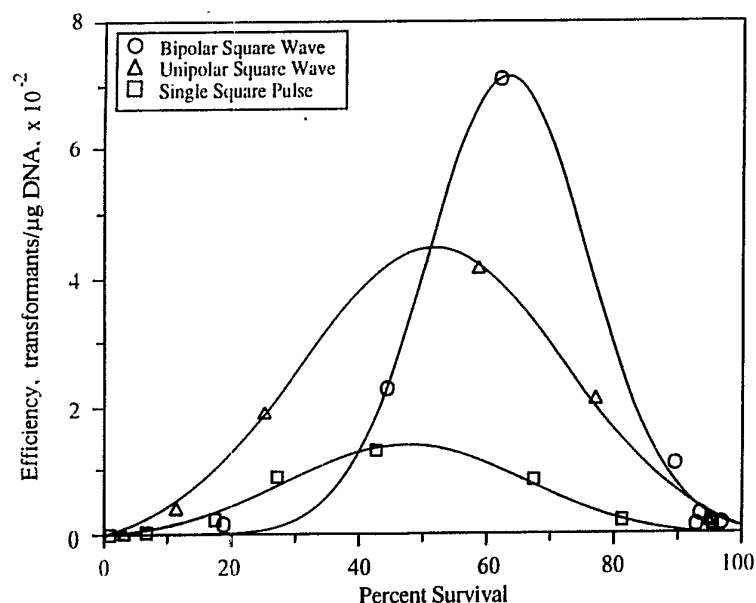




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(54) Title: METHOD OF ELECTROPORATION USING BIPOLAR OSCILLATING ELECTRIC FIELDS

**(57) Abstract**

A method of electroporation of biological particles which involves applying a bipolar oscillating electric field of sufficient strength to biological particles to cause poration of the particles, the bipolar oscillating electric field having either a square bipolar waveform or other bipolar periodic waveforms. The electroporation method increases cell survival and transfection efficiency. The method causes symmetrical poration at the two hemispheres of the cells which face the electrodes which apply the electric field. This method also provides similar advantages for cell fusion.

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METHOD OF ELECTROPORATION USING
BIPOLAR OSCILLATING ELECTRIC FIELDS

Technical Field

The present invention relates to the field of poration and fusion of biological cells. More particularly, the present invention relates to a method of electroporation and fusion which utilizes high frequency bipolar oscillating electric fields to permeabilize biological cells.

10 Background Art

Electric field induced permeabilization of cell membranes is an important technique for gene transfection and cell hybridization. Mechanistic studies of this process revealed that the uptake of fluorescent indicator by plant protoplasts occurs predominantly on the hemisphere facing the positive electrode, while in erythrocyte ghosts the probes exit through the hemisphere facing the negative electrode. To reconcile these observations symmetrical pore formation and a mechanism of molecular exchange by electroosmosis has been proposed.

Transfection of cells by DNA for studies of gene expression and regulation has been by far the major application of the electro-permeabilization technique.

25 Since the demonstration of the uptake of a plasmid DNA containing the thymidine kinase gene and subsequent expression of the gene in cultured mouse fibroblast cells by electro-permeabilization (Newmann et al, EMBOJ., 1, 841-845 (1982) and Wong et al, Biochem. Biophys. Res. Commun., 107, 584-587 (1982), numerous other works have reported similar success (using different genes) in other mammalian, bacterial, yeast and plant cells. In recent years, however, attention has focused on improving and optimizing the transfection

efficiency by manipulating one or several experimental parameters. Typical parameters reported to have improved the efficiency of transfection in some cell types include lowering of the temperature during and after the electric pulse, use of linearized plasmids, adjustments of the 5 ionic strength of the pulsing medium, addition of carrier DNA, use of actively growing cells, and post-pulse chemical treatment. While some of these optimization techniques have resulted in considerable improvements in 10 the cell lines used, others have, nevertheless, been shown to have either no or the opposite effect when used on other cell types.

Electric field of sufficient magnitude and duration has been shown to create transient pores in 15 cellular membranes. This method has been widely used for introducing DNAs, allosteric effectors, antibodies and other macromolecules into living cells either by molecular exchange between intra and extracellular media or by cell to cell fusion. In many instances, these 20 field methods have proved to be superior to the more conventional methods of chemical or viral-induced cell transfection and fusion. Some studies further suggest that electric fields could be used to modulate the activities of membrane enzymes in transducing biological 25 signal and energy.

Besides the various parameters mentioned above, manipulation of the electrical variables (i.e., field strength, pulse width, frequency, waveform) to improve gene transfections have not, as yet, been fully 30 exploited. In a recent report along these lines, a d.c. shifted RF (radio frequency) a.c. pulse was shown to be more efficient than a d.c. square pulse of similar amplitude and duration (Chang, Biophys. J., 56, 641-652

(1989).

The present invention is an improvement over prior electro-permeabilizations which allows for a number of advantages over prior methods.

5 Disclosure of the Invention

It is accordingly one object of the present invention to provide an improved method of electroporation and fusion of biological particles.

Another object of the present invention is to
10 provide a method of electroporation which increases cell survival and transfection efficiency.

A further object of the present invention is to provide a method of electroporation and fusion which utilizes bipolar oscillating electric fields having
15 square bipolar waveform, or other bipolar periodic waveforms.

A still further object of the present invention is to provide a method of electroporation and fusion which permeabilizes cell membranes symmetrically.

20 According to the present invention there is provided a method of causing poration of biological particles, comprising the steps of:

positioning a plurality of biological particles between electrodes; and

25 applying a bipolar oscillating electric field of sufficient strength to the plurality of biological particles to cause poration, wherein the bipolar oscillating electric field is either a square bipolar waveform, or other bipolar periodic waveform.

30 The present invention further provides a method of causing poration of biological particles, comprising the steps of:

positioning a plurality of biological particles

between electrodes; and

subjecting the plurality of biological particles to an electric field having a sufficient strength to cause fusion of at least a portion of the
5 cells.

Brief Description of Drawings

The present invention will be described in reference to the annexed drawings which are given by way of non-limiting examples only, in which:

10 Figure 1a is a block diagram illustrating an electro-permeabilization apparatus according to one embodiment of the present invention. Fig. 1a shows the connections arranged to produce bipolar pulses.

15 Figure 1b shows oscilloscope traces of the different waveforms used in the present study.

Figure 2 shows results of a typical electro-permeabilization experiment of adherent cells on a culture dish.

20 Figure 3 shows reversible electro-permeabilization of cells utilizing different wave forms including the preferred waveform of the present invention.

25 Figure 4 shows the relationship between transfection efficiency and percent survival for various waveforms including the preferred waveform of the present invention.

Figure 5 shows selected videoframes of electro-permeabilized cells subjected to electric field of various waveforms according to the present invention.

30 Best Mode for Carrying out the Invention

The electroporation method of the present invention involves applying a bipolar oscillating electric field of sufficient strength to biological

particles including cells in suspension and cells attached in a cell culture to cause poration of the particles. The bipolar oscillating electric field is unique in that it can easily permeabilize cells at multiple positions and retain high survivability.

