THE APPLICATION OF
THE PULSED RUBY LASER
TO EXPERIMENTAL MICROSURGERY
AT THE CELLULAR AND INTRACELLULAR LEVEL

A
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1. INTRODUCTION

1.1 The nature and scope of the thesis

Conventional microsurgery using microneedles is a very useful technique for the developmental biologist. However, there are some limitations imposed on this technique because of both practical physical considerations and the time required for an operation. Very elaborate equipment is necessary for the most delicate work and the nature of the equipment is such that the variety of usable experimental material is somewhat limited. Work with microneedles at the tissue level is often a difficult proposition and the necrosis of cells adjacent to a puncture can be a difficult problem to overcome with certain types of material. At the levels requiring greatest precision even the micromanipulator has one serious limitation in plant micrurgy. In order to perform an intracellular operation, one must first alter the integrity of the plant cell wall.

With the advent of the laser beam a new approach to microsurgery was suggested. Laser microsurgery showed great promise as a supplement to conventional techniques. The possibility of using focused laser light as a tissue, cell or organelle disrupting agent suggested immediate application to studies in cytology and developmental biology. Theoretical considerations showed that one could expect to have a range of destructive capability from altered volumes of material at the cubic micron level up past altered volumes of the order of cubic centimeters. The unusual properties of the laser light might
even allow one to perform an operation on material surrounded by other material without affecting the intervening material.

Before such research could be attempted it was necessary to design a system whereby one might align target material for a predetermined operation and observe the effects. This necessitated an extensive study of the properties of laser light and the application of these with respect to a given physical system designed for a specific range of operations. Thus, an instrumentation had to be developed and a technique evolved for practical use of the laser in studies of a cytological nature. It was very important that the overall limitations of the technique be defined. Prior to this, the nature of the laser effects had to be determined and the relative contributions of the controllable variables to producing an effect had to be ascertained. A description of all the components of the system was required as well as a description of the reproducible effects obtained with living material.

With the instrumentation and techniques established, it was also desired to demonstrate the applicability of laser microsurgery to the solution of a purely biological problem with a view to demonstrating the ease and versatility of this new technique. A "model tissue" used to determine the relative contributions of energy input, objective focal length and degree of defocusing in producing a laser effect was found to be of great utility in this work. Thus, three distinct researches were undertaken: the development of the instrumentation, the study of the laser effects and the solution of a
biological problem using the laser technique. Because of the interdisciplinary nature of the work, an elaboration of laser action and the properties of laser light as related to their application is introduced at this point.

1.2 Laser action as related to the pulsed ruby laser

A laser is a device which emits coherent, extremely monochromatic light through the utilization of the phenomenon of stimulated emission of radiation. In fact, the word laser is an acronym for light amplification by stimulated emission of radiation. Stimulated emission is not to be confused with fluorescence which is a spontaneous emission random in direction and phase. Lasers emit in the visible and near visible range and may emit continuously or in pulses, depending on the type. The process of stimulated emission of radiation is a result of an interaction between exciting electromagnetic waves and excited atoms and, in reality, is just the reverse of absorption. The emitting material in the laser is a synthetic ruby rod having a controlled concentration of 0.05% trivalent chromium in an aluminum oxide crystal structure. The ends of the rod are polished flat to an extremely small tolerance and coated with dichroic filters, one end being coated so as to be approximately 100% reflecting and the other end coated so as to be approximately 98% reflecting at the laser wavelength.

The ruby rod acts as a medium in which the excitable chromium atoms are regularly spaced and separated from each other so that they have discrete energy levels. An intense
illuminating source such as a flash tube is used to excite the chromium ions. The illuminated, coated ruby can be considered as a collection of regularly spaced emitters between two parallel reflecting plates. When many chromium ions are raised to a high energy metastable state by a pulse of flash tube radiation, some of these ions will spontaneously drop to a lower energy level and discharge a photon. The photons produced in this way bombard the many chromium ions still remaining at the higher unstable energy level, stimulating these ions to revert to the lower energy level with an associated photon emission which is in phase with the stimulating photon (see figure 1). Thus, each chromium ion, in making a discrete jump between distinct energy levels, discharges a photon of a specific wavelength with this in turn causing the discharge of more photons of the same wavelength from other chromium ions. This results in a chain reaction or cascade of photons oscillating back and forth between the reflecting plates. If the amplification in traversing the length of the crystal is greater than the loss on reflection, the light wave will build up into a coherent oscillation with some of it emerging through the partially transparent reflecting end plate as a beam. Since only a small percentage of the flash tube radiation falling on the ruby is absorbed, and since all the emitted photons not travelling parallel to the axis of the ruby escape from the sides of the rod, the process is quite inefficient. Nevertheless, the photon discharge which does occur through the partially transparent reflecting end plate is very intense and has very unusual properties.
The ruby prior to excitation. The dots represent chromium atoms in the ground state.

Excitation of the chromium atoms by flash tube emission. The arrows represent photons and the circles represent excited atoms.

The occurrence of a spontaneous emission followed by a subsequent stimulated emission parallel to the axis of the ruby.

The buildup of an oscillation.

The oscillation upon reflection by the 100% reflecting plate.

The emergence of the usable laser energy. This energy emerges after each complete oscillation until the termination of laser action within the ruby.

Figure 1

A simplified model of stimulated emission

This model was adapted from Schawlow (1961).
1.3 The properties of the laser beam

The essential property of the laser beam is coherence which is of two kinds, spatial and temporal. Spatial coherence means that, at any given instant, the phase of the wave front is uniform at all points of a large surface perpendicular to the direction of propagation of the wave. Temporal coherence means that a periodic relation exists between the amplitude of a wave at a particular time and its amplitudes at later instants. Because of their spatial coherence, laser beams have an extremely small divergence and are, therefore, very highly directional. In fact, coherent light is so highly directional that it will deviate from a single direction of propagation only by effects of diffraction from the aperture of the emitting surface. The angle of divergence of a laser beam in radians due to diffraction limitations is approximately

\[
\frac{1.22 \lambda}{D}
\]

where \( \lambda \) is the wavelength of the light emitted and \( D \) is the diameter of the ruby rod emitting surface (Solon, 1961). Thus, the spot size achievable by focusing a laser beam with a good lens system depends on how close the angular divergence of the laser light comes to the theoretical diffraction limit. It can be shown theoretically, and possibly demonstrated in practice, that one can achieve a spot size comparable in size to the smallest object resolvable by the high power lens of an optical microscope. Because of the discrete energy transition of the chromium ions, the emitted photons are all of the same wavelength and, thus, a given type of laser operating at a given temperature under given conditions produces a specific
emitted wavelength which, in the case of the pulsed ruby laser operating at room temperature, is 6943 Å. Because a laser beam can be focused to a very small spot whose diameter is of the order of one wavelength of the laser light itself (Malt and Townes, 1963), one can achieve enormous energy densities in portions of the beam approaching the focal point. Because of the extremely short pulse duration which is approximately one third of one millisecond for the particular laser used, one can also achieve enormous power densities at the focal spot of the radiation. Since the electric field strength of an electromagnetic wave is proportional to the square root of its intensity, the field at the focal spot of the laser beam can be millions of volts per centimeter (Levine, 1963). Thus, there must be considerable ionization about the focal point of the radiation at any energy output not closely approximating zero. As an approximation, the radiation focused on a homogeneous target can be considered to be uniformly absorbed within a small sphere of a radius equal to that of the true spot radius, although conduction of the generated heat may undergo relatively considerable spread peripherally from the surface of this sphere and, thus, yield an affected sphere of much greater radius. We have found that this enlargement of spot size or sphere of effect is related to the laser energy output with the energy levels available to us and, thus, to achieve experimental spot sizes approaching one with the minimum radius as limited by diffraction, one can only use very low output energies. This regulation of energy can be achieved
by taking advantage of the remaining special property of laser light, its polarization. (The emission from a ruby laser may or may not be polarized, depending upon the orientation of the optical axis of the crystal.) With a polarized output, a rotatable polarizer situated between the emitting surface and the target is a simple means of attenuating the beam.

1.4 Microsurgical use of the laser beam with regard to its special properties

Because of the unique collection of properties of laser light, one can utilize a laser beam in ways not readily accessible to light beams from other sources. The energy density of laser light can be made much greater than that of light from any other source known. Indeed, the energy density can be so great as to allow for a focused parallel beam to vaporize completely any appropriately thin layer of any material placed in its path (Schawlow, 1961). Because of its coherent properties, the laser light can be focused in practice to an extremely small spot size of the same order of magnitude as the smallest discrete objects discernible by the light microscope, and this allows for concentration of the energy density into objects of very small size to destroy them. Increasing the energy can yield increasingly greater spot sizes for destructive purposes up to the point where the energy output of the laser is so great that one can use the unfocused beam for destruction of relatively large objects. Decreasing the resolving power of the lens can also yield increasingly greater spot sizes. As previously mentioned, a lower limit
is placed on the minimum spot size achievable by diffraction considerations. But, for practical purposes the spot size an experimentalist is limited to is that spot size in which an alteration occurs but which requires enough energy such that there is a peripheral spread of effect which cannot be demarcated from the spot size which might have been achieved had the area covered by the spot been isolated from all surrounding conductive material. Despite this peripheral heat spread, one may still achieve very tiny volumes of target material destruction with a focused laser beam, small enough so that intracellular microsurgical operations can be performed on relatively large cells. Very few other techniques can affect volumes of living material as small as those which can be affected by focused laser light. There is some suspicion that a distinct effect may be produced at a molecular level because of the degree of coherency of the radiation, but so far none has been defined. Actually, there is really no reason to predict specific effects from coherence itself because during absorption the molecules undergo various random disturbances that largely annul the regularity or coherence of the absorbed light. Precise changes about the focal spot because of the electric field or ionization phenomena produced at extreme energies have not been defined.

In addition to the effects one can produce utilizing the highly collimated character or coherency of laser beams, there are many effects which can be produced because of the extreme monochromaticity of the light. At appropriately moderate
energies, the laser beam can only affect regions which will absorb the light to some appreciable extent. Transparent material may completely transmit the radiation without showing any observable changes either at the time of the pulse or afterwards, although this does not necessarily mean a complete absence of effect. This absorption specificity allows the experimenter to destroy an appropriately pigmented object located in a near-transparent medium without appreciably affecting the medium even though the object may be smaller than the spot size normally attainable under similar experimental conditions. If the output energy of the laser is made sufficiently low and if the cytoplasmic medium is sufficiently transparent and of high heat capacity, then one should be able to use focused laser light to destroy tiny cell organelles independently of other nearby organelles despite very close proximity between them. Not only does pigmentation allow one to be very specific in the destruction of a tiny volume, but it also allows for great selectivity of effect through a considerable depth of biological material. Theoretically, with an intense beam of large spot size, one should be able to affect thin alternating layers of pigmented and transparent material in such a way as to yield great destruction to the pigmented layers with only minimal or negligible effects to the intervening transparent tissues. This effect has been noted experimentally. Not only are layers of destruction sharply demarcated from each other in some tissue experiments but the affected pigmented tissue is sharply demarcated from
the adjacent pigmented material outside the region of effect.

The high power density also allows for special application. Microsurgical operations best performed only by a pulsed laser beam are immediately suggested by the extremely short time interval of the pulse. The ruby laser utilized in this research had a pulse time of only one third of one millisecond duration. Thus a target area affected by this pulse can, theoretically, be destroyed without appreciable effect on the thermal equilibrium of surrounding tissue. This localization of effect, in time, is a great factor in producing minimal disruption in adjacent material regardless of its absorption characteristics or heat capacity. Another advantage with respect to time of operation is that rapidly dividing cells, cells readily damaged under microscope lighting conditions, and cells and cell organelles with appreciable movement can easily be operated on by a laser pulse, whereas other more time-consuming techniques might not be at all applicable.

