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Title
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Permalink
https://escholarship.org/uc/item/6tp2v299

Journal
Journal of Assisted Reproduction and Genetics, 9(6)

ISSN
1058-0468

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Publication Date
1992-12-01

DOI
10.1007/bf01204247

Peer reviewed

LASER

Microscope-Delivered Ultraviolet Laser Zona Dissection: Principles and Practices

INTRODUCTION

Since their introduction, the techniques of zona drilling (ZD) and partial zona dissection (PZD) have drawn considerable attention as potential tools in gamete and embryo micromanipulation. ZD involves the application of small volumes of acidic Tyrode's medium, which, through its digestive properties, creates small holes in the zona pellucida (ZP). This process was originally described by Gordon and Talanski (1). PZD is a mechanical technique for breaching the ZP and was first described by Malter and Cohen (2). It makes use of a pointed micropipette which pierces the ZP and is then sheared against a larger pipette, thus creating a breach in the zona pellucida. Indeed, most currently accepted methods of micromanipulation of oocytes and embryos are based on the use of mechanical techniques or the use of an acidified medium solution (3,4).

In 1989, Tadir *et al.* (5) suggested the incorporation of laser technology to achieve accurate incisions in the ZP as well as sperm micromanipulation through the use of optical traps. In addition, laser-induced selective destruction of extra pronuclei was also attempted (6). In these studies, the interaction of the fundamental wavelength Q-switched Nd:YAG laser (15-nsec pulses of 1.064 μm), the Nd:YAG harmonics (at 532 and 355 nm), and a nitrogen-pumped dye laser (600-psec pulses, at 366 nm) were all tested.

In an effort to determine the most suitable parameters for selective laser interaction with the various components within oocytes and embryos, we have been studying additional laser systems (7). Since cells in general, and oocytes and embryos in particular, are relatively transparent and contain high

amounts of water, we searched for laser systems emitting radiation where water is characterized by relatively high absorption.

While other researchers have elected to pursue wavelengths of high absorption coefficient (8) and have recently reported alternative methods for delivering these particular laser pulses to the zona target by either using glass micropipettes filled with positive air pressure (with the 193-nm radiation) (9) or using specialized fibers or micropipettes (in the case of the 2.94-μm erbium:YAG laser) (10), we have chosen to focus our attention on other wavelengths. Possible difficulties with contact methods such as energy delivery to the target, the need for making use of a glass or salt-based instrument (some of them are toxic), and the necessity for maintaining physical contact between the delivery media (pipette or fiber) defeat most of the advantages in using laser light and essentially reduce the laser-based techniques to a mechanical/contact mode, similar to conventional PZD methods. Additional complications arise from the need for sterilization of optical fibers or micropipette tips. This requires constant adjustments in the delivery system and disposal of relatively expensive fiber segments. The tip quality must also be carefully controlled in order to achieve a good beam quality, a consideration which is sure to slow down the procedure. A final problem is due to the fact that some laboratories make extensive use of mineral oil cover over the targeted medium. As we discovered, droplets of oil tend to adhere to optical fibers when they are inserted through the oil layer into the host medium. These oil droplets, in turn, absorb the fiber output radiation, accumulate heat, expand (thereby mechanically disturbing the micromanipulated object), and completely block any additional radiation from arriving at its target.

Based on the above considerations, we have elected to search for a set of laser parameters which will allow the laser light to be delivered to the

target through liquid media and yet be characterized by strong enough absorption in the zona glycoproteins (or other cell components), sufficient to produce localized interaction and the desired selective damage. To achieve this, one of two alternative approaches was used: (i) selecting laser wavelengths which are weakly absorbed by water (i.e., could be transmitted by the relatively diluted media yet be absorbed more strongly by the glycoprotein in the zona pellucida) or (ii) using highly localized (in space and time) radiation with wavelengths which are normally transmitted by the transparent media, which through nonlinear absorption processes, result in the desired selective damage.

After taking into consideration our experience with five wavelengths for gamete micromanipulation (266, 308, 355, 366, and 532 nm), we have concluded that the best results were observed with a XeCl excimer, emitting short pulses of 308-nm radiation.

Excimer (acronym for "excited dimers") lasers (11) are a class of gas lasers which are formed by the excitation of rare earth gases and their subsequent reaction with halide atoms such as chlorine or fluorine. Since these molecule exist only in their excited state and dissociate instantly upon emission of radiation, they constitute an ideal system for population inversion, which is the precondition for lasing action. Because their relaxation mechanism involves transition between electronic states with relatively large energy differences, excimer laser radiation is always in the ultraviolet (UV) range of the electromagnetic spectrum.

Growing interest among assisted reproductive technology (ART) scientists and engineers in the capabilities and potential of laser technology raises the need for a better understanding of laser interaction principles and potential techniques for applying its radiation. It also requires that professionals in the field become familiar with the advantages and disadvantages of each method as they become available.

The purpose of this article is to present a study on the application of two XeCl laser systems as a tool for gamete and embryo microsurgery and micromanipulation. The first is a low-power, high-repetition rate excimer laser with a varying pulse duration, while the second is a lower-repetition rate laser with shorter pulses (15 nsec) but with a higher energy per pulse. A discussion of the critical laser parameters responsible for achieving various cut configurations and a comparison of the advantages of these systems and techniques of application to alternative lasers are also presented.

MATERIALS AND METHODS

Three hundred sixty-four oocytes that failed to fertilize following insemination in 120 IVF cycles were used 2–3 days following retrieval. The oocytes were stored at room temperature in HEPES-buffered nutrient media. For drilling purposes, the oocytes were placed in drops of nutrient media on top of a quartz glass (Dynatech Electro Optics, CA) secured in a standard rose tissue culture chamber.