As discovered during the course of the present invention the bipolar oscillating electric field may have a frequency of about 10 kHz to about 1000 kHz. However, a frequency of about 10 kHz to about 250 kHz is preferred, with a frequency of about 10 kHz to about 100 kHz being more preferred, and a frequency of about 60 kHz being most preferred for present applications.

The strength of the bipolar oscillating electric field may be between about 0.8 kV/cm to about 12.5 kV/cm, with a strength of about 2.2 kV/cm being preferred for present applications.

The square bipolar waveform may have a peak-to-peak amplitude of up to 5kV and a pulse duration of about 50 ns to about 10ms, with a preferred pulse duration being about 400 μ sec.

The electroporation method of the present invention has been found to be particularly advantageous in that the optimum transfection efficiency is obtained at a relatively high cell survival when utilizing a bipolar oscillating electric field having a square bipolar waveform. Cell survival is increased probably because the cell membranes are not polarized for extended periods of time and hence are not susceptible to irreversible damage. Transfection efficiency is increased because it has been discovered that when utilizing a bipolar oscillating electric field having a square bipolar waveform poration occurs symmetrically at the two hemispheres of the cells which face the

electrodes.

Most biological membranes in vivo maintain a resting membrane potential difference which may range from about -30 to -180 mV. An applied field causes an induced membrane potential which adds vectorially to this. Because the lipid bilayer is several orders of magnitude less conductive than the aqueous medium, ions of opposite signs accumulate at the interior and exterior surfaces. Consequently, an applied field is greatly magnified (with the magnification proportional to the radius of the cell) within the membrane. For an oscillating electric field, the frequency dependence of the induced membrane potential is given by¹

$$V_m = \frac{3/2 R E}{\sqrt{1 + (2\pi f \tau)^2}} \cos(\theta) \quad (1)$$

where τ is the relaxation time, f the frequency of the applied field, R the radius of the cell, E the electric field strength, and θ is the angle between the field direction and any point on the cell membrane. For d.c. fields, $f=0$ so that the denominator of eq.(1) is reduced to 1.

If the total imposed membrane potential (i.e., that due to the field coupled to the resting potential) reaches some critical value, electrical breakdown occurs and it becomes permeable to otherwise impermeable ions and molecules. Equation (1) shows that the maximum electrical effect would be at the two poles of the cell membrane facing the electrodes (i.e., $\cos(\theta) = 1$ for $\theta = 0$, and -1 for $\theta = \pi$). In the course of the present

invention it was discovered that membrane breakdown is essentially asymmetric with unipolar a.c. or single d.c. electric fields and that symmetrical permeabilization occurs only with bipolar a.c. fields. This discovery indicates that the resting membrane potential may play a role in electro-permeabilization of the cell membrane and electroosmosis is not the dominant mechanism in the cellular uptake of molecules in electroporation. In addition, taking the advantages of bipolar a.c. fields, it has been shown that one can obtain a significant improvement in the efficiency of DNA transfection using a bipolar a.c. field electroporation technique.

For the purposes of the present invention an electric pulse apparatus capable of producing several types of waveforms with frequencies up to 1MHz was developed. The block diagram of the instrument arranged to deliver bipolar oscillating electric pulses is shown in Fig. 1a.

The electric pulses were produced by serially gating several square wave generators as follows: The total on-time of the bipolar a.c. burst is controlled by a single square pulse generator (HP-8011A) of variable pulse duration (amplitude 5V) whose output gates the pulse frequency generator (Wavetek 183) equipped with a frequency dial adjustable from d.c. to 1MHz. The rising edge of each incoming single square pulse from the pulse frequency generator (amplitude 10V) triggers the high power positive pulse generator (amplitude 10V) triggers the high power positive pulse generator (Cober 606P). The amplitude and duration of the pulse from the Cober is independently adjustable from 0 to +2.5 kV, and 50 ns to 10ms, respectively (for both unipolar and bipolar a.c. pulses, however, the period is limited by the repetition

rate selected on the pulse frequency generator). The falling edge of a synchronous 10V square pulse from the Cober is then allowed to trigger the second high power pulse generator (Cober 606P) with similar adjustable
5 output (but with amplitude of 0 to -2.5kV). Combination of these pulses finally produces the desired bipolar waveform of peak-to-peak amplitude of up to 5kV. The maximum delay in the trigger mechanism between the two high power generators is less than 0.5 μ s. The magnitude
10 of the electric pulses were measured across the electrodes with a 1000:1 probe (Tektronix P6015) and monitored on a storage oscilloscope (Tektronix 7704A).

Single square pulses of either polarity were produced by disabling the pulse frequency generator and
15 one of the high power pulse generators. Similarly, unipolar oscillating pulses could be obtained by disabling any one of the high power generators depending on the polarity desired. Typical examples of the waveforms obtained with the present instrument measured
20 across a 500 Ohm load resistor are shown in Fig. 1b.

The electrodes for the instrument were constructed out of 50mm long stainless steel and were held fixed on a Kel-F support in grooves with a separation of either 1 or 2mm. A culture dish containing
25 cells grown in monolayer or suspended in droplets was placed on a platform which could be moved vertically to make contact with the electrodes. In addition, horizontal movement of the platform allows for up to twelve experiments (using 2mm electrode separation) to be
30 performed on the same culture dish (8cm diameter).

Another cell holding chamber was constructed using a pair of stainless steel electrodes sandwiched between two microscope slides (electrode separation

0.75mm) with a capacity to hold 25 μ l of cell suspension. The chamber was mounted on a ZEISS (ICM405) inverted low light fluorescence microscope. The increase in fluorescence intensity, monitored at 610nm (excited at 520), that results from the binding of ethidium bromide (Sigma) to cytosolic DNA or RNA was used as indicator of electro-permeabilization. Real time images of these events were acquired with an image intensifier (Videoscope International, KS-1381) attached to a CCD camera (COHU, 4815) and recorded on a videotape (Sony, VO-5600). The time resolution of the videoscope recordings was 33ms. The recorded images were later transferred to a digital disk recorder (Panasonic, TQ2028F) and analyzed with a digital image processor (Recognition concepts, 55/48Q) using the RTIPS software library from TAU Inc. Some of the video images in the time series were then displayed on a color monitor and photographed by transferring to a freeze frame recorder (Polaroid Corp.)

In experiments conducted during the course of the present invention to investigate electric field effects, wild type NIH3T3 cells were grown to approximately 50-70% confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum at 37° in a 87% humidified, 5% CO₂ incubator (Forma). Adherent cells were removed from the culture dish with 0.25% trypsin solution and washed three times with a pulsing buffer which consisted of 250mM sucrose, 10mM phosphate, 1mM MgCl₂, pH 7.2. The number of harvested cells was determined in at least three readings using a hemocytometer chamber and the concentration was adjusted by appropriate dilutions to be 10⁶ cells/ml. Cells were kept at ice-cold temperature just prior to both DNA

transfection and permeabilization experiments (using ethidium bromide). For experiments performed on cells adherent to the culture dish, the growth medium was substituted with 4ml of cold pulsing buffer. The
5 viability of cells suspended in the pulsing buffer was not altered for up to two hours.

