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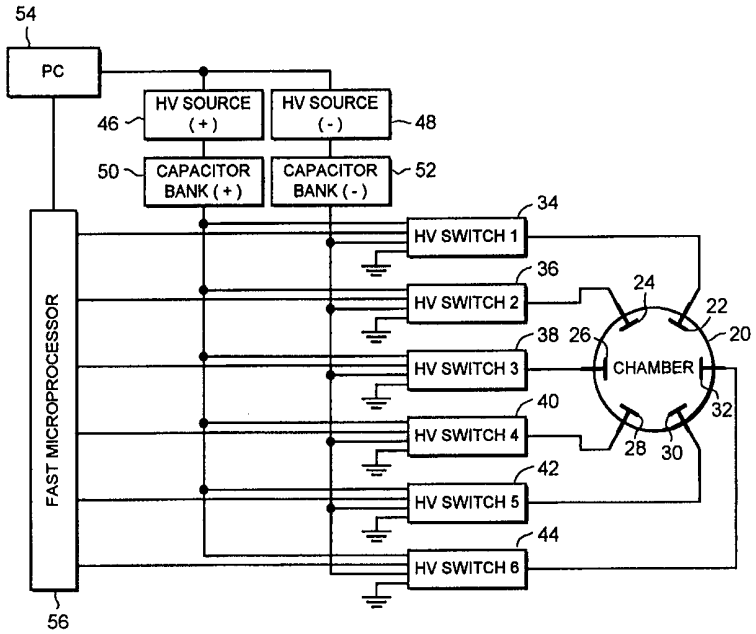

(57) Abstract

An electropermeabilisation apparatus provides a central chamber for receiving biological cells, surrounded by a ring of six electrodes (20, 22, 24, 26, 28, 30). The cells may be immersed in a perfusion fluid. Each electrode may be connected to a high positive voltage, a high negative voltage, or to earth by means of a respective one of a bank of switches (34, 36, 38, 40, 42, 44) controlled by a PC (54) and a microprocessor (56). Positive and negative voltages are then applied to different opposed pairs of electrodes in sequence, in order to expose the cells to an electropermeabilising electric field, the direction of which moves during exposure.

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Electropermeabilisation Method and Apparatus

The invention relates to an electropermeabilisation method and apparatus, with particular application to the electropermeabilisation of the membranes of biological cells.

Background of the Invention

The plasma membrane of a biological cell is a continuous macromolecular structure composed of a lipid bilayer with immersed proteins. It defines the outer dimensions of the cell, and possesses anchor points for the cellular cytoskeleton, thus providing structural integrity. As it is essentially composed of lipid, the plasma membrane is virtually impervious to water-soluble molecules and ions. They can usually only traverse the plasma membrane via specific channels formed by proteins located in the bilayer. The study and control of cellular processes often requires manipulation of the intracellular environment. Methods currently in use to allow the introduction of non membrane-permeant molecules include micro injection and patch-clamping of cells. These techniques are limited in that they are time-consuming and allow only a relatively few cells to be manipulated. Alternative techniques, which permit many cells to be manipulated simultaneously, involve permeabilisation of the membrane using weak amphipathic detergents, liposome-mediated delivery, scrape-loading and electric fields (known as electropermeabilisation or electroporation).

Exposure of cells to high-intensity electric fields causes transient localised disruptions, or rupture, of the lipid bilayer (the outer cellular membrane), which give rise to short-lived pores through which molecules can pass relatively unhindered. This is a widely-used technique in

laboratory and clinical environments, to enable rapid and selective introduction of non-membrane-permeant materials. The efficiency of electrical permeabilisation is essentially related to two parameters: (i) the area of the cell membrane which has been permeabilised and (ii) the properties of the created pores, in particular pore diameter and life-time.

In conventional electropermeabilisation a cell is placed in a uniform electric field generated, for example, by applying a voltage between two electrodes, a cathode and an anode. The field interacts with the cell membrane and causes changes in the membrane potential. If these changes exceed several hundred millivolts, membrane breakdown occurs and non-selective pores start to open (electropermeabilisation). The pore life-time and the pore dimensions are directly affected by the strength of the field. The electric field strength must be increased to increase the permeabilisation effect.

