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Technique for Cellular Microsurgery Using the 193-nm Excimer Laser

Daniel Palanker, MSc, Shoshanit Ohad, MSc, Aaron Lewis, PhD, Alex Simon, MD, Joseph Shenkar, MD, Shmuel Penchas, MD, and Neri Laufer, PhD

Hadassah Laser Center and Division of Applied Physics, Hebrew University of Jerusalem, Givat Ram 93707 (D.P., A.L.); In Vitro Fertilization Unit, Department of Obstetrics and Ginecology, Hadassa University Hospital, Kiryat Hadassa Ein Karem, (S.O., A.S., J.S., S.P., N.L.), Jerusalem, 91120 Israel

A new cell surgery technique has been developed to produce well-defined alterations in cells and tissue without detectable heating and/or other structural damage in the surroundings. The technique involves the use of an argon fluoride excimer laser, in the deep ultraviolet (UV) region of the spectrum at 193 nm, which is guided through a glass pipette filled with a positive air pressure. To demonstrate the method, holes were drilled in the zona pellucida of mouse oocytes. The diameter of the drilled hole was determined by the pipette tip size, and its depth by an energy emitted per pulse and number of pulses. Scanning electron microscopy of the drilled mouse oocytes showed uniform, round, well-circumscribed holes with sharp edges. Oocytes that had their zona pellucida drilled with this new method fertilized in vitro and developed to the blastocyst stage in a rate similar to that of control group. These results demonstrate the nonperturbing nature of this cold laser microsurgical procedure. In addition to the extension of our results for clinical in vitro fertilization purposes, such as enhancement of fertilization and embryo biopsy, there are wide-ranging possible uses of our method in fundamental and applied investigations that require submicron accuracy in cellular alteration.

Key words: laser surgery, microsurgery, photoablation, zona drilling

INTRODUCTION

For the past 20 years, a variety of lasers have been used as microbeams to perform cell surgery. The first results in this area were obtained with the ruby laser [1] one year after this laser was developed. After the argon ion laser was introduced it became the microbeam of choice in cellular applications [2]. With the development of the tunable flash lamp dye laser, a microbeam system was built using this laser as the light source [3]; subsequently, the Nd:YAG laser [4] and the Nd:YAG pumped dye laser were introduced as potential tools in subcellular surgery [5].

The possible mechanisms by which these lasers interact with biological material are either heat generation caused by linear or multiphoton absorption processes [6,7] or dielectric breakdown of the molecular constituents caused by the very high peak power of a pulsed laser focused to a small spot. The dielectric breakdown of the molecules cause shock waves in the material that may be damaging to the surrounding. An example of this effect is the well-known observation of the explosive detachment of the retina caused by a pulsed, highly focused Nd:YAG laser [8].

An ideal tool for microsurgery with the least damage to the surrounding material would be a laser that would interact with tissue in a photochemical rather than thermal or dielectric breakdown mode. This has been difficult to achieve because biomolecules are composed of bonds be-

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Address reprint requests to Dr. Aaron Lewis, Division of Applied Physics, Bergman Blvd., Hebrew University of Jerusalem, Givat Ram, Jerusalem, 93707 Israel.

tween carbon, oxygen, and nitrogen atoms which have energies of dissociation in a regime that corresponds to the deep ultraviolet (UV) region of the spectrum where no lasers were available. In fact, with the introduction of the argon fluoride excimer laser, such photon energies can readily be achieved. The wavelength of this laser is 193 nm, which is the shortest available laser wavelength that can propagate in air. It has been argued [9,10] that such a wavelength raises biomolecules to a dissociative excited state. From this state, the molecules enter a photochemical pathway in which there is direct breakup of the molecular bonds. In principle, all the energy of the photon goes into this ablation process, breaking up molecules rather than heating the material.

In fact, excimer lasers exhibit many emissions, some at longer and others at shorter wavelengths. The longer wavelengths at 248 nm and 308 nm result in ablation but with increasing thermal effects and depth of penetration [10]. In addition, these wavelengths are known to cause considerably greater damage to genetic material than does 193 nm radiation [11–13]. Furthermore, the 193-nm wavelength is the shortest that can propagate in air. A problem with the argon fluoride excimer laser for microbeam applications is the lack of aberration-free lenses that are capable of focusing this beam to submicron dimensions. In addition to these optical limitations, there is a further problem in the application of the ArF excimer laser within biological aqueous solutions caused by the strong absorption of the 193 nm radiation by such media.

Because of these limitations, the principal application of the ArF excimer laser in biology and medicine is in the field of refractive surgery. In this application, both the lack of heating of the surrounding tissue and the ability of submicron control of the ablation depth are used for corneal sculpturing [14–16]. However, this use of the excimer laser does not require a micron-size beam and does not involve the delivery of this laser beam into a liquid surrounding, which are of crucial importance for cellular microsurgery.

In this paper, we apply for the first time the precision ablation without heat deposition qualities of this laser to cellular surgery. This is accomplished by combining the unique properties of this laser beam with micropipette technology that is widely used in cell biology. As a sensitive assay of this combination of technologies for cell surgery, in vitro fertilization of mouse oocytes was employed. Specifically, small holes were drilled in the zona pellucida (the 8-μm-deep outer wall) of the oocyte and the subsequent fertilization and development rate was monitored in comparison with the already existing method of fertilization enhancement using mechanical zona pellucida disruption [17–20]. A typical result of such mechanical zona pellucida disruption is shown in Figure 1. Thus, the object of this study was (1) to morphologically characterize these holes as an important step to the general application of this new technique in cellular microsurgery, and (2) to evaluate possible damaging effects of the method with the extremely sensitive process of mouse oocyte fertilization and development in vitro.

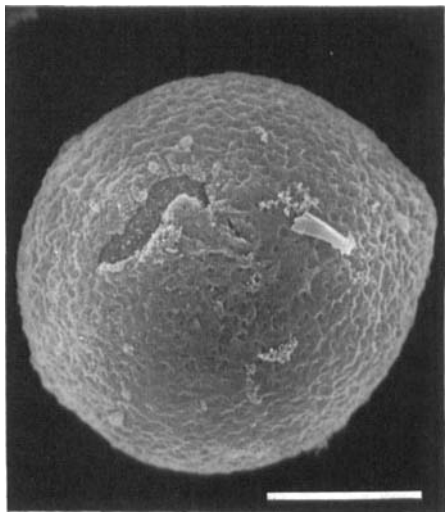

Fig. 1. Scanning electron microscopy of mouse oocyte with a mechanically disruptured zona pellucida. Bar = 20 μm. ×1,700.

MATERIALS AND METHODS

Laser Equipment

A diagrammatic representation of the drilling system used in this experiment is depicted in Figure 2. A model 103 MSG, Lambda Physik (Gottingen, West Germany) argon fluoride (ArF) excimer laser with a 193-nm wavelength was used. The absorption depth of biological materials at 193 nm is extremely small (<1 μm) [10]; therefore, with each pulse of the laser a thin layer of material may be removed. This characteristic of the laser–tissue interaction permits very precise control of the drilling process.

The difficulties with the use of this laser are its inability to penetrate aqueous biological media and the limitations in focusing its radiation

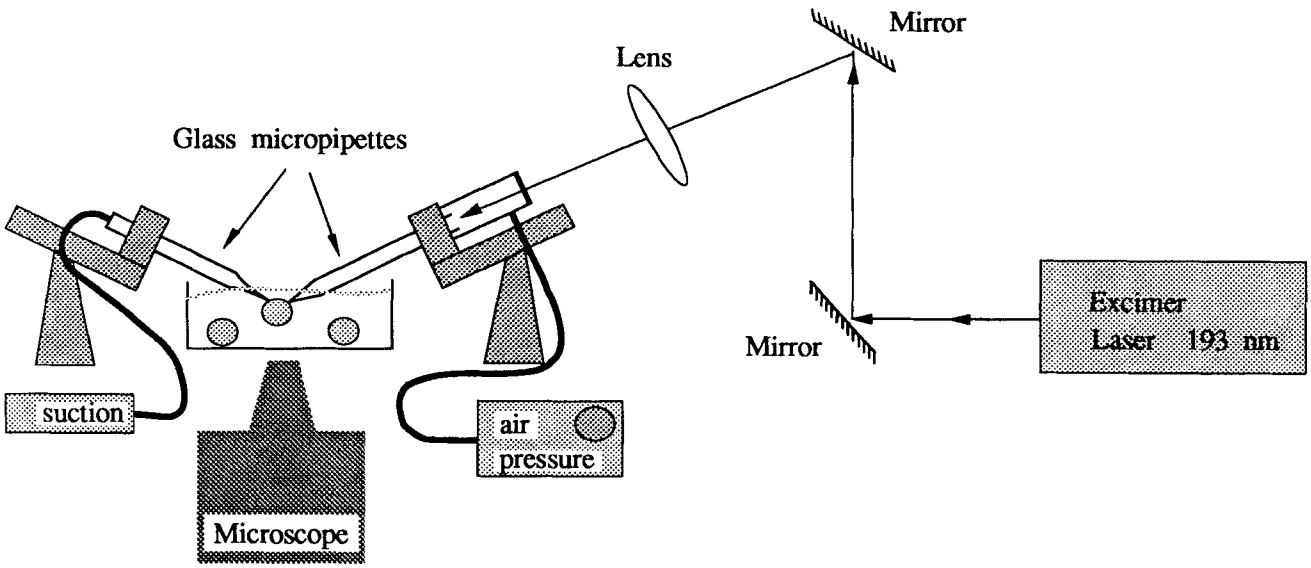

Fig. 2. Diagrammatic representation of the argon fluoride excimer laser-drilling system.

with lenses to a small accurate spot of required diameter. Our solution of these optical problems was to combine the unique properties of the ArF laser with micropipette technology that is already being used in cellular micromanipulation. In this combination, the laser beam is directed by series of mirrors and a long focal ($f_L = 1{,}000$ mm) lens through the large end (0.5 mm) of a glass micropipette placed in the aqueous media. The pulses of 193 nm light with an energy fluence of about 5–10 mJ/cm^2 emanate from the pipette in which a constant air pressure is maintained. The air pressure should be enough to prevent a liquid entering into the pipette and not so high that the air bubbles will exit the pipette tip. Because of high absorption of the laser radiation by liquid, the pipette was brought into near contact to the zona pellucida, and up to four pulses of the laser beam were required until the zona pellucida was penetrated to form a hole. The glass walls of the pipette and the surrounding liquid protect the oocyte from the laser light very effectively, so the spot size is determined only by the diameter of the pipette tip.

The alumina silicate pipettes used in our experiments were pulled from glass capillaries with a 1-mm outer diameter (O.D.) and 0.58-mm inner diameter (I.D.) (Sutter Instrument Co., Novato, CA). The micropipettes used had tip diameters of 3–5 μm. These pipettes were pulled to the required dimensions by a Flaming/Brown Micropipette Puller, model P-80/PC (Sutter Instrument Co.).

The energy of the laser pulse that exits from the pipette tip is extremely small (~1 nJ), making it difficult to measure an energy fluence directly. To circumvent this problem, the power that exits pipette tips of 150–250 μm was measured at a pulse repetition rate of 100 Hz and with a voltage of 24 kV, which was considerably higher than the 21 kV used in our experiments with the smaller pipettes. The relationship of the laser output at 24 kV that we used for the energy fluence measurements and at 21 kV that we used in the experiments allowed us to calculate the energy fluence in the pipette tip. The measurements were accomplished with a Laser Power/Energy Meter model DGS with a model 03AP head, Ophir Optronics Ltd. (Jerusalem, Israel). Thus, the total energy per pulse that exits from a 5 μm pipette tip in our experiment is about 1–2 nJ. After completion of laser drilling, both drilled and control oocytes were inseminated and cultured as described.

Sperm Cells and Oocyte Recovery

Sperm cells and oocytes were obtained from C57Black × BALB C hybrid mice. Superovulation was induced in 6-week-old females by intraperitoneal injection of 10 IU of pregnant mare serum (PMSG) (Gestyl; Organon, Oss., Holland) followed 48 hr later by 10 IU of human chorionic nonadotropin (hCG) (pregnyl; Organon). On the morning following hCG injection, animals were sacrificed; oocytes were recovered and placed in Hoppe and Pitts culture medium [21]. Eggs were denuded of cumulus cells by treatment with hy-

aluronidase (1 mg/ml) for 5–7 min. After the cumulus cells were dispersed, oocytes were washed, and some were subjected for laser drilling.

Three-month-old male mice were sacrificed by cervical dislocation; the sperm were expressed from the vasa deferentia and caudae epididymides into 2 ml of culture medium. A 2-hr incubation time followed to allow for sperm dispersal and capacitation. The laser-drilled oocytes and the nonmanipulated oocytes were placed in 1 ml of culture medium and inseminated with sperm, to yield a final count of 5×10^5, or 5×10^4 sperm/ml.

Culture Media

Micromanipulation was carried out in a phosphate-buffered saline (PBS) solution supplemented with 0.4% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO). Following laser drilling, in vitro fertilization was carried out in two steps. For the first 24 hr, oocytes were inseminated and cultured in a modified Hoppe and Pitts medium in which sodium lactate was omitted and sodium chloride concentration was 6.4 gr/L. Later on, the embryos at the two-cell stage were transferred to a high lactate medium [22] for further development. Both media were supplemented with 0.4% BSA. Embryo development to the two-cell and blastocyst stages was assessed after 24 and 72 hr, respectively.

Scanning Electron Microscopy

Following laser drilling, oocytes were immediately fixed in 1% glutaraldehyde for 1 hr; an additional fixation step was carried out in 2% glutaraldehyde solution for 1 hr. Following fixation, oocytes were placed on a coverslip precoated previously with poly-L-lysine hydrobromide 1 mg/ml (Sigma Chemical Co.) and fixed with 1% osmium tetraoxide for 1 hr [23]. Coverslips with the attached oocytes were then dehydrated by washes in increasing ethyl alcohol concentrations. Specimens underwent a critical point dehydration with CO_2 and were subsequently coated with gold in a Polaron ES-100 sputter coater and examined with a JFM-200 CS scanning electron microscope.

RESULTS

Laser Drilling and Its Morphological Characterization

The drilling procedure by the laser beam was accomplished in a stepwise fashion. The pipette was brought into contact with the zona pellucida, and a hole was formed with several controlled laser pulses. An example of the smooth and narrow hole in the zona pellucida as seen in a light microscope is shown in Figure 3. The hole being bored by the laser light looks like a narrow white strip in front of the pipette tip. The width of the hole was determined by the size of the pipette tip and the depth by the energy of the laser beam used, as well as the number of pulses.

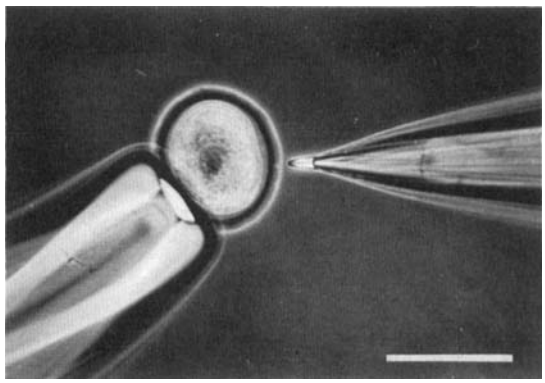

Fig. 3. Phase-contrast light micrograph of the mouse oocyte after the laser-drilling procedure. The drilling pipette points at a smooth and narrow hole generated by laser irradiation. Bar = 50 μm. ×320.

We found that in order to produce accurate holes with sharp edges in the zona pellucida and to avoid any damage to the ooplasm, 3–5 pulses with an energy fluence of 5–8 mJ/cm^2 per pulse were found to be optimal. To ensure that the ooplasm was not damaged, the hole was drilled at the point where the zona pellucida was separated by at least a few microns from the ooplasm. It is interesting to point out that microscopic observation of the ablation process indicated no recoil of the cell wall or other movements in the cell. The ablated material disappears in a very slow fashion (~1 sec). The process looked like a dissolution of the ablation products in the surrounding liquid without any gas bubble formation.

In order to better characterize the drilled holes and the effect of laser on the integrity of the oocytes, scanning electron microscopy (SEM) was performed. Figure 4a shows a SEM photograph of a mouse oocyte treated by the laser procedure. Two 3 μm holes in the zona pellucida can be seen, and the zona pellucida structure is fully preserved. This result demonstrates the possibility to perform numerous holes in the zona pellucida in order to enhance sperm interaction with the ooplasm and still retain the integrity of both the zona pellucida and ooplasm. Higher magnifica-

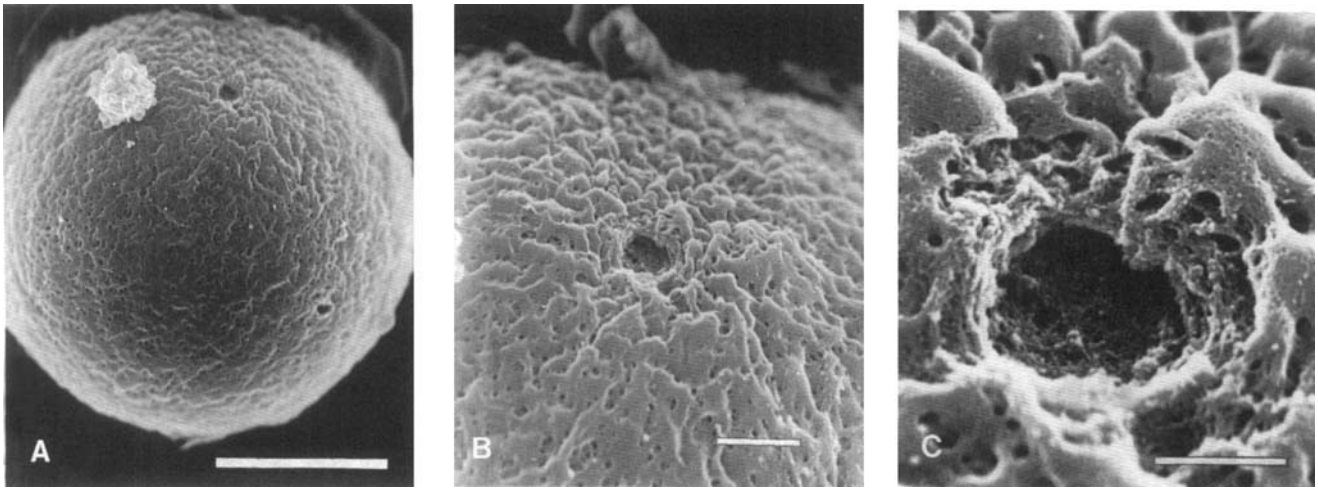

Fig. 4. Scanning electron microscopy of mouse oocytes drilled by a laser beam. **A.** Two holes in an oocyte. The webbed mesh architecture of the zona pellucida is preserved. Bar = 20 μm. ×2,000. **B.** Sharp circular edges of a drilled hole 3 μm in diameter. Bar = 5 μm. ×5,000. **C.** Laser-drilled hole showing the unaffected zona pellucida structure. Bar = 2 μm. ×20,000.

tions (Fig. 4b,c) illustrate the circular and sharp edges of the holes and demonstrate that the damage to the zona pellucida is local without any extension.

Effect of Drilling on Fertilization and Embryo Development In Vitro

The development stages after insemination of laser-drilled oocytes and controls is shown in Table 1. For each sperm concentration, the fertilization rate of the laser-drilled oocytes was comparable to that of the control. The ability of the fertilized oocytes to develop into the blastocyst stage, as assessed 72 hr after insemination, did not differ between the two groups and suggests that the laser-drilling procedure did not interfere with normal development. No effect of enhanced fertilization was observed in this experiment because the drilled holes were one half the diameter of the sperm cells, so no sperm penetration was possible. Comparing the blastocyst morphology at 72 hr, it was observed that a significantly ($P < 0.002$) higher rate have hatched in the laser-drilled group (73%, 11/15) than in the nontreated embryos (17%, 5/29). Thus, at these sperm concentrations, laser drilling does not seem to have an adverse effect on embryo development processes.

DISCUSSION

It was first reported in 1982 that when pulsed UV laser radiation falls on the surface of an organic polymer, the material at the surface is etched at depth of 0.1 μm to several microns [10]. The main features of this phenomenon that distinguish the interaction of UV laser pulses from visible or infrared laser pulses are the control that can be exercised over the depth of etching and the lack of thermal damage to the substrate. The ablation depth of the polymer is a function of the energy flow deposited during a single pulse and the number of pulses fired at the surface. This potential of the 193-nm excimer laser to ablate biopolymers precisely and without heat deposition is already in use in medicine in the field of refractive surgery [15,16].

This study is the first to describe the use of excimer laser technology in the field of cell surgery exampled by zona pellucida drilling. Our results demonstrate that an excimer laser at 193 nm is capable of producing extremely accurate and well-circumscribed holes with sharp edges; also, the well-preserved structure of the zona pellucida around the etched area provides further evidence for the capacity of this laser to ablate materials with a high degree of precision and minimal damage to the surrounding structures.

The possibility of DNA damage by UV radiation during excimer laser treatment has previously been investigated in a few experimental models. Yeast cells demonstrated a significant amount of DNA repair after excimer irradiation at 193 nm, thus suggesting injury to nucleic acids [24]. By contrast, it was shown that 193 nm radi-

TABLE 1. In Vitro Fertilisation of Laser-Drilled and Untreated Oocytes in Two Sperm Concentrations Used for Insemination

	Sperm/ml inseminated	No. oocytes inseminated	Two-cell stage after 24 hr (%)	Blastocysts after 72 hr (%)	Hatching after 72 hr (%)
Control	5×10^5	50	22 (44)	17 (77)	2 (13)
Laser drilled	5×10^5	20	11 (55)	8 (72)	6 (75)
Control	5×10^4	47	18 (38)	12 (66)	3 (25)
Laser drilled	5×10^4	21	12 (56)	7 (57)	5 (71)

ation is not mutagenic in a mammalian cell mutagenesis assay [11] and the scattered radiation into tissue during ablation does not initiate unscheduled DNA synthesis [25]. Another study indicated that 193 nm light did not transform mouse fibroblasts in culture [12]. Our work, employing mouse gametes, demonstrates that fertilization and development to the blastocyst stage were not adversely affected by laser drilling and further substantiates the notion that this treatment is safe and has no cytotoxic effects.

Our observation that laser drilling enhanced blastocyst hatching corroborates similar observations in mouse and humans following mechanical drilling [26,27]. It was previously found that the hatching process of embryos following partial zona dissection by a mechanical disruption was altered. The zona of drilled oocytes did not thin as it did normally during blastocyst expansion and hatching occurred earlier and at a higher frequency. Since hatching is impaired in human IVF and since only one in four fully expanded blastocysts derived from IVF will hatch, it was suggested that hatching could be enhanced by creating artificial slits in the zona pellucida, a procedure termed *assisted hatching*. Thus, creating holes in the zona pellucida is known to enhance hatching. This is also seen in our excimer laser method.

It is concluded that it is feasible to perform zona pellucida drilling in a reproducible and extremely precise manner with an argon fluoride excimer laser at a wavelength of 193 nm. This treatment seems to be safe and does not interfere with the rate of fertilization and development in vitro up to the blastocyst stage. Future experiments will investigate whether such zona drilling facilitates fertilization at low sperm concentrations. The effect of this radiation on development in vivo requires thorough examination before its clinical application is considered.

In view of the results that have already been obtained, several additional applications of this new approach to microsurgery seem to be possible. In the area of research, we can see applications for the accuracy of the procedure we have described in the field of developmental biology in which critical regions of fertilized eggs are removed in order to relate specific regions of the fertilized egg to the resulting development of the organism. In the area of diagnostics, it seems likely that embryo biopsy could be successfully undertaken with the precision and the technical flexibility of our new method. Finally, in the growing field of cloning for improvements in animal husbandry, the accuracy that we provide may increase the efficiency of these procedures.

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REFERENCES

1. Bessis M, Gires F, Mayer G, Nomarski G. Irradiation des organites cellulaires l'aide d'un laser a Rubis. C R Acad Sci 1962; 255:1010–1112.
2. Berns M, Rounds D. Cell surgery by laser. Sci Am 1970; 222:98–110.
3. Berns M. Partial cell irradiadiation with a tunable organic dye laser. Nature 1972; 240:483–485.
4. Tao W, Wilkinson J, Stanbrige E, Berns M. Direct gene transfer into human caltured cells facilitated by laser micropuncture of the cell membrane. Proc Natl Acad Sci USA 1987; 84:4180–4184.
5. Joussot-Dubien J, ed. Lasers in Physical Chemistry and Biophysics. Amsterdam: Elsevier, 1975; pp 389–401.
6. Berns M, Salet C. Laser microbeams for partial cell irradiation. Int Rev Cytol 1972; 33:131–154.
7. Berns M. A possible two-photon effect in vitro using a focused laser beam. Biophys J 1976; 16:973–977.
8. Mosier M, Champion J, Liaw L, Berns M. Retinal effects of the frequency-doubled (532 nm) YAG laser: Histopato-