The basic experimental apparatus is illustrated in Fig. 1: the laser beam was directed through a mechanical shutter into an input port of an Axiomat inverted microscope (Carl Zeiss, Oberkochen, Germany). The electronic shutter driver/timer (Uniblitz Model SD-1000) was set to determine the exposure time duration. An energy meter was moved in and out of the beam's path to measure the pulse energy. Oocytes on the quartz slide were placed on a motorized x–y stage, which allowed their accurate positioning with respect to the stationary laser beam. A videocamera and a monitor screen allowed continuous monitoring and recording throughout the procedure for further evaluation on the computerized image processor (Imaging Technology Inc. Series 151, Model S7V807F8-841; IBM PC-AT).

Two laser systems were used. The first (system I) was a 15-nsec XeCl excimer laser (Lumonics HyperEx-400, Quebec, Canada). The laser emits 308-nm radiation at pulse repetition rates (PRR) ranging from 1 to 100 Hz. Incisions in the zona were performed using laser energy (measured at the microscope input) of 2, 10, and 25 mJ, although zona ablation could be observed at energies below 1 mJ. Since the beam's total energy loss, after passing through the system, was measured at 98%, the pulse energy at the zona target was about 40, 200, and 500 μJ, respectively. Energy at the laser output was measured with Gentek Ed-500 and Ed-100A energy meter heads (Quebec, Canada). The second laser (system II) was a XeCl excimer laser (Potomak Model GX-1000, Lanham, MD) at 308 nm with a pulse duration ranging from 50 to 250 nsec. Pulse repetition rates (PRR) ranged from 1 to 2000 Hz. Incisions in the zona were obtained by using laser energy (measured at the microscope input) of 25 mJ per pulse. The actual energy at the target
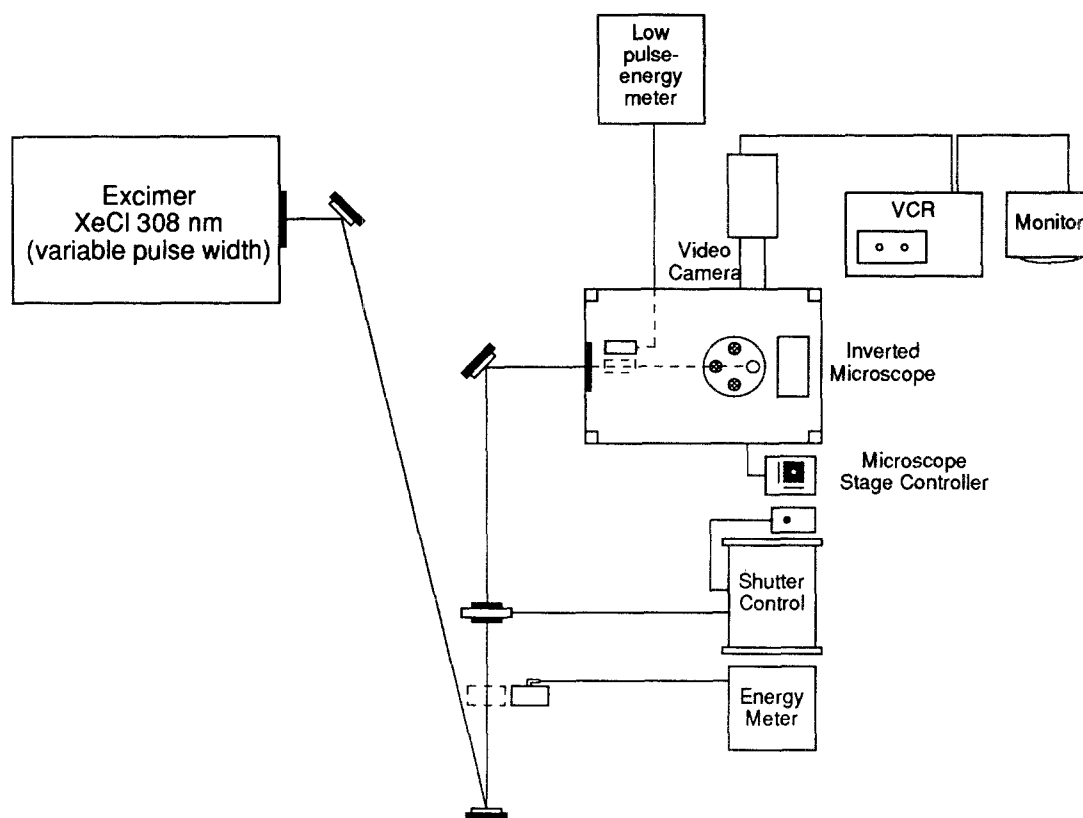
Fig. 1. Experimental setup.

was, therefore, about 500 nJ. Energy at the laser output was measured with a power meter Scienteck Model 360001, Boulder, CO) and, at the target, with the Gentek Ed-100A energy meter head.

A measure of the beam spot size at the objective output and its dependence on the laser and the optical system parameters were obtained by using a highly absorbing black ink.

Each set of laser parameters was used to create four cuts per oocyte in three oocytes, for a total of 12 cuts per parameter. The effects of the following laser parameters on the drilled hole sizes were evaluated.

1. Laser pulse repetition rates. The total number of pulses delivered was held constant at 500. This dose was delivered at 1, 5, 10, 20, 30, 50, 75, 100, 150, 200, 250, 300, 400, and 500 Hz, while the total time of exposure was adjusted accordingly. For example, at a PRR of 250 Hz laser irradiation lasted 2 sec, while at 50 Hz the exposure time was 10 sec. All other laser parameters were maintained constant. The size of the holes produced in the zona pellucida was measured with the image processor, then averaged, and the standard deviation of the

mean was calculated. These dimensions were then correlated to the laser PRR. This experiment was performed both on a highly absorbing ink (for calibration purposes) and on the zona pellucida of the oocyte.