The circular plasmid DNA, pSV2-neo, (~8kb) was kept frozen prior to use. Powdered antibiotic G-418 sulfate was obtained from GIBCO. Its specific activity
10 was 516.7 μ g per mg of powdered compound. A stock solution of selection media was prepared by first dissolving the antibiotic in serum free media which was then filtered using 0.2 micron filter. The filtered solution was later supplemented with 10% calf serum. The
15 final concentration of G-418 was 400 μ g of active antibiotic per ml of medium.

All transfection experiments described herein were performed typically within about 20 minutes of freshly harvested cells. Appropriate volume of the
20 plasmid DNA (1 μ g/ μ l) was added to a suspension of cells (10^6 cells/ml) in the pulsing buffer to give a final concentration of 10 μ l/ml of plasmid DNA. In other experiments, 1, 5, and 20 μ g DNA per ml were used to test the dependence of transfection efficiency on DNA
25 concentration. All solutions were thoroughly mixed and allowed to stand on ice for 10 minutes before electric pulse application.

An aliquot of the homogeneous cell suspension (100 μ l) was carefully placed as a thin strip on an empty
30 culture dish mounted on the movable platform (see Fig. 1a). After proper alignment, the platform was moved vertically towards the fixed parallel electrodes and upon contact the strip of cell suspension was evenly

distributed between the electrodes (separation 2mm) through capillary action. The electrodes were sterilized with absolute alcohol and blow dried prior to each use.

After application of the electric pulse of
5 desired amplitude and duration, the platform was gently lowered and the culture dish was immediately removed, covered, and placed on ice-water temperature for 25-30 minutes. Cells were then diluted with 5ml of growth medium on the same culture dish and distributed in a 96-
10 well flat bed chamber (Costar) at 10^3 cells per well and kept in the incubator for 6-8 hours. After this incubation period, the medium was aspirated off and replaced with selection medium at $100\mu\text{l}$ per well. Every 2 days the selection medium was replaced with fresh
15 medium and after 14 days the number of G-418 resistant colonies was counted in each well.

Permeabilized sites on the membrane were identified using the increase in fluorescence intensity resulting from the binding of ethidium bromide to
20 cytosolic DNA/RNA as described earlier. The concentration of ethidium bromide ranged from 0.01% to 1%. Further studies on field induced transient permeabilization were performed in the course of the present invention using adherent cells grown in monolayer
25 on a culture dish. The reversibility of the permeabilization was monitored using trypan blue stain (GIBCO, final concentration = 0.04%) exclusion method as a function of time. The survival of cells after the electric pulses were assayed using trypan blue exclusion
30 and counting the number of stained cells in a hemocytometer chamber. All counting was done at least three times and averaged.

The relative efficiency of transfection of many

cell types may depend on a multitude of parameters (i.e., temperature, form of DNA, ionic strength, etc.). However, in most reported experiments involving transfections, the critical parameters have generally
5 been the electric field strength and duration. Therefore, comparative studies of transfection efficiency obtainable between different waveforms were based on these parameters.

Initial experiments focused on the electric
10 field strength dependence of permeabilization and its reversibility as well as viability of cells after the electric pulses as a function of different waveforms.

A typical representative example of electro-permeabilization of cells grown in a culture dish as a
15 function of electric field is shown in Fig. 2. In Fig. 2 the dark stripes show cells that lay between the two parallel electrodes that have been stained with trypan blue. The dye was introduced 1 hour after the termination of the electric pulse and labeling was
20 continued for 30 minutes after which the cells are washed and photographed against a white background. The waveform utilized was a d.c. pulse having a pulse width of 400 μ s.

For qualitative identification of the
25 electrical parameters (e.g., critical membrane breakdown voltage, pulse width dependence, etc.) this procedure referred to in reference to Fig. 2 above was found to be quick and simple with minimal disturbance to the adherent cells prior to the electric field application. Moreover,
30 this procedure allows for single or multiple experiments and the control to be performed on the same culture dish.

Utilizing the basic procedure discussed in reference to Fig. 2 above, cell survival was examined as

13

a function of electrical parameters. The results are partially summarized in Table 1 below.

TABLE 1
Summary of Permeabilization and Transfection Efficiency as a Function of Various Parameters

Electric Field (kV/cm)	Frequency (kHz)	Pulse width (μsec)	NCBEP $\times 10^{-5}$	Plasmid A (μg/ml)	% Survival			Efficiency Transformants/μg		
					BAC	UAC	DC	BAC	UAC	DC
0.4	60	400	1.1	10	96.8	94.7	95.4	15	19	14
0.8	60	400	1.2	10	93.4	95.3	81.2	29	21	20
1.2	60	400	1.1	10	92.8	77.0	42.6	13	213	129
1.6	60	400	1.2	10	89.4	58.6	27.1	108	417	87
2.2	60	400	1.2	10	62.0	25.1	17.4	710	191	22
3.0	60	400	1.1	10	44.3	11.3	6.7	229	39	3
4.0	60	400	1.0	10	18.7	3.2	<1	15	2	0
1.6	60	400	1.1	1	-	-	-	30	70	10
1.6	60	400	1.2	5	-	-	-	98	392	94
1.6	60	400	1.1	20	-	-	-	115	408	96
1.6	60	1000	1.0	10	81.4	53.4	25.7	103	359	71
1.6	60	5000	1.1	10	41.2	27.8	20.8	48	92	14
1.6	250	400	1.0	5	93.4	68.4	-	22	112	-
1.6	1000	400	1.2	5	92.1	78.4	-	16	48	-
0	0	0	1.2	10	95.7	95.8	98.1	0	0	0
1.6	60	400	1.1	0	85.8	61.4	37.7	0	0	0
0	0	0	1.0	0	95.4	98.4	95.8	0	0	0

eviations: NCBEP (Number of cells before electric pulse); BAC (Bipolar A.C.); UAC (Unipolar A.C.); DC (D.C.)
ie electric field for the bipolar a.c. field is evaluated from the peak-to-peak applied voltage.

Viability (% survival) was quantitated by counting the percentage of cells excluding trypan blue that was introduced 1 hour after the termination of the electric pulses. In a separate experiment, it was found
5 that no further improvements in viability for cells incubated for longer than 1 hour.

For all three waveforms the viability of cells was found to decrease with increasing electric field. Cells were, however, found to survive more in the
10 oscillating pulses than the d.c. pulse of the same peak amplitude. For the bipolar a.c. pulse, the survivability data shown in Table 1 corresponds to electric field values computed from the peak-to-peak applied voltage. If these field values are recalculated from the amplitude
15 of the half cycle, the survivability is found to be still better than in the d.c. pulse and lower but comparable to the unipolar a.c. pulse.

The effect of pulse width and frequency on the survivability of cells was also examined at a fixed field
20 strength. For field strengths below about 1.6kV/cm and a frequency of 60kHz, increasing the pulse width from 100 μ s up to 1ms did not appreciably change the viability of cells. Increasing the pulse width to 5ms and above (field strength 1.6kV/cm), however, resulted in sharp
25 increases in cell death (Table 1).

The frequency dependence of cell viability showed the opposite effect. At 1.6kV/cm, for example, cells pulsed with the unipolar pulse recovered by about 20% when the frequency was raised from 60kHz to 1 MHz
30 (Table 1). Similar recovery was also obtained with the bipolar pulse when the electric field was fixed at 2.5kV/cm. When other parameters are fairly constant, increases in the frequency from 20kHz to 80kHz did not

appreciably affect cell viability for the pulse width (400 μ s) used in the present experiments.

The transient behavior of electric field induced permeabilization was monitored through time course measurements. When the electric field is not exceedingly high or the pulse width too long, permeabilization of the cell membrane was found to be reversible. This is demonstrated in Fig. 3 for the three waveforms studied. In Fig. 3 cells were permeabilized using the optimum field strength found for transfection for each waveform shown. Pulse width: 400 μ s. Frequency: 60kHz. a) Bipolar a.c. field: 2.2kV/cm. b) Unipolar a.c. field: 1.6kV/cm. c) d.c. field: 1.2kV/cm. Trypan blue was added after the electric field application at the times shown. Permeabilized cells were quantitated by counting the number of stained cells.

The recovery time course for both unipolar and bipolar waveforms appeared to be similar if not identical. They were fitted with the same exponential function. When the two curves are extrapolated to the y-axis, they suggest that almost 100% of the surviving cells are permeabilized at these field strengths. The time course for the bipolar and unipolar waveforms extrapolation to $\approx$ 110% permeabilization may indicate that initially the recovery is proceeded with a lag time. The data also show that maximal recovery is achieved within 30-35 minutes.

One of the primary objectives of transfection experiments is to obtain the optimum number of stable or transient transformants for a given amount of plasmid DNA. In most cases the availability of the plasmid is usually limiting and comparative experiments are thus conveniently expressed in terms of their efficiency of

transfection which is defined as the number of transformed cells obtainable per μg of DNA. The results showing the dependence of the transfection efficiency as a function of the electric field, waveform, frequency and pulse width are summarized and shown in Table 1.

The optimum transfection were found at electric field strength of 1.2, 1.6 and 2.2kV/cm for d.c., unipolar and bipolar pulses respectively. At these field strengths, about 60% of the cells were viable for both unipolar and bipolar pulses but only about 40% were viable for the d.c. pulse. Under these optimal conditions for each waveform, the transfection efficiency with the bipolar a.c. pulse was 1.7 and 5.5 fold greater than the unipolar and d.c. pulses respectively.

When the frequency (60KHz) and the pulse width (400 μs) were held constant, the efficiency was found to very low if either cell survival was above 80% or below 20% for all three waveforms. It is likely that at low electric field strength (even though the membrane is permeabilized (e.g., at 0.8kV/cm)) the pore sizes may not be large enough to allow passage of the plasmid thus decreasing the efficiency. On the other hand, cell death may be the factor for the decreased efficiency at higher electric field strengths.

To ensure these comparative experiments were not done at limiting or saturating levels of the plasmid DNA, the transfection efficiency was tested as a function of the plasmid concentration at a field strength of 1.6kV/cm with the frequency and pulse width set at 60KHz and 400 μs , respectively (Table 1).

The number of transformants obtained was found to increase linearly over a four fold range of plasmid concentrations (5 $\mu\text{g}/\text{ml}$ to 20 $\mu\text{g}/\text{ml}$). The efficiency,

therefore, remained relatively constant over this concentration range. At $1\mu\text{g/ml}$ of DNA the efficiency decreased sharply for all waveforms thus most of our experiments were done with plasmid concentrations ranging
5 from $5\mu\text{g/ml}$ to $10\mu\text{g/ml}$. The effect of cell density on the efficiency was also examined and slight but random variation was observed when the concentration was raised from 1×10^6 cells/ml to 3×10^6 cells/ml.

Increases in the pulse width or frequency were
10 also found to affect the transfection efficiency. When the pulse width was increased to 1ms, the efficiency was only slightly lower compared to the efficiency with a pulse width of $400\mu\text{s}$ when the electric field and frequency were fixed at 1.6kV/cm and 60KHz , respectively.
15 However, the efficiency dropped significantly when the pulse width was increased to 5ms under the same conditions. When the frequency was raised from 60KHz to 1MHz at a fixed field strength and pulse duration, the efficiency was again found to decrease although cell
20 survival was found to increase.

The effectiveness of the three types of waveforms studied here in permeabilizing the cell membrane was examined using ethidium bromide as a permeabilization indicator probe. In the absence of
25 electric field, ethidium bromide can diffuse into the cells but only at a slow rate (typically complete staining was observed in 20 to 30 minutes). Experiments were thus performed within 5 minutes after the mixing of the dye. In these experiments, distinct qualitative
30 differences between the bipolar and unipolar or d.c. pulses in their ability to permeabilize the cell membrane were discovered.

Fig. 5 depicts selected video frames of both

the location and time course of electro-permeabilized NIH3T3 cells subjected to various waveforms of different amplitude and frequency. In Fig. 5 the positive electrode is at the bottom and the negative electrode is at the top of each frame displayed. The first row for each waveform shows the prepulse image where the nucleus is relatively bright due to the slow uptake of the ethidium bromide during sample preparation. In subsequent frames showing events after the pulse, the prepulse image has been subtracted. The fluorescence is color coded in increasing intensity in the following order: blue, purple, gray, yellow, red, and white. For each waveform displayed, time increases from top to bottom. Pulse duration = 0.400 msec. From left to right, i) Unipolar a.c. pulse: Electric field, $E = 1.10 \text{ kV/cm}$, Frequency = 250 KHz. Time series of frames: 0.528, 1.508, 2.730, 6.303, 8.745 seconds. ii) d.c, square pulse: $E = 4.95 \text{ kV/cm}$. Time series of frames: 0.297, 0.825, 1.419, 2.013, 8.948 seconds. iii) Bipolar a.c. pulse: $E = 2.25 \text{ kV/cm}$ (peak to peak). Frequency = 250 KHz. Time series of frames: 0.132, 0.396, 0.891, 1.848, 3.267 seconds.

Although not expected from theory, the data clearly show that the cell membrane is permeabilized at either one hemisphere (that facing the positive electrode) or asymmetrically when the applied electric pulse is unipolar or d.c.

The left column in Fig. 5 shows results from an unipolar a.c. field with a frequency of 250KHz and electric field magnitude of 1.1 kV/cm , which is near the field strength required for membrane breakdown (about 0.8 - 1.0 kV/cm). No qualitative difference was observed in the experiments between unipolar a.c. and single d.c.

pulses. The data clearly show that only the hemisphere of the cell membrane facing the positive electrode is permeabilized.

When the applied electric field is gradually
5 increased. often by about 100 to 300V/cm, one also observes the entry of the fluorescent probe, at a much reduced quantity, from the hemisphere facing the negative electrode. The additional voltage required to initiate the second entry site can be attributed to the field
10 strength needed to overcome the resting membrane potential, which is calculated from eq.(1) to be in the range of -80 to -210mV.

Although the entry positions on the cell surface remain unchanged, the permeabilized area
15 increases with increasing electric field. For example, when the applied electric field is greatly enhanced, significant permeabilization is observed at both hemispheres facing the electrodes. The permeabilization however, remains asymmetric with the effect being more
20 pronounced at the site facing the positive electrode than the other. This was demonstrated using either d.c. or unipolar a.c. fields.

The results from a 0.400msec time window, 4.95kV/cm single d.c. field is shown in the middle column
25 of frames in Fig. 5. Under these conditions, the first available frame after the pulse (33ms) show that the asymmetric permeabilization occurs simultaneously on both sides.

On the right column of frames in Fig. 5, it is
30 shown that, unlike those observed with either d.c. or unipolar a.c. fields, the bipolar a.c. field induces symmetric permeabilization of the cell membrane.

All experiments reported above were also

performed using wild type Chinese Hamster Ovary (CHO) cells and similar results were obtained.

The results reported here are consistent with the hypothesis that the negative resting potential interacts vectorially with the imposed electric potential. A negative resting potential means that the equilibrium electric field in the membrane is directed inwards. When an external electric field is applied, the sign of the imposed membrane potential is (+) inside and (-) outside on the pole of the cell membrane facing the negative electrode and the opposite is true on the other pole facing the positive electrode. Thus, when the applied electric field alone is nearly equal to or slightly greater than that required for membrane breakdown, the field vectors are additive and greater than V_c at the membrane site facing the positive electrode, while they cancel on the membrane site facing the negative electrode and resulted with a net potential being less than V_c . Once the applied potential overcomes this cancellation, a second permeabilized site is observed at the hemisphere facing the negative electrode.

The fact that ethidium bromide is able to enter from both hemispheres at high electric fields suggests that electrophoretic or electroosmotic mechanisms cannot be the predominant factors since both processes are fundamentally unidirectional.

Electro-permeabilization of cell membranes for gene transfection is increasingly being preferred over other chemical techniques partly because of its ease of operation and increased yields. The present invention provides a new and improved method of electro-permeabilization using bipolar a.c. pulses. The efficiency of transfection using the present method

provided improvements of 1.7 and 5.5 fold over the conventional unipolar a.c. and the d.c. square pulse techniques, respectively under optimal conditions.

For all three waveforms studied here, the electric field strength, and to a relatively lesser extent the pulse width and frequency, were found to be the critical factors with the greatest influence on the transfection efficiency. When both the frequency and pulse width were held fixed, the transfection efficiency was sensitive in a narrow window of electric field strength (about 1 to 1.6kV/cm; bipolar pulse expressed from half height). Moreover, transfection data (60KHz and 400 μ s) at this critical electric field region appears to correlate with cell survivability. This situation was observed when the pulse width was increased to 5ms.

Comparison of the efficiency at this survival rates to the efficiency of those with comparable survival rates under other conditions was markedly different. When the survivability of cells as a function of waveforms was compared under similar electrical parameters, d.c. pulse treated cells suffered more in comparison to unipolar or bipolar a.c. pulses. This could be due to the fact that with the d.c. method, the membrane is polarized for extended period of time and hence susceptible to irreversible damage. Similarly, the increased survivability that was observed both unipolar and bipolar pulses with increasing frequency may also be partially explained by the same effect in addition to the fact that the induced membrane potential responsible for breakdown is inversely proportional to the frequency.

If it is assumed that the transfection efficiency is proportional to the amount of plasmid DNA introduced into the cell when comparable number of

transfectable cells are available, then it is reasonable to expect the bipolar a.c. pulse to be more effective than the unipolar a.c. or d.c. method since it has been shown that the present method permeabilizes the membrane
5 symmetrically. Together with the qualitative and quantitative results presented in the course of the present invention, it was concluded that permeabilization of the membrane at multiple sites without affecting cell viability may account for the observed improvements in
10 the transfection efficiency (i.e., the number of cells transformed per μg of DNA used) of electro-permeabilized NIH3T3 cells with bipolar a.c. fields compared to single d.c. or unipolar a.c. fields or other waveforms.

Although the invention has been described with
15 reference to particular means, materials and embodiments, from the foregoing description, one skilled in the art can easily ascertain the essential characteristics of the present invention and various changes and modifications may be made to adapt the various uses and conditions
20 without departing from the spirit and scope of the present invention as described by the claims which follow.

Claims

1. A method of causing poration of biological particles, comprising the steps of:
positioning a plurality of biological particles
5 between electrodes; and
applying a bipolar oscillating electric field of sufficient strength to said plurality of biological particles to cause poration of said particles, said bipolar oscillating electric field having a square
10 bipolar waveform or other bipolar periodic waveforms.
2. A method of causing poration of biological particles according to claim 1, wherein said bipolar oscillating electric field has a frequency of about 10 kHz to about 1000 kHz.
- 15 3. A method of causing poration of biological particles according to claim 2, wherein said bipolar oscillating electric field has a frequency of about 10 kHz to about 250 kHz.
- 20 4. A method of causing poration of biological particles according to claim 3, wherein said bipolar oscillating electric field has a frequency of about 10 kHz to about 100 kHz.
- 25 5. A method of causing poration of biological particles according to claim 4, wherein said bipolar oscillating electric field has a frequency of about 60 kHz.
6. A method of causing poration of biological particles according to claim 1, wherein said square bipolar waveform has a peak-to-peak amplitude of up to
30 5kV.
7. A method of causing poration of biological particles according to claim 1, wherein the strength of said bipolar oscillating electric field is about 0.8

kV/cm to about 12.5 kV/cm.

8. A method of causing poration of biological particles according to claim 7, wherein the strength of said bipolar oscillating electric field is about 2.2
5 kV/cm.

9. A method of causing poration of biological particles according to claim 1, wherein the pulse duration of said square bipolar waveform is about 50 ns to about 10 ms.

10. A method of causing poration of biological particles according to claim 9, wherein the pulse duration of said square bipolar waveform is about 400
10 μ sec.

11. A method of causing poration of biological
15 particles according to claim 1, wherein said biological particles are biological cells suspended in solution.

12. A method of causing poration of biological particles according to claim 11, wherein said biological particles are biological cells attached in a cell
20 culture.

13. A method of causing poration of biological particles, comprising the steps of:

positioning a plurality of biological particles between electrodes; and

25 subjecting said plurality of biological particles to an electric field having a sufficient strength to cause poration of at least a portion of said cells at two hemispheres which face the electrodes.

14. A method of causing poration of biological
30 particles according to claim 13, wherein said poration of at least a portion of said cells at two hemispheres which face the electrodes is symmetrical.