logical comparison with argon laser. Lasers Surg Med 1985; 5:377–404.
9. Yeh J. Laser ablation of polymers. J Vac Sci Technol A 1986; 4:653–658.
10. Srinivasan R, Braren B. Ultraviolet laser ablation of organic polymers. Chem Rev 1989; 89:1303–1316.
11. Green H, Boll J, Parrish J, Kochevar I, Oseroff A. Cytotoxicity and mutagenicity of low intensity 248 and 193 nm eximer laser radiation in mammalian cells. Cancer Res 1987; 47:410–413.
12. Trentacoste J, Thompson K, Parrish R, Hajek A, Berman M, Gangei P. Mutagenic potential of a 193 nm excimer laser on fibroblasts in tissue culture. Ophthalmology 1987; 94:125–129.
13. Kochevar I, Buckley L. Photochemistry of DNA using 193 nm excimer laser radiation. Photochem Photobiol 1990; 51:527–532.
14. Trokel S, Srinivasan R, Braren R. Excimer laser surgery of the cornea. Am J Ophthalmol 1983; 96:710–715.
15. L'Esperance F, Warner J, Telfair W, Yoder P, Martin C. Excimer laser instrumentation and technique for human corneal surgery. Arch Ophthalmol 1989; 107:131–139.
16. Taylor D, L'Esperance F, Del-Pero R, Roberts A, Gigstad J, Klintworth G, Martin C, Warner J. Human excimer laser lamellar keratectomy. A clinical study. Ophthalmology 1989; 96:654–664.
17. Gordon J, Grunfeld L, Garrisi G, Talansky B, Richards C, Wiczyk H, Rappaport D, Laufer N. Fertilization of human oocytes by sperm from infertile males after zona pellucida drilling. Fertil Steril 1988; 50:68–73.
18. Cohen J, Malter H, Wright G, Kort H, Massey J, Mitchell D. Partial zona dissection of human oocytes when failure of zona pellucida penetration is anticipated. Hum Reprod 1989; 4:435–442.
19. Gordon J. Use of micromanipulation for increasing the efficiency of mammalian fertilization in vitro. Ann NY Acad Sci 1989; 541:601–613.
20. Depypere H, McLaughlin K, Seamark R, Warnes G, Matthew C. Comparison of zona cutting and zona drilling as techniques for assisted fertilization in the mouse. J Reprod Fertil 1988; 84:205–211.
21. Malter H, Cohen J. Partial zona dissection of the human oocyte: a method using micromanipulation to assist zona pellucida penetration. Fertil Steril 1989; 51:139–148.
22. Laufer N, Pratt B, DeCherney A, Naftolin F, Merino M, Markert C. The in vivo and in vitro effect of clomiphene citrate on ovulation, fertilization and development of cultured mouse oocytes. Am J Obstet Gynecol 1983; 147: 633–638.
23. Nogues C, Ponsa M, Vidal F, Boada M, Egozcue J. Effect of aging in the zona pellucida surface of mouse oocytes. JIVF-ET 1988; 5:225–229.
24. Seiler T, Bende T, Winckler K, Wollensak J. Side effects in excimer laser corneal surgery. DNA damage as a result of 193 nm excimer laser radiation. Grafes Arch Clin Exp Ophthalmol 1988; 226:273–276.
25. Green H, Margolis R, Boll J, Koshevar I, Parrish J, Oseroff A. Unscheduled DNA synthesis in human skin after in-vitro ultraviolet excimer laser ablation. J Invest Dermatol 1987; 89:201–204.
26. Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer M, Wiemer K. Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. Hum Reprod 1990; 5:7–13.
27. Malter H, Cohen J. Blastocyst formation and hatching in vitro following zona drilling of mouse and human embryos. Gamete Res 1989; 24:67–80.