2. Laser pulse energy. Pulse repetition rate and total time of exposure were maintained constant while the energy was changed (either by attenuating the light with filters or by changing the excimer gas fill). Holes in the zona were measured as described above and correlated to the pulse energy.

3. Laser pulse duration. This experiment was performed with the low-pulse energy laser only. All parameters were maintained at a constant level while the pulse duration was varied from 50 to 250 nsec.

4. Focal plane position. The effect of changing the position of the microscope focal plane with respect to the target (by moving the microscope objective) on the ablated spot size was measured.

Once the effect of the optical system and the laser parameters on the spot size was determined, we could choose the precise manner by which a cut in the zona would be generated. The drilled site

could be created in one of several ways: first, by gradually removing small external portions of the zona, thus generating "trenches" (Figs. 2A and B); second, by aiming the unactivated laser beam at a desired location (to enhance this process one may use a secondary, weaker, "aiming beam"), then turning on the laser radiation and drilling "tunnels" in the zp (Fig. 2A); and, finally, by carving out an entire section by cutting along a plane (of any shape), thus disconnecting a portion of the material from the main cell mass and allowing it to drift away (see "shear" in Fig. 2B).

Some of these configurations were demonstrated by cutting trenches and tunnels of different shape and sizes. The trench depth was carved out of the zona by moving the stage (and the oocyte on top of it) into the beam path and was varied for each laser parameter to 25, 50, or 90% of the total zona thickness was removed at the drilling sites.

Immediately following laser microsurgery the treated oocytes were fixed in 3.5% glutaraldehyde in 0.1 M cacodylate for 1 hr, then stored in 0.1 M cacodylate under refrigeration. After further processing in 1% osmium tetraoxide for 30 min, they were attached to 12-mm round coverslips (Fisher) that had been coated with poly-L-lysine hydrobromide (MW >300,000; Sigma), 2 mg/ml, at 4°C overnight. The oocytes were dehydrated in acetone, critical point-dried in CO_2, gold-coated to an approximate film thickness of 10 nm, examined with a scanning electron microscope (Phillips 515), and photographed.
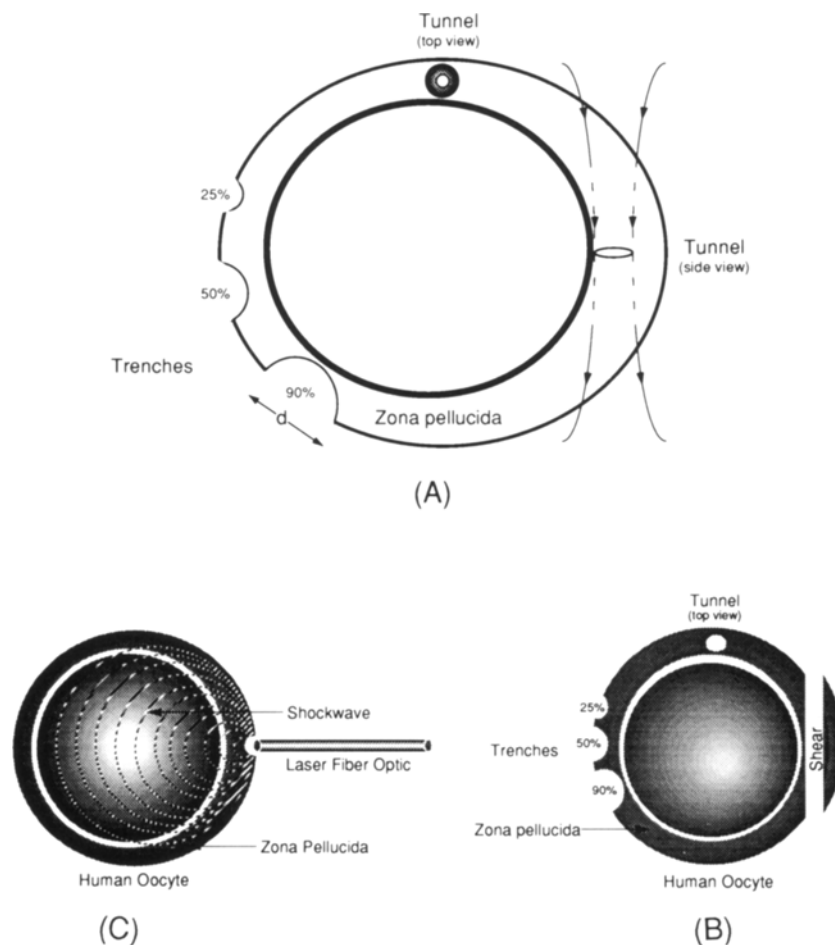

Fig. 2. Laser zona cutting configurations: (A) tunnels and trenches (d is the trench diameter), (B) shearing cut, and (C) drilling in the radial configuration, showing potential generation of mechanical disturbances (shock waves, dashed spherical lines) propagating within the oocyte following ablation at high energy densities with a highly absorbed laser wavelength.

RESULTS

There was a direct relationship between the hole diameter (HD) and the laser pulse energy (Figs. 3A and B). The high pulse energy system was tested for an energy range from 2.5 to 100 μJ per pulse (laser PRR was maintained constant at 5 Hz). Initially the trench size increased rapidly with increasing energy, then reached a plateau (around 40 μJ/pulse). No additional increase in size was observed even if the energy per pulse more than doubled. In the low-energy unit (system II, Fig. 3B), the pulse

energy was smaller by a factor of 100 and ranged from 300 to 800 nJ (laser PRR was maintained constant at 200 Hz). Here the HD ranged from just over 2 to just under 4 μm. HD initially demonstrated an approximately linear increase with energy, which tended to level off above 600 nJ.