In summary, the use of a focused laser beam can be a valuable tool in microsurgery because of: the very precise localization of effect even when extremely small volumes of material are affected, the complete absence of ionization effects at moderate energy outputs except for restricted regions within and very near the focal spot, and at a microscopic level, the phenomenon of transmission through materials to affect a region within another with only negligible effects to the intervening transmitting material. The possibility of affecting regions located at great depths beneath surface
tissue layers using focused laser radiation, with minimal effects to the intervening layers despite the great depth of transmission, also offers exciting applications.
2. A REVIEW OF THE LITERATURE

The physics of laser action and the design of lasers is a subject which has undergone a tremendous explosion in the technical literature. The monographic work by Lengyel (1962) encompasses all the major developments which took place initially in this field. Schawlow's 1963 article in Scientific American gives a good account of laser action and lasers in general in a non-technical manner. The paper by Levine (1963) gives a similar treatment but at a deeper and more expansive level. Dacey (1962) in a concise article gives a brief account of the history and development of lasers, the principles involved in laser action, and a comparison of the properties of different types of lasers.

Speculation about the application of laser light to biological research began very shortly after the first successful operation of a laser. Townes (1962) discusses the focused beam with respect to the phenomena occurring about the focal point and speculates upon the obvious immediate uses of the laser beam in many avenues of research. He relates the properties of laser light to its applications and speculates upon the application of focused beams to microsurgery. Malt and Townes (1963) discuss the properties of laser beams and relate these properties to many possible applications, with great attention being focused upon intracellular surgery. Mention is made of the biological work which had been done up to the time of writing, especially the work of Bessis who had irradiated living material with beams of 1.5 microns diameter.
With a rather complex system, Bessis et al. (1962) had demonstrated the possibility of selectively destroying cell organelles and had shown the selectivity of effect one could achieve in irradiating cells of different pigmentation. Recently, a lengthy review of the literature on applications of laser beams to biology and medicine was presented by Fine, Klein and Scott (1964). They discussed the effects of the beam on biologically active molecules, studies on skin lesions, microsurgery, laser irradiation of tumors, beam-tissue interaction factors and eye hazards.

Much work has been done on the effects of laser radiation on the eye. Zaret et al. (1961) described retinal and iris lesions produced in the rabbit eye by a pulsed ruby laser. In an excellent article in 1963, Zaret et al. described these rabbit retina lesions in great detail and sorted out the effects produced by pigmentation, energy output and degree of defocusing. Also documented was the peripheral wave of destruction which would follow a pulse focused on a heavily pigmented retina. Campbell et al. (1963) compared laser photocoagulation with the most modern xenon arc photocoagulator and discussed the practical aspects of the use of the laser in photocoagulation work on the eye in a review which related the properties of laser beams to the clinical effects desired. Kapany et al. (1963) discussed the heating effect upon the pigmented tissue layers in focus at the back of the eye, and related varying degrees of damage to energy output in demonstrating a non-linear relationship between lesion diameter and incident energy.
In recognizing that the versatility of a laser retinal photo­coagulator would be very limited if restricted to one wavelength, provision was made in their instrument so that the active medium could be either a ruby rod emitting at 6943Å or a neodymium-doped glass rod emitting at 10,600Å.

The effects of laser radiation on the skin have also received much attention in recent months. Fine et al. (1963) made studies of effects produced on the skin of Syrian hamsters by high energy beams in the 20 to 50 joule range from a neodymium-doped glass laser. L. Goldman et al. (1963) described skin lesions produced by a ruby laser and discussed the effect of pigmentation on the severity of the lesion. A very promising laser application was suggested by the discovery of McGuff et al. (1963) that the ruby laser light had a selective effect upon some tumors, killing them while leaving adjacent normal tissue relatively unaffected. Both delayed and immediate effects were noted. Fine et al. (1964) compared the skin lesion effects produced by lasers emitting at 6328Å, 6943Å, and 10,600Å at energy levels exceeding 500 joules. They found a selective interaction with melanin and a sharp demarcation of irradiated areas from adjacent non-irradiated areas. Free radicals were found following irradiation in vivo and in vitro.

A brief review by L. Goldman (1964) discusses the latest developments in lasers with respect to the possible contribution these might make in biological and medical studies. In this review, he refers to the most recent work of McGuff
which demonstrates that some unpigmented cancers are very sensitive to laser radiation and indicates that some type of antibody response may be set up as a result of laser tissue destruction. J. Goldman et al., refer to the transmission of laser beams through fiber optics in producing both skin damage and internal damage. In work with the skin, he has found threshold energy density values for red cell damage. L. Goldman et al. (1964) were able to damage red blood cells without affecting white blood cells in their midst. J. Goldman et al. (1964) discuss the usefulness of a laser-fiber optic complex as a medical instrument where low energies could supply illumination for observation and then high energies could be supplied for therapy through the same bundles.

Some attention has been given to the use of vital stains in the selective laser destruction of living biological material. Saks and Roth (1963) in an excellent paper describe the use of methylene blue chloride for the micropuncture of cell walls. Bessis et al. (1962) demonstrated the usefulness of Janus green B in the destruction of mitochondria. In this thesis is described a novel experiment with Janus green B. The work of L. Goldman et al. (1964) has produced a list of useful vital stains. In their work on skin irradiation they found the most practical vital stain was Evans blue. It produced the least debris and artifacts of the stains tested. Eriochrome Black T was found to be very useful and Trypan blue also showed promise. One should keep in mind the fact that vital stains may play a role other than that of an absorbing
material in laser cytoplasm interactions. Rounds et al. (1964) have found that a previous laser injury makes cells sensitive to Janus green toxicity. A complete understanding of this phenomenon would contribute to the understanding of laser-cytoplasm interaction at the molecular level.

Considerable work at the molecular level has been undertaken in recent months. Rounds (unpublished) has demonstrated that laser light can be used to inhibit some biochemical transformations and has uncovered some interesting phenomena regarding the interaction of laser light with DPNH. Fine, Klein and Scott (1964) discuss the laser beam generation of free radicals in living tissue and the effects on some proteins which indicate alterations in reactivity differing from those of thermal denaturation. They refer to studies on blood group substances which indicate that laser irradiation may enhance the biological reactivity of a molecule rather than decrease its reactivity. Also they refer to studies which are being carried out to determine whether laser radiation can differentially affect one or more activities of enzymes catalyzing more than one transformation, in comparison with the laser effect on the remaining activities.

Little work on single cells has been done. Rounds et al. (1964) reported that a line of rabbit aortal endothelium showed an increased frequency of nuclear abnormalities and giant cell formation following laser irradiation. The modal value for this line shifted from 42 chromosomes to 40 in the third subculture of irradiated cell populations. Dicentric
chromosomes and chromatid breaks were often observed in irradiated populations with none appearing in the control cells. Saks and Roth (1963) using a pulsed ruby laser were able to perform microsurgery on the internal structures of Spirogyra cells without damaging the cell wall. They give very good descriptions of the effects produced on Spirogyra cell organelles with beams of very low energies of the order of one milli-joule and less. Nuclear changes were described and threshold values for coagulation of different cell components were determined. With the advent of ultra-violet laser beams (Rounds, unpublished) (Heard, 1963) intranuclear effects are open to study. Booth et al. (submitted for publication) reported the selective destruction of chloroplasts in the cell of a higher plant with a pulsed ruby laser and Bessis et al. (1962) claim the achievement of selective destruction of stained mitochondria.

Studies involving the laser irradiation of tissues have shown considerable promise. Rounds et al. (1964) compared similar tissues with different pigmentation as to the laser effects. Some work on the irradiation of excised chick embryo hearts and isolated beating chick cardiac cells was done by them to compare the recovery in each case and study the effect on contraction rate. Chick embryos were also studied by Lang et al. (1964) in an article which also compared the helium-neon laser with the pulsed ruby laser. It is noteworthy that they were able to produce a radiation effect on chick embryos which, at the time of operation, were completely enclosed by
The use of closed circuit television in laser investigations has been a recent development (Fine et al., 1964) allowing the investigators to study laser-tissue interactions at the moment of interaction. Some unique studies on tissues have been initiated by the newly developed technique of laser spectroscopic ultramicroanalysis (Rosan et al., 1963). This technique consists essentially of focusing a laser through a microscope objective to vaporize a small portion of tissue, the elemental gases of which are analyzed by an ingenious system. The gases caused by the pulse are raised to emission levels by a spark traversing the gap between carbon electrodes situated lateral to the irradiation site. Elemental analysis is then performed on these excited gases by conventional spectroscopy. Analysis for trace elements appears to be practical because the analytical limits allow some ions to be detected in concentrations of the order of $10^{-10}$ moles.

Some very interesting experimental studies on entire organisms have been carried out by Witt et al. (1964). They have studied the web building activity of the spider, Araneus diadematus Cl. and compared the normal activity with the web building of spiders having a laser lesion in their central nervous system. The authors feel that the laser beam may be a means for objectively relating spider behaviour changes to a specific damage site in the central nervous system. Fine, Klein and Scott (1964) reporting on work done with hamsters describe some unique effects brought about by the use of very high energy beams. They discussed the case of one hamster...
which died from a laser burn and showed a pattern of injury completely different from that of a thermal burn. The superficial layers of the skin were vaporized, the peritoneal layers were intact and apparently unaffected, and the internal organs in the beam path were severely damaged. Histological findings conclusively showed that the radiation path through the hamster was sharply delineated and that this path contained alternating layers of injured and uninjured tissues.

Findings similar to those just mentioned have been described in a number of papers and much concern has been felt about the dangers inherent in laser experimentation. A discussion of these dangers may be found in a paper by L. Goldman (1963) in which a list of protective measures is given. Solon (1963) also discusses safety precautions especially with regard to eye hazards. These papers really relate to experimental work done with very high energy lasers and may not really be applicable to work with lasers whose maximum output is measured in millijoules, although certain eye hazards are inherent in this work regardless of the laser exit energy. The safety devices for a low energy laser as described in this thesis were felt to be adequate.
3. INSTRUMENTATION

3.1 Description of the laser and associated mechanisms as utilized in this research

3.1.1 The crystal and regulation of its energy output

The ruby crystal used in this research is a cylindrical solid rod of three inches length and one quarter inch diameter. At room temperature it emits radiation at 6943 Å. It is precisely positioned along one focal line of an elliptical cylinder reflector. A straight flash tube has a position along the other focal line of the elliptical cylinder reflector as shown in figure 2. This design served the purpose of introducing a maximum amount of exciting white light into the ruby from the flash tube. Geometrical considerations will show that virtually all light emitted from one focal line of the ellipse in the above system must pass through the other focal line upon reflection by the enclosing reflector system (Felstead, 1964).

Figure 2
The elliptical cylindrical reflector
Figure 3
A diagrammatic representation of the laser system as utilized in this research.
Upon excitation, the ruby emits a variable energy output over a time span of approximately one third of one millisecond. The pulses can be spaced as closely as ten seconds apart, each pulse being controlled by switch manually. The energy output of the flash tube and thus the energy output of the ruby itself can be varied by varying the voltage to which the capacitor banks supplying the flash tube are charged. The minimum voltage to which the 400 microfarad capacitor could be charged in order to pulse the flash tube with this particular laser for a concomitant ruby discharge was 950 volts. Charging the capacitors past 1600 volts would exceed the rating of the flash tube and either explode it or damage it. Thus, using a voltage control alone to vary the laser output allows one to gain usable output energies over only a very limited range. The maximum output attainable at maximum workable voltage was found to be about 57 millijoules as ascertained by averaged thermopile readings and the minimum consistent output was about 10 millijoules using a 1150 volt setting. Only in the range between 1550 volts and 1150 volts could a linear and reproducible relationship between applied voltage and energy output be achieved. Voltage settings between 950 and 1150 could not be depended upon to give consistent energy outputs and sometimes failed to cause the ruby to discharge. The relationship between voltage setting and laser output is diagrammed in figure 4.

One can obtain reduced output energies by using a voltage setting within the range between 1150 volts and 1550
The relationship between voltage setting and laser output.

The relationship between the polarizer setting and the energy output with the voltage set at 1500.

The dichroic filter was removed for these measurements as was also the case for figure 4.
volt and interposing a polarizer between the emitting face of the ruby and the exit barrel. Rotation of the polarizer allows for a selection of energies down to fractions of a millijoule (see figure 5) and the use of appropriate light filters as attenuators can further reduce the exit energy to a very tiny fraction of a millijoule. Using these methods, though, one should carry out periodic checks on the polarizer and filters so that they may be replaced when alterations have been brought about in them by constant use over time.