To model this effect, an electrically-stimulated cell may be considered as a thin-walled sphere made of pure dielectric. A uniform electric field applied to the cell then induces a change of the membrane potential (ΔV) which varies with position on the cell surface according to the following simplified equation (1):

$$\Delta V = 3/2\ E r \cos\phi \qquad (1)$$

where E is the electric field intensity, r is the radius of the cell, and ϕ is the angle between the field direction and the radial vector from the cell centre to the cell surface at the position where the membrane potential is considered (the cathode lies at $\phi=0°$). The total membrane potential V consists of the resting

potential V_{rest} (which exists when no external field is applied) and the superimposed field-induced ΔV, as expressed by equation (2):

$$V = V_{rest} - \Delta V \qquad (2)$$

In practical terms, the external field E causes a hyperpolarisation (V_{hyp}) at the cell hemisphere nearest the anode and a depolarisation (V_{dep}) at the opposite cell hemisphere, nearest the cathode. The most pronounced potential change occurs at those membrane portions that directly face the electrodes, where ϕ is 0° and 180° respectively, and $\cos \phi$ reaches its maximal values of ± 1.

Little or no change is induced by the electric field in the cell wall along the circumference between the hemispheres described above. These portions of the membrane are perpendicular to the electrical field ($\phi = 90°$ and $270°$), and so the membrane potential remains unchanged ($\cos \phi = 0$), i.e. $V(\phi)$ equals V_{rest}.

Figures 1 and 2 attached hereto show the membrane potential $V(\phi)$ as a function of the angle ϕ in polar coordinates in unexposed (Fig. 1) and exposed (Fig 2) spherical cells. Each figure illustrates the voltages in the cell membrane around a section through an ideal, spherical cell. The cell is shown schematically at the centre of each drawing. The origin of the co-ordinates is at the centre of the cell. Since the resting voltage is negative for most cells, hyperpolarising voltages are shown in the radially outward direction and depolarising voltages in the radially inward direction.

In unexposed cells ($\Delta E=0$; $\Delta V=0$) the membrane potential is constant and equal to the resting potential all over the cell membrane ($V(\phi)=V_{rest}$). This is illustrated by means of the circle 2 in Figure 1 concentric to the origin. When an external electric field E is applied, $V(\phi)$ becomes an eccentric circle 8 shifted towards the anode as shown in Figure 2. This indicates that one side of the cell is hyperpolarised (the left side in Figure 2) and the other side is depolarised (the right side in Figure 2). In Figure 2, $V(\phi)$ is rotationally symmetrical about $\phi=0°$, which is the direction of the electric field E.

In Figures 1 and 2, the membrane potential levels at which permeabilisation first occurs, i.e. the threshold levels, are shown as outer and inner circles 4, 6 respectively. Since V_{rest} is negative for most cells, the total membrane potential during electric field exposure has the highest absolute values at the hyperpolarising side of the cell. This results in practice in a highly uneven permeabilisation effect between the two sides of the spherical cell; membrane disruption will always be higher at the end of the cell facing towards the anode.

Making certain assumptions, it is possible to model quantitatively the electropermeabilisation effect. The following calculation is based on the following assumptions and parameters: a) the cell is spherical, b) the membrane is made of pure dielectric, c) the electric field is homogenous, d) the resting potential is -100mV, and e) the critical threshold potential is +/-200mV. It is then possible to calculate that when the intensity of an applied electric field exceeds the threshold level by 15%, permeabilisation will occur on approximately 17% of the membrane facing towards the anode. This area is

approximately circular and subtends an angle of approximately 60° about the centre of the cell (see Fig. 9). (No permeabilisation occurs facing the cathode under these conditions).

To double the permeabilised membrane area, the field intensity has to be increased by at least 200%.

Although these figures are calculated on the basis of a model, experiment indicates that the conclusions are valid.

A significant limitation of conventional electropermeabilisation arises from these figures. An increase in electropermeabilisation effect may be achieved by increasing the intensity of the electric field applied to the cells. On average, this increases the area of the cell membrane permeabilised, the diameter of the pores and the pore life-time. However, such an increase in field strength does not necessarily lead to an increased efficiency of electropermeabilisation, since high-intensity electric fields can lead to a loss of cell viability by creating irreversible pores that do not re-seal. Therefore, although the chances of permeabilising cells are higher with more intense electric fields, the actual efficiency of the process may be lower because the cells die.

A further significant drawback of conventional electropermeabilisation is that unlike, for example, chemical permeabilisation, where the entire cell membrane is rather evenly affected, the electric field causes permeabilisation primarily restricted to the areas of the cell facing the electrodes (particularly the hyperpolarising side of the cell which faces the anode).

This means that cell shape is a very important determinant of electropermeabilisation efficiency. Only perfectly spherical cells undergoing spontaneous movement allow a homogeneous electropermeabilisation of populations of cells, and unfortunately not all cells can be grown under conditions in which they retain a spherical shape. Instead, many cells need to adhere to a basal surface to maintain their characteristic morphology and physiology. Such adherent cells usually flatten considerably and occupy unpredictable irregular shapes.

A common finding with adherent cells is that the sensitivity of various regions of the cell membrane to electropermeabilisation is not uniform. This means that the chances of permeabilisation depend upon the orientation of the cells relative to the direction of the electric field. Therefore, a significant proportion of cells may not be permeabilised at all if they are in the wrong orientation.

As an extension of the prior art systems described above in which a uniform electrical field is applied between an anode and a cathode, attempts have been made to carry out electropermeabilisation by applying alternating sinusoidal or square wave voltages between two electrodes. With reference to the model of electropermeabilisation described above, this may cause symmetrical electropermeabilisation in the cell membrane facing each electrode rather than the electropermeabilisation being greater on one side of the cell, opposite the anode. An example of an investigation into the use of alternating electric fields is described in published International patent application No. PCT/US91/06884.

Summary of the Invention

The invention provides an electropermeabilisation method and apparatus as defined in the appendent independent claims. Preferred or advantageous features of the invention are defined in dependent subclaims.

The invention preferably uses low-intensity, high-frequency, multipolar electric fields to achieve more effective electropermeabilisation at lower field strengths than in the prior art.

Advantageously, the invention may apply a moving or rotating electrical field to cells. The electrical field may advantageously move or rotate stepwise. This may allow more extensive, or more uniform, electropermeabilisation of cells at lower field strengths than if a uniform or unidirectional alternating field is used as in the prior art.

The preferred rate or frequency of movement of the electric field may vary depending on the type of cell being electroporated, on the electric field strength applied and on the duration, or length, of the electric field pulse applied in each direction. Large cells, such as mammalian cells, are easier to porate than small cells, such as bacteria, and so may require lower frequencies, lower field strengths and/or shorter pulse durations. Smaller cells may require higher-energy poration. Higher energy poration may also advantageously be used to uptake larger molecules into porated cells.

Given these factors the invention may advantageously use frequencies of about 1kHz or less, such as from about 100Hz up to radio frequencies. For higher frequency applications, frequencies of 100 kHz or more may be used.

(Frequency is defined with relation to the time period taken to complete a cycle of operation of the invention, when the field has been applied in all available directions, equivalent to a 360° rotation if a rotating field is used). The invention may also advantagsouly use electric field strengths of about 100 V/cm up to about 1000 V/cm or more. Particularly preferably field strengths of 500 V/cm to 1000 V/cm may be used. Advantageously, pulse lengths of less than microsecond to about 1 ms may be used, although shorter pulses are preferred, such as about 1 to 10 microseconds.

Preferably, the electric field may be applied in each of a number of different directions in the form of a square pulse. Particularly preferably, opposite voltages may be applied to electrodes, or pairs or groups of electrodes, on opposite sides of a cell or cells to be electropermeabilised. If such voltages are applied across single pairs of electrodes, equal and opposite voltages may advantageously be applied to respective electrodes.

Compared with the prior art, the invention may allow an advantageously larger area of a cellular membrane to undergo permeabilisation, