cm²). These changes were not observed after one hour of irradiation (142J/cm²), whilst the number of cell in mitosis increased at this dosage. ³H-thymidine incorporation rate into DNA decreased transiently for 6-9 hours after one hour of He-Ne irradiation. Glucose consumption was also lowered for 10 hours after irradiation while lactate production increased. Prostaglandin E₂ production was reduced, which was also demonstrated by Shimizu *et al.* (1995) (previously mentioned).

3.10 Laser Parameters

Large differences in treatment parameters pervade the literature, and severely limit the ability to compare results from different studies. Few studies have been carried out comparing the effects of different parameters and exposure regimes, with much conflicting evidence regarding the most effective and efficient dosage regimes.

It was suggested that daily treatment with low level laser may be required to provide the maximal benefit (Walsh 1997). Low level laser therapy every second day is said to provide little benefit (Kuliev *et al.* 1991; Chentsova *et al.* 1991). Others, however, have shown positive results utilising this regime (Trelles *et al.* 1987; Takeda 1988; Lyons *et al.* 1991; Lee *et al.* 1993).

Anders *et al.* (1993) investigated the effect of low level laser irradiation on regeneration of the rat facial nerve. Preliminary experiments were carried out to determine the most effective wavelength, laser power, length of irradiation, and treatment schedule. The wavelengths evaluated were 361, 457, 514, 633, 720, and 1064nm. The laser power output ranged from 8.5 to 40mW, and lengths of irradiation time from 13 to 120 minutes. Irradiation treatment was carried out daily, on alternating days and on the first 4 days post-injury. The most effective laser parameters included daily irradiation with a Helium-Neon or Argon pumped tunable laser, a wavelength of 633nm, power of 8.5mW, for treatment duration of 90 minutes (45.9 Joules, 162.4 J/cm²).

Effects of varying wavelength on healing of open skin wounds in rats were also investigated (Kana *et al.* 1981). The Helium-Neon laser (wavelength of 632.8nm) and Argon laser (wavelength of 514.5nm) at a constant power density of 45mW/cm² were compared. An incoherent monochromatic light source was used, with an inbuilt cooled lamp and interference filters of 630nm for red light and 540nm for green light, and maximal output of 150mW. No infrared component of the spectrum was present in the filtered light. Energy densities of 4, 10 and 20J/cm² were utilised. Helium-Neon irradiation of 4J/cm² significantly accelerated the rate of wound closure in rats, with a slight deceleration observed at 20J/cm². Irradiation with an Argon laser or incoherent green light did not cause significant acceleration of wound closure. Collagen production was maximally enhanced with the Helium-Neon laser at a dose of 4J/cm² daily. Argon laser irradiation and the unirradiated control (contralateral wound on same rat)

exposed to incoherent green light both significantly enhanced collagen synthesis. The Argon laser had its maximum effect at $10\text{J}/\text{cm}^2$, and the incoherent green light at $20\text{J}/\text{cm}^2$. No qualitative difference in the effect on wound healing between the incoherent light and laser of the same wavelength was detected. It was therefore proposed that there is some degree of wavelength specificity based on a photobiological action of the laser irradiation of respective wavelengths. This concept is further discussed in section 3.11.

In a study of low level laser therapy effects on clonal osteoblasts, Yamada (1991) investigated the effects of various energy densities (0.01 , 0.1 and $1.0\text{ J}/\text{cm}^2$) on one day or daily for four days, using a He-Ne laser. DNA synthesis was found to increase in a dose dependent fashion, with irradiation at $1.0\text{J}/\text{cm}^2$ in the growing phase resulting in a 32% increase over controls. ^{45}Ca accumulation in the cells and matrix layer increased with four sessions of laser irradiation at $1.0\text{J}/\text{cm}^2$.

The effects of a single versus multiple applications of low level laser therapy have been investigated *in vitro* in a human gingival fibroblast model (Pourreau-Sneider *et al.* 1989). The cells were irradiated (He-Ne laser, 632.8nm , $10\text{mW}/\text{cm}^2$) one (day 0), two (day 0 and 7), or three (day 0, 7 and 11) times, with a total exposure of $1.2\text{J}/\text{cm}^2$ per irradiation. The biostimulative effect of low level laser treatment on the growth of fibroblasts after a single irradiation were found to be transitory, growth declining in the one-dose group from days four to seven. When the cells were administered a second laser treatment on day seven, an additional stimulation of growth occurred; this was again observed following the third treatment on day eleven. By day fourteen, cells that had received multiple doses were found to be twice as numerous as those that had received one dose.

Hrnjak *et al.* (1995) compared single and double irradiation effects with the He-Ne laser on fibroblast proliferation. The single dose of He-Ne irradiation exhibited a significant stimulation effect on human fibroblast proliferation.

Different wavelengths and energy levels have varying influences on different cell types. Fibroblasts and lymphocytes have been found to be preferentially affected by green light (Argon laser) whilst fibroblasts and epithelial cells are affected by red light (Helium-Neon laser). Leukocyte phagocytic activity *in vitro* is found to be stimulated by both Helium-Neon laser and dye lasers emitting green light (534 to 558nm) (Mester *et al.* 1979). Therefore, all

laser parameters need to be investigated in relation to all individual cell types and different tissue systems of the body.

Further studies are presented in table format to give an indication of the broad range of research, different lasers in use, energy levels and dosage regimes. The effects of laser irradiation within these parameters are also presented.

Table 3.1 Effects of low level laser irradiation on Wound Healing.

Author	Species	Wound	Laser	Power Density (mW/cm ²)	Dose (J/cm ²)	Regimen	Compared	Control	Effect
Kana et al (1981)	Rat	Open	HeNe 632.8nm Argon 514.5nm	45	4,10,20	1x daily	Rate wound closure Collagen conc ^o	Yes	No sig. Diff. ↑collagen at 4J/cm ²
Lee et al (1993)	Rat	Open Infected	GaAs 904nm	27W, 2mW	76.4mJ/cm ²	Every 2 days for 1week	Area healed	Yes	↑ healing ↓ oedema
Lyons et al (1991)	Mice	Sutured	HeNe 632.8nm	4.05	1.22	Every 2 days for 2 months	Tensile strength collagen content	Yes	↑ strength ↑ collagen
Utsunomiya (1998)	Dog	Exposed dental pulp	GaAlAs	300mW			Histopath. changes, collagen, lectin	Yes	Accel ^d wound healing, lectin & collagen expression
Malm & Lundeberg (1991)	Human (42 cases)	Leg ulcers	GaAlAs				Rate of healing	Yes, placebo	No differences
Malm & Lundeberg (1991)	Human (46 cases)	Leg ulcers	HeNe	6mW	4J/cm ²	2x/week for 12 weeks	Ulcer area, proportion healed	Yes, placebo	No differences

Table 3.2 Effects of low level laser irradiation on pain.

Bulow et al. (1994)	29 cases	Knee osteoarthritis	GaAIAs		22.5J/txn	Txn in weeks 4, 5 & 6, total 9 txns	Pain levels Analgesia req'd Strength	Double blind	No sig. diff. Placebo effect
Heussler et al. (1993)	25 cases	Rheumatoid arthritis of hands	GaAIAs		12	12 txns for 4 weeks	Movement Grip strength Analgesia, etc	Double blind randomised	Powerful placebo effect
Asada et al. (1989)	53 cases, 102 joints	Rheumatoid arthritis	GaAIAs 830	3W/cm ²	10-20 secs /irradiation	Single exposure at multiple sites	Pain score & range of motion	Uncontrolled	No ↑ in ROM Effective pain removal.
Moore et al. (1992)	20 cases	Elective cholecystectomy post-op pain	GaAIAs 830	3W/cm ²	24J/cm ² /point	6-8mins immediately post-surgically	Demand for analgesia	Double blind	↓oral analgesia required
Kemimoto et al. (1991)	63 cases	Post-herpetic Neuralgia	GaAIAs 830	1.2-3W/cm ²	10-20mins, 10secs/spot	2-3x or 4-6x /week	Pain scores	Double blind	↓pain scores, also strong placebo effect
Lim et al. (1995)	39 cases	Ortho post-adjustment pain	GaAIAs 830	60mW/cm ²	15, 30 & 60 secs & placebo	5 days	Pain scores	Double blind with placebo	No stat sig diff between placebo & txns

Table 3.3 Effects of laser irradiation on bone healing.

David et al. (1996)	Rat (62)	Bilateral tibiae osteotomies	HeNe 632.8			0,2,4	Every 2 days for 2-6 weeks	Radiological biomechanical histological	Yes	No effect, biomechanically weaker
Luger et al. (1998)	Rat (30)	Tibial bone fracture with fixation	HeNe 632.8	35mW		30mins	1x/day for 14 days	Structural stiffness, maximal load	Yes, randomised blind	Enhanced bone healing
Trelles & Mayayo (1987)	Mice	Tibia fractures	HeNe 632.8			2.4J	1x every 2 days, 12 treatments	Microscopic changes	Yes	↑vascularisation denser trabecular net
Asanami et al. (1993)	Rabbits	Hydroxyapatite implants	HeNe 632.8	6mW		10 mins	1x/day for 4 days	Bonding of implants, inflammation	Yes	Better bonding, ↑osteogenesis, ↓inflammation
Takeda (1988)	Rats	Extraction site wounds	GaAs 904	25mW/cm ²		20J/cm ²	0,2,4, and 7 days	Fibroblasts, osteoid & new bone	Yes	Beneficial effect on initial healing
Barushka et al. (1995)	Rat	Tibia hole injury	HeNe 632	6mW		31J/cm ²	1x/day on days 5 & 6 post-injury	ALP, calcium, histological	Yes	Enhanced bone repair, time, energy & frequency dependent

Table 3.4 Effects of low level laser irradiation on nerve healing.

Midamba and Haanaes (1993)	Human (15 cases)	Mental and lingual n.	GaAlAs 830nm	437.5		4.9	3x/week for 1-8 weeks	Improved sensation	No	Improved sensation
Midamba and Haanaes (1993)	Human (40 cases)	Mental and lingual n.	GaAlAs 830nm	437.5		6.0	3x/week until satis. result	Improved sensation	No	Improved sensation
Khullar et al (1996)	Human (13 cases)	IDN	GaAlAs 820nm			6.0 (x4 sites)	20 treatments	Improved sensation	Double blind with placebo	↑ Subjective & objective sens ⁿ
Anders et al. (1993)	Rat	Facial nerve crush injury	361nm, 457nm, 514nm, 633nm, 720nm, 1064nm	8.5-40mW		6.63-288J (162.4J/cm ² gave best results)	Daily, alternate days, and first 4 days.	Horse radish peroxidase labeled neurons in motor nucleus	Yes	Increased rate of regeneration

Table 3.5 Effects of low level laser irradiation on cell cultures.