15. A method of causing poration of biological particles according to claim 13, wherein said electric field comprises a bipolar oscillating electric field having a square bipolar waveform or other bipolar
5 periodic waveforms.

16. A method of causing poration of biological particles according to claim 15, wherein said bipolar oscillating electric field has a frequency of about 10 kHz to about 100 kHz, a strength of about 1.2kV/cm to
10 about 3.0 kV/cm and a pulse width of about 200 μ sec to about 400 μ sec.

17. A method of causing poration of biological particles according to claim 16, wherein said bipolar oscillating electric field has a frequency of about 60
15 kHz, a strength of about 1.2 kV/cm to about 3.0 kV/cm, and a pulse width of about 400 μ sec.

18. A method of causing poration of biological particles according to claim 13, wherein said biological particles are biological cells suspended in solution.

20 19. A method of causing poration of biological particles according to claim 13, wherein said biological particles are biological cells attached in a cell culture.

20. A method according to claim 1, further
25 including the step of fusing porated cells.

1 / 5

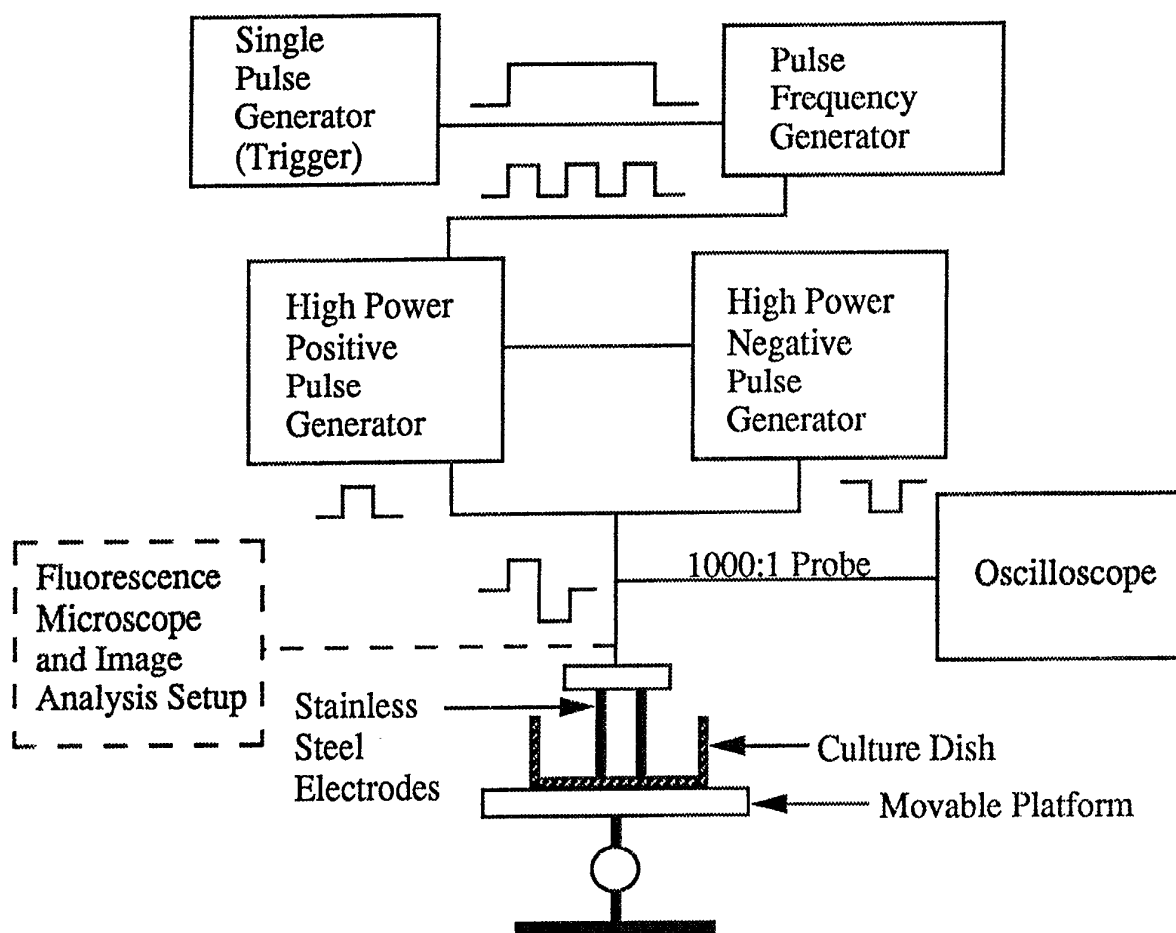


FIG. 1A

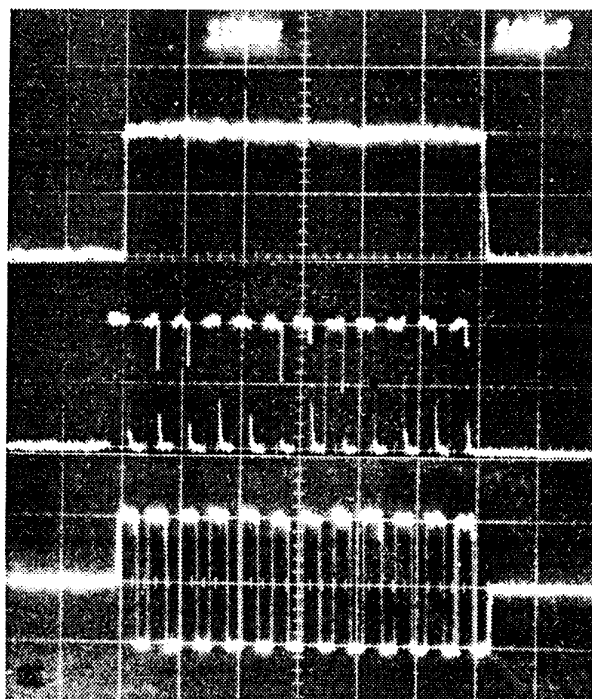
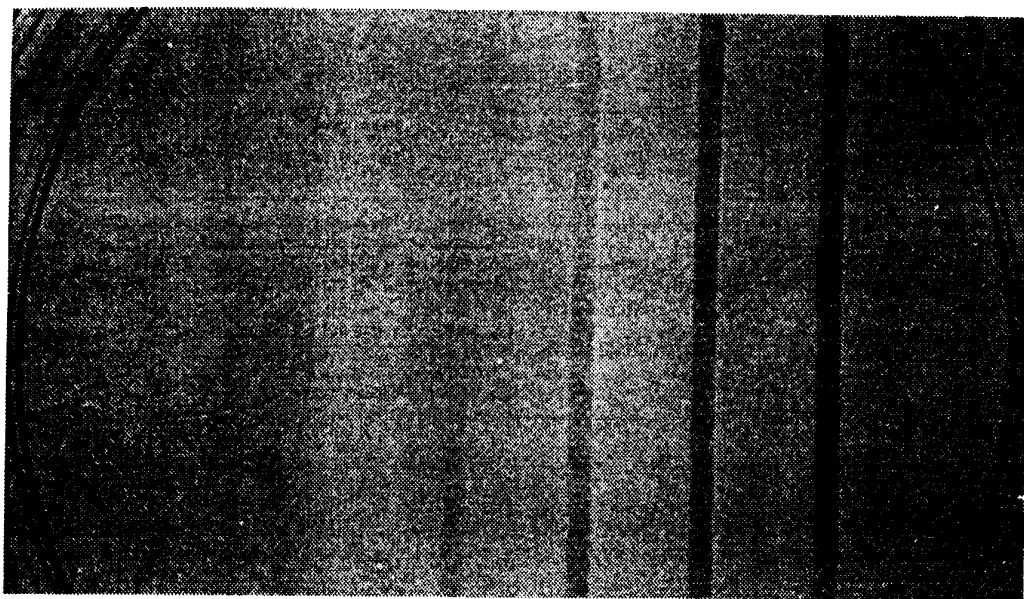