The ablated spot sizes on the laser pulse repetition rate (from 1 to 1700 Hz) are shown in Figs. 4A and B. The tunnel diameter (μm) dropped initially at the very low PRR but was consistently insensitive to variation in the PRR. A similar lack of dependence on PRR was observed in the high-energy system as well.

Figure 5 shows the effect of changing the position of the objective focal plane on the trench diameters. Energy per pulse was maintained at a con-
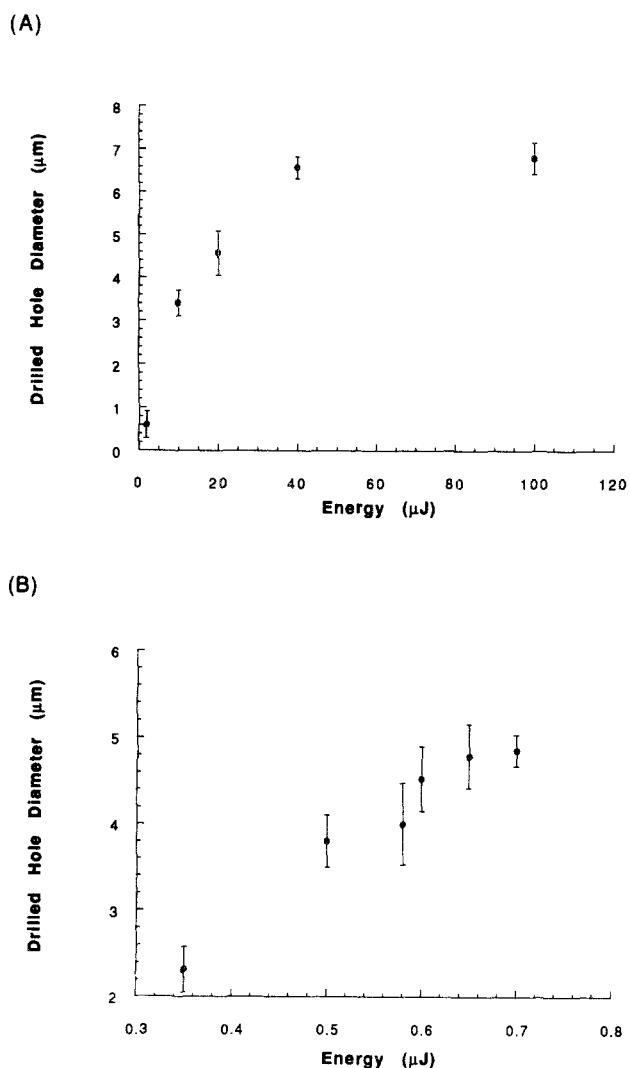
(A)

(B)

Fig. 3. Tunnel diameter (μm) as a function of pulse energy (μJ) in (A) the high-energy system (the error bars represent the standard deviation of the mean taken by averaging six drilling sites ablated with the same energy) and (B) the low-pulse energy system (the error bars represent the standard deviation of the mean of $n = 5$ drilling sites per energy setting).
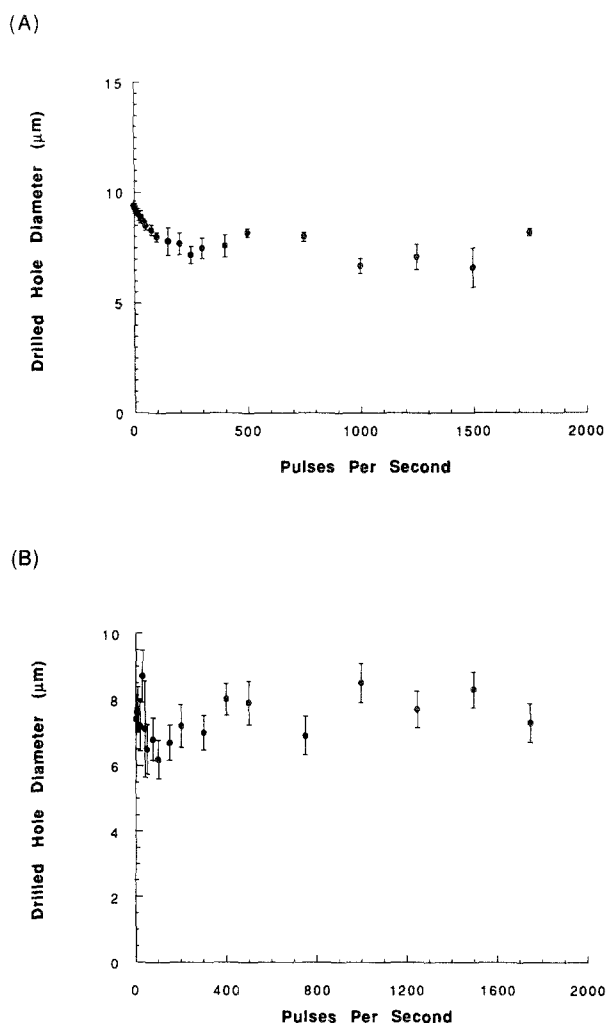
(A)

(B)

Fig. 4. Tunnel diameter (μm) as a function of changing PRR in the low-pulse energy system: (A) ablation of black ink and (B) ablation of zona pellucida. The error bars correspond to the standard deviation of the mean of $n = 6$ drilling sites.
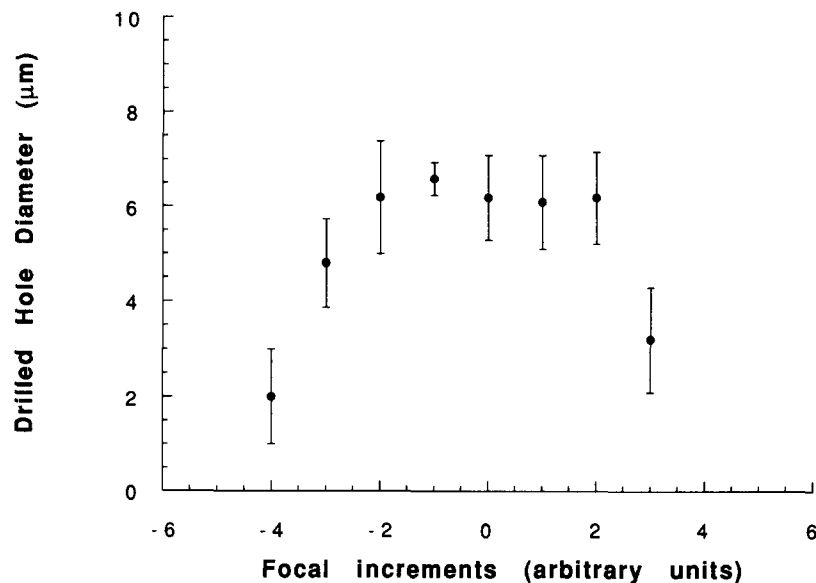
Fig. 5. Tunnel diameter (μm) as a function of the microscope objective focal plane position (arbitrary units) at 200 Hz and 700 nJ/pulse. The error bars correspond to the standard deviation of the mean of $n = 5$ drilling sites.

stant level of about 700 nJ and the laser PRR was 200 Hz. The figure shows that the largest ablation diameter was actually obtained when the beam waist (the narrowest part of the laser beam; Fig. 6A) was placed at the targeted area. Since the microscope and laser were parfocal, this position coincided with the condition of a clear focused image, which we designated 0 point. The defocusing units are arbitrary but show that as the image is defocused, the size of the ablated tunnel decreases symmetrically. Each objective setting was tested three times. Figure 6B explains (in terms of the beam characteristics) the origin of the decrease in tunnel size with defocusing of the laser. The common characteristics of laser "Gaussian" beams, which are important in determining the quality and size of the ablated holes, are illustrated in Figs. 6A and B).

Images of the trenches generated in the zona pellucida are shown in Fig. 7. A relatively narrow trench (3–4 μm in diameter), which may be used for enhanced fertilization purposes, is shown in Fig. 7A, while Fig. 7B shows shearing of a large zona segment, which theoretically may be beneficial for assisted hatching. In both figures, the structure of the zona pellucida adjacent to the irradiated area appears free of damage.

A more complete SEM study was undertaken to characterize the effect of tangential trench cutting and the effect of the interaction on the integrity of

the oocytes. Figure 8 demonstrates two laser-generated cuts in a human oocyte. The incision is about 10 μm wide. No thermal damage is observed and the zona pellucida appears to be intact. Higher-magnification SEM demonstrated the sharp edges of the trenches, the smooth "floor" at the bottom of the crater, and the localized nature of the damage zone.

Finally, interaction thresholds in the zona pellucida for the low-energy system were determined by gradually attenuating the laser energy until no effect was detected. PRR was constant at 200 Hz. Attenuation was achieved by introducing successive layers of partially absorbing slides. The interaction threshold (i.e., the minimum pulse energy at which zona removal could be achieved) was determined to be 100 nJ. No threshold determinations were performed at other PRR.

DISCUSSION

Laser light constitutes a highly accurate and versatile tool for interaction with biological materials. It can cut, coagulate, fuse, ablate, vaporize, and even trap and manipulate cells or subcellular organelles. Several laser and delivery system characteristics will ultimately determine the radiation effect at the target. In this regard, the results from our
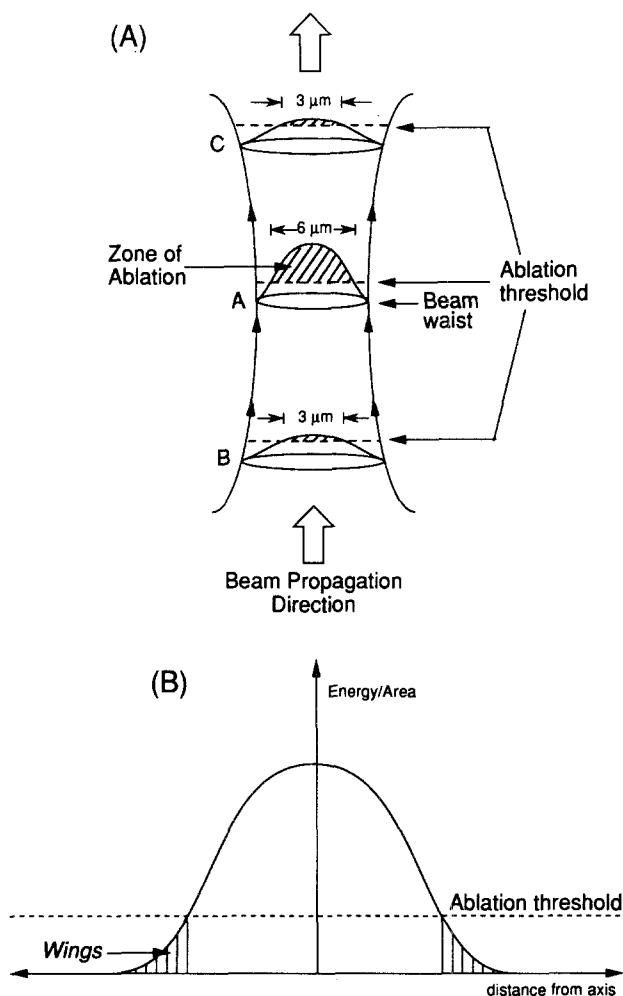
Fig. 6. (A) Beam profile explaining the dependence of the tunnel diameter on the objective focal plane position. Planes A, B, and C represent the ablation threshold level and the cross-hatched area is the segment of the beam capable of ablation. (B) A characteristic (Gaussian) laser beam profile.

study serve two purposes: (i) they define the laser parameters necessary to achieve the microsurgical objectives, and (ii) they illustrate the potential advantages of laser light as a micromanipulator when applied in the noncontact mode.