Author (Year)	Cell Type	Laser Type	Power (mW/cm ²)	Dose (J/cm ²)	Frequency	Duration	Effects	Significance
Yumada (1991)	Clonal Osteoblasts	HeNe 632.8nm	3.03mW/cm ²	0.01-1.0J/cm ²		For 1 day or 4 consecutive days	Growth rate DNA synthesis Ca accumulation Alkaline phos.	Yes
Yu et al. (1994)	Fibroblasts	660nm		2.16 3.24			Cell proliferation bFGF production	Yes
Shimizu et al. (1995)	Stretched PDL cells (human)	GaAAs	60mW	10.8-36.0J		1x/day	PGE2 & IL-1 beta levels	Yes
Skinner et al. (1996)	Fibroblasts (human)	GaAs (pulsed)		0-1		1-4 days	Hydroxyproline DNA synthesis	Yes
Hrnjak et al. (1995)	Fibroblasts (human)	HeNe 632.8		0.5, 1, 1.5, 2		Single & double	Cell number	Yes
P-Schneider et al. (1989)	Fibroblasts (human, gingival)	HeNe 632.8	10mW/cm ²	1.2J/cm ²		1, 2 or 3 doses	Cell number Ultrastructural modifications	Yes
Loevschall (1994)	Fibroblasts (human)	GaAAs 812	4.5mW/cm ²	0, 4.5, 13.5, 45, 144, 450, 1422, & 4500mJ/cm ²		1 dose	DNA synthesis	Yes
Rigau et al. (1991)	Fibroblasts (human)	HeNe 632.8	10mW			1x/12 hrs for 2 days	Cell number Metabolic rate	Yes
Hallman et al. (1988)	Fibroblasts (Human)	HeNe 633	24.7mW/cm ²	60secs		1x/day for 5 days	Cell number	Yes

3.11 Biostimulation

Biostimulation, including bioinhibition, is the use of low-energy laser light on tissues to achieve a clinical effect. Experimentally supportable mechanisms for the alleged photo-biostimulatory effects of photo-irradiation are limited. Possible mechanisms have been proposed that may be capable of accounting for both the stimulating action of visible and infra-red lasers on cell cultures at small dose, and the inhibitory or damaging action at larger doses (Basford 1995). Multiple biological targets of low level lasers have been described and investigated (Karu 1997). Examples of these include stimulation of ionic movements between intracellular compartments (Kubasova *et al.* 1984), action on mitochondria via cytochrome oxidases (Rounds *et al.* 1968; Bosatra *et al.* 1984; Karu 1989), photochemical effect on protein synthesis (Abergel *et al.* 1984; Mester 1983; Lam *et al.* 1983), increase in RNA synthesis (Abergel *et al.* 1984), and action by resonance, on DNA (Fine *et al.* 1965; Goldman 1966).

The basic mechanism for biostimulation occurs at the molecular level. In all living organisms, biological processes are based on selective interactions between specific biomolecules (Cosic 1994). These interactions mostly involve and are driven by proteins, which are the main conductors of living processes within an organism. Knowledge of the physical nature of these interactions is limited. It is assumed that biomolecular interactions, particularly protein-protein and protein-DNA interactions, are electromagnetic in their nature (Cosic 1994). Certain periodicities within the distribution of energies of localised electrons along a protein molecule are shown to be critical for protein biological function, i.e. interaction with its target. With the introduction of protein conductivity, a charge moving through a protein backbone can produce electromagnetic irradiation or absorption with spectral characteristics corresponding to energy distribution along the protein. These spectral characteristics are found to be in the range of infrared and visible light (Cosic 1994). Experimentally obtained frequency characteristics of some light-induced biological processes have proven these theoretically calculated spectra. The function of some proteins is said to be directly related to the absorption of visible light of defined wavelengths. The light is absorbed effectively on a prosthetic group bound to the protein, with frequency selectivity in this process being defined by the protein itself (Cosic 1994).

Considerable evidence exists to suggest that the induced change of the energy states of biomacromolecules (such as the effect of ultraviolet and visible light on primary photoacceptors) leads to the modulation of some biological processes in cells (Karu 1987). There is also evidence to suggest that low intensity light irradiation at precisely defined

wavelengths (frequencies) can produce defined, frequency-dependent effects on living systems *in vivo* or *in vitro*. The correlation between the frequency selectivity of light-induced biological processes and a Resonant Recognition Model's characteristics of biomacromolecules involved in these processes have been described for cell growth and proliferation (Cosic 1989).

Table 3.6 Light irradiation frequencies which have been shown to affect cell growth are compared with the characteristic Resonant Recognition Model frequencies for different groups of growth factors normally involved in these cell proliferation processes (Cosic 1994).

Growth factor group	Effect observed	Characteristic RRM frequency f_c	Expected wavelength λ_c (nm)	Wavelength of maximum effect (nm)
IGFs	DNA synthesis	0.492±0.008	406±6.5	400
FGFs	DNA synthesis Therapeutic effects	0.453±0.004	441.5	441.6
Insulins	DNA synthesis	0.344±0.16	581.4±10	552
Growth factors (EGF, CSF, GH, PLF)	DNA synthesis ATP synthesis Therapeutic effect	0.293±0.016	682.6±35.4	633 650 633
PDGFs	Therapeutic effect	0.242±0.008	826±26	830

The second column represents the biological effect observed under laser irradiation of particular frequency presented in the fifth column.

There are further cell studies to support this. The irradiation of Chinese hamster fibroblasts with blue (404nm) or red (632.6nm) light resulted in changes in the intracellular cAMP level, whereas irradiation with light at 546 or 700nm was shown to have no appreciable effect (Karu *et al.* 1985b, 1987b). Some similarity is found to exist between the wavelengths effective for DNA and RNA synthesis stimulation, which result in the variations in cAMP level (Karu *et al.* 1989).

Data indicates that irradiation causes a rearrangement of cell metabolism, with light being only in the role of a trigger. It is said that this is the reason that doses needed for inducing biostimulation effects are comparatively low and the radiation times needed short (Karu 1989).

It has been shown (Karu 1989) that there are two groups of active spectral regions. The first group includes light with wavelengths (364, 404, 434nm and 890nm) found to have a stimulative action 10 to 100 times lower than doses from the second group of wavelengths (454, 560, 633, 680nm and 750nm). The same effect can thus be achieved with light in the near UV and blue, as well as the far-red region, using one-tenth the doses required as with red light.

It has been found (Karu 1989) that metabolic processes in the cell can be enhanced by irradiation with monochromatic visible light in the blue, red and far-red regions, the photobiological effect being dependent on the wavelengths, dose and intensity of the light.

Biological processes can be induced or modulated by irradiation with characteristic frequencies, which as evidence suggests (Karu 1987; Biscar 1976), is caused directly by light-induced changes of the energy states of molecules and, in particular, of proteins. Consequently, it may be inferred that biological processes can be influenced by an external radiation of defined frequency (Cosic 1994).

3.11.1 Laser-induced Ca²⁺ release in the cytoplasm

Clinically significant effects of various types of electrotherapy have been shown to be due to changes in membrane permeability to various ions such as calcium (Mortimer *et al.* 1988). A variety of external signals can result in changes in cytoplasmic calcium concentrations. Cytoplasmic calcium fluctuation enables cellular activity adaptation in response to changes in the immediate environment (Young *et al.* 1990). Low level laser may affect cellular behaviour via its effect on the permeability of the cell membrane to calcium ions.

Cells have extreme sensitivity to changes in intracellular Ca²⁺ concentration. A number of systems are involved in regulating Ca²⁺ concentration (Lubart *et al.* 1992) including the mitochondria (Babcock *et al.* 1976; Bradley *et al.* 1980), the plasma membrane ATP-

dependent Ca^{2+} pump (Bradley *et al.* 1980; Breitbart *et al.* 1983), the Na^{+} - Ca^{2+} anti-port (Bradley *et al.* 1980) and the voltage dependent Ca^{2+} channel (Babcock *et al.* 1987).

Accelerated fibroblast proliferation after laser irradiation is proposed to be due to transient changes in the Ca^{2+} concentration in the cytoplasm (Lubart *et al.* 1992).

Macrophages have been found (Young *et al.* 1990) to increase calcium uptake upon low level laser irradiation with a GaAlAs laser at varying wavelengths (660, 820 and 870nm). Maximum calcium intake was achieved in the energy density range of 4-8J/cm², with diminishing calcium influx above and below this range.

Lubart *et al.* (1992) investigated Ca^{2+} uptake in bull sperm cells utilising a He-Ne laser (632nm) and a 780nm-diode laser. Accelerated Ca^{2+} uptake was observed at low energy doses and a decrease after high doses, with maximum stimulation achieved in the energy density range of 6-18 J/cm² when the He-Ne laser was used, and 3 J/cm² with the 780nm diode laser.

It is proposed that the cellular calcium influx may be as a result of a direct modification of the cell membrane causing it to become more permeable to the ion (Young *et al.* 1990).

Alternatively, laser irradiation may intensify the formation of a transmembrane electrochemical proton gradient in mitochondria. This may occur by either singlet oxygen formation by endogenous porphyrins 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). Efficiency of the proton motive force may thus be increased and more calcium is 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+} is released at higher doses, with hyperactivity of the Ca^{2+} -ATPase calcium pumps resulting which exhausts the ATP reserves of the cell. The intramolecular osmotic pressure becomes larger than that of the medium resulting in water influx, which produces blebs extruding from the cell membrane. At even higher laser doses, more Ca^{2+} influx may completely exhaust the cell's energy and the intracellular osmotic pressure may explode the cell (Malik and Lugaci 1987).

Breitbart *et al.* (1996) later found that there is an accelerated Ca^{2+} uptake by the mitochondria after low power He-Ne irradiation and inhibition after high power. Flux of Ca^{2+} from the mitochondria and ATP-dependent Ca^{2+} uptake by bovine plasma membrane vesicles were both unaffected by the He-Ne irradiation.