The polarizer attenuates the laser beam by approximately $\cos^2 \theta$ according to Malus' Law, where $\theta$ is the angle between the light's direction of polarization and the polarizer's direction of polarization. Because the polarizer absorbs in the red there is an absorption effect as well as a direct attenuation effect such that only approximately 30% of the light entering the polarizer is actually affected by rotating it. The remaining 70% is absorbed on its path through the polarizer. Inserting the dichroic filter at $45^\circ$ for use in conjunction with the pilot light attenuates the energy by about 40% largely by reflection. Thus the energy output of the laser at maximum applied voltage but with the dichroic filter in and the polarizer set in place for maximum output is approximately 10 millijoules. It is wise to check the exact transmission values for the filter and polarizer periodically as well as the relationship between applied voltage and ruby energy discharge so that one may always know exactly the energy output leaving the barrel at any given combination of settings.
For high experimental accuracy the voltage setting should be held constant at 1500 volts and the polarizer varied.

One other possibility exists for energy output control. This is the use of a diaphragm for changing the diameter of the beam. Never should this manner of control be used for experimental work of this nature. The energy is not evenly distributed across the diameter of the beam nor is it evenly distributed among the quadrants of a cross section of the beam. Furthermore, the use of a diaphragm increases the diffraction effects tending to disrupt the coherent properties of the beam.

It should be kept in mind that the energy outputs one records are not those entering the object in focus but those leaving the exit barrel. Many sites are present within the microscope's optical system for further energy attenuation. The beam splitter sends a readily detectable beam out of the eyepieces and the beam more than fills the back focal plane of the objective, thus resulting in appreciable loss. The optical cement in microscope objectives is believed to be altered by beam absorption (Weber, 1962) and the many glass air interfaces must certainly attenuate the energy to some degree. No attempts have as yet been made at measuring the energy as it leaves the microscope objective.

3.1.2 Proper alignment of the beam

The beam must enter the microscope parallel and as coaxial as possible with the optical system through which it must pass. Whenever the laser is taken apart, great care must be taken in the positioning of the ruby and the elliptical
cylindrical reflector so that the beam exits parallel to the axis of the barrel which is coupled by a metal sleeve to the vertical ocular tube of the microscope. The pilot lamp is so placed that an image of its filament is focused at infinity through the barrel by means of the collimating lens and dichroic filter (see figure 3). This beam is not parallel; its angle of divergence is much greater than that of the laser light and so if the two beams are properly aligned the laser light should illuminate a small area within the circle of illumination of the pilot lamp. When using a microscope, one knows that one is pulsing parallel with the optical axis of the objective lens if the pilot lamp filament may be focused to a small circle centrally located within the field of view. One should never test for proper alignment of the laser path by direct observation through the inclined oculars.

3.1.3 Safety mechanisms

There is a shutter system interposed between the polarizer and the dichroic filter which is always shut except during the laser pulse. This prevents any spontaneous discharges from leaving the barrel. The pulse trigger itself operates this shutter. The electrical aspects of the trigger action are such that the shutter is snapped open just before the flash tube is fired.

A second safety shutter is located between the laser proper and the exit barrel. A manually controlled aperture was set up here to enable the experimenter to be able to charge up the capacitors and then visually line up the object
to receive the pulse without any fear that accidental triggering will allow the radiation to enter his eye.

3.2 The microscope

3.2.1 Description of the microscope

The microscope used was a Cooke, Troughton, and Simms Ltd. M15 model with a triocular head. It was attached to the laser barrel by a sleeve of very tight fit which could be screwed on to the laser and when properly aligned above the microscope could be slid over the vertical ocular tube. A special suspension arrangement held the laser so that it placed an almost negligible weight on the microscope. The laser-microscope coupling arrangement is shown in figure 6. Figure 7 is a generalized drawing of the microscope and figure 9 shows the triocular head. The data for the objective lenses and the periplan oculars may be found in tables 1 and 2 respectively.

3.2.2 The camera attachment

The camera used was an Asahiflex 35 millimeter camera with the lens removed (see figure 6). A microscope adapter of a type which clasps the ocular was attached to the camera and inside this adapter was fitted a 6 x Leitz periplan ocular. A 10 x Leitz periplan ocular was also tried but the quality of the photographed image was found to be very poor with this lens with the technique used. The quality of the image with the 6 x was little altered when the ocular was inserted only part way into the tube; thus one could adjust the object in focus visually with one ocular all the way into the
Figure 6

The instrument as used for laser microsurgery.
Figure 7
A generalized drawing of the microscope.

This diagram was taken from page 7 of the Cooke Microscope Manual, 4th Ed.
Table 1
Data for the objective lenses.

<table>
<thead>
<tr>
<th>Magnifying power</th>
<th>Numerical aperture</th>
<th>Semiangle of cone</th>
<th>Depth of focus</th>
<th>Diameter of field (6x ocular)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3x</td>
<td>0.10</td>
<td>5.7°</td>
<td>55μ</td>
<td>4370μ</td>
</tr>
<tr>
<td>20x</td>
<td>0.50</td>
<td>30.0°</td>
<td>2.0μ</td>
<td>650μ</td>
</tr>
<tr>
<td>40x</td>
<td>0.65</td>
<td>40.5°</td>
<td>1.2μ</td>
<td>340μ</td>
</tr>
<tr>
<td>100x oil</td>
<td>1.30</td>
<td>59.1°</td>
<td>0.37μ</td>
<td>130μ</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Working clearance</th>
<th>Combined effective length</th>
<th>Focal length</th>
<th>Distance from object in focus to objective shoulder</th>
</tr>
</thead>
<tbody>
<tr>
<td>3x</td>
<td>33530μ</td>
<td>33mm</td>
<td>43.87mm</td>
<td></td>
</tr>
<tr>
<td>20x</td>
<td>1350μ</td>
<td>8585μ</td>
<td>43.87mm</td>
<td></td>
</tr>
<tr>
<td>40x</td>
<td>613μ</td>
<td>4216μ</td>
<td>43.87mm</td>
<td></td>
</tr>
<tr>
<td>100x oil</td>
<td>356μ</td>
<td>1819μ</td>
<td>43.87mm</td>
<td></td>
</tr>
</tbody>
</table>

The objectives were all Cooke, Troughton, and Simms Ltd. achromats.

These data were taken from material supplied by Vickers Instruments Ltd., York, England.

Table 2
Data for the periplan oculars

<table>
<thead>
<tr>
<th></th>
<th>Field of view in eyepiece</th>
<th>Pupil distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>periplan 6xB</td>
<td>18.0mm</td>
<td>7.4mm</td>
</tr>
<tr>
<td>GF10x wide field</td>
<td>18.0mm</td>
<td>9.6mm</td>
</tr>
</tbody>
</table>

The field of view in eyepiece is the virtual diameter of the eyepiece diaphragm (making allowance for the action of the field lens). The pupil distance is the distance between the pupil of the eye and the uppermost lens surface of the eyepiece. These data were taken from page 8 of the pamphlet, The Optics Of The Microscope, by C. H. Claussen.
tube while taking a high quality picture through the other
despite the necessity of having the other ocular partially
out of the tube. The 10 x ocular would not permit this tech-
nique because its imaging properties were just too sensitive
to changes in optical tube length. With this system the ex-
posure time could easily be judged by eye with practice.
Although these techniques are not refined they do permit pro-
duction of excellent photographs. The ground glass image
resolution is often not adequate for use of the camera image
in selecting for desired detail at the proper focal depth in
the field of view and thus it is necessary to use a pair of
oculars with imaging properties appropriate to the technique
outlined above.

3.2.3 Illumination of the object

Very small objects may be placed in water on a glass
slide with cover slip applied and illuminated via the conden-
sser in the usual manner. Larger objects which transmit little
light may be placed on a glass slide with or without water and
illuminated by the laser pilot lamp from above. No tissue
should be exposed to the pilot lamp for any time longer than
the minimum time required for the operation because of its
intensity and glare. Very large specimens such as plant apices
with their associated stem or rhizome structure may be mounted
in plasticine on the condenser stage and illuminated either
with incident light from lamps arranged about the microscope
stage or by the laser pilot lamp from above. Only the 3 x and
20 x objective lenses have been found useful with the incident
Illumination technique because of the difficulties involved in getting enough incident light reflected from the specimen into the objectives of high numerical aperture. Incident illumination is preferable to the pilot lamp illumination for plant apices even though the intensity of the latter may be attenuated by filters. Using transmitted light, the visibility of intracellular detail is very limited in specimens which are grown on filter paper or thick agar films mounted on a glass slide in preparation for an operation. This is due mainly to the light scattering produced by these media. Often these media must be resorted to in order to maintain proper moisture conditions for specimens mounted on slides and exposed to the drying action of the condenser light when the nature of the experiment precludes the use of a cover slip.

3.3 The laser light path

It is essential that the coherent laser light enter the objective lens system parallel to the optical axis of the lens system. It is also essential that the laser light either fill the back focal plane of the objective or enter the back focal plane coaxial with the optical axis of the microscope. With the apparatus used here, the filling of the back focal plane depends upon the increase in the diameter of the beam resulting from what little natural divergence occurs and also the divergence brought about by the lens and prism system in the triocular head. For the ruby employed, the divergence of the beam was found to be approximately one quarter of one degree from a ruby face of one quarter inch diameter.
Geometrical considerations show that the divergence angle alone will allow for the filling of the back focal plane with the system used.

To check on the diverging or converging effects produced in the head (see figure 9), a photographic film was placed in the back focal plane of the objectives and irradiated by a laser pulse in the dark. The negatives showed that this plane was filled in all cases and in fact the beam covered a much larger area than that predicted, thus causing a considerable energy loss in the system. The significance of this feature of the illuminating system is indicated by the following considerations. If the back focal plane is filled, the aiming of a defocused beam is not very sensitive to imperfect coaxiality. But, if the plane is not filled it can be seen in figure 8 that making use of vertically misaligned beams can be a serious problem when the beam is not perfectly centered within the objective.

![Figure 8](image)

The path of a narrow beam through the objective lens.
Figure 9

The triocular head.

The above was taken from material supplied by Vickers Instruments Ltd., York, England.
The coverage of the back focal plane also determines to some extent the minification of laser spot size achievable with a given objective lens and ruby face to lens distance. When this plane is filled, as is the case with the instrument used, the periphery of the lens acts as an aperture, thus introducing a new source of diffraction and yielding a slightly larger minimum spot size. Achieving the greatest degree of minification of the laser focal spot depends upon a number of primary factors, the elaboration of which are essential to this thesis. The mathematical considerations to be presented are referable to figure 10.

Figure 10
A diagrammatic representation of the optical considerations involved in achieving minimum spot size.
The laser emits with a divergence angle $\phi$.

$$\phi \text{ theoretical minimum} = \frac{2.44 \lambda}{D_L}$$

where $\lambda$ is the wavelength of the laser radiation in microns and $D_L$ is the diameter of the emitting face of the ruby in microns. This is the Fraunhofer diffraction relationship (Solon et al., 1961) and $\phi$ here has a value of 0.00027 radians. The actual value of $\phi$ for the ruby used was found to be 0.004 radians. The spot size produced by the laser beam can be considered to have the diameter $h$ in microns.

$$h = fA$$

where $f$ is the focal length of the objective lens in microns and $A$ is the angle in radians denoted in figure 10. Angle $A$ can equal $\theta$, $\phi$ or $\beta_{\text{minimum}}$ depending on the theoretical considerations to follow.

$$\beta_{\text{minimum}} = \frac{2.44 \lambda}{D_E}$$

where $D_E$ is the diameter of the lens whose back focal plane is filled with laser light. This will be the case when the laser is effectively at infinity and the absolute minimum spot size diameter possible can be given by the equation:

$$h_{\text{minimum}} = f \beta_{\text{minimum}}$$

When the laser cannot be considered as a point source, the following cases prevail:

$$h = f \phi \text{ when } \phi \text{ is less than } \theta.$$  

or $$h = f \theta \text{ when } \theta \text{ is less than } \phi.$$
where both $\theta$ and $\phi$ are greater than $\beta_{\text{minimum}}$ and their size relationship to one another is determined by $D_L$ and the distance between the lens and the emitting face of the ruby. If either $\theta$ or $\phi$ is less than $\beta_{\text{minimum}}$, the spot size is given by $h_{\text{minimum}}$ as in the equation $h_{\text{minimum}} = f \beta_{\text{minimum}}$.