FIG. 1B



0.2 0.4 0.8 1.2 1.6 2.0

Electric field (kV/cm)

FIG. 2

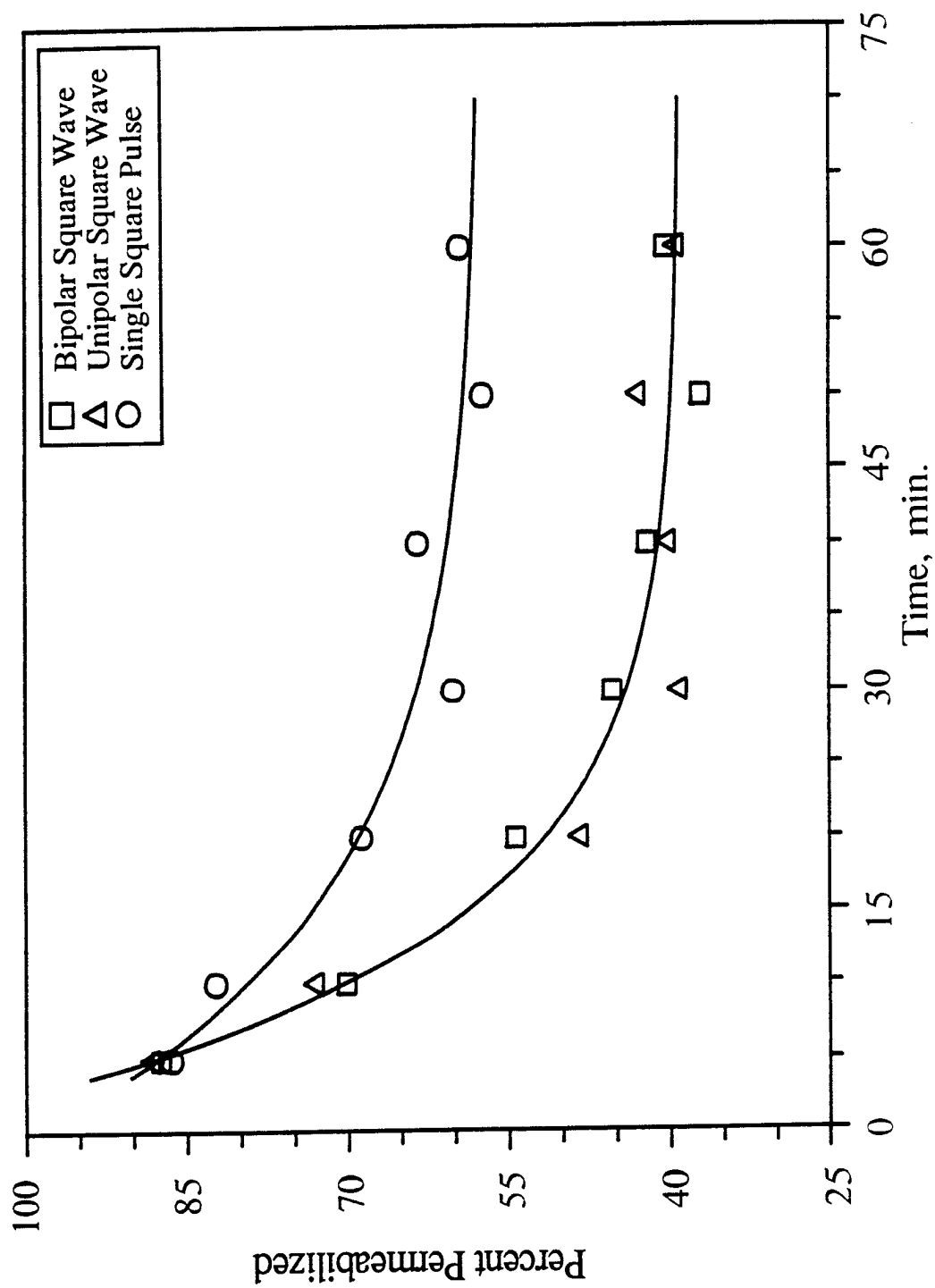


FIG. 3

4 / 5

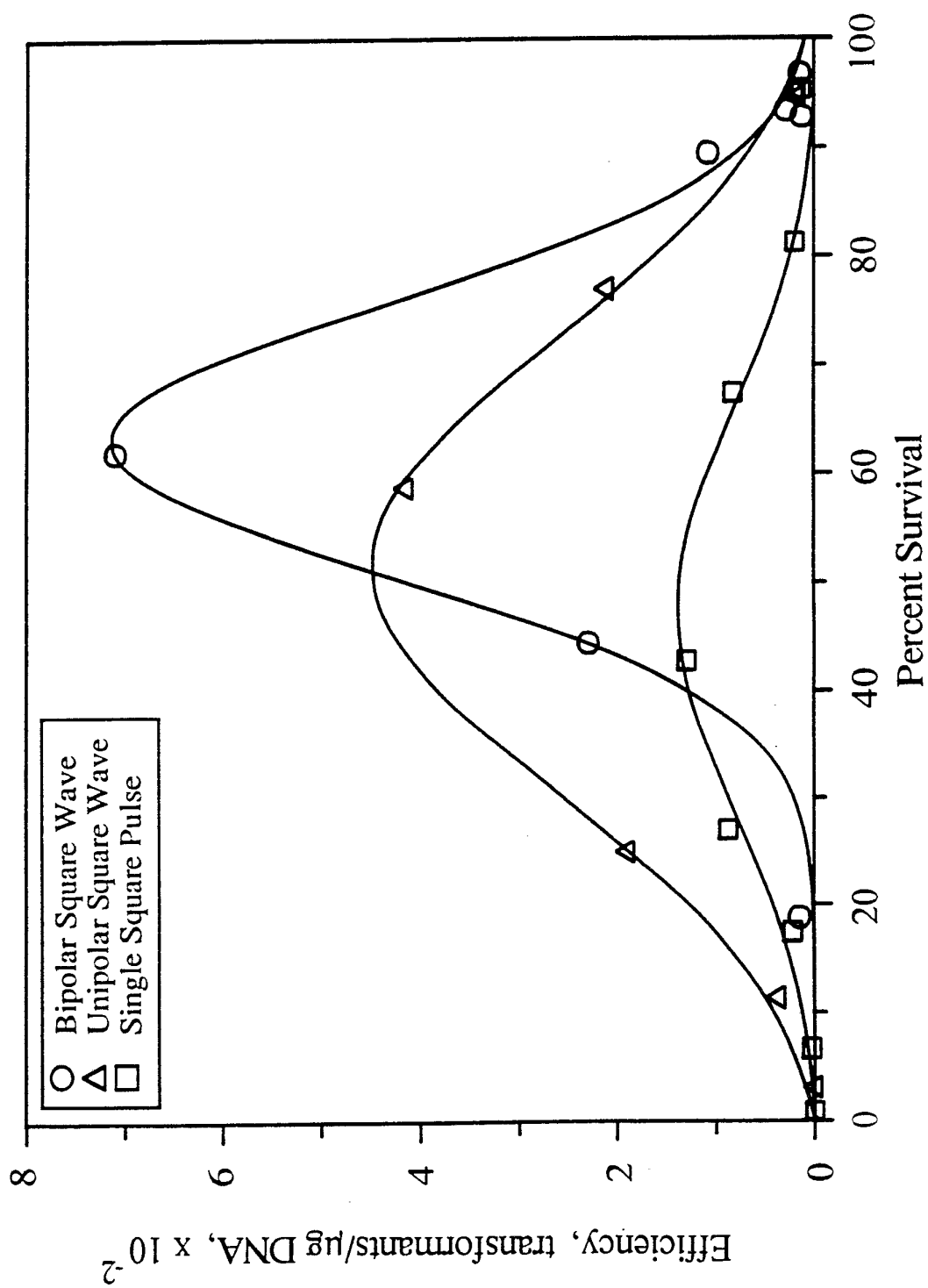


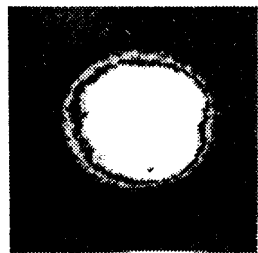
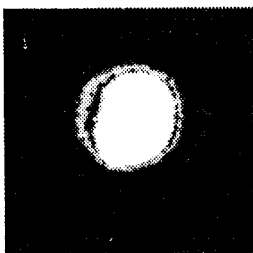
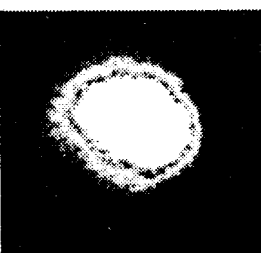
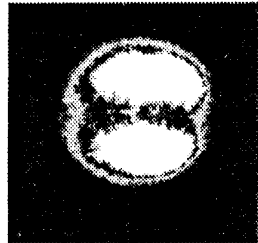
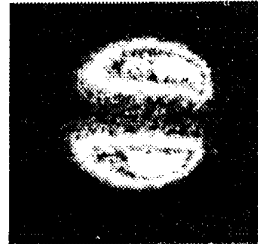
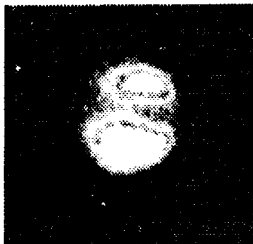
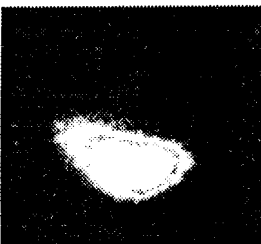
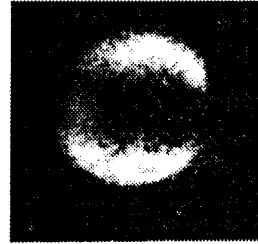
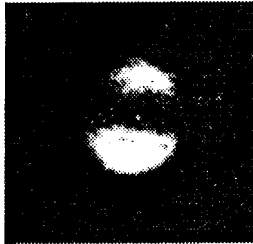
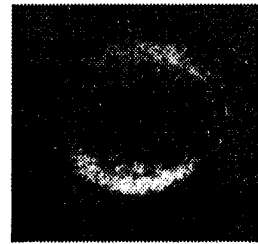
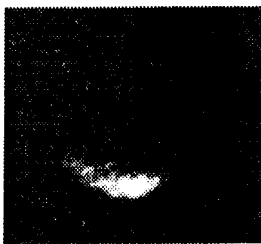
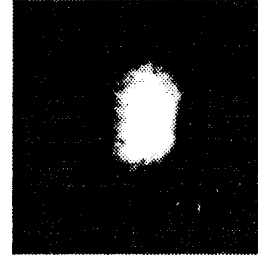
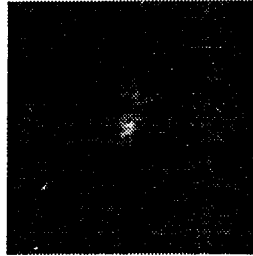
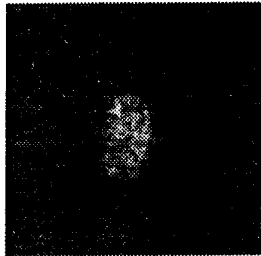
FIG.4

SUBSTITUTE SHEET

5 / 5
FIG. 5B

FIG. 5A

FIG. 5C



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06884

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶	
According to International Patent Classification (IPC) or to both National Classification and IPC	
PCT(5): C12N 13/00, 15/00	
USCL: 435/172.2, 173	
II. FIELDS SEARCHED	
Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
USCL:	435/172.2, 173
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸	
CAS PERSONAL FILES BIOSIS	

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X Y	Plant Cell REP.. Volume 8. issued May 1990. Joersbo et al.. "Direct Gene Transfer to Plant Protoplasts by Electroporation by Alternating Rectangular and Exponentially Decaying Pulses". pages 701-705. see entire document.	1 1-19
X,P Y	Chemical Abstracts. Volume 114. No. 3 issued 21 January 1991. Xie et al.. "Study of Mechanisms of Electric Field-Induced DNA Transfection II. Transfection by Low Amplitude, Low Frequency Alternating Electric Fields" see page 210. column 1. abstract No. 18900. <u>Biophys J.</u> 58(4). 897-903.	1 1-19
X,P	US.A 5.007.995 (Takahashi et al.) 16 APR 1991, see entire document.	1-20

- * Special categories of cited documents: ¹⁰
- "A" document defining the general state of the art which is not considered to be of particular relevance
 - "E" earlier document but published on or after the international filing date
 - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 - "O" document referring to an oral disclosure, use, exhibition or other means
 - "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

10 DECEMBER 1991

International Searching Authority

ISA/US

Date of Mailing of this International Search Report

27 JAN 1992

Signature of Authorized Officer

Jon P. Weber

tf

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹⁴ not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹⁵, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-19, drawn to electroporation method, classified in class 435, subclass 173.

II. Claim 20 drawn to electrofusion method, classified in class 435, subclass 172.2

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only these claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.