Enhanced intracellular calcium levels and fertilising potential of mouse spermatozoa by irradiation with a He-Ne laser (632nm) has been detected (Cohen *et al.* 1998). The effect of light on calcium transport and on fertilisation rate was observed to be abolished in the absence of Ca^{2+} during the time of irradiation, indicating that the effect of laser irradiation is Ca^{2+} -dependent. The stimulatory effect of laser light on Ca^{2+} uptake was eradicated in the presence of a voltage-dependent Ca^{2+} -channel inhibitor nifedipine, indicating the involvement of a plasma membrane voltage-dependent Ca^{2+} channel. In addition the stimulatory effect of light was observed to be completely inhibited by the mitochondrial uncoupler FCCP, indicating that laser irradiation might affect the mitochondrial Ca^{2+} transport mechanisms.

The proton motive force also increases ATP production, which activates Na^+ , K^+ -ATPase and other ion carriers. Intracellular K^+ levels are therefore increased and the Na^+ concentration and membrane potential are decreased; factors also influencing cell proliferation (Cone 1971).

It is accepted that there exists a causal relationship between variations in the concentrations of cAMP and Ca^{2+} on the one hand and the rate of DNA and RNA synthesis on the other at early stages of regenerative processes (Martelly and Franquinet 1984).

3.11.2 Primary Photoacceptors

The action spectrum of visible light on various growth stimulating and damaging effects of certain organisms and the absorption bands of respiratory chain components of these organisms, such as the flavins and the cytochromes, have been found to be correlated (Karu 1988).

Irradiation has been found to cause considerable activation of the respiratory chain components, NADH dehydrogenases and cytochrome c oxidase (Karu 1989) (see Figure 3.6).

Proteins have been proposed as the major absorbing structures for the red visible and infra-red laser wavelengths used in low level laser therapy. The exact identity of the photoreceptors

responsible for the biological effects of low level laser therapy has not been determined (Karu 1997).

Studies suggest that either elements in the mitochondrial cytochrome system or endogenous porphyrins in the cell are the possible energy-absorbing chromophores (Labbe *et al.* 1990; Lubart *et al.* 1992).

It is proposed that laser light penetrates through tissue and strikes a chromophore, or photosensitive molecule, which, in tissues is the cytochrome contained within mitochondria. Other existing photosensitive molecules include rhodopsin needed for vision, chlorophyll for photosynthesis, and hematoporphyrin derivative (HpD) used in Photodynamic therapy (PDT). Mitochondrial cytochromes are responsible for converting adenosine diphosphate (ADP) to adenosine triphosphate (ATP), thus driving cellular metabolism by supplying energy to the cell. The absorption spectrum of biomolecules and tissue optics determines the ability of the laser light to affect target molecules (Miserendino and Pick 1995) (see Figure 3.6).

3.11.2.1 Cytochromes

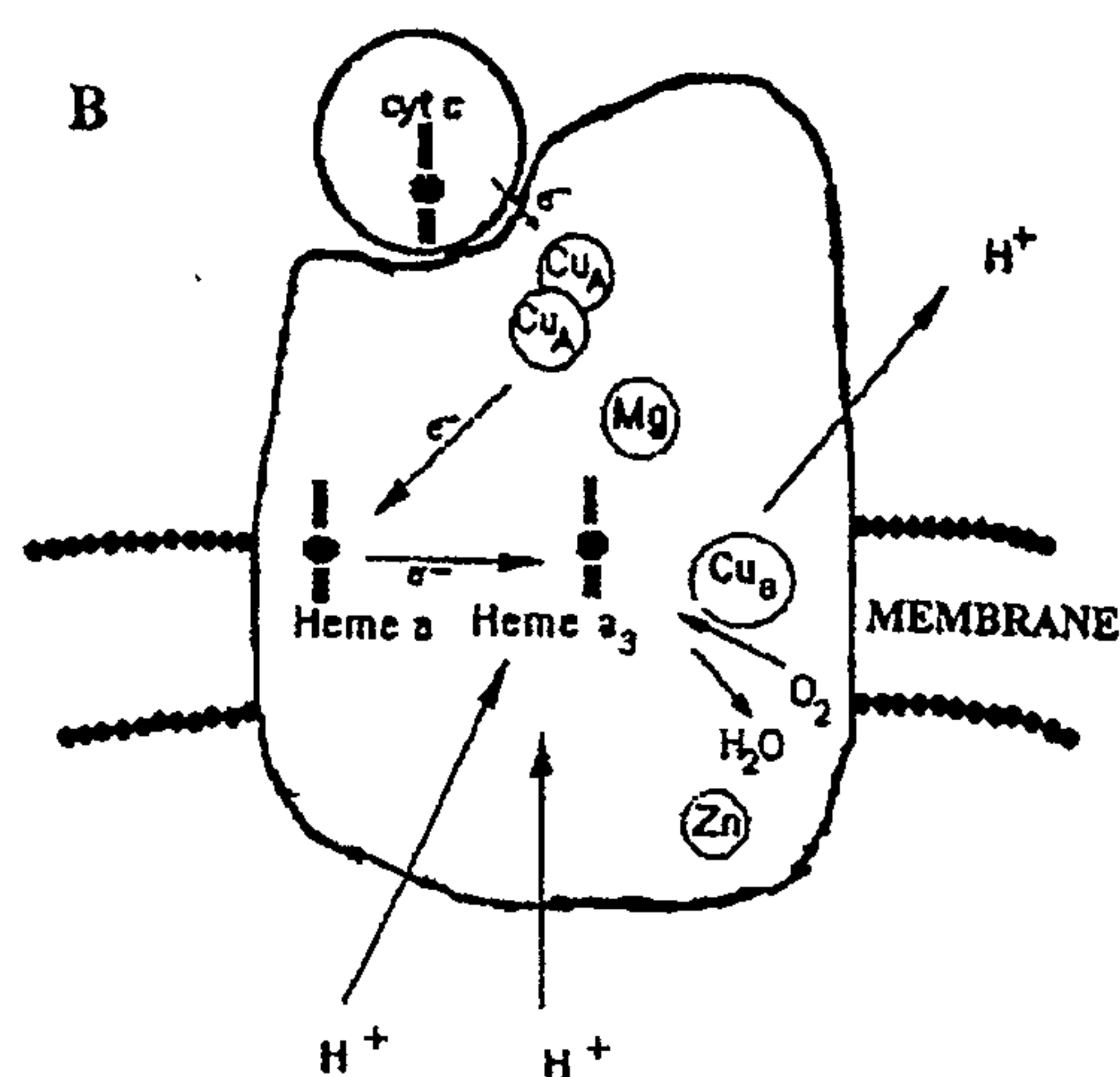
Cytochrome c oxidase has an absorption spectra of 800 to 900nm (Chance and Leigh 1977; Beinert *et al.* 1980), a range within which the laser we are utilising falls. Cytochrome oxidase is the terminal electron acceptor of the mitochondrial respiratory chain and is responsible for the great majority of oxygen consumption in the body. It is essential for the efficient generation of cellular ATP.

Cytochrome c oxidase of mammalian cells is a large multicomponent membrane protein displaying considerable structural complexity, the overall picture of cytochrome c oxidase function being extremely complicated and controversial (Karu 1997).

An action spectrum is said to follow the absorption spectrum of the photoacceptor molecule (Hartman 1983). More recently HeLa cells have been utilised to determine action spectra after irradiation with low level laser (Karu 1997). Generalised action spectra in the range 580-860nm were found to consist of two series of doublet bands in the range 620-680nm and 760-895nm with well-pronounced maxima at 620, 680, 760 and 825nm. These bands were identified by concordance with the metal-ligand systems spectra characteristic of this spectral range (Wilkinson *et al.* 1987; Hughes 1987). It is suggested that enzymes containing multiple nuclei of Cu(II) (copper (II)) are participating (Hughes 1987). The photoacceptor molecule is

proposed to have these different types of centers (or nuclei) containing Cu(II) with absorption spectra in the ranges 420-450nm and 760-830nm. It is concluded that all bands in the action spectra may be related to cytochrome c oxidase (Karu 1997), as this enzyme contains active copper centres. Within the cytochrome c oxidase structure are two heme moieties (heme a and heme a₃), two redox active copper sites (Cu_A and Cu_B), one zinc and one magnesium site (See Figure 3.5). The enzyme structure thus contains four redox active metal centres. Cytochrome c oxidase can be fully oxidised, fully reduced, or partially reduced (Karu 1997). It is suggested (Karu *et al.* 1995) that the 820nm band of the action spectrum belongs to the oxidised Cu_A, the 760nm band to the reduced Cu_B, the 680nm band to the oxidised Cu_B, and the 620nm band to the reduced Cu_A. The binuclear Cu_A centre was also found, by Cooper and Springett (1997), to have a strong absorbance in the near-infrared.

Figure 3.5 A scheme illustrating the catalytic cycle of cytochrome c oxidase.



A cellular study by Yu *et al.* (1997) has been carried out to investigate this concept further. The study was carried out to determine whether oxidative metabolism and electron chain enzymes in rat liver mitochondria could be modulated by photo-irradiation. Mitochondrial enzyme activities were analyzed to assess the specific enzymes that are directly involved with the photo-stimulatory process. An argon-dye laser, wavelength 660nm and power density of 10mW/cm², was used as a photon source. Photo-irradiation was found to significantly increase oxygen consumption, phosphate potential, and the energy charge of the rat liver mitochondria and enhanced the activities of NADH ubiquinone oxidoreductase, ubiquinol ferricytochrome c oxidoreductase and ferrocyanochrome c oxygen oxidoreductase. The

activities of succinate ubiquinone oxidoreductase, ATPase, and lactate dehydrogenase were not found to be affected by photo-irradiation.

3.11.2.2 Porphyrins

Minute amounts of porphyrins, naturally present in cells, may be among the primary photoacceptors in addition to the pigments of the respiratory chain (Lubart *et al.* 1990).

Enhanced ATP synthesis in isolated mitochondria following irradiation at appropriate wavelengths supports the assumption that the primary photoacceptors are pigments located in the mitochondrion (Kato *et al.* 1981). It has been suggested that the action of the laser light on the pigments of the respiratory chain may be similar to the photophysical and photochemical processes triggered in photosynthesis (Friedmann *et al.* 1991).

Utilising electron paramagnetic resonance in cell culture models (Moan 1979; Rochkind *et al.* 1992) it has been found that at low radiation doses, singlet oxygen is produced by energy transfer from porphyrin, and not cytochrome as commonly assumed, which is known to be present in the cell. Singlet oxygen has been shown to be a significant biochemical intermediate in biochemical processes, and thus important in biostimulation (Moan *et al.* 1987). At low concentration singlet oxygen can modulate biochemical processes taking place in the cell and trigger accelerated cell division. Whereas, at high concentration, singlet oxygen damages the cell (Moan *et al.* 1987).

In further support of this concept, laser therapy has also been compared to oxygen therapies (Klima 1988). In oxygen therapies the light (632nm) emitted by singlet oxygen has a therapeutic effect. It is assumed (Klima 1988) that Helium-Neon laser light (632nm) plays the same role as singlet oxygen emission in immune regulation. Work by Lubart *et al.* (1990) has shown that low level laser light generates small amounts of singlet oxygen in the irradiated cells. The singlet oxygen was demonstrated to be produced by the endogenous porphyrins produced by the cells which are photo-excited by the Helium-Neon light. They also proved that neither cytochrome c or hemin produced singlet oxygen after irradiation.

It is yet to be determined as to whether the photobiological effects observed to occur with low level laser therapy are specific to the monochromatic coherent laser energy, or may also be produced by conventional light sources emitting non-coherent energy over a similar range of wavelengths and frequencies (Walsh, 1997).

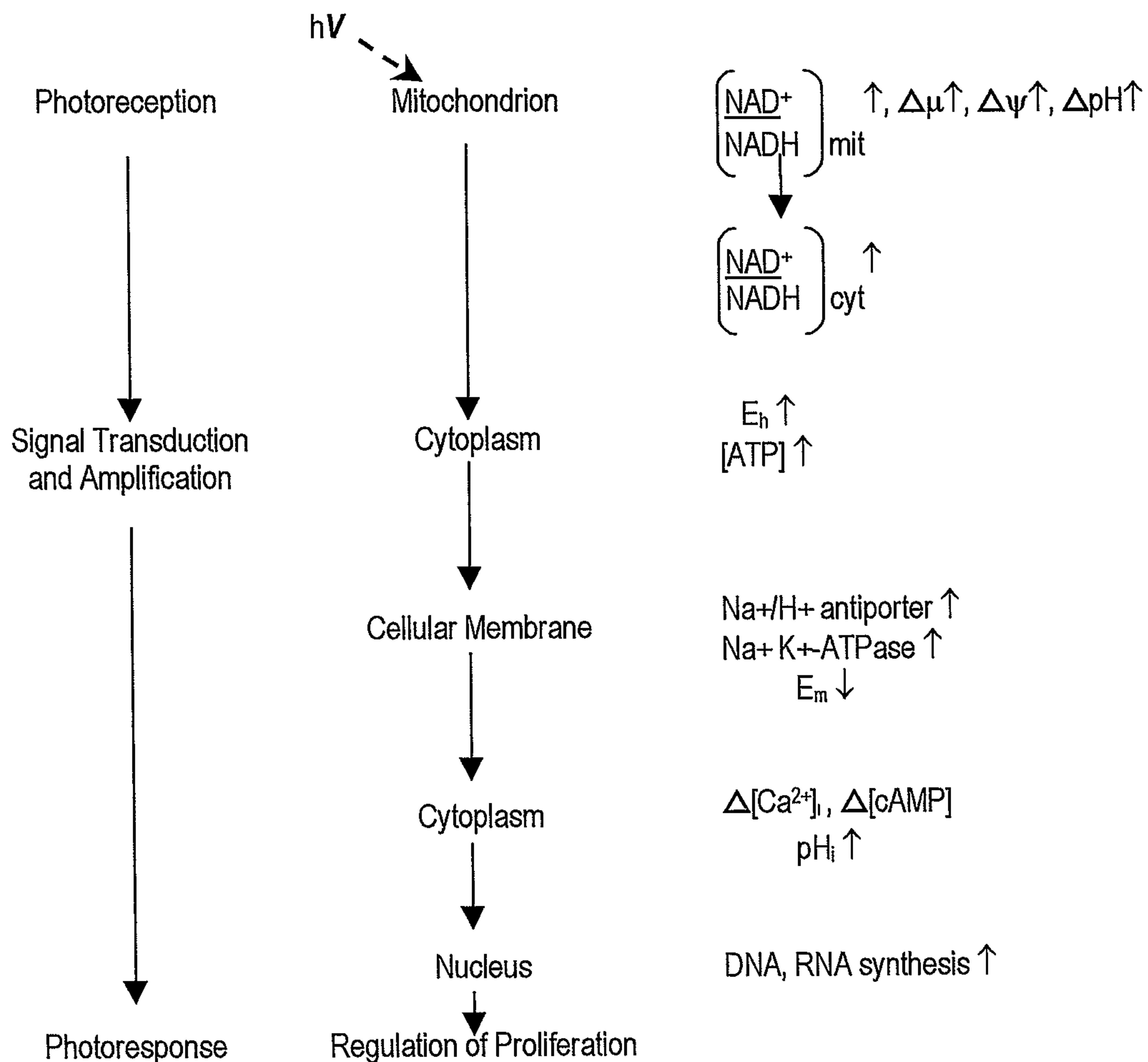


Figure 3.6 Possible photosignal transduction chains for proliferation stimulation (Karu 1989).

$\Delta\mu \uparrow, \Delta\psi \uparrow, \Delta\text{pH} \uparrow$ -- increase in photomotive force, proton gradient and electrical potential of mitochondrial membrane.

$[\text{ATP}] \uparrow$ -- increase in ATP concentration.

$E_h \uparrow$ -- increase in cellular redox potential (change into more oxidised direction)

$\left(\frac{\text{NAD}^+}{\text{NADH}}\right)_{\text{mit}} \uparrow \rightarrow \left(\frac{\text{NAD}^+}{\text{NADH}}\right)_{\text{cyt}} \uparrow$ -- oxidation of NADH pool in mitochondrion, causing changes in NADH pool of cytoplasm.

Na^+/H^+ antiporter $\uparrow, \text{Na}^+ \text{K}^+$ -ATPase \uparrow -- activation of these enzymes

$E_m \downarrow$ -- decrease of electrical potential of cellular membrane.

$\text{pH}_i \uparrow$ -- decrease of H^+ concentration in the cell.

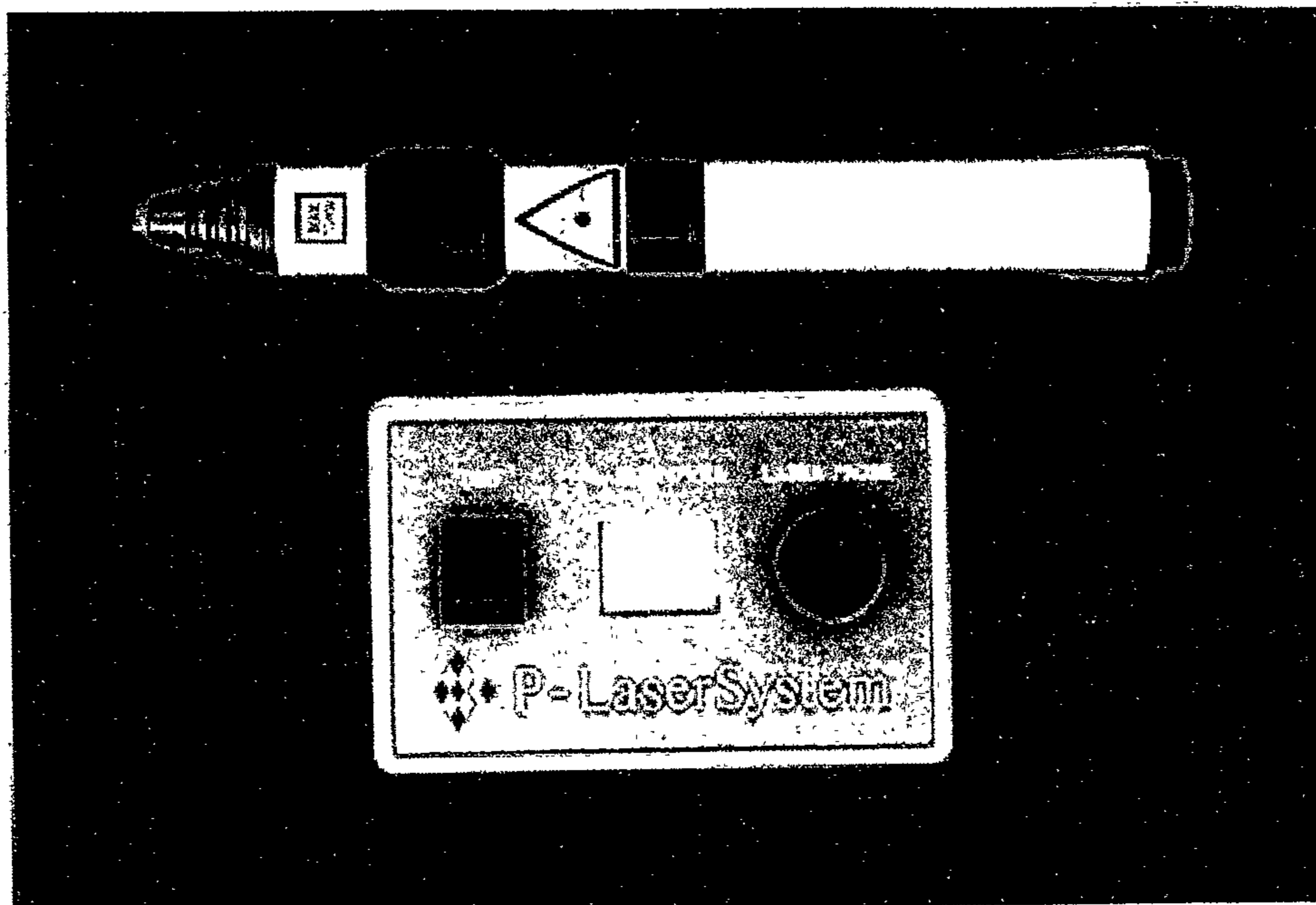
$\Delta[\text{Ca}^{2+}]_i, \Delta[\text{cAMP}]$ -- changes in intracellular concentration of Ca^{2+} and cAMP.

DNA, RNA synthesis \uparrow -- activation of nucleic acid synthesis.

4.1 P-Laser System®

The P-Laser system (Liberato Lasers, Copenhagen, Denmark) utilised for this research is a Class 3B laser pen laser.

Figure 4.1 P-Laser System® (top) and digital powermeter (bottom).



Laser Generator

The GaAlAs (Gallium, Aluminium, Arsenide) laser operates at a wavelength of 830nm and 70mW net effect. Its optical system of mirrors and lenses mixes red targeting light with the laser beam for visibility for accurate placement on the desired point of application and optimum energy absorption. The lens ensures very low beam divergence and therefore maximal penetration. A microprocessor constantly monitors laser function and triggers a rapid beeping signal if there is a malfunction. The laser has an acoustic bleep for On/Off and beeps every 0.5 seconds when it is running. It also has a failure alarm, which warns the user when the battery is running low.

Electronic feedback circuitry constantly monitors the laser beam, ensuring that the energy emitted is kept at a constant, high level. Energy levels were monitored at intervals during each irradiation period using the powermeter provided with the system.

Control Ring

The black control ring on the laser generator provided two modes of operation. If the ring was squeezed once, and held, the laser would run until its timer stopped it. The timer could be interrupted and the laser stopped by releasing the pressure on the control ring. If the control ring was squeezed twice in rapid succession, the laser would continue to run to the end of its timer setting even if finger pressure was released from the ring. The laser could be stopped early by squeezing the control ring a third time.

Alcohol was used for cleaning the laser and battery parts for aseptic purposes before irradiating the cells.

Fiber Optic Tips

Fiber optic glass tips transmit the beam. The tips available for use were said to provide a typical effective beam transmission rate of 75-95%, which introduced an error to the irradiation dose applied.

There were three different kinds of glass fiber tips available. For these experiments the larger 8mm diameter tip was used as this had approximately the same diameter as a well in the 96-well microplates. However, the laser beam diameter for the 8mm and 4.5mm tips was almost the same. The laser light is thus concentrated in the central part of the 8mm tip, only the red target light is distributed throughout the total diameter of the tip. The smaller beam diameter necessarily meant that not all cells in a well were exposed to irradiation. This would reduce the measured effect that low level laser irradiation may have had on the cells.

The tips can be autoclaved (140°C) or disinfected using alcohol.

Battery Pack

The battery pack provided 40 minutes (70mW version) of laser energy and took 15 hours to charge fully. This restricted the number of experimental groups that could be run at any one time and greatly prolonged the time to complete a series of experiments. The use of energy levels higher than 4 joules was impractical, as the laser did not have enough battery longevity to complete all plates for an experimental group.

The battery pack could not be over charged. When battery power was too low to generate the maximal laser power, a sound signal indicated that recharging was necessary and the laser light / targeting light would be discontinued.

The unit's timer and safety lock were located at the base of the battery pack. The timer settings were 15, 30, 60, and 120 seconds.

A key was used to move the switch from the locked position -0- to the -off- position. From this position switching to different positions could be done directly by hand, and returned to the -0- position itself.

The -0- position provides a safety feature, preventing the use of the laser by unauthorised persons or children when the unit is left unattended.

Powermeter

The "test" button is pressed while irradiating the "test" eye. Measurement of the output power has an accuracy of +/- 20%. The reading should therefore not be less than 60mW for the 70mW version. This inherent inaccuracy is another potential source of error.

A low power reading could be caused by dirt on the mirrors in the optical system, or from the tips not being cleaned properly.

The laser malfunctioned once during the period of this research, for which it required repair of the generator unit of the laser. When the laser was returned the output power as determined by the digital powermeter had increased from 70mW to 90mW. The output readings varied during the irradiation period by up to 7mW, and were always higher than the proposed 70 or 90mW. Calculation of actual energy utilised could not therefore be entirely accurate.

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).

Hazards

The P-Laser System is classified as a class 3B laser and is, therefore, potentially hazardous to the eyes if the laser beam is focused on the retina. The beam should, therefore, never be looked into or directed into the eyes of another person.

Further technical data on this laser can be seen in Appendix 1.

4.2 Calculating Laser and Treatment Parameters

Laser therapy devices are generally specified in terms of the average output power (milliwatts) of the laser diode, and the wavelength (nanometres) of light they emit. To accurately define the parameters of the laser system the area of the laser beam (cm²) at the treatment surface must also be taken into account.

The output power (milliwatts) of the laser refers to the number of photons emitted at the particular wavelength of the laser diode.

Power density measures the potential thermal effect of those photons at the treatment area. It is a function of Laser Output Power and Beam area.

The total phototonic energy delivered into the tissue by a laser diode operating at a particular output power over a certain time period is measured in Joules.

The distribution of the total energy over the treatment area is measured as Energy Density (Joules/cm²). Energy density is a function of Power Density and Time in seconds.

Calculation of Power Density

$$\text{Power Density (W/cm}^2\text{)} = \frac{\text{Laser Output Power (W)}}{\text{Beam area (cm}^2\text{)}}$$

$$\text{Beam area (cm}^2\text{)} = \text{Diameter (cm)}^2 \times 0.7854$$

$$\text{Or : Beam area (cm}^2\text{)} = \text{Pi} \times \text{Radius (cm)}^2$$

Calculation of Energy

$$\text{Energy (Joules)} = \text{Laser Output Power (Watts)} \times \text{Time (Seconds)}$$

Calculation of Energy Density

$$\text{Energy Density (Joules/cm}^2\text{)} = \frac{\text{Laser Output Power (Watts)} \times \text{Time (Seconds)}}{\text{Beam Area (cm}^2\text{)}}$$

Or : $\text{Energy Density (Joules/cm}^2\text{)} = \text{Power Density (W/cm}^2\text{)} \times \text{Time (Seconds)}$

Calculation of Treatment Time for a particular Dosage

$$\text{Treatment Time (Seconds)} = \frac{\text{Energy Density (Joules/cm}^2\text{)}}{\text{Output Power Density (W/cm}^2\text{)}}$$

Or : $\text{Treatment Time (Seconds)} = \frac{\text{Energy (Joules)}}{\text{Laser Output Power (Watts)}}$

4.3 Dosage guide

Beam area = 0.159cm²

Power density: For 70mW = 0.44 W/cm²
 For 90mW = 0.566 W/cm²

Table 4.1 Dosage parameters utilised.

Energy (Joules)	Energy Density (Joules/cm ²)	Treatment Time for 70mW (seconds)	Treatment Time for 90mW (seconds)
0.5	3.14	7.14	5.56
1.0	6.29	14.3	11.1
2.0	12.57	28.57	22.22
4.0	25.13	57.1	44.4

4.4 Cells

Related to the complexity of osseous tissue and the limitations attendant to the manipulation of bone *in vitro*, a cell culture system was used to investigate the nature of osseous cell responses to laser irradiation.

The human osteogenic sarcoma cell line Saos-2 was used for this work.

The origins and characteristics of this cell line can be found in ATCC (American Type Culture Collection) Cell Lines and Hybridomas, 8th ed. 1994. The cell line is catalogued as ATCC HTB-85 Saos-2 (human osteogenic sarcoma, primary). It is one of an extensive series of human tumour lines isolated and characterised by Fogh and Trempe (1975).

This cell line is grown as a monolayer, and transferred 1:3 weekly. Its morphology is epithelial-like and is reported to have a viability of 82%. The growth rate of Saos-2 cells in 10% foetal bovine serum has been shown to exhibit logarithmic growth with a doubling time of 37 hours (Rodan *et al.* 1987).

The Saos-2 human osteosarcoma cell line was chosen for use in this research as it has been found to provide a useful model for studies of phenotypical expression of human osteoblastic cells (Yamane 1985). It is a cell model that exhibits some of the well-characterised features of the osteoblastic phenotype (Murray *et al.* 1987, Rodan *et al.* 1987).

Established cell lines such as Saos-2 provide unlimited amounts of cells. Established cell lines also provide the advantage that many of their characteristics are already known (Fogh *et al.* 1982). These cells are also easily manipulated *in vitro*.

Minimal problems with infection in culturing these cells were encountered. The culture medium contained antibiotics to combat any bacterial infections. The one infection we had an initial problem with was environmental yeast from the incubator. The incubator was fumigated and cleaned, after which no further problems were encountered.

4.5 Cell Culture

To avoid culture contamination all cell culture work was performed in a laminar flow cabinet (Email Westinghouse, NSW, Australia) using correct aseptic technique.

Cell suspensions were prepared and manipulated at room temperature. Suspensions were occasionally cooled on ice to reduce the metabolic requirements of the cells.

Osteosarcoma cells were cultured in sterile tissue culture flasks or plates. 100ml flasks (Sarstedt, Newton, N.C., USA) with a culture surface area of 75cm² were used for bulk cultures of volumes between 15 and 20 ml. Standard flat-bottomed 96-well microplates (Sarstedt-Group, Adelaide, South Australia) were used to culture small volumes of 200µl per well for all experiments if cells were to be irradiated and analysed.

A potential problem with attempting to culture cells was the risk of contamination of cultures by organisms. Contamination was avoided by supplementing media with antibiotics and by employing aseptic technique. Aseptic technique aimed to minimise the introduction of microbes to the culture. It involved:

- Maintaining a barrier between the environment and the culture.
- Ensuring that all materials, equipment and media that came into contact with the culture were sterile.
- Manipulating cultures, materials and equipment such that there was no contamination from the environment or the operator.

To help achieve this all culture work was performed in an Email Airhandling Biological Safety Cabinet; Class two (Email Westinghouse, NSW, Australia). This vertical laminar flow cabinet protects the operator against biological hazards and protects experiments against external contamination. The work area was kept aseptic by wiping it down regularly with 70% alcohol and by ensuring that gloved hands were clean and that anything introduced to the cabinet was also wiped with 70% alcohol.

All material used in tissue culture was either obtained sterile or sterilised. Sterilisation was achieved by:

- Autoclaving. Material resistant to heat was autoclaved under 240kPa at 121°C for 15 minutes. This included glassware, pipette tips, troughs and phosphate buffered saline used for sterile cell culture work. Glassware was sealed using aluminium foil and autoclave indicator tape. Troughs were sealed in autoclave bags.
- Filtration. Media was filtered using a glass filter (Millipore). Smaller quantities of liquid including media, serum and other media supplements, trypan blue and MTT were

sterilised by filtration through sterile 0.2µm Ministart filters (Sartorius, Germany). Before use such reagents were checked visually for sterility.

Cultures were also routinely checked microscopically for contamination.

Cell cultures were grown in an incubator (Sanyo, Japan, Supplied by Quantum Scientific, Australia) at 37°C in 5% carbon dioxide, which provided the optimal conditions for cell growth.

The conditions necessary for cell cultures include:

- An atmosphere sealed from the outside so as to reduce the risk of culture contamination.
- A constant temperature of 37°C, the temperature that cells experience *in vivo*.
- A high humidity level to avoid evaporation of culture media.
- An atmosphere of 5% carbon dioxide in air. All cultures were vented to this atmosphere.

The purpose of this carbon dioxide enriched atmosphere is to prevent spontaneous alkalinisation of the media. Cells were cultured in media at approximately a physiologic pH, around 7.2. Excessively basic or acidic conditions would have been toxic to the cells. Culture media was thus buffered with bicarbonate to maintain a relatively constant pH to counteract the carbon dioxide production by the metabolising cells. However, bicarbonate buffered media undergoes spontaneous alkalinisation if left in air alone as carbon dioxide diffuses out into the atmosphere. Therefore excess carbon dioxide from the atmosphere in the incubator dissolves into the media to prevent alkalinisation.

Subculturing Osteosarcoma Cells

Confluent osteosarcoma cell cultures were passaged by dispersion with trypsin-EDTA. After dispersion, the trypsin-EDTA was neutralised with serum free medium and the resultant mix was centrifuged to isolate the cells after removal of the supernatant. The cells were resuspended in fresh cell growth media containing foetal bovine serum and split during passaging in a ratio of one to three or four and placed into new culture flasks (Appendix 2).

4.6 Media

Preparatory

Sterile phosphate buffered saline adjusted to pH 7.2, was used for cell preparation for tissue culture. This was prepared by dissolving ten phosphate buffered saline tablets (Oxoid, Hampshire, England) in 1 litre of deionised water, adjusting the pH with hydrochloric acid or sodium hydroxide as required, and sterilising by autoclaving at 121°C for 15 minutes. For non-sterile work general laboratory phosphate buffered saline was used. This was prepared by dissolving 8g NaCl, 0.2g KCl, 0.2g KH₂PO₄ and 1.15g NaH₂PO₄.2H₂O in 1 litre of deionised water (pH to 7.2).

Culture

Osteosarcoma cells were cultured as a monolayer in sterile Dulbecco's Modification of Eagles Medium (DMEM) (ICN Biomedicals Inc., Aurora, Ohio). DMEM and 10% foetal bovine serum used for culturing Saos-2 cells has been described previously (Farley *et al* 1991). To one litre of DMEM was added 20ml of 7.5% sodium bicarbonate solution (ICN) and 10ml Penicillin/Streptomycin solution (ICN) (5000 IU/ml solution -Penicillin 50 U/ml, Streptomycin 5mg/ml). DMEM was supplemented with 10% foetal bovine serum (Trace Biosciences, Australia) and 2mM L-glutamine (BDH Chemicals, Victoria, Australia) as required. The medium, serum and L-glutamine were all filter sterilised.

4.7 Cell Freezing

Cells were frozen in liquid nitrogen in order to maintain a supply of available cells to use if required (Appendix 2). After normal cell harvesting, the cells were centrifuged to form a cell pellet. The supernatant was removed and the pellet resuspended in a freezing mixture containing a high concentration of foetal bovine serum and dimethyl sulfoxide. Cells were frozen in in vials of 2 ml volumes containing 10⁶ cells / ml, which was equivalent to a confluent 75 cm² flask. Suspensions were cooled to -70°C and then stored in liquid nitrogen.

Thawing was carried out by removal from the liquid nitrogen and warming the vial under warm running water. Thawed suspensions were diluted in cell growth media containing foetal bovine serum and centrifuged. This allowed the rapid removal of the dimethyl sulfoxide, which is toxic but essential to stop ice crystal formation during the freezing process. After centrifugation the cells were resuspended in growth medium and transferred to a flask.

4.8 Cell Counting

Osteosarcoma cell counts were routinely performed using an improved Neubauer Haemocytometer (Weber, England) (See Appendix A.4). The number of cells in a known volume was counted; hence the concentration of the cell suspension could be calculated using the following formula:

$$\text{Osteosarcoma concentration} = \frac{\text{cells counted} \times \text{dilution factor} \times 10^4}{\text{number of } 1\text{mm}^2 \text{ areas counted}}$$

4.9 Irradiation

Osteosarcoma cells to be irradiated were seeded in 96-well microplates. The cells were seeded in wells such that experimental and control groups were not in adjacent wells (at least two wells separation), eliminating the possibility of irradiation scatter reaching these cells. The plates were also placed on a black surface when being irradiated to eliminate the possibility of reflection of the output beam.

All irradiation was carried out in a vertical laminar flow cabinet (Email Westinghouse, NSW, Australia) using aseptic techniques. Surfaces of the cabinet were wiped with 70% alcohol before use. The lids were removed from the plates and placed on a sterile surface. The fiber optic laser tips were cleaned with 70% alcohol. The laser was hand held, the tip resting on the rim of the wells.

Ten plates were seeded for each experimental group (one plate for each day) from the same cell suspension, one plate being analysed daily for a period of ten days. Each of the ten plates was irradiated once or on a daily basis until analysed.

The energy levels utilised were 0.5, 1.0, 2.0 and 4.0 joules (see table 4.1). Cells were irradiated once or daily, and analysed over a period of ten days. The energy output was monitored during the irradiation period using the digital powermeter. The tips used were the same diameter as the wells and so rested easily on the rims of each well. The laser was hand held for all experiments, with the vertical distance from the cells being standardised by the height of the wells. The reduction in beam output reaching the cells due to the distance and medium in the wells was minimal (Powermeter reading range 85-78mW).

Table 4.2 Output changes observed over half an hour, measured every five minutes.

Time (minutes)	Output Beam (mW) (Proposed 70 mW)	Output Beam (mW) (Proposed 90 mW)
0	84	98
5	82	96
10	82	94
15	78	97
20	76	96
25	78	94
30	77	92

Cells were irradiated at least 3 hours after seeding. Stimulation has been noted when the interval between irradiation and plating is 30 minutes or more (Karu *et al.* 1984c).

Irradiation of cells that were analysed by confocal microscopy was carried out by placing the laser under the stage of the microscope and irradiating through the plastic slide on which the cells were seeded. The laser's coherence remains after passing through media of different density and composition. Placing a slide over the powermeter eye and measuring the output with the laser beam passing through the slide determined the output power in this situation. Minimal change in power output was observed (Powermeter reading range 88-79mW).

4.10 Assays to Determine Cell Population Changes

4.10.1 Cell Counts and Cell Viability –Trypan Blue Exclusion

Osteosarcoma cells were harvested from 75cm² flasks by trypsinisation and resuspended in fresh medium. They were plated at an initial seeding density of 10,000 cells per well in a volume of 200µl per well in 96-well microplates, and incubated at 37°C in 5% CO₂ for a total period of ten days. Volume measurement inaccuracies and uneven cell distribution between the wells contribute to errors. The cells were therefore seeded using a multipipetter (Transferpette, Merck), with the wells being loaded from the top row downwards so that

experimental and control wells in each column were seeded from the same pipette to reduce this inter-sample seeding error.

Figure 4.2 The cells were distributed in the plates as illustrated.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Expt	Expt		Expt	Expt		Expt	Expt		Expt	Expt	
C	Expt	Expt		Expt	Expt		Expt	Expt		Expt	Expt	
D												
E												
F	Control	Control		Control	Control		Control	Control		Control	Control	
G	Control	Control		Control	Control		Control	Control		Control	Control	
H												

Medium was changed every third day.

Trypan Blue (Sigma) was made to a solution of 0.4% with phosphate buffered saline. The solution was first filtered through two layers of filter paper and then through a series of three (0.8µm, 0.8µm, 0.2µm) and stored at 4°C in a foil wrapped bottle.

The procedure for carrying out the cell counts involved removal of the medium, containing 10% foetal bovine serum, by inversion of the plates. The cell layer was gently washed twice with phosphate buffered saline. The cells were trypsinised with 50µl of Trypsin. The cells were then returned to the incubator for three to four minutes until they were in suspension. 50µl of serum free Medium (DMEM) was added to each well. Serum free medium is used as Trypan Blue has a greater affinity for serum proteins than for cellular protein. Each well was pipetted vigorously and the 100µl of cell suspension added to Eppendorfs containing 100µl of Trypan Blue. This created a dilution factor of two. After day five the dilution factor was increased to four to make cell counting more manageable and to keep the number of cells per 1mm square less than one hundred.

The cells were counted within one hour after the dye solution was added. If cells are exposed to Trypan Blue for extended periods of time viable cells begin to take up dye as well as non-viable cells.

With a cover slip in place, using a pasteur pipette, a small amount of the Trypan Blue-cell suspension, was transferred to a chamber on the haemocytometer. This is done by carefully touching the edge of the cover slip with the pipette tip and allowing the chamber to fill by capillary action. Before being transferred to the haemocytometer the cells were dispersed by vigorous pipetting in the Trypan Blue suspension.

Each of the four wells from each experimental group was counted separately using one chamber of the haemocytometer for each sample.

Cells were counted in the 1mm center square and the four corner squares of the haemocytometer, non-viable cells staining blue, viable cells remaining opaque. A separate count was kept for viable and non-viable cells.

Each square of the haemocytometer (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 1ml, the subsequent cell concentration per ml (and the total number of cells) was determined using the following calculations:

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

Total cell number = 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.

4.10.2 Cellular activity and Proliferation –MTT assay

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma), was dissolved at a concentration of 5mg per ml in sterile phosphate buffered saline at room temperature. The solution was sterilised by filtration and stored at 4°C in a foil wrapped bottle. It was prepared freshly each month.