It should be emphasized that the minimum spot size refers only to the area affected by a laser beam in focus with the object. The formulation assumes that the distribution of emitted energy across the face of the emitting surface is uniform and it should also be emphasized that this assumption is not right. Also, it should be pointed out that as the refractive index of the oil for the 2 mm lens is increased, the spot size is decreased.

It is known that only the core of the ruby rod participates to a maximal extent in emitting the laser beam and thus there is a gradient of energy distribution across the diameter of the beam (Buddenhagen et al., 1962). Superimposed on this radial energy gradient is another energy gradient brought about by the particular oscillation properties of the laser rod. The rod used in this project yielded a more intense energy output from one half of the emitting face than the other. This was determined by the same photographic technique used to determine the degree of illumination of the back focal plane of the objective. At low output energies there is some evidence to show that this emission anomaly has an effect on the shape of the area of destruction brought about by a minified beam. Rather than obtaining an affected area shaped
like a disc or a crater with a circular rim, one often obtains a U-shaped area of destruction.

3.4 Use of the beam path as a tool

3.4.1 The objectives

It was found by use of a stained protein film that when the film surface was in perfect focus with transmitted light, the laser beam was divergent upon the film surface. Geometrical considerations place the laser beam focus within the cone of white light described by the object focal point and the outer surface of the first objective lens. The laser light enters the rear of the objective as a parallel beam and consequently achieves focus at a minimal distance from the frontal lens surface (see figure 11).

Figure 11

The vertical misalignment of the laser beam focus with the white light focus.
The fact that the laser light is monochromatic means that it will virtually have a point of focus whereas white light when focused even by the best color corrected objectives actually has a focal depth. This is illustrated in figure 12.

Figure 12
A comparison of the foci of laser light and white light.

The laser light being of relatively long wavelength will leave the objective at a steeper angle than would light of any other color and thus come to a focus at a point further from the objective and closer to the white light focus than would parallel white light leaving the objective. All these considerations make it extremely difficult to locate the laser focus with respect to the white light focus by mathematical
considerations. However, it is a fact that the most intense area of destruction would occur in a thin absorbing film when the laser beam was focused exactly on its surface. Because the laser beam was divergent at the object focal plane it was a simple matter to place a thin protein film mounted on a glass slide in this plane, irradiate it, and then move it in steps progressively toward the objective with an irradiation at each step. This movement was performed by using the microscope stage fine adjustment and the vertical movement was measured as was the diameter and severity of the affected film area. This experiment was performed many times with each objective and very good agreement was obtained in every case for the vertical location of the laser beam focus for each objective. The figures in table 3 were those found for the vertical misalignment of foci when the inclined oculars had been set at an all-the-way-in position. The inclined oculars are adjustable so that moving them outward alters the optical tube length and thus focuses one at a slightly greater depth. Thus it can be seen that varying the optical tube length by adjusting the oculars (see figure 13) gives one a different distance of vertical misalignment of foci because the laser will focus at the same point no matter how the oculars are set. It should be noted in table 3 that because of the great variation in cover slip thickness, no cover slips were used for the determinations. The percentage error for the figures in table 3 is probably high because of the difficulty in making accurate measurements of such small distances, but these figures are
set ocular position  ocular adjusted outwards

objective lens

object plane  new object plane

Figure 13
The relation of the object plane level to the position of
the oculars

Table 3
Vertical misalignment distances for the different objectives

<table>
<thead>
<tr>
<th>objective</th>
<th>vertical misalignment of the laser focus with the white light focus</th>
</tr>
</thead>
<tbody>
<tr>
<td>33 mm</td>
<td>could not be determined with technique used</td>
</tr>
<tr>
<td>8 mm</td>
<td>approximately 260µ</td>
</tr>
<tr>
<td>4 mm</td>
<td>approximately 60µ</td>
</tr>
<tr>
<td>2 mm</td>
<td>approximately 0µ</td>
</tr>
</tbody>
</table>
3.4.2 The "model tissue" and its relationship to laser microsurgery

A "model tissue" was constructed to relate area and severity of damage to the three principal instrumentation variables. The "model tissue" was a hydrated protein film of 25 to 30 microns thickness, having 92% transmission to a wavelength of 695 millimicrons and was mounted on a glass slide without cover slip. The film was stained bright green and had an exact composition prior to irradiation of 1% gelatin in 0.05% aqueous fast green dye. A number of irradiations was performed with each objective using approximately 36 mj, 23 mj, and 6 mj pulses. The diameter and approximate depth (a measure of severity of effect) of the craters formed in the film were tabulated for each objective with regard to each energy setting. Then the vertical alignment of the laser focus with the film in the object plane was varied so that the film was bombarded by a series of increasingly divergent or convergent beams with a given objective and energy setting. The results of the tables were graphed in such a way that one could see the relationship between the effects produced and the factor varied (see figures 14, 15, and 20).

With the laser focused on the surface of the film it was found that the smallest crater diameter at each energy was obtained using the 2 mm objective, a not unexpected result considering that minification of the focal spot diameter is directly dependent on the focal length of the lens. It should
The inclined lines represent the focused laser beam. The length of the horizontal lines represents the diameter of the crater formed in the "model tissue" when its surface was set at varying vertical distances from the laser focus. The thickness of the lines is a measure of the depth of the crater and thus the severity of laser effect. A dot signifies no visible effect.

Figure 14
Energy and vertical misalignment effects with the 2 mm objective
be mentioned that the craters were depressions created in the film, presumably by vaporization, without bleaching of the pigment. There really was, however, very little difference in the diameters of the craters formed in the film using the 2 mm, the 4 mm, and the 8 mm objectives. Lateral heat conduction probably masked considerably the difference in diameters resulting from the direct impact of the beam. The 33 mm objective was found to be completely inapplicable to these studies when using the energy range available and it will not be considered further. There was little difference in crater diameter as the energy was varied from 6 mJ to 36 mJ, although the effect of changing the energy was readily seen when the laser beam was vertically misaligned with the target. It should be stated here that this misalignment was always brought about by moving the microscope stage with the fine adjustment and experiments with other materials showed that the special physical properties of the particular protein in the gel had no bearing on the results obtained.

The charts in figure 14 summarize the measurements obtained with the 2 mm objective for varying degrees of vertical misalignment at three energy levels. The inclined lines in figure 14 represent the illuminating beam and in this particular case may also be considered to approximate the laser beam path because the focal point separation was effectively zero. Oil was used but without a cover slip in making the measurements. The length of the lines is an indication of severity of effect. It should be noticed that by positioning
The cross represents the laser focal level. The length of the horizontal lines represents the diameter of the crater formed in the "model tissue" when its surface was set at varying vertical distances from the laser focus. The thickness of the lines is a measure of the depth of the crater and thus the severity of laser effect. A dot signifies no visible effect.

Figure 15
Energy and vertical misalignment effects with the 4 mm objective
an object at the appropriate vertical distance from the laser focus, one can obtain craters of extremely small area. This is because the energy density in the core of the beam is greater than at the periphery (Buddenhagen et al., 1962), and so as the beam diverges the energy density sufficient to produce an effect becomes more and more restricted to the axial portion of the beam until the beam energy is attenuated so greatly by divergence that even the energy density at the axis is insufficient for destructive purposes. Increasing the energy increases the vertical distance about the focus through which the beam can have an effect and also increases the diameter of the affected region. The apparent asymmetry of the graphs in figure 14 is not readily explained. The problem of measuring the short vertical distances involved is complicated by the differences in refractive index of the media through which the beam passes. Perhaps there was a real difference between the effects produced by the converging beam and those produced by the diverging beam.

It should be noted that the field of utilizable laser energy about the focus, as defined by the physical properties of the irradiated material, is very compressed with the high magnification 2 mm objective; this effect being a result of the great beam divergence at very small vertical distances from the focal point. With the 4 mm objective (see figure 15) the field of utilizable energy about the focus is a tube of rather distinctive shape. The effect of changing the energy input into the 4 mm objective was to lengthen the tube and
increase the tube diameter at the levels where it was maximal. At maximum diameter, the severity of the laser effect is diminished but the effect is extended over a very large area. Again there is a dropoff in the area of utilizable energy with great vertical misalignment such that very tiny craters could be formed in the film when the pulse was focused at great distances above the film. A consideration of the overall series of effects with the 2 mm and 4 mm objectives led to a theoretical mapping of the tube of utilizable radiation (see figure 16) with respect to intensity patterns within the tube. All the aforementioned phenomena as well as this resultant theoretical map were put to the test on appropriate biological material and the model was conclusively found to be of great application. Furthermore, experiments with stained agar and black friction tape also allowed for the description of tubes of utilizable radiation which were essentially comparable to those obtained with the gelatin film.

Consider the tube as represented by the 23 mj output pulsed through a 4 mm objective. A pulse directed at a thin colored film of material placed at the level of the laser beam focus yields a very severe effect of relatively small diameter. Moving this object away from the focus of the beam vertically causes the area of severe effect to be decreased although the total area affected increases. An experimental study of this intensity phenomenon allows one to draw the graphic representation of intensity patterns within the tube of effect as shown in figure 16. A similar pattern is to be
tube of utilizable radiation for 23 mj pulsed through the 4 mm objective (measurements in microns)

Figure 16
A graphic representation of intensity patterns within the "tube of effect"
tube of utilisable radiation for the 4 mm objective using 36 mj

Ulva layer irradiated at level AA', severe damage

Ulva layer irradiated at level BB', moderate damage

Ulva layer irradiated at level CC', slight damage

Figure 17
A demonstration of laser intensity patterns on biological material
found using the 2 mm objective.

A followup to this approach to demonstrating intensity patterns was undertaken with a specimen of green plant tissue because the organization of the tissue allowed for exact measurement of intensity patterns produced by pulses which disrupted this organization. Tissue portions of the green alga, Ulva, which grows in thin sheets were placed at certain levels with respect to the laser focus as shown in figure 17. Their appearance after irradiation with a single pulse of 36 mJ is represented in figure 17. From this operation and from many other demonstrations it became clear that regardless of the true spot size at the focal level, a vertically misaligned beam could be used as a tiny surgical needle with a diameter of the order of microns. If, for a tiny puncture or lesion in a given material, the degree of vertical misalignment required at any energy level was so great that inaccurate horizontal aiming of the beam became a problem, then the process of merely decreasing the energy enabled the operator to be able to carry out the operation with a lesser degree of vertical misalignment required. This "needle" was readily adapted for rupturing organelles when utilized with both the 2 mm and 4 mm objectives. Figure 18 illustrates the "puncture" obtained with a 4 mm objective using a defocused beam on a chloroplast of a gametophyte cell of Osmunda cinnamomea L. Figure 19 illustrates some "needle effects" one can produce with the same material using the 2 mm objective with oil. In these demonstrations the chloroplasts
Figure 18

The use of the 4 mm objective in producing a "needle puncture" effect

The cell shown in figure 18 was that of a gametophyte of the fern Osmunda cinnamomea L. Figure 18A shows a large green body, tentatively identified as a chloroplast, in the upper cytoplasmic layer of the target cell. Figure 18B shows the same organelle shrivelled by a pulse of 33 mj through the 4 mm objective. It still appeared bright green, thus precluding a carbonization effect. The interpretation at the time of operation was that fluid had been expressed from a rupture of the organelle membrane. Perhaps a vapor bubble had been formed within the organelle to burst it. It should be noted that despite the close proximity between the organelle and the upper cell wall, the wall was not ruptured.
The use of the 2 mm objective in producing minimal lesions

In this demonstration, using Osmunda gametophyte cells, a "needle effect" was achieved by decreasing the energy of a vertically misaligned beam. With the 100x oil objective the cell shown in figure 19A was irradiated with a pulse of 36 mj to produce the effect shown in figure 19B. The cytoplasm of an adjacent cell as shown in figure 19C received a pulse of 9 mj to produce the effect shown in figure 19D. The diameter of the severely injured region at 36 mj was about 15μ whereas the diameter at 9 mj was about 5μ.
effectively represented extremely thin protein films.

It should be noted that all the misalignment distances are really the distances through which the stage was moved and not really distances through which the laser focus was misaligned. The vertical distance between the point of focus in transmitted white light and the laser light focus is determined by the refractive indices of the media through which the light passes after it leaves the objective. Consequently this distance is not constant. It has been established for the condition where the object is viewed in air. Deviation from this value for particular liquid mounting media and cover slips could be established by trial and error. When this is done, the object focal plane level always should be altered such that the misaligned beam striking that plane is divergent.

The accuracy of the fine adjustment over its entire range of turns is questionable and when making measurements it was found to be good technique to select beforehand a range of turns of the fine adjustment which yielded accurate measurements at all points within this range. Furthermore there is some dispute over the exact calibration of the fine adjustment. The Cooke Microscope Manual states that each small unit represents 2 microns. Experimental measurements on the microscope used showed that a value of 3 microns per small unit was correct and all vertical measurements mentioned in this work were derived from the latter figure. The workings of the fine adjustment used are described in the book
The cross represents the laser focal level. The length of the horizontal lines represents the diameter of the crater formed in the "model tissue" when its surface was set at varying vertical distances from the laser focus. The thickness of the lines is a measure of the depth of the crater and thus the severity of laser effect. A dot signifies no visible effect.

Figure 20
Energy and vertical misalignment effects with the 8 mm objective
Microscope Design and Construction.

As has been shown previously, both an increase in energy and a decrease in the objective magnifying power bring about an elongation of the tube of utilizable radiation. The 8 mm objective attests to the role of the latter factor. The tube as shown in figure 20 at maximum energy has a length of the order of millimeters. The diameter of this tube is remarkably insensitive to energy changes and at no point in the energy ranges tested is the diameter much greater than that at the focal plane. This tube is not well suited for cellular operations but is readily applied to tissue operations on a small scale. The depth of effect producible with the 8 mm objective has many disadvantages associated with it for microsurgical purposes but the elongated tube does have some application.

3.5 Additional technical considerations

Objective lenses with a minimum of spherical aberration are necessary to obtain maximum benefit from the laser's monochromatic output in reducing the spot size. Only the best quality objectives should be used despite the fact that the beam's high power density may affect the objectives adversely with time.

Horizontal misalignment with defocusing operations is a problem because of imperfections in the microscope and deformation of the triocular head by any pressures exerted by the laser's weight on it. When the microscope stage is lowered a great deal to take advantage of the "needle effect", 
the area affected by the laser beam tends to shift laterally from the target as was centrally located in the object plane. Reducing the energy and thus the amount of defocusing required allows one to overcome this to a great extent but for best performance of the instrument all factors contributing to horizontal misalignment of the light path should be corrected as fully as possible. The factors responsible for this also are responsible for distorting the beam somewhat so that the focal spot is not circular. At low energies the laser itself does not produce a circular spot in the object focal plane but the two factors affecting circularity of the spot can be sorted out. A method whereby one may demonstrate the relative contribution of laser and microscope to distortion of the laser spot is outlined below.

Mount an appropriate absorbing material such as black friction tape or a film of stained protein gel on a glass slide so that the surface of this absorbing material is in the object focal plane. Then pulse the laser at a low energy such as a few millijoules through the 4 mm objective. A U-shaped crater will often be formed. Rotate the microscope 90° and pulse again. The U-shaped crater produced will have taken up a position in the field of view which gives it the same directional orientation as the laser. Another rotation confirms that the position of the U-shaped crater configuration is dependent on the position of the laser. More correctly, the shape of the crater at the low energy is a result of some anomalous behaviour of the ruby emission. If
the energy is turned up to a maximum and the experiment is repeated, the crater formed is oval-shaped. With rotation of the microscope this shape is found always to have the same orientation in the field of view. Thus the lack of circularity at the higher energy results primarily from an imperfect microscope optical system. Therefore two different factors contribute to an irregular spot shape and their relative contribution depends upon the energy density of the beam.

Often the radiation comes to a focus which is not quite in the center of the field of view. This is mainly because of slight coaxial misalignment between the laser and the microscope because of a not-quite-perfect fit of the coupling sheath over the upright ocular. Provided that the laser spot is not too far off center (less than about five percent of the diameter of the field of view) there is no real need to attempt to align it perfectly. Before use, however, one should find out where the laser focus lies in the field of view so that one can position the material to be irradiated in the proper manner with the stage manipulators and ocular micrometer.

Internal reflections within the optical system of the microscope may create some difficulties. They may greatly increase the affected area of material and may decrease the amount of energy impinging on the predetermined target to a considerable degree. Reflected beams emerging from the oculars, the exact cause of which are unknown, are demonstrated occasionally by photographs taken during the laser pulse (see figure 21). They appear to have little or no destructive
Figure 21

Photographic evidence of internal reflections

This photograph was a five second exposure with the pulse occurring after the first half second. The before and after photographs are figures 19A and 19B.
effect; their large area and apparent intensity being misleading in this respect. Possibly very little reflected laser light reaches the object, with most of what is photographed being the result of reflections directed into the oculars from the prism system in the head (see figure 9). With respect to figure 21, the before and after photos are shown in figure 15A, and the retraction of pigmented material from the site of the pulse should be noted.
4. LASER MICROSURGERY

4.1 General aspects

4.1.1 Minimum spot size

Much emphasis has been placed on achieving a minimum spot size with focused beams and an area of minimal effect with defocused beams. These are important considerations when microsurgery is to be performed within single cells but the value of achieving these minima must be viewed with an eye to other considerations. There is no advantage in perfecting the instrumentation to the point where the diameter of the area illuminated by the beam is so small as to be of the order of a fraction of a micron, if so little energy reaches the object in view that no effect can be produced. In many cases there is also no advantage in producing a laser effect confined to a volume of the order of a micron diameter if the destruction of this volume sets off a secondary effect leading to the complete disruption of a much larger volume. Some experiments with the obliteration of chloroplasts of two to three microns diameter were very successful with respect to containing the total primary destructive effect within a very tiny volume (see figure 19D). However, it was frequently observed that the destruction of a few chloroplasts at one pulse led to a release of cytoplasmic tension or a pulling away of the cytoplasm from the affected region such that many other chloroplasts were extensively damaged even though they were peripheral to the area of primary laser effect. One definitely cannot always depend on containing the region of effect within the
area of laser illumination. On the other hand, one may in appropriate situations affect visibly a volume of pigmented material of much smaller diameter than that of the area of illumination, a feature which was dealt with previously (see section 1.4).

Irradiating a heavily pigmented tissue at a high energy level may often bring about a wave of changes peripheral to the primary region of laser impact. These changes are primarily a result of heat conduction and the degree of peripheral spread of destruction is a function of laser beam energy density and of the pigmentation and heat capacity of the affected tissue. These peripheral waves of destruction have been very well described in the rabbit retina by Zaret et al., (1963) and have been observed here in the cytoplasm of single cells of the gametophyte of the fern *Osmunda cinnamomea* L.

4.1.2 The cytoplasm

At relatively moderate energies such as those obtainable with this laser at maximum output, transparent material such as the cytoplasm of the amoeba *Pelomyxa carolinensis* could not be visibly affected by the beam. Cytoplasmic coagulation was effected in a pigmented gametophyte cell of *Osmunda cinnamomea* L. by pulsing into the cell such that the region of utilisable energy as defined by the 2 mm objective at 33 mj would occupy a volume which included the cell contents. The cell could be observed over time and the configuration of the intact organelles could be checked daily. Their immobility over a period of days was ample evidence that the cell's
cytoplasm had been coagulated (see figure 37).

This effect (the coagulation of transparent cytoplasm) introduces a complication into the measuring of affected volumes. One can visually determine the extent of damage of a pigmented body but only detailed observation over time can allow one to assess the structural alteration of transparent cytoplasm peripheral to a disrupted pigmented object. When using the dry objectives, there is no way of immediately assessing the amount of cytoplasmic coagulation within the "tube of effect". Also, one cannot accurately know the extent to which one has altered biologically active molecules within this "tube" and the enzymatic activity of a cell is certain to be affected by laser radiation. However, it is obvious that if only a few pigmented organelles within a region of many are disrupted, there certainly can be little effect on nearby transparent cytoplasm. There is much evidence in the literature to indicate that only minor or negligible effects may be produced within this tube at appropriately low energy levels and so an experiment was devised to demonstrate this.

Gametophytes of *Osmunda cinnamomea* L. which can be considered as tissue layers one cell thick were placed in pairs one above the other and the double layer formed in each case was irradiated with the laser focus being located deep within the second layer (see figure 22).
It was found that considerable damage (with respect to wall obliteration and protoplast disruption) could be done to the underlying tissue layer with only minor or negligible protoplast disruption damage to the overlying tissue (see figure 22). Repeating the experiment by pulsing into the upper tissue layer yielded an effect whereby the upper tissue layer could be greatly damaged with minor or negligible damage to the underlying tissue layer.

Figure 22
Irradiation of a tissue layer through another tissue layer interposed between it and the beam
Simple experiments of this type and observation of transparent streaming cytoplasm which had been irradiated indicate that one can affect the cytoplasm minimally or negligibly by passing the beam through it, although localized coagulations may be produced near obliterated pigmented regions. Regardless of cytoplasmic resistance to the laser path, one must be concerned in intracellular work with the laser effects on pigmented structures within the intense energy density zone of the "tube of effect". There are great advantages in using highly vacuolate cells for the study of minimal laser effects on the cytoplasm. In cells of this type much of the intense portion of the beam can be aligned so as to pass through the vacuole (see figure 22). Provided the vacuolar fluid is colorless, that portion of the beam capable of greatest damage will not be absorbed at all.

Lack of visible effect by beams in the 10 mj to 30 mj range on some cells may be attributed to more than just transparency. Very granular cytoplasm as is found in the aleurone cells of *Avena fatua* L. and in the pollen grains of *Tradescantia paludosa* L. was very resistant to observable damage by the beam. Cell wall reflection may play a role here but a possible major factor, aside from the weak pigmentation of these materials is the lack of water in the protoplast. Many of the obliterator effects have been seen to be intimately tied in with the instant production of a water vapor bubble within a structure (see figures 23A and 23B). Whether the bubble contributes directly to the obliteration or is produced
Figure 23

Water vapor bubble formation caused by a laser pulse

The target structure as shown in figure 23A was a rhizoid cell of an Osmunda gametophyte. The target area contained considerable brown pigment and some red. Figure 23B was taken during a pulse of 36 mj through the 4 mm objective. The cell wall appears to be collapsing at one place and completely missing over most of the target area. Note the small vapor bubble rising up the vacuole. Within seconds the large bubble (figure 23B) had contracted greatly (figure 23C) and the small bubble had disappeared from the field of view. Mitochondria could be seen streaming out of the hole. Seconds later, the large bubble had almost entirely disappeared and the rim of the hole was clearly defined as shown in figure 23D. The diameter of the hole was 20μ.
in addition to it is disputable.

The pigmentation of the target material plays a great role in vapor bubble formation. Very thick cell walls such as are found in the aleurone cells of *Avena fatua* L., which could not be ruptured by irradiation, were often seen to have a huge vapor bubble of the order of hundreds of microns diameter form at their surface after a pulse had been focused on some pigmented debris situated on the outer surface of the wall. Tiny intracellular bubbles were sometimes seen during immediate examinations of irradiated weakly pigmented material which appeared little affected. Blue, green, brown and black pigments as found in a wide range of biological material tested were maximally affected by the radiation, whereas orange and yellow pigmentation usually was completely resistant to radiation effects. Red pigments were inconsistent; the red cells of some red algae being unaffected by a given pulse whereas the reddish pigmented rhizome cells of the gametophyte of *Osmunda cinnamomea* L. were ruptured by a pulse of the same energy.