Six parameters determine the laser cutting characteristics: wavelength, pulse duration, energy per pulse, pulse repetition rate, total time of radiation exposure, and beam spot size.

Excluding a few systems such as dye lasers, titanium:sapphire, and free electron lasers, most laser systems emit radiation at only a few, very narrow frequency bands. Thus, discussing a wavelength of choice also implies selection of a laser system. Previous studies and the knowledge of wavelength-dependent characteristics led to the

study of the near-UV part of the spectrum. More specifically, the 308-nm excimer lasers allow both optical fiber delivery as well as direct delivery through microscope objectives, without the need for mediating instruments.

One of the first questions that must be answered in determining the optimal beam properties is What are the objectives of a laser micromanipulation procedure? Theoretically, one may expect ZD for enhanced fertilization (where narrow trenches are preferred in order to minimize the risk of polyspermy) to be altogether different from zona cutting for assisted hatching purposes (where a significant portion of the zona must be removed). Thus, in ZD for fertilization procedures a minimization of the beam spot size is required. In this case we would prefer to work with the beam waist (see Fig. 6A) as a "knife tip" to cut a tangential trench and completely remove the zona at only a single contact point (Fig. 2A). An alternative configuration in ZD applications, which should also help reduce polyspermy, is illustrated in Fig. 2A. Here the laser is used to drill a tangential tunnel through the zona, only one point of the tunnel makes contact with the cell membrane, and a minimum amount of zona is removed.

For AH procedures it is suggested that a larger portion of the zona should be removed. A large gap will be created to avoid potential entrapment of the emerging blastocyst in a narrow opening during the hatching process (4). In this case, one can use the laser to "nibble" at the zona by working the laser beam tangentially from the outside inward, until the desired curved shape is achieved. (See shearing in Fig. 2B.) Alternatively, we may want to use the beam waist to sever a portion of the zona mass along a line of any desired shape. (By grabbing the oocyte with a holding pipette and rotating it with respect to the stationary beam, one may "shave" the zona along its natural contour.) In either case the size of the beam can be adjusted for the desired convenience, accuracy, and speed of operation. (A larger beam will remove a larger volume but at the expense of some accuracy.) The actual cutting motion can be accomplished by either manipulating the motorized stage or manipulating the gamete itself (held by a micropipette) with respect to the stationary beam. The accuracy of the minimal increment, in this case, will depend on the precision of the manipulators (some motorized stages are precise to within 50 nm).

Finally, the ZD with the radial irradiation configuration (Fig. 2C) uses lasers at a highly absorbed
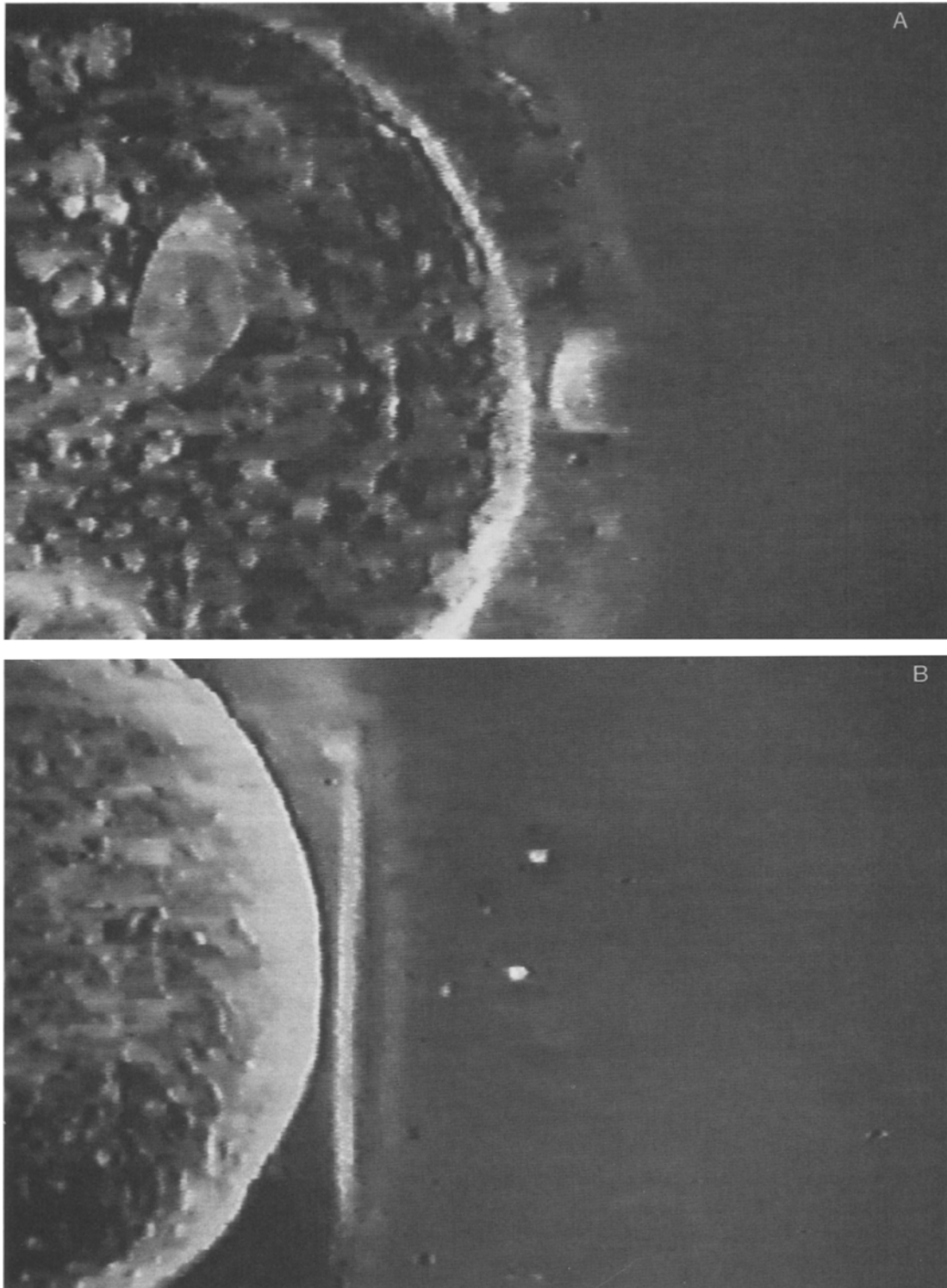
Fig. 7. Video images of zona pellucida microsurgery: (A) a trench 12 μm wide; (B) a tangential shear close to the cell membrane.
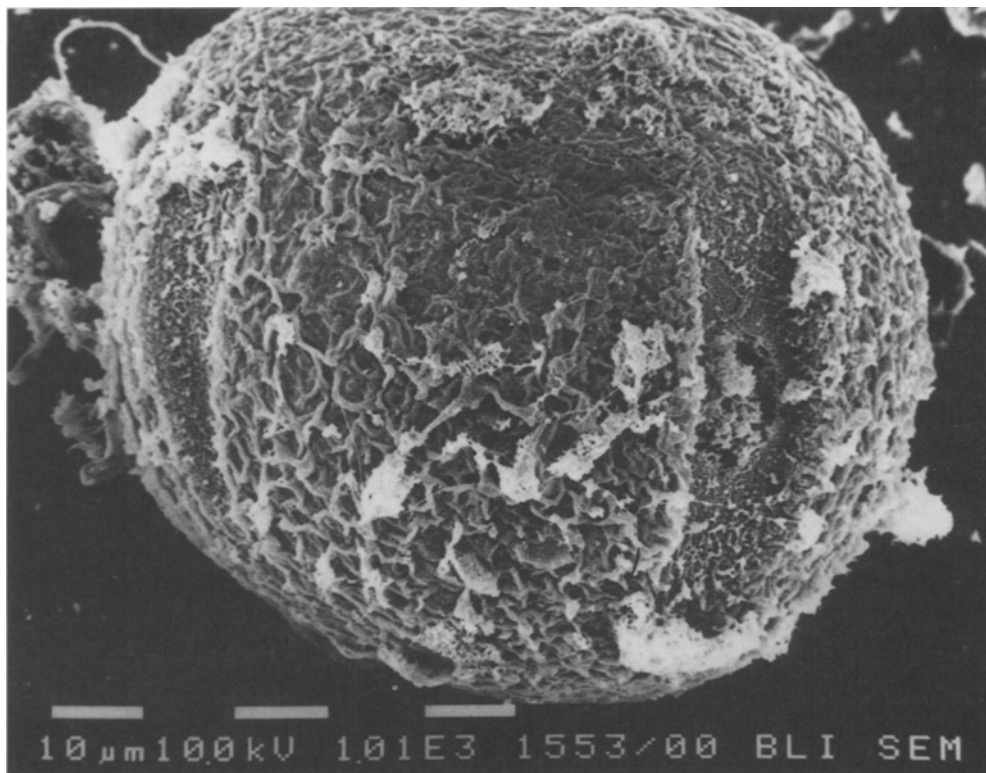
Fig. 8. SEM of two tangential trenches in the zona pellucida ablated by the high-energy system.

wavelength and is, perhaps, conceptually closer to "drilling." This configuration may be more hazardous to the oocytes and embryo because any remnant radiation must continue to propagate in its original radial direction. As a result, greater exposure of intracellular components may occur. Tangential irradiation schemes avoid this problem. Additionally, the combination of a tightly focused beam and a highly absorbing wavelength results in highly localized power densities. Consequently, explosive ablation might occur which could send damaging mechanical disturbances throughout the oocyte or embryo.

As stated above, the actual ablation spot size of a stationary laser beam/target configuration is a function of the laser parameter as well as the position of the microscope objective focal plane. From our measurements and knowledge of laser beam properties, the following specific characteristics can be assigned to the effects of each parameter.

PULSE ENERGY. As shown above, the minimum attainable spot size in black ink was between 3 and 5 μm when a 32× objective was used with the high-pulse energy system. In the ablation of the zona

pellucida, on the other hand, tunnels as small as 2 and 1 μm were regularly obtained. The smaller holes can be explained in terms of weaker absorption characteristics in the zona pellucida in comparison to the ink. The high absorption properties of the ink means that the extremities of the beam (its "wings"; see Fig. 6B), where the light energy density is low, are now able to retain more light, thus bringing the photon density (photons absorbed per unit volume) over the required ablation threshold for this material. Because the zona pellucida is much more transparent to the near-UV radiation, less light is retained and only the portion of the beam around its center (where the energy density is highest; Fig. 6B) is above the required ablation threshold.

As demonstrated in Fig. 3A, the actual dependence of the drilled site diameters (DSD) on the pulse energy is characterized by a rapid initial increase followed by a gradual leveling off at the higher energy levels. Again, the beam profile characteristics (Fig. 6B) can explain this dependence: The initial increase in DSD corresponds to activation of the beam wings with increased energy as they are brought above the ablation threshold. When all of the unobstructed beam is above the

energy threshold, the spot size ceases to increase and a leveling off of the curve is observed.

A similar effect was observed with the second laser system (Fig. 3B), where an initial linear increase in the DSD is followed by leveling off around 0.6 μJ. With this system, the smallest ablation spot sizes in the zona pellucida are between 1 and 2 μm (32× magnification). With the 100× objective, smaller ablation spot sizes can be achieved.

The parameters which distinguish the two XeCl systems are energy (a pulse from the high-energy system is about 100 times stronger than the one from the low-energy system) and pulse duration (the high-energy system is shorter by about a factor of 10). Since the peak power is the ratio of energy over pulse duration, the high-energy system has a peak power performance roughly a thousand times greater than that of the lower-energy high PRR system. This, in turn, leads to two clearly distinct modes of operation: the low-energy system appears to operate in a sub-ablation threshold mode, which is qualitatively different from that of the higher-pulse energy laser. (Note that "ablation threshold" is different from "interaction threshold.") With the former we observe a situation where the interaction of several pulses must accumulate before any effect is observed. The removal of material occurs as a dissociation of the irradiated portions from the rest of the zona pellucida. This is contrasted with the high-energy system, where a single pulse is sufficient to remove a similar mass of the zona pellucida but in a more violent ablation process. Roughly 10 250-nJ pulses from the low-energy system were needed to achieve the effect of a single 50-μJ high-energy laser pulse.

PRR. An increased PRR means that more pulses are incident per unit time and, thus, that more energy is absorbed. The effect of an increased laser pulse repetition rate is most often associated with heating, although it can also result in enhanced mechanical effects. Increased heating can, therefore, result in damage to surrounding areas and in enlarged ablation spot sizes due to the diffusion of heat or explosive ejection of debris. Indeed in macroscopic ablation we often find that a high PRR results in a significantly increased thickness of the zone of thermal damage where cracking, charring, and carbonization occurs. In the case of the zona pellucida one may expect a zone of protein denaturation and melting when significant heat is accumulated. However, no such increase in ablation

spot size was observed in either laser system tested, and no significant thermal damage was detected by SEM (Fig. 8).

Laser PRR did not influence the ablated spot diameter (Figs. 4A and B). The slight drop in spot size noted at a low PRR is due to a corresponding energy drop. As the PRR is increased further, the energy output stabilizes.

The lack of visible PRR dependence is probably due to the low pulse energies at the objective output (in both systems), which can dissipate quickly. However, the fact that no obvious changes are detected does not mean that the increased PRR has no other adverse effects. Some heat is always generated in light interaction processes (both directly, by molecular absorption of the light energy and its subsequent dissipation, or through the transfer of energy from the expanding ablation by-products). An increased PRR means that a larger amount of energy is deposited per unit time and, also, that pulse-to-pulse thermal relaxation becomes incomplete as heat accumulates in the target. A small yet significant elevation in temperature can, therefore, take place and result in irreversible damage to the micromanipulated cell or organelle.

FOCAL PLANE POSITION. Changing the focal plane position with respect to the target also affects the beam spot size at the target (Fig. 6A). Thus, varying this parameter amounts to optically modifying the beam profile. While not changing the energy in each pulse, it does affect the energy density distribution. Figure 5 shows the dependence of the spot size on this parameter. The spot size has a maximum at position 0 in our arbitrary units, which corresponds to the focal position of the optical images. The curve then drops symmetrically as the beam is defocused (up or down) from this position.

The mechanism which explains these observations is illustrated in Fig. 6A: The effect of focusing the beam waist (plane A) on the zona pellucida is to place the smallest spot of the beam also at this position. Since the energy density is the highest at the focal plane, most of the beam profile is above the ablation threshold (dashed lines in Figs. 6A and B). In the defocused planes, the beam waist is moved above or below the targeted zona pellucida, and a larger portion of the beam is below threshold levels (Fig. 6A, planes B and C). The net result is that a smaller region of the beam is capable of ablation and the tunnel size decreases, which is what was observed (Fig. 5).

PULSE DURATION. Pulse duration effect was difficult to evaluate because, in the low-energy system, varying the pulse duration resulted in corresponding changes in energy. Therefore, the effect of this parameter was not discussed beyond the general observations (see above) regarding the differences in interaction modes (ablative vs subablative) between the two systems.

TOTAL TIME OF EXPOSURE. In the low-energy system, a small incubation time (corresponding to several pulses) was necessary in order to initiate a hole. In the high-energy system a hole could be obtained with a single pulse, but the final size of the hole was obtained after several pulses. In either system no effort to monitor the volume of material removed per pulse was made. However, once the final spot was formed, the DSD was unaffected by additional exposure.

CONCLUSION

Excimer lasers of 308 nm operating in a short pulse mode (15 to 250 ns) are effective microsurgical tools for achieving removal, in a noncontact mode, of a portion of the zona pellucida. At this particular wavelength, the optical absorption is strong enough to allow selective interaction with the zona pellucida, yet weak enough to minimize accumulation of heat or explosive ablation. In addition, 308-nm radiation can be delivered through slides, microscope objectives, optical fibers, and fluid or oil. It can facilitate easy, accurate, and highly reproducible material removal without the need for handling and maintaining a contact delivery system. The critical role of various lasers and beam parameters in controlling ablation has been illustrated and quantified on human oocytes. This convenient and highly accurate technique could replace other modalities in micromanipulation procedures, since as the demand for greater accuracy increases, a corresponding rise in the need for this high-precision technology will follow.

ACKNOWLEDGMENTS

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