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A Novel Method of DNA Transfection by Laser Microbeam Cell Surgery

M. Tsukakoshi, S. Kurata*, Y. Nomiya, Y. Ikawa, and T. Kasuya

The Institute of Physical and Chemical Research, Wako, Saitama 351, Japan

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Abstract. A new technique is presented to incorporate exogeneous gene materials (DNA) into cells with a microbeam irradiation from an uv pulsed laser. A frequency-multiplied Nd:YAG laser, 355 m wavelength, 5 ns pulse duration, punches a self-healing hole of submicrometer aperture in cell membrane under selected irradiation conditions. It takes a fraction of a second for the aperture to close, long enough to allow the foreign DNA, contained in the medium, to slip into the cell. The method offers a clear advantage over existing methods: increases the success rate of DNA transfection as well as the efficiency of cell modification by orders of magnitude.

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The introduction of gene materials into cells of different organism has usually been accomplished by either chemical or manual method.

The chemical method, established by Graham and Van Der Eb [1] is based on phagocytosis of DNA precipitated in calcium phosphate. The method was applied with success in detecting oncogene in tumor cells [2], and is at present a prerequisite in the research of those genes associated with phenotype expressions of carcinogenic and other characters. However, an extremely low probability of DNA introduction, say about one in 10^5 success rate, is a major problem of the method: cells are very often damaged by high-concentration chemical substances used for operation.

The manual method, developed by Capecchi [3] in a transformation of the cultured Tk^- cell, is based on a direct injection of DNA into the host cell through a fine needle with an aid of an optical microscope [4]. The method was later developed by Yamamoto et al. [5] into the "pricking method" with which the foreign genes contained in the medium slip into the pricked cells, the instant the needle is plucked. The manual approach allows an introduction of DNA with much higher efficiency, but demands a great deal of skill and predigious concentration on the part of the operator. At best it can produce only about 3×10^3 doctored cells an hour. A new technique of DNA transfection, therefore, is highly desired by which an improved success rate of DNA transfection is combined with a high efficiency of cell modification.

Owing to the recent development of the laser microbeam technique [6], the microsurgery in subcellular detail such as the dissection of particular cytoplasmic organelles in single cells has been put into practice [7–9]. This is very suggestive of possible use of highly focused laser light as a microscalpel to stimulate DNA transfection into cultured cells.

In this paper we present a new technique of DNA transfection as a practical alternative of the above-described conventional methods. The present method employs ultraviolet laser light of nanosecond pulse length as an "optical" needle to prick cells. It perforates a self-healing hole of submicrometer diameter in the cell membrane, through which the foreign DNA, contained in the medium, has a chance to slip into the cells. In its current, semi-automated form, the laser method, as examined by splicing genes from Escherichia coli (Eco-*gpt*) into rat kidney cells, proved capable of modifying around 10^3 cells a minute, tenfold efficiently compared to the manual method, with a

* Present address: Laboratory of Human Genetics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