The role of pigment in producing gross morphological changes naturally suggested the use of vital stains for microsurgery and the use of protein stains for mapping the path of the beam through non-vacuolate cells. Janus green B was found to be effective for laser work on transparent material. Even when transparent protoplasm, such as that of the amoeba *Pelomyxa carolinensis*, was stained so weakly that little coloration could be seen to be imparted to it using Janus
green B, it was found that the stained protoplasm was greatly affected by the radiation whereas the unstained amoeba could not be visibly affected at all with pulses ranging up to 36 mj. Considerable work has been done by other investigators on the irradiation of stained material and there is some evidence to suggest that the stain plays a role in addition to that of absorption in producing a laser effect (Rounds et al., 1964).

Attempts were made to produce a path of coagulated protoplasm through aleurone cells of *Avena fatua* L. so that the shape of the coagulated region might be determined by use of the native protein stain Naphthol Yellow S (Deitch, 1955). The aleurone tissue was stained after irradiation and the sections examined to find no staining differences which would indicate the radiation path. Another experiment was tried where the aleurone tissue was stained first with Naphthol Yellow S and then irradiated, but to no avail.

4.1.3 The plant cell wall

The possibility of performing intracellular surgery on a given plant material depends greatly on the properties of the cell wall. It has already been demonstrated in this thesis and elsewhere that intracellular surgery can be achieved without rupturing the cell wall (see figures 18, 19A and 19B), and that the cell wall can be ruptured to allow the protoplast to escape (figures 23A and 23B). It has also been demonstrated in this work that absorption of laser energy may also allow for a plasticizing effect on a plant cell wall. The experiment
to be described next demonstrates this effect and also demonstrates the importance of plastid pigment as absorbing material.

The alga *Closterium littorale* Gay, mounted in water under cover slip and observed with the 2 mm objective, was positioned peripherally in the field of view and then moved centrally 5 microns at a time with a pulse being given centrally after each movement. None of the pulses resulted in any morphological effect, even the pulse given to the transparent tip of the alga, until a portion of the pigmented material of the alga, the axial chloroplast, was centrally positioned in the field. The first pulse given to the alga once the chloroplast tip was in the path of the beam resulted in the tremendous morphological change as shown in figure 24. The area of the cell wall can be seen to have been increased considerably in the region of the pulse, presumably because of a superheating phenomenon. This cell wall at the time of the operation did not appear to be ruptured and thus the photographs making up figure 24 are evidence of a plasticizing effect on the wall brought about by the beam.

Many plant cells appear to explode when irradiated, ejecting their protoplasts many cell diameters away (see section 4.3). Possibly the explosion is a turgor effect brought about by the rupturing of a cell wall which is under great strain, but the instantaneous creation of water vapor from cell water could very well be the main factor in bringing about the explosive effect.
Figure 24A

The plasticizing of a plant cell wall

Figure 24A is a specimen of the green alga *Closterium littorale* Gay mounted in water under coverslip. Figure 24B is the same specimen immediately after having received a pulse of 33 mj through the 100x oil objective. Presumably, the heat generated by the pulse plasticized and expanded the cell wall. It is interesting to note that no rupture of the cell wall could be detected.
Some rather interesting phenomena frequently were found to be associated with a cell which had received a large burst of energy but had not ruptured. Often its cell wall was found to bulge considerably into a neighboring cell. In filaments of cells (such as the protonemal filament of *Bryum stenotrichum* C.M.) it was found that the bulging of a wall into a neighboring cell might set up a wave of destruction reminiscent of a shock wave. The situation as illustrated in figure 25 was once observed in a protonema when too much energy was used to explode the target cell.

![Figure 25](image)

*A laser shock wave effect*

The phenomenon illustrated in figure 25 points out the necessity of using appropriate energy levels. It was frequently observed in experimenting with moss protonemata that the cell structure in cells adjacent to the target cell would collapse so that the cytoplasm would immediately be
pulled away from the cell wall and form a shrunken green ball after a pulse. This did not appear to be solely a direct radiation effect, because the chloroplasts displayed no evidence of dispersal or fragmentation such as is usually found about the outer margin of an irradiated region. The interpretation of this can only be speculated upon at this time but a shock wave effect causing the tearing of cytoplasmic strands is not an unlikely explanation.

4.2 Studies with the laser beam on cells

4.2.1 The nucleus

The focused laser beam appeared to be an excellent tool for the study of the nucleus and nuclear-cytoplasmic relations. It was found that the nucleus of gametophyte cells of *Osmunda cinnamomea* L. could be exploded with a laser pulse despite its transparency and lack of pigmentation by using high energy levels. Perhaps the presence of nearby chloroplasts allowed for enough heat conduction to the nucleus to burst it. Possibly there are even soluble pigments in the cytoplasm having a sharp absorption peak of about 6900 Å to 7000 Å but which are present in visually undetectable amounts. This could conceivably allow for cytoplasmic absorption causing the nuclear disruption. The work of Saks and Roth (1963) indicates, however, that nuclear damage may be a result of direct absorption of the laser energy by the nucleus. A surprisingly great range of damage effects can be produced on the nucleus, depending on the energy used. At times one can observe the production of a small rupture in the nuclear
Figure 26

Complete obliteration of a nucleus

Figure 26A shows the nucleus of an *Osmunda* gametophyte cell. Figure 26B shows the result of a 36 mj pulse focused on the nucleus using the 100x oil objective. It should be noted that the cell wall is intact despite the obliteration of the organelles on its surface.
membrane with a resultant spurting of nuclear material through the opening, the entire phenomenon lasting many seconds. Saks and Roth (1963) describe some novel effects produced with the use of energy outputs of the order of one millijoule. At the other energy extreme, the nuclear structure could be completely dispersed as is shown in figure 26, effectively yielding an enucleation without rupturing the cell wall. In this particular experiment there was enough energy used so that widespread damage was caused to the cell; thus the advantages of a dispersed nucleus approach to enucleation over micromanipulator removal of a nucleus are doubtful when considering the trauma involved. Nevertheless, the use of focused laser beams of low energy in "enucleation" experiments could conceivably have great application.

4.2.2 The vacuole

Because the integrity of the vacuole is an essential consideration in the performance of cytoplasmic operations it was decided to investigate the immediate alterations encountered when the pulse was aligned so as to affect the vacuolar membrane directly. Rupture of the tonoplast had been observed in gametophyte cells of Osmunda cinnamomea L. in this study. This was difficult to document photographically, and so an attempt was made to record this phenomenon in another organism. It was decided to irradiate the food vacuole of the amoeba Pelomyxa carolinensis. Repeated pulses at maximum energy and with each objective had no visible effect on the vacuole or intervening cytoplasm.
A dye solution made up of one drop of one percent Janus green B in fifty cubic centimeters of glass distilled water was mixed with an equal quantity of the amoeba's culture water. A drop of this mixture containing an amoeba was placed on a glass slide without cover slip and left for one hour. After this time interval the amoeba was found to be actively streaming and lightly stained with a greyish coloration in the granular portions of its cytoplasm. Some portions of the hyaline cytoplasm were bright blue but this coloration appeared to be confined to the surface membrane. Presumably the mitochondria had some dye associated with their structure. The fluid in the food vacuole did not appear colored. The pulse was aligned such that the contents of the food vacuole were brought into focus using the 8 mm objective and a pulse of 36 mJ was directed through the stained cytoplasm overlying the vacuole (figure 27). The plasmalemma was ruptured and some granular cytoplasm ejected with the food vacuole enclosed within. The plasmalemma immediately sealed itself over, leaving the food vacuole with its apparently unaffected membrane and intact contents outside the amoeba. This experiment was repeated twice more with the same result, the vacuole floating away in each instance. However, one of these vacuoles encountered some debris in its path and was ruptured, the membrane collapsing like a flaccid balloon. All attempts at bursting the floating vacuole with the laser failed.

An interesting aspect of this experiment is the ease with which the vacuole was removed from its cellular environment.
Isolation of a food vacuole

Figure 27A shows a pseudopod of the amoeba Pelomyxa carolinensis containing a food vacuole. Figure 27B shows the ejection of cytoplasmic material immediately after a pulse of 36 mj through the 8 mm objective. Prior to the pulse the amoeba had been stained vitally with Janus green B. Figure 27C was taken a few seconds after figure 27B. It can be seen that the rupture had sealed itself over quickly. The free-floating spherical object is the food vacuole as shown in figure 27A. All attempts to burst the membrane of this vacuole with laser pulses failed. However, the membrane did collapse when it was torn in an encounter with some debris as is shown in figure 27D.
This isolation technique may have great applicability to the study of vacuoles.

4.2.3 The response of cells to laser beam damage

It was decided to operate on a single cell and conduct a photographic study of the cell's response to the damage. A gametophyte of *Osmunda cinnamomea* L. was mounted in water on a glass slide and studied under the 2 mm oil objective. A single large cell was selected for irradiation with the purpose of destroying a large number of the chloroplasts in the upper layer of cytoplasm with minimal damage to the remainder of the cell. The alignment of the laser focus with respect to the cell to achieve this purpose is illustrated in figure 28. Changes in the cytoplasm adjacent to the irradiated site were documented as well as changes in the nucleus over the life of the cell.

The pulse was of 23 mj and was focused directly on the upper layer chloroplasts as shown in figure 29A. It should be noted that the primary damage site was of 30 microns diameter and no rupture of the upper wall or side wall occurred (see figure 30A). Much of the debris brought about by chloroplast obliteration was instantly snapped into place along the side wall opposite the nucleus (figure 30B). The nucleus quickly became swollen and rounded and appeared to snap away from the wall. A comparison of figures 29B and 30B shows the changes undergone by the nucleus within the first three minutes.
Figure 28
Laser focus alignment for irradiation of the upper layer cytoplasm of a vacuolate cell

By eight minutes after the pulse, mitochondrial motion had ceased throughout the whole of the upper cytoplasmic layer except along the upper sidewalls remote from the target area. Mitochondrial motion in the adjacent cells remained unchanged throughout the duration of the experiment.
Figure 29

The Osmunda cell before the pulse

Figure 29A shows the upper cytoplasmic layer prior to the pulse. Figure 29B shows the interior of the cell at the level of the nucleus prior to the pulse. The upper cytoplasmic layer was about 45μ above the level of the nucleus.

Figure 30

The Osmunda cell shortly after the pulse

Figure 30A shows the upper cytoplasmic layer one minute after a pulse of 23 mJ was focused directly on one portion of it by the 100x oil objective. The primary damage site was of 30μ diameter. Figure 30B shows the nuclear level three minutes after the pulse. It should be noted that much debris was present at this level, apparently the result of cytoplasmic retraction.
Until the final changes leading to cell death after 193 minutes, there appeared to be no alteration of the lower cytoplasmic layer although progressive inward movements of the sidewalls occurred. At ten minutes the side wall adjacent to the nucleus suddenly bulged greatly inwards. At twelve minutes the nucleus jerked away from the side wall again. Possibly this jerking movement was a result of the snapping of cytoplasmic strands. Peripheral plastids in the upper layer appeared to be shriveling. At sixteen minutes, the nucleus was very dark and granular. It had progressively darkened and had assumed an oblong shape. At eighteen minutes no mitochondrial motion at all could be detected in the upper layer. At twenty-four minutes a clear transparent vesicle formed on the nucleus and started expanding on the side facing the apparently intact vacuole. At twenty-six minutes the vesicle was greatly expanded with a diameter of about eight microns as compared to the nuclear diameter of twelve microns, although the vesicle appeared to be continuous with the nucleus. The nucleoli had become very distinct partly at least because the nucleus had become much less granular. The upper layer chloroplasts were becoming very diffuse in appearance as they were losing their form and pigmentation. This corresponds to some degree to the wave of peripheral destruction referred to earlier, although these changes in the gametophyte cell took considerably more time. By twenty-eight minutes the nucleus had become transparent with very prominent, sharply defined nucleoli, and the upper layer was becoming progressively more translucent. The entire
upper half of the cell at 31 minutes was very translucent and the few intact chloroplasts appeared to be degenerating. The nucleus itself was becoming opaque although the nucleoli remained distinct (figure 31). From this point onwards the nuclear vesicle appeared to expand and contract slowly. At 36 minutes the translucent upper layer exhibited little evidence of structure (figure 32). At 43 minutes all the upper layer chloroplasts appeared to be fragmented or dispersed and the cytoplasm was translucent to a very great degree. By 180 minutes the adjacent cells had greatly compressed the irradiated cell by swelling into its sidewalls as a comparison of figures 33A and 33B will show. The nucleus appeared granular and was in a state of compression. A photograph (see figure 34) was taken of the level half way between the upper wall and the nucleus at 180 minutes. This showed that the upper wall had not been deformed while also showing the constriction of the central portion. Exactly at 193 minutes after irradiation the cell completely collapsed, having been, in effect, obliterated by its neighbors. What little remained of its contents was represented by a compressed bright green mass of material (see figure 35).

Despite the extensive spread of altered cytoplasm after the pulse it appears that an osmotic imbalance may have played a role in bringing about cell death. The radiation damage may have had some effect upon the cell's membrane structure, causing the cell to lose its water. Nuclear changes were considered to be a response to the irradiation rather than
Figure 31

The nucleus with its vesicle

This photograph was taken 31 minutes after the pulse

Figure 32

The irradiation site 36 minutes after the pulse
Figure 33

The shrinkage of the irradiated cell

Figure 33A was taken 43 minutes after the pulse. Figure 33B was taken 180 minutes after the pulse.
Figure 34
The cell 180 minutes after the pulse
This photograph was taken with the object focal plane level being halfway between the upper wall and nuclear levels.

Figure 35
The cell 193 minutes after the pulse
a primary laser effect. Thus this experiment conclusively demonstrated the feasibility of studying the response of a single cell to cytoplasmic damage.

A second single cell experiment was set up whereby a very small amount of cytoplasm was irradiated with a 33 mj pulse through the 4 mm objective. The beam was brought to a focus above the irradiation site so that the impact beam was divergent in order to make use of the "needle effect". This cell was studied over time with a view to discovering whether it could recuperate and regenerate the small volume of altered cytoplasm. The experiment also served the purpose of determining whether the irradiation of a single cell led to mitotic inhibition in adjacent cells.

Three days later the approximate irradiation site was placed under observation. Despite the growth which had occurred, there was no doubt that the portion of the gametophyte subjected to intense examination was definitely that portion which had been in the field of view during the pulse. No trace of a dead cell, a necrotic cell, or a cell containing debris could be found. Conceivably the irradiated cell could have been completely obliterated without a trace but this appeared to be unlikely. Numerous mitoses had occurred in the region and all the cells were normal in appearance. Assuming that the irradiated cell had not been totally obliterated, only cytoplasmic recovery and regeneration could explain these observations.
The production of a tiny lesion in an *Osmunda* cell

Figure 36A shows the target cell. Figure 36B shows the immediate effect of a 33 mJ pulse focused above the irradiation site by the 4 mm objective in order to cause minimal damage. Note the retraction of the pigmented debris to one corner of the cell.
This result suggested performing an "enucleation" to discover the cell's response to nuclear dispersion and the pattern of cell death in a cell whose nucleus had been destroyed without any hole being created in the cell wall; an operation not easily performed by any other technique on green plant cells. Again an Osmunda gametophyte cell was used. A pulse of 36 mj was directed through the 2 mm objective, with the laser beam focus being aligned so that the beam could cause maximum damage, in order that the nucleus be completely dispersed. The energy proved to be much too great as the protoplast was instantly coagulated. The cell bulged considerably (figure 37B) and then retracted. All unobliterated chloroplasts were locked in place instantly. It is interesting to note that the side wall adjacent to the nucleus was not ruptured despite the obliteration of all chloroplasts on its surface.

Six days after the operation, an extensive examination showed no changes in the position of the chloroplasts although a loss of pigmentation was clearly evident (figure 37C). Even the debris formed by the pulse was still in place. Adjacent cells, however, were altered considerably in appearance, a major factor in this alteration being a fungal invasion. Much cytoplasmic deterioration and infection was present in all the nearby cells but the coagulated cell appeared to be completely infection-free. Some work on nuclear disruption was being done by Saks and Roth (1963) at this time and so this investigation of response to nuclear
Figure 36A shows the Osmunda target cell immediately prior to the pulse. Figure 37B shows the effect of a 36 mj pulse focused into the cell interior on the nucleus with the 100x oil objective. Figure 37C shows the target cell six days later. The debris had maintained a constant position over this time as had all the nearby chloroplasts. The omega configuration of chloroplasts should be noted in all three photographs. Also, the bulging of the irradiated cell in figure 37B is noteworthy.
Figure 38A shows an Osmunda gametophyte apex prior to irradiation. Figure 38B shows the debris ejected from the irradiation site, the apical cell, after a pulse of 36 mj through the 40x objective. Cellular damage was confined entirely to the apical cell and a small cell adjacent to it. Figure 38C shows the extent to which mitoses had occurred within 25 hours after the pulse.

Figure 39

The ejection of a protoplast through a ruptured cell wall

This Osmunda gametophyte apex was irradiated with a pulse of 36 mj focused with the 4 mm objective.
dispersion was terminated. The relationship of the coagulated cell to the fungal infection is of interest here, however.

An apical cell of an Osmunda gametophyte was punctured in an attempt to determine whether or not the immediately adjacent tissue would quickly become necrotic or whether it could exhibit its normal mitotic behaviour after the pulse. This is an important consideration because the laser beam affords an excellent tool for the study of apical dominance provided that the material used for the study is not extremely dependent upon apical cell factors for proper function.

The ejected debris as shown in figure 38B was bright green and appeared to be a collection of fused chloroplasts. Cellular damage was confined entirely to the apical cell and a small cell adjacent to it. The ejection of a protoplast as it occurs may be better seen in figure 39. This is a photograph of another irradiated gametophyte taken for the purpose of showing the ejection of the protoplast through the ruptured cell wall. Both the number of cells and the pattern of cells proximal to the apex changed after the irradiation of the apical cell as shown in figure 38. Thus mitotic activity had occurred following the pulse. The relative amounts of the tissue shown in figures 38A and 38C indicate the degree of elongation of the gametophyte after irradiation. All the sub-apical cells were of normal appearance.

4.3 The isolation of single cells; application of the laser to a biological investigation

The precision with which one can puncture a plant cell wall or burst a plant cell with a laser beam suggested
experiments involving the isolation of a single cell from a multicellular plant tissue by bursting its neighbors. This is difficult to do with conventional microsurgical techniques. The laser microsurgical technique, however, showed promise of achieving single cell isolations with ease, presumably leaving the isolated cell in a completely undamaged state. The culture of single cells isolated from multicellular tissue can yield much useful information to the developmental biologist and so laser beam isolations followed up by the culturing of the cells was attempted on an organism of simple structure. The protonema of the moss *Bryum stenotrichum* C.M. was selected as the experimental material because of its large uniform cells, simplicity of form and ease of culture. Filaments comprised of only three cells were selected. These had been grown on a special sugarless culture medium layered on a glass slide (see table 5).

The spores were dusted on the medium-coated slide in preparation for germination in a humid chamber. The slide was only removed from the chamber for the operations and subsequent observations because the film of medium on the slide progressively lost its moisture over the duration of the experiment. In fact the experiment was automatically terminated when the film lost so much moisture that it effectively provided no substrate at all. Previously it had been found that thicker films greatly scattered the transmitted illumination, thus preventing good viewing of the material, and so the use of thicker films was not advisable for increasing the total
culture time. In all, ten days were required to produce an ample supply of three-celled filaments, the growth being so uniform that nearly all of the filaments on the slide were comprised of exactly three cells.

For the operation, a pulse of 15 mj was directed through the 4 mm objective sixty microns above the upper level plastids in order to rupture the target cell's wall and disperse the protoplast. Many times, much of the protoplast was ejected to a position tens of microns away from the irradiation site. Fifteen apical cells were isolated by disruption of the second cell of the filament (figure 43B). As a comparative study ten operations were carried out in which the second or subapical cell was isolated by disruption of the first and third (see figure 40).

Within six days, ten of the fifteen isolated apical cells had undergone at least one mitosis, with three of these apical cells having produced a filament of three. The first cross-wall formation had occurred in four cells within 49 hours. These initial mitoses yielded cells of normal appearance and in all cases the cross-wall was produced without the formation of a protuberance from the side-wall. Table 4A summarizes the experiment involving the fifteen isolated apical cells.

Within six days, seven of the ten isolated sub-apical cells had undergone one mitosis with a protuberance having been initiated on an eighth cell. The production of a protuberance from the side-wall was accompanied later by
Figure 40A shows a three-celled protonema of the moss *Bryum stenotrichum* C. M. prior to irradiation. Figure 40B shows the bursting of a cell after a pulse of 15 mj brought to a focus 60μ above the upper cell wall by the 4 mm objectives. Figure 40C shows the disruption of the apical cell by the same technique, thus isolating the subapical cell.
cross-wall formation in isolated apical cells

cross-wall formation as seen in some isolated subapical cells

Figure 41
Cross-wall formation in isolated cells
formation of a transverse cross-wall (see figures 41 and 42) and thus the cross-wall did not serve the purpose of cutting the protuberance off from the rest of the isolated cell. Often the bulging of the side-wall was not very prominent. Whether there is a distinct protuberance produced before cross-wall formation in some instances and not in others or whether there is a gradient of protuberance sizes could not be deter-mined with the small sample size. The protuberance could be formed at any point along the side-wall of the isolated cell. Within 33 hours a protuberance had been formed on each of two isolated subapical cells, one proximal and one distal, with the cross-walls appearing within 49 hours. The total results of the subapical cell isolations may be found in table 4B.

The protonemal cells obviously had a considerable degree of totipotency with no real difference being detected between the apical cell and the subapical cell with respect to capacity to undergo a mitosis after isolation. Comparable studies were attempted with a tiny metal needle but all attempts to puncture the cell wall failed. The walls were very tough and elastic. Needle pressure from above either caused the protonema to slip laterally or deformed the cell wall until enough pressure was applied so that the protonema was pressed into the medium. No puncture with associated discharge of protoplast could be effected. It would be interesting to attempt the experiment with a micromanipulator for a compara-tive study but certainly the speed and ease of operation could not be duplicated.
Figure 42

Evidence for mitosis in an isolated subapical cell

Figure 42A shows a subapical Bryum protonema cell isolated by the technique described in figure 40. Figure 42B is a picture taken six days later. Note the cross-wall and the protuberances.
The isolation of an apical cell and subsequent regeneration

Figure 43A shows a three-celled Bryum protonema. Figure 43B shows the isolated apical cell. The subapical cell was burst by a 15 mj pulse focused 60μ above the upper cell wall using the 4 mm objective. Figure 43C was taken six days later. The cross-wall is clearly evident.
Table 4A
The isolated apical cells

<table>
<thead>
<tr>
<th></th>
<th>49 hours</th>
<th>73 hours</th>
<th>127 hours</th>
<th>146 hours</th>
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<tbody>
<tr>
<td>1</td>
<td>little change</td>
<td>little change</td>
<td>first cross-wall formed</td>
<td>little change</td>
</tr>
<tr>
<td>2</td>
<td>first cross-wall formed</td>
<td>little change</td>
<td>second cross-wall formed</td>
<td>little change</td>
</tr>
<tr>
<td>3</td>
<td>great cell elongation</td>
<td>first cross-wall formed</td>
<td>little change</td>
<td>little change</td>
</tr>
<tr>
<td>4</td>
<td>first cross-wall formed</td>
<td>little change</td>
<td>second cross-wall formed</td>
<td>little change</td>
</tr>
<tr>
<td>5</td>
<td>little change</td>
<td>little change</td>
<td>first cross-wall formed</td>
<td>little change</td>
</tr>
<tr>
<td>6</td>
<td>first cross-wall formed</td>
<td>little change</td>
<td>little change</td>
<td>little change</td>
</tr>
<tr>
<td>7</td>
<td>damaged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>little change</td>
<td>little change</td>
<td>little change</td>
<td>first cross-wall formed</td>
</tr>
<tr>
<td>9</td>
<td>damaged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>damaged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>little change</td>
<td>little change</td>
<td>little change</td>
<td>little change</td>
</tr>
<tr>
<td>12</td>
<td>little change</td>
<td>little change</td>
<td>little change</td>
<td>little change</td>
</tr>
<tr>
<td>13</td>
<td>little change</td>
<td>little change</td>
<td>first cross-wall formed</td>
<td>little change</td>
</tr>
<tr>
<td>14</td>
<td>first cross-wall formed</td>
<td>little change</td>
<td>little change</td>
<td>second cross-wall formed</td>
</tr>
<tr>
<td>15</td>
<td>great cell elongation</td>
<td>first cross-wall formed</td>
<td>little change</td>
<td>little change</td>
</tr>
</tbody>
</table>
Table 4B
The isolated subapical cells

<table>
<thead>
<tr>
<th></th>
<th>little change</th>
<th>great cell elongation</th>
<th>little change</th>
<th>first cross-wall formed, each cell had protuberance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>damaged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>little change</td>
<td>proximal protuberance formed</td>
<td>little change</td>
<td>first cross-wall formed, each cell had protuberance</td>
</tr>
<tr>
<td>4</td>
<td>little change</td>
<td>little change little change</td>
<td></td>
<td>first cross-wall formed, protuberance bisected by cross-wall</td>
</tr>
<tr>
<td>5</td>
<td>little change</td>
<td>little change little change</td>
<td></td>
<td>first cross-wall formed, no protuberance</td>
</tr>
<tr>
<td>6</td>
<td>damaged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>little change</td>
<td>medial protuberance formed</td>
<td>little change</td>
<td>little change</td>
</tr>
<tr>
<td>8</td>
<td>distal protuberance formed</td>
<td>first cross-wall formed</td>
<td>little change</td>
<td>little change</td>
</tr>
<tr>
<td>9</td>
<td>proximal protuberance formed</td>
<td>first cross-wall formed</td>
<td>little change</td>
<td>little change</td>
</tr>
<tr>
<td>10</td>
<td>little change</td>
<td>little change proximal protuberance formed</td>
<td></td>
<td>first cross-wall formed, no sign of protuberance</td>
</tr>
</tbody>
</table>

In this table proximal and distal refer to position with reference to the apex.
Table 5
The moss culture medium

Knudson's stock solution
- trace elements B5
- ferric citrate
- K$_2$HPO$_4$
- glass distilled water
- agar (Difco Bacto-agar)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knudson's stock solution</td>
<td>250 cc</td>
</tr>
<tr>
<td>trace elements B5</td>
<td>1 ml</td>
</tr>
<tr>
<td>ferric citrate</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>glass distilled water</td>
<td>750 cc</td>
</tr>
<tr>
<td>agar (Difco Bacto-agar)</td>
<td>8 gm</td>
</tr>
</tbody>
</table>

Knudson's stock solution
- Ca(NO$_3$)$_2$.4H$_2$O
- (NH$_4$)$_2$SO$_4$
- MgSO$_4$.7H$_2$O
- glass distilled water

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(NO$_3$)$_2$.4H$_2$O</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.5 gm</td>
</tr>
<tr>
<td>glass distilled water</td>
<td>1000 cc</td>
</tr>
</tbody>
</table>

Trace elements B5
- H$_2$SO$_4$ (specific gravity 1.83)
- MnCl$_2$.4H$_2$O
- ZnSO$_4$.7H$_2$O
- CuCl$_2$.2H$_2$O
- H$_3$BO$_3$
- CaCl$_2$.6H$_2$O
- Na$_2$MoO$_4$.2H$_2$O
- glass distilled water

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$SO$_4$ (specific gravity 1.83)</td>
<td>0.5 cc</td>
</tr>
<tr>
<td>MnCl$_2$.4H$_2$O</td>
<td>2500 mg</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>50 mg</td>
</tr>
<tr>
<td>CuCl$_2$.2H$_2$O</td>
<td>15 mg</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>2000 mg</td>
</tr>
<tr>
<td>CaCl$_2$.6H$_2$O</td>
<td>30 mg</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>25 mg</td>
</tr>
<tr>
<td>glass distilled water</td>
<td>1000 cc</td>
</tr>
</tbody>
</table>

Ferric citrate solution
- FeC$_6$H$_5$O$_7$.5H$_2$O
- glass distilled water

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tr>
<td>FeC$_6$H$_5$O$_7$.5H$_2$O</td>
<td>2.5 gm</td>
</tr>
<tr>
<td>glass distilled water</td>
<td>100 cc</td>
</tr>
</tbody>
</table>

K$_2$HPO$_4$ solution
- K$_2$HPO$_4$
- glass distilled water

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$</td>
<td>25 gm</td>
</tr>
<tr>
<td>glass distilled water</td>
<td>100 cc</td>
</tr>
</tbody>
</table>
It was decided to isolate apical cells and culture them in a sealed petri dish half-filled with the sugarless culture medium (table 5) in order to observe the protonemal growth over a long period of time. Over a time span of many weeks, all five apical cells isolated with the laser beam regenerated and produced an advanced protonema with no indication of cessation of further development.

Thus the apical cell in a very young stage of the protonema of the moss *Bryum stenotrichum* C.M. was shown to be totipotent, the subapical cell was shown to have extensive regenerative capacity with no polarity impressed upon initial protuberance formation, and the isolation technique was demonstrated to be simple and quick. The ease of operation as shown in this work cannot be duplicated by other techniques.

4.4 Discussion of laser microsurgery

The cell studies were carried out with the purpose of demonstrating the problems and considerations involved in laser microsurgery. The usefulness of achieving extremely small spot sizes has already been discussed as have some of the factors involved in laser-cytoplasm interactions. The role of vapor bubble formation in producing obliteratorive effects was mentioned as well as the role of cell pigments, and studies on the plant cell wall proved to be very interesting. A remarkable plasticizing effect was demonstrated and it was found that tiny localized coagulations could be created within living cells without rupturing the cell wall. It was shown that a nucleus could be completely obliterated without bursting
the cell wall and although the biological application of this "enucleation" technique was not investigated it was the opinion of this writer that the laser-microscope complex affords a very promising new tool for research on nuclear-cytoplasmic interactions. The single cell study involving the response of the nucleus to extensive cytoplasmic damage is ample proof of the applicability of the particular instrumentation used and certainly the vacuole work yielded a surprising result. In the literature may be found descriptions of operations performed on nuclei or a portion of one using extremely low beam energies as well as studies on individual organelles. Despite the great interest shown in laser studies on the cell, however, the biochemical aspects of laser beam-cytoplasmic interactions are completely open to speculation. Nevertheless, laser microsurgery at the intracellular level shows promise of making a great contribution to cytology and developmental biology in general.

At the cell and tissue level the laser-microscope complex may also be a valuable tool. The studies on response to damage indicate that slightly damaged cells may recover and that one cell surrounded by many may be killed without the death of its neighbors. Certainly the problem of deranged or inhibited mitotic ability in cells adjacent to an irradiated site did not prove to be a problem at all in the researches undertaken. These features leave future embryological work with laser beams open to great speculation because now the embryologist has a simple new tool which will allow him to
remove specific cells from a developing embryo while conceivably reducing trauma to an absolute minimum. Because of many unknown quantities, however, a note of caution should be injected into this type of speculation. Apical dominance and morphological work with plant meristems may also be an important area for intensive study with the laser beam. Preliminary studies by this author have already demonstrated that a fern gametophyte apical cell may be destroyed without causing any immediate necrosis of the apex or mitotic inhibition of sub-apical cells. Also, it has been found that a lesion can be created within the apical cell of a sporophyte of the ostrich fern, Matteuccia struthiopteris (L.) Todaro with good accuracy using a pulse of ten millijoules focused with the eight millimeter objective. Comparative studies with the micromanipulator and microbeams of various types are in order but it is the opinion of the author that the laser-microscope complex will find its place as a valuable tool for the embryologist and morphologist.

For obvious reasons laser microsurgery cannot replace the classical microsurgical techniques nor can it entirely supplant microbeams as biological tools. It can, however, act as a supplement to these other techniques and can also allow the experimenter to enter new avenues of research such as the study of the cell's response to coherent light coagulation damage. A laser allows the researcher to use light having a degree of coherency, monochromaticity and energy density which previously was impossible to achieve. Given the appropriate
technique a focused beam can be used with great versatility to perform cellular and intracellular surgery accompanied by only very minor effects on intervening material. A comparison of traumatic side-effects produced during microsurgery between a good laser technique and the best micromanipulator is yet to be done. The possibility does exist, however, that in some cases the laser beam would prove superior as a tool and certainly in many cases the focused laser beam could be shown to be a better research tool for a problem in question because of ease and speed of operation. This feature is amply illustrated by the work with moss protonemata.

Microsurgery is not the only biological field which may benefit greatly from laser technology. The medical sciences and cancer research fields are showing great interest and one result of this interest is the recent design of machines for laser photocoagulation of the eye. Biochemical studies on laser beam interactions with living material and important biological molecules must certainly be carried out. For this avenue of investigation high energy density unfocused beams may provide an important research tool. Indeed, some work of this nature has already been initiated and was dealt with in the literature review. The future of all biological work with the laser rests on scientists of different disciplines working cooperatively together so that the problems involved may be attacked directly and the results fitted harmoniously into an interdisciplinary context.
5. SUMMARY

An instrument was assembled which consisted essentially of a pulsed ruby laser utilized in conjunction with a triocular, multi-objective microscope to yield a versatile tool for microsurgical experimentation. The components of this instrument were described in detail as was the laser light path through the system, especially the vertical misalignment of the laser beam focal spot with the object plane in focus. The theory of laser action as related to the pulsed ruby laser was dealt with in an elementary manner, the properties of the laser beam were discussed and the reasons for making use of a focused laser beam were outlined. The versatility of the instrument was elaborated upon in great detail, with the area and intensity of the laser effects produced related to the lens magnification, energy input into the microscope and the degree of vertical misalignment of the laser beam focal spot with the tissue or cell in focus in the object plane.

A model, consisting of a very thin film of stained protein gel mounted on a glass slide, was constructed from which one could predict, in advance, the effect that a particular combination of lens magnification, energy, and vertical misalignment would have with regard to the size and severity of regions of destruction produced in thin layers of green plant material. With slight modifications the model also allowed one profitably to operate on relatively large specimens such as fern apices and also enabled one to plan
intracellular operations such as organelle destruction or localized cell wall disruption.

Photographic studies were undertaken to gain some insight into the primary and secondary effects produced by focused laser radiation on living material. By primary effects are meant the destructive effects such as water vaporization and membrane and cell wall disruption at the site of absorption. Secondary effects include all factors leading to cell death which resulted from the primary destruction. This includes cell death resulting from the loss of nucleus, inability to maintain proper osmotic relations, and other such phenomena. These studies were expanded to encompass the various operations such as cell wall puncture, chloroplast coagulation, bursting of the nuclear membrane and other such operations which could be performed at will and independently of each other.

Some attempts were conducted experimentally to gain a knowledge of the limitations imposed on the particular instrumentation used with regard to both the energy range available, problems inherent in the operating technique and the selection of appropriate biological material. Studies were made of such factors as specimen pigmentation, tissue and cell characteristics, restriction of destructive effect, achievable "useful" spot size, and the depth of effect to illustrate the many factors one must consider in planning a particular operation using the model as a guide. This knowledge was utilized when some simple biological problems were undertaken with a view to demonstrating the immediate
applicability of the instrument and the ease of the techniques employed as well as successfully attaining the desired results. In addition, a purely biological study involving the isolation of single cells with laser beams was undertaken with very satisfactory results.

Present attainments by other workers using laser systems on biological materials were discussed and possible future developments speculated upon. In the introduction and literature review the special properties of laser light were related to each particular application thus far shown to be of promise in biological research.
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