Cells were harvested from 75cm² culture flasks by trypsinisation, resuspended in fresh medium, and seeded in 96-well microplates. Cells were seeded at a density of 10,000 cells per well, in a volume of 200µl per well, as determined by the initial optimal plating efficiency experiments for the MTT assay.

Plates were seeded from the top row of wells downwards with control groups in the same columns as the experimental groups so that control and experimental wells are seeded from the same pipette in the multipipetter to reduce potential errors. Other avoidable technical errors may have arisen from inaccurately measuring volumes and poorly mixing suspensions so that the cell distribution was uneven. Initial seeding of the 96-well microplates also contributes to errors due to volume measurement inaccuracies and uneven cell distribution between the wells. Cells may also stick to the sides of wells and to the pipettes. Some cells may be lost due to rinsing, or at the time of tipping out the medium and MTT prior to addition of absolute ethanol. Analysing multiple samples reduced these errors.

The cells were seeded using a multipipetter, with the wells being loaded from the top row downwards so that experimental and control wells in each column were seeded from the same pipette to reduce inter-sample error. The plates were incubated at 37°C in 5% CO₂ in air. Medium was changed every third day by inverting the plates and shaking out the medium and refilling the wells to 200µl.

Figure 4.3 Cellular distribution for the MTT assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Column											
B	Blank		Expt	Expt	Expt	Expt		Expt	Expt	Expt	Expt	
C			Expt	Expt	Expt	Expt		Expt	Expt	Expt	Expt	
D												
E												
F			Control	Control	Control	Control		Control	Control	Control	Control	
G			Control	Control	Control	Control		Control	Control	Control	Control	
H												

Cells were irradiated with a single dose of 0.5, 1, 2, or 4 Joules, or daily irradiation of 0.5, 1, 2, or 4 Joules, at least four hours after seeding. Plates were irradiated in the laminar flow cabinet.

The procedure for running the MTT assay involved removal of the medium, containing 10% foetal bovine serum, by inversion of the plates. The cell layer was washed gently twice with phosphate buffered saline. The medium was replaced with 100µl serum-free DMEM to which 10µl of the 5mg/ml MTT solution was added. Medium and MTT are also added to the blank control column. The plates were returned to the incubator (37°C, 5% CO₂) for 3.5 hours. The Titertek® Twinreader Plus was turned on to warm up for approximately 45 minutes.

After incubation the medium is gently tipped out and 100µl of ethanol added to each well. Control wells containing medium only (no cells) were processed in exactly the same manner as the rest of the plate. The plates were analysed immediately after the addition of ethanol.

The Plates were analysed consecutively for ten days. The 96-well microplates were read in a single channel vertical light photometer (Titertek® Twinreader Plus, Flow Laboratories). The photometric recordings were conducted using the 540nm and 690nm filters. The concept is based on passing a light beam through the whole sample and the light absorption is proportional to the amount of light absorption by material in the well. Absorbance values were measured in a range of 0.001 to 2.000 absorbance units. Values greater than 2.000 were considered unreliable. The cells were analysed daily for ten days.

The plates are analysed in the Titertek® Twinreader Plus, with an initial shaking period of 30 seconds. Absorbance was measured at 540nm, with a background subtraction at 690nm. Absorbance at 690nm was used as the reference wavelength for detecting artifacts in the plastic plates (Ford *et al.* 1989). The additional MTT/medium blank control was also used. The mean absorbance for these wells was subtracted from the absorbance values in the other wells.

Spectrophotometers have inherent reading errors of ± 10 percent. A result of 10-20% or greater was required to prove a significant effect to rule out the possibility of error.

4.10.2.1 Plating Efficiency

The most appropriate seeding density for the MTT assay over a ten day period in the 96-well microplate format was determined. The cells were seeded at different densities (5000, 10000, 15000 and 20000 cells per well) in a volume of 200 μ l, and the plates analysed daily for a period of ten days. In the first group the medium was not changed and in the second group it was changed every three days. Plating efficiency was found to be best at 10, 000 cells per well, with the media being changed every three days. If the media is left for longer at this seeding density it becomes too acidic and the cells start to deteriorate.

96-Well Microplates

The 96-well microplates (Starstedt, Disposable Products PTY Ltd, Ingle Park, South Australia) were flat-based, 6.7mm in diameter and 11mm high with a growth surface area of 0.35cm². Each well could hold a volume of up to 0.38ml

4.10.3 Cellular proliferation –CFSE labelling

The 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE) (Molecular Probes, Eugene, Oregon, USA) is used to monitor the division of osteosarcoma cells after single dose and daily irradiation of predetermined doses of low level laser irradiation *in vitro*.

Most *in vitro* methods of determining cellular proliferation merely quantify at a gross level the overall proliferation within a culture. CFSE can be used to specifically analyse the division history, determining any differences in the number of cellular divisions. This is used as an adjunct to the results found with the MTT assay.

CFSE was stored as 20 μ l aliquots at –70°C in anhydrous Dimethyl Sulphoxide.

For initial labelling osteosarcoma cells were harvested from a confluent 75cm² flask by trypsinisation and washed twice with serum-free medium (DMEM). Labelling was carried out in serum free medium, otherwise the dye can be quenched by the protein (Lyons and Parish, 1994).

They were then counted using Trypan Blue and a haemocytometer and resuspended to the desired concentration. The cells were returned to the incubator and warmed for 5 minutes at

37°C. CFSE was added to the cell suspension at a final concentration of 10 μ M. The cells were then returned to the incubator for a further 10 minutes and inverted every 3 to 4 minutes. The reaction was stopped with cold medium (DMEM) containing 10% foetal bovine serum and the stained cells then washed twice with serum free medium and centrifugation.

Cells were seeded in the 96-well microplate format. The available laser running time limited the number of wells that could be seeded for irradiation. Wells were therefore seeded at initial densities of maximum cell numbers, such that there would be sufficient cells by the day they were to be analysed (see Table 4.3). Wells were seeded so that cells would proliferate sufficiently without cell death, or wells becoming too acidic by day ten. Cells were analysed daily for ten days.

Ten wells were seeded for each experimental group. Control samples were seeded in the same plates utilizing the same number of wells and seeding densities.

Table 4.3

<i>Day</i>	<i>Cell number per well</i>
1	120,000
2	120,000
3	60,000
4	60,000
5	30,000
6	30,000
7	15,000
8	15,000
9	7,500
10	7,500

Irradiation was carried in the laminar flow cabinet using predetermined energy levels. These were 0.5, 1.0, 2.0 and 4.0 Joules daily or as a single dose. Seeding control samples in the same plates meant that they were necessarily exposed to the same environmental conditions for the same time period as the experimental group.

Cells were harvested from the wells for analysis by flow cytometry on consecutive days for ten days. The cells from the ten wells were combined to make up one sample. To sample multiple cell suspensions individually on the FACScan used would have been very labour intensive

and a much greater cell number required. The assay was thus limited to one experiment per energy level to be investigated.

Harvesting was carried out by initially rinsing the wells twice with phosphate buffered saline, and then trypsinising the cells. Sodium Azide, a metabolic inhibitor, is added with the transfer pipette and the cell suspension vigorously pipetted, transferred to a trough and then to the respective FACS tubes. The cells were washed twice with sodium azide containing 2% foetal bovine serum. After the final centrifugation the cells were resuspended in the residual fluid and 300 μ l of fixation solution, paraformaldehyde, added. The cells were then analysed by a FACScan flow cytometer (Becton Dickinson, Mountain View, California, USA) equipped with an argon laser, labelled cells being excited at 488nm.. Samples were gated on forward scatter (FSC) versus side scatter (SSC) to exclude dead cells, debris and clumps (Fujioka *et al.* 1994, Graziano *et al.* 1998). Acquisition and analysis of data were performed using CELLQuest 3.1f software (Becton Dickinson, California, USA).

4.11 Heat Shock Response

The heat shock-70 family of nuclear proteins was chosen as they are commonly expressed in mammalian tissue.

Using an indirect immunofluorescence assay combined with analytical flow cytometry with respect to HSP70, quantitative analysis of the responses of cell populations was carried out. Response patterns were established by heating cultured cells at 42°C for one hour, followed by sequential measurement of the HSP70 profile (Richter *et al.* 1986).

Responses to laser irradiation were assessed against the heating profile.

For the examination of heat shock protein-70 expression, large cell numbers were required (1 x 10⁶ cells per FACS tube). Cells to be irradiated were densely seeded in the 96-well microplate format. Again, the number of wells that could be utilised for the experiment was limited by available laser battery time. This factor also restricted the energy level that could be utilised, being limited to a maximum of 2 Joules.

Logistically it was difficult to recover the cells from the 96-well plates in which the cells are irradiated for analysis.

Osteosarcoma cells to be heated were seeded in 25cm² flasks to a confluent monolayer at least 24 hours before running the experiment. Cells to be heated were placed in a temperature controlled water bath set at 42°C for one hour. The caps of the flasks were sealed well with parafilm to prevent any leakage or contamination from the water bath. After heating the cells were returned to the incubator until they were to be analysed at predetermined time intervals. An initial series of experiments was conducted to establish a profile of the heat shock-70 response in osteosarcoma cells. This involved the establishment of the production of heat shock protein-70 by osteosarcoma cell cultures, and the time after 42°C incubation when this was maximally expressed.

Cells to be irradiated were densely seeded (175,000 cells per well) in 96-well microplates 24 hours prior to irradiation and analysis. The number of wells that could be seeded, and therefore the seeding density, was determined by the time limitations that the laser imposed. The cells were irradiated at an energy level of 2 Joules and returned to the incubator. The irradiation level was set at 2 Joules, because logistically it was the highest dosage that could be used within the time constraints of the laser and the number of cells required for the experiment. 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. A further control group was left in the incubator in order to compare the effects of exposing the cells to the environment at room temperature versus leaving the cells in the incubator. The irradiation level was set at 2 Joules, as this was the maximum energy level that could be used for the number of wells to be irradiated for the experiment. The cells were removed from the incubator at predetermined time intervals for analysis. The time intervals were 0, ½, 1, 1½, and 2 hours as established.

Logistically it was also difficult to recover the cells from the 96-well plates, in which the cells are irradiated, for analysis.