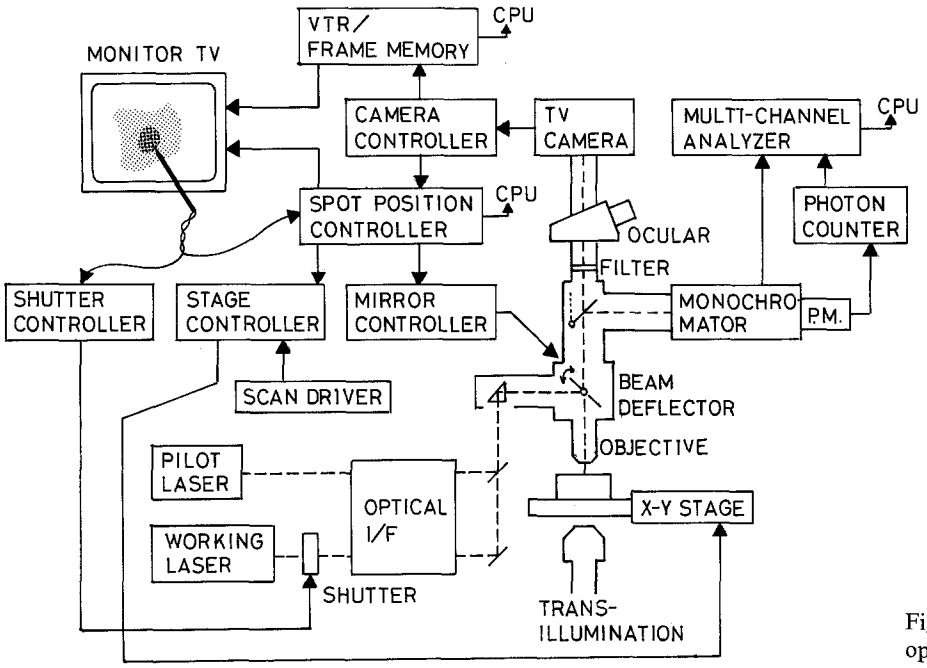

Fig. 1. Block diagram of electric and optical arrangement

success rate of at least one in 10^2 cells, higher than the chemical method by three orders of magnitude.

1. Instrumental Arrangement

The system is essentially a microscope optics (based on OLYMPUS, BHT-NE) interfaced to an uv laser subjected to a fine electric control, as shown schematically by the block diagram in Fig. 1. The third-harmonic wave of a Nd : YAG laser (QUANTA-RAY, DCR-1A), 355 nm wavelength and 5 ns pulse duration, is employed as the working laser for microbeam cell irradiation. An auxiliary light beam of a He-Ne laser is superimposed by a beam splitter for alignment of the optical set-up and for aiming. Both laser beams are collinearly focussed through an incident-light microscope objective (CARL ZEISS, ULTRAFLUAR 32 X) onto the specimen.

The focussed irradiation at any desired site of the specimen is given by moving either the light spot or the specimen. The spot movement is accomplished by deflecting the beam off a pair of rotatable dichromatic mirrors prior to focussing down through the objective. The rotation is provided by a galvanic mirror-driving unit (GENERAL SCANNING, G-102 PD) with angular sensitivity of 0.1 deg/V and linear response of up to 400 Hz. The speciment movement of subcellular precision is achieved by a motor-driven $x-y$ stage of 1/8 μm step, which allows the target to be brought into the fixed focus of the laser beam.

The image of the specimen under trans-illumination is visually observed or displayed on a TV monitor screen through a uv-cut filter, or registered in a video tape recorder and a frame memory (HAMAMATSU PHOTONIX, C1901). The focal spot of the pilot laser is superposed on the specimen image in comparable resolution. The monitor unit interfaces the spot-position controller (HAMAMATSU PHOTONIX, C1055) which provides an analogue output of the $x-y$ coordinates of the point indicated by a light-pen on the monitor screen. The output signal is delivered either to the galvanic mirror-driver or to the $x-y$ stage controller. The precision aiming of a subcellular structure is, therefore, carried out either by directly indicating the target image with a light-pen or by driving the $x-y$ stage relative to the pilot marker with the aid of a control stick.

The ignition pulses of the working laser, 10 Hz repetition, are sent to the shutter controller which, on receiving the cue from the light-pen, releases the shutter of the uv laser for a given period of time so that the prescribed number of pulse shots are to be delivered to the identical portion of the specimen.

The microscope optics used is compensated to infinity. This arrangement is advantageous in designing optical interface to the lasers. This is because the para-axial incidence to the objective is less critical to optical mode matching than the usual incidence for which focussing on the intermediate image plane is required. A nearly diffraction-limited spot size of 0.5 μm is available with ease for both laser foci by adjusting zoom lens connected with the optical interface.

As shown in Fig. 1, the present system is also equipped with a photon detection system for the measurement

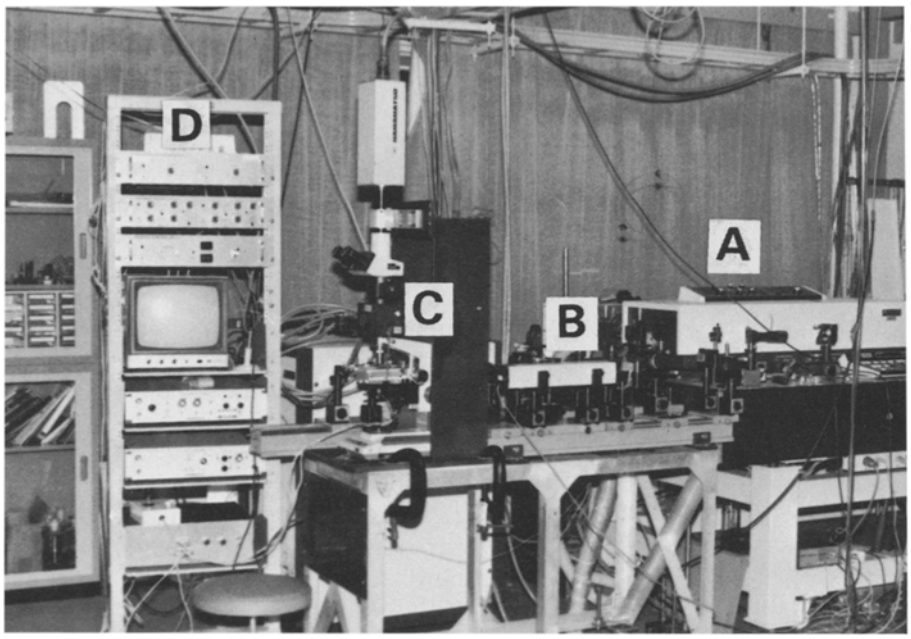

Fig. 2. Photographic overview of experimental apparatus (A: YAG laser system (QUANTA-RAY, DCR-1A) B: Optical interface C: Laser microscope D: Control system)

of, for example, localized luminescence from a particular organelle in a single cell. The photographic overview of the apparatus is shown in Fig. 2.

2. System Operation for Microperforation of Living Cells

The living cells treated in the present experiment are normal rat kidney (NRK) cells cultured in the Dulbeco MEM (DMEM) medium with 10% FBS (GIBCO) at 5% CO_2, 37° C environment. The cells are inoculated 20 h in advance on a quartz plate, 0.1 mm thick and 18 mm square, to a concentration of roughly $2 \times 10^5/cm^2$. The plate is then turned over to cover the aperture, 10 mm diameter, of the upper wall of the culture flask, and is clamped with an autoclaved rubber packing, as shown in Fig. 3. This arrangement facilitates the focussed irradiation through an objective of a large numerical aperture and of a short working distance.

Cells are destructed with irradiation of above a rather sharply defined threshold of intensity. At a selected irradiation condition, the uv absorption leads to a perforation in cell with a hole of submicrometer aparture during a single shot of 5 ns burst of laser pulse. In practice, the highly localized hole is most efficiently obtained when the focussing of the working laser is slightly within the cell. The working and pilot laser foci are, therefore, independently regulated to allow an appropriate relative distance. The diameter of the hole, as observed on the monitor screen, is a strongly nonlinear function of incident power and energy density. A minimal diameter of a fraction of the laser

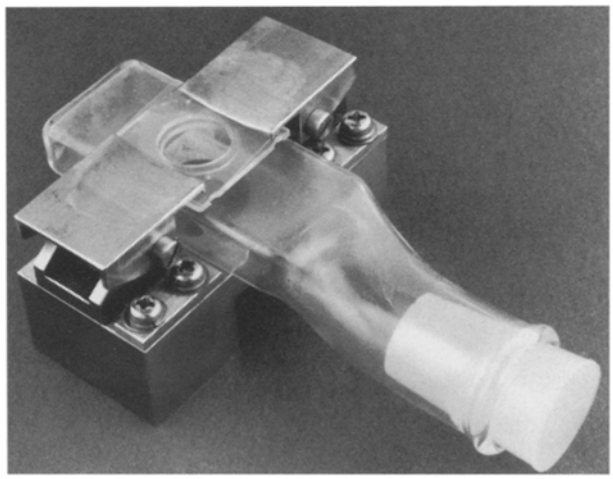

Fig. 3. Flask employed in laser perforation of cultured cells

spot-size, say 0.3 μm, is available at a critical irradiation of around one mJ, nearly the lowest possible for perforation.

The striking finding is that the well localized hole can be obturated within a fraction of a second. Figure 4 is the photograph of an NRK cellular nucleus representing the self-healing process of laser microperforation.

For microperforation of inoculated culture cells, the present system operates in either "pointing mode" or "scanning mode". In the former mode of operation, the light-pen is linked to both the shutter and the mirror controller, as described in Sect. 1. Then one can perforate a selected portion of each cell by indicating

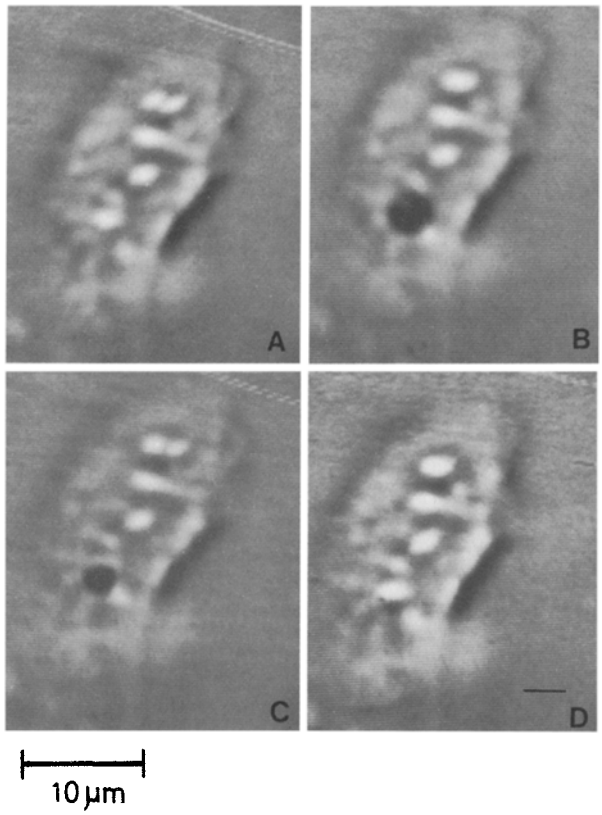

Fig. 4A–C. Self-healing process of perforation on NRK cellular nucleus. Photographs of large aperture are selected for demonstration. uv laser, 355 nm wavelength, 10 ns pulse duration, 1 mJ energy, is irradiated through objective, ZEISS ULTRAFLUAR 32X, 0.4 NA. (A) Before irradiation, (B) immediately after irradiation, (C) initiation of hole obturation, (D) hole almost obturated. Required time for steps (B) through (D) is about 0.5 s

one by one with the light-pen (Appendix). While in the latter, the shutter is left released, and the $x-y$ stage automatically traces the prescribed path so that a given area relative to the fixed laser spot is scanned over. The successive single-spot irradiation in the scanning mode allows a sequential perforation of a given interval on cells in the scanning coverage. The scanning rate is adjusted to an appropriate value for the cell concentration.

3. DNA Transfection by Laser Method

The basic idea of the present laser method of DNA transfection is to let the gene materials contained in the culture medium slip into the cell through the laser-aided self-healing hole. That the introduced gene materials are held securely by the cell is positively supported by the experiments to subject cells to control under which those containing no foreign DNA will die out so that only those with DNA transfected by laser irradiation survive and grow. Some particular cloned genes called "dominant positive selective marker" works as the antibiotic resistant allowing growth under such control, among which Eco-*gpt* gene, the pSV-2 EcoRI-Pvu II fragment cloned on pBR 322, is typical [10]. The Eco-*gpt* gene codes for enzyme converting xantine to xantine monophosphate, so that those cells in which the gene functions will survive in the selection medium charged with mycophenolic acid (MPA), an inhibitor of reaciton from inosine monophosphate to xantine monophosphate.

The first success of DNA transfection with laser irradiation is demonstrated as follows. The NRK cells are treated in the scanning mode of laser irradiation in the medium containing Eco-*gpt* gene, cultured for four days in the above-described selection medium, and subject to microscopic observation (Photographic pictures are shown in Fig. 5). A number of cells within the laser-scanning coverage (all over in Fig. 5A and bottom half in Fig. 5B) survive in the control. This is in contrast to those cells irradiated in the absence of Eco-*gpt* gene which dwarf on the verge of death, as shown in Fig. 5C. The DNA transfection by the laser perforation is evidently supported by the above observation that the cells growing in the control medium are found exclusively out of among those treated with laser in the presence of Eco-*gpt* gene.

A more direct proof of integration of Eco-*gpt* gene within the nuclear genomes is given by the following experiment. Several colonies derived from a single clone are allowed independent proliferation, from each of which a DNA molecule is extracted. After being digested with EcoRI, it subjects to the Southern hybridization with a probe of PvuII-EcoRI fragments of pSV-2 [11]. As a result, Eco-*gpt* genes are found to be integrated in the genomes in all of above clones, although the number of transfected genes and their genomic sites are different between colonies.

The success rate of DNA transfection R is given by the survival rate S/S' devided by the proliferation factor Q during a given period of culture:

$$R(\%) = 100 \times S/S'/Q, \tag{1}$$

where S is the number of cells survived in the control medium and S', that of cells subjected to laser operation.

Several specimen plates with a large number of cells ($S' \sim 5 \times 10^4$) are laser-treated in the scanning mode in the medium of each different DNA concentration. The survival ratio is measured after 4 days of controlled culture for which Q is found to be 3.8 by a separate set of observation. As plotted against the DNA concentration in Fig. 6, the success rate R saturates to its maximum 0.6% for the DNA concentration of above

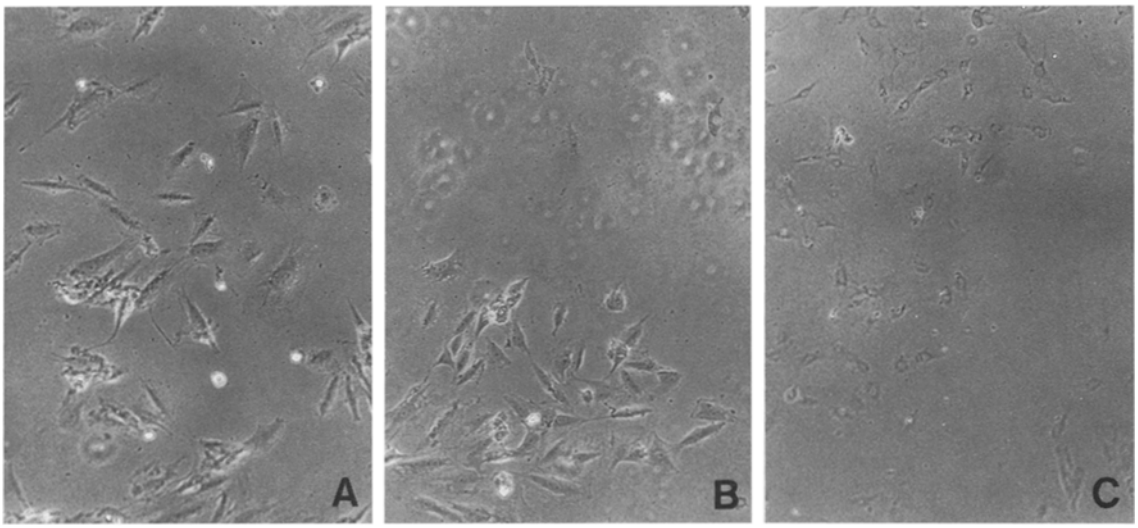

Fig. 5A–C. Microscopic photograph of NRK cells ($5 \times 10^4/cm^{-2}$) day 4 of culture at 37 °C with 5% CO_2 in the selection medium, DMEM 10% FBS, containing 35 μg/ml MPA, 2 μg/ml aminopterin, 250 μg/ml xantine, and 15 μg/ml hypoxantine. (A) All over and (B) bottom half of the look of the picture are within the scanning overage of laser perforation in the presence of Eco-*gpt*. (C) Scanned all over in the absence of Eco-*gpt*

10 μg/ml. A strong dependence of the success rate on the subcellular site of laser irradiation is revealed by the pointing mode of operation: an awfully improved success rate of 10.2% is obtained with the nuclear irradiation in marked contrast to almost nothing rate with cytoplasmic irradiation.

Those cells which integrate the exogenous DNA in their genomes will continue to proliferate, while those of transient transfection will not hold it any more than several cycles of proliferation [12, 13]. The former cells are distinguishable from the latters by the individual colony formation during sufficiently long term culture of thinly scattered surviving cells. The "complete" transfection rate R^* is, therefore, given by multiplication of the rate R achieved in the pointing mode of operation with the rate of colony formation out of among surviving cells:

$$R^* = RC/C', \tag{2}$$

where C is the number of colonies among C', the number of surviving cells subjecting to observation. As a result of experiment, the rate R^* is found to be 0.066%.

The rate of transfection described in this section is tentative to our particular case of NRK cells, which is expected to be improved by a careful optimization of irradiation conditions. More detailed description on the biological treatment will appear elsewhere.

In conclusion, the laser microbeam irradiation provides a new method of introducing exogenous gene materials into cells. The method offers a clear advantage over the conventional methods both in the rate of transfection and in the speed of operation. The method promises a good versatility owing to the wide controllability of the laser-beam intensity, to the large flexibility in optical manipulation with respect of focal spot size and depth in a cell, as well as to the wide choice of laser wavelength and pulse width. The method is expected to be applicable to cells whose walls are too hard to introduce foreign DNA by conventional methods, or to those which lack efficient plasmid vectors. Future versions of the system will be fully automated in the pointing mode of operation, which should greatly increase throughput as well as success rate. Furthermore, the method might have a wide application in the field of cell biology as a practical method to transfer cytoplasmic materials.

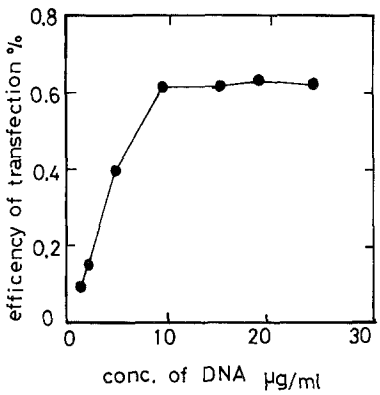

Fig. 6. Dose response for transfection of NRK cells with laser irradiation

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Appendix

Resolution of Laser Microspot Moving on the Specimen

The optimum design of the incident optics of the laser microscope requires an analytical expression for the behavior of a focussed spot on the image plane. The practical coverage of the moving spot, for example, depends on the extent of the spot deformation by lateral displacement. Various properties of the moving spot are derived, in principle, from the treatment of a Gaussian beam transfer [14] through a thick lens equivalent to a microscope objective. The essential results of the treatment are described as follows in the approximation to the first order of deflection angle of the incident beam.

The lateral displacement χ of the beam spot on the image plane is proportional to the off-axis deflection angle θ of the incident beam:

$$\chi=\zeta\theta. \tag{A.1}$$

The proportionality coefficient ζ is given by

$$\zeta=\left(1-\frac{h_2}{f}-\frac{z_2}{f}\right)S+\frac{d}{n^2}+\left(1-\frac{h_1}{f}\right)z_2, \tag{A.2}$$

where n is the index of refraction of the lens material, d, the thickness, $h_{1,2}$, the principal plane parameters, f, the focal length of the lens, S, the axial distance of the point of deflection beyond the incidence, and z_2, the distance of the beam waist produced behind the emergence. The distance z_2 in (A.2) is written in terms of the incident waist position z_1 and waist size ω_1, and of the characteristic angle ϕ of the beam transfer:

$$z_2=f\left[1+\frac{h_2}{f}-\left(1-\frac{h_1}{f}-\frac{z_1}{f}\right)\cos^2\phi\right] \tag{A.3}$$

with

$$\tan\phi=\frac{\pi\omega_1^2}{\lambda}\left[\left(1-\frac{h_1}{f}\right)f-z_1\right]^{-1}. \tag{A.4}$$

The beam spot produced on the image plane is deformed with its lateral displacement so that the radii $W_{\parallel}$ parallel and $W_{\perp}$ perpendicular to the displacement are, respectively, expressed in the form,

$$W_{\parallel}=\omega_2(1+\eta_{\parallel}\theta)$$

and

$$W_{\perp}=\omega_2(1+\eta_{\perp}\theta), \tag{A.5}$$

where ω_2 is the on-axis waist spot size at z_2 given by

$$\omega_2=\frac{\lambda f}{\pi\omega_1}\sin\phi. \tag{A.6}$$

The energy density within the spot is, therefore, reduced down with the displacement by a factor $[1-(\eta_{\parallel}+\eta_{\perp})\theta]$. The coefficients $\eta_{\parallel}$ and $\eta_{\perp}$ are expressed, respectively, as

$$\eta_{\parallel}=\eta_{\perp}-2a\sin^2\phi$$

and

$$\eta_{\perp}=-2bh_1\left[\frac{1-n}{R_1}\sin^2\phi-\left(1-\frac{h_1}{f}-\frac{z_1}{f}\right)^{-1}\cos^2\phi\right], \tag{A.7}$$

where

$$a=\frac{n^2-1}{2n^2}\left(\frac{S}{R_1}+1\right)^2,$$

$$b=\frac{1}{2n^2}\left[(1-n)\frac{S}{R_1}+1\right]^2. \tag{A.8}$$

In our present model of the laser microscope, the practical extention in which the laser spot moves with required resolution for efficient perforation is limited to about a half the diameter of microscopic field. With an objective lens of 100 X, for example, the extention is roughly 60 μm diameter on the specimen, long enough to bring the spot into any desired portion of a single cell.

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