Cell suspensions were first treated with the metabolic inhibitor, sodium azide, and then fixed with paraformaldehyde. After incubation for an hour they were permeabilised with 0.2% Tween-20 detergent. One tube for each group was left unstained, a second, the matched isotype control, was stained with a primary monoclonal antibody mouse IgG₁, (Dako, clone DAK-GO1, X0931). The remaining suspensions were stained with a primary antibody for the localisation heat shock protein-70, Monoclonal Anti-Heat Shock Protein 70 (HSP70) (Sigma,

isotype mouse IgG₁, clone BRM-22, H5147). All cell suspensions were labelled with a fluorescent secondary detection antibody, FITC (fluorescein isothiocyanate) conjugated to sheep anti-mouse IgG₁, (Sigma, F2266). [See Appendix 2 for detailed protocol]. Cell suspensions were then analysed Flow Cytometrically using a FACScan (Becton Dickinson). Acquisition and analysis of data were attained using CELLQuest 3.1f software (Becton Dickinson).

4.12 Flow Cytometry

Flow cytometric analysis of cell suspensions was performed using a FACScan system (Becton Dickinson, California, U.S.A.). The computer software programme CELLQuest 3.1f was used for cell acquisition and analysis (See Appendix A.2.9).

Flow cytometry allows the simultaneous measurement of many physical properties of single cells. This is repeated over a large number of cells, to establish the physical profile. In this series of experiments the relative cellular size, internal granularity, and relative fluorescence intensity was measured. To create a more homogeneous cell population free from dead cells and other culture debris, a gating was placed around the population to exclude this material. Parameters for this gating were first determined on untreated osteosarcoma cells and the same parameter was used throughout the cytometric evaluation. After the isolation of a relatively homogeneous cell population, in relation to cellular size and internal granularity, the relative fluorescence of the cell population was examined. The intensity of fluorescence is directly related to the quantity of secondary fluorescent antibody that binds to the primary marker antibodies.

In flow cytometry cells were identified and characterised as they passed through a laser beam one at a time. The light scatter and fluorescence of each individual cell in the sample was measured until 30,000 cells were gated. Specifically forward light scatter, side light scatter and three different spectral regions of fluorescence were looked at. Stable fluorescent labels, fluorochromes, were used as non-radioactive tags to identify and characterise cells while still viable. A combination of flow cytometry and the use of fluorochromes and, in the case of heat shock protein analysis, specific monoclonal antibodies against cell surface determinants provides a very powerful methodology for the resolution of cell populations (Stewart 1986).

The fluorochromes and labelled products used were:

1. 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE) (Molecular Probes)
2. Monoclonal Anti-Heat Shock Protein 70 (HSP70), Mouse Ascites Fluid (Sigma),
Isotype: Mouse IgG₁, Clone number: BRM-22
Monoclonal Anti-Heat Shock Protein 70 reacts specifically against HSP70 and may be used for the localisation of Heat Shock Protein 70 by immunocytochemistry. It was used at a titer of 1:5,000. Immunofluorescent staining demonstrates a rapid and reversible accumulation of the HSP70 protein within the nucleus of heat-stressed (42°C, 1 hour) human cells.
3. Monoclonal antibody mouse IgG₁ (Dako) was used as the matched isotype control.
4. FITC, fluorescein isothiocyanate (Butcher and Ford 1986). FITC conjugated to sheep anti-mouse IgG₁, f(ab')₂ fragment of antibody (Sigma) was used as a secondary detection antibody.
FITC: excitation and absorption 495nm, emission 525nm (green, FL-1).

4.13 Intracellular Calcium flux

The fluorescent probe Oregon Green 488 BAPTA-2 (1,2 bis (o-amino phenoxy)ethane-*N,N,N',N'*-tetraacetic acid) was used to monitor changes of intracellular Ca²⁺ by confocal microscopy. The probe could be loaded into viable adherent cells, thus allowing analysis of intracellular calcium flux in living cells following laser irradiation in relatively physiologic conditions over a period of time rather than just at a point in time.

Oregon Green™ 488 BAPTA-2 is a visible light-excitable probe derived from Oregon Green 488. This probe is prepared in a cell-permeant acetoxymethyl (AM) ester form. Upon binding to calcium, this indicator exhibits an increase in fluorescence emission intensity with little shift in wavelength. For intracellular calcium measurements by confocal scanning microscopy, utilising the argon-ion laser for excitation, Oregon Green 488 BAPTA indicators are the preferred reagents due to the spectral properties of these indicators permitting the use of lower dye concentrations. This also means that the dye is phototoxic. The fluorescence of Oregon Green 488 BAPTA-2 is enhanced at least 37-fold at saturating Ca²⁺. Oregon Green™ 488 BAPTA-2 exhibits very low fluorescence in the absence of Ca²⁺.

The Saos-2 cells were seeded to a highly confluent monolayer on 4-well chamber slides (Nunc) at a predetermined seeding density of 400,000 cells per well, 24 hours before labelling with the fluorescent dye and analysis.

The Oregon Green BAPTA-2 AM ester was stored desiccated at -20°C as a stock solution of 0.1mM in DMSO (*anhydrous* dimethylsulfoxide).

Loading of Oregon Green AM Ester involved washing the cells twice by centrifugation with serum-free medium (DMEM) and returning them to the incubator to warm for 5 minutes at 37°C to return to physiologic temperature. Oregon Green was diluted in warm serum-free DMEM buffered with HEPES and added to the cells at a final concentration of $10\mu\text{M}$. The cells were incubated with the dye for 25 minutes at 37°C . After this time the medium was gently tipped out of the chambers and a large volume of cold serum-free medium, with HEPES buffer, gently added to the cells serum free medium. The cells were incubated for a further 30 minutes to regain good adhesion. The cells were gently rinsed with several volumes of cold serum free DMEM, HEPES buffered DMEM containing 1.7mM calcium chloride (Vacca *et al.* 1997; Cohen *et al.* 1998) was added to the chambers and the cells returned to the incubator until needed (within 3 hours). After addition of calcium chloride to the media, the cells were left for at least 5 minutes prior to analysis. This allowed for the cells to equilibrate as previously described (Cohen *et al.* 1998).

The cells are maintained as a living, adherent and confluent monolayer on the slides. A slide of viable stained cells was placed on the microscope stage. Cells were located and an initial image captured. This served as the control image. The same cells were irradiated through the slide by placing the laser under the microscope stage and directing the laser beam at these particular cells. The lens of the microscope was used as the target at which to direct the laser beam.

Only two different fields in each slide chamber (one at each end) could be irradiated in order to avoid pre-exposure of cells to be investigated. Only alternate wells in the chamber slides were utilised. Three experimental groups were analysed for each condition investigated. Ten cells being individually analysed for each condition.

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. A positive control was established initially and the degree of photobleaching was investigated.

Experiments were performed in a warmed environment (30-37°C) to mimic physiologic temperature as much as possible.

At predetermined time intervals, x-y images were acquired on a confocal laser scanning system (Optiscan, Model F900e, Victoria, Australia) which is fitted with an argon ion laser and a Leitz Orthoplan (Wetzlar, Germany) inverted microscope, and lens magnification size x50.

The power source used was high at 902-1000, single xy acquisitions were used to capture the images.

Positive Control

Calcium Ionophore A23187 (Calcimycin) was used as the positive control for this series of experiments. Its working concentration was 2mM. Calcium Ionophore A23187 is an antibiotic possessing weak *in vitro* antimicrobial activity as well as functioning as a divalent cation ionophore. Calcium Ionophore A23187 rapidly transports calcium into cells. It is commonly used to increase intracellular Ca²⁺ levels in intact cells, and for *in situ* calibrations of fluorescent calcium indicators, to equilibrate intracellular and extracellular Ca²⁺ concentrations.

Photobleaching

Under conditions of high-intensity illumination, the irreversible destruction or photobleaching of the excited fluorophore becomes the factor limiting fluorescence detectability. Oregon Green™ dyes are new fluorescein substitutes providing improved photostability. Photobleaching rates are dependent on the fluorophore's environment.

A control was run to observe the decay rate (amount of photobleaching) for this particular probe. The control is scanned a number of times to observe the amount of fade of the fluorescence.

Another potential problem with this technique is that spontaneous and sustained increases in fluorescence signal intensity have been observed in association with confocal microscopy. HeLa cells preloaded with fluo-3/AM (1.5µM) and exposed to laser scanning have been shown to develop Ca²⁺ fluorescence signals which increased progressively with time, without

the benefit of added stimuli. It was further discovered that only illuminated cells were affected in this way, while all other cells outside of the area exposed to laser light had only background fluorescence (Lui *et al.* 1997). It is proposed that the excitation laser light of the confocal microscope generates heat that enhances the conversion of residual non-fluorescent acetoxymethyl (AM)-esterified indicator to the fluorescent form, thus giving rise to erroneous signals.

Temperature Maintenance For Confocal Microscopy

To maintain as normal as possible living environment for the cells and to maintain their adherence and viability for as long as possible it was decided to heat the microscope stage to approximately 37°C and thus mimic the temperature of the incubator. To achieve this an electric fan heater was placed at the level of the microscope stage and a thermometer was attached to the stage.

To control the pH of the media when out of the 5 percent carbon dioxide atmosphere of the incubator, Hepes buffer was added to the media.

Cells were observed to be still viable 3-4hours after labelling if kept in the incubator. The probe is eventually eliminated from the viable cells and cannot therefore be left for a prolonged period of time before analysis, unlike fixed cells.

Many constraints were inherent in utilising this method of intracellular calcium analysis. These included time and reliance on another person to operate the equipment, availability and cost of the fluorescent dye, and the logistics of the experiment itself. Although cells were seeded at a maximum seeding density for the chambers, very few cells could be observed in a field at any one time. This was partly due to the magnification that was required to clearly see the cells. All of these factors meant that only a small sample size could be analysed.

Average brightness values were determined for individual cells or entire images using the computer software program Adobe Photoshop version 4.0 (Adobe Systems Incorporated, San Jose, California, USA). The brightness values were determined by the number of pixels in the image or selected area.

4.14 Treatment of Data

A paired two-tailed Student's t test was used to assess the significance of any difference between the means of two samples. It was used when samples contained more than three observations from which both the mean and standard deviation could be calculated. The levels of significance were set at 1% ($p < 0.01$) and 5% ($p < 0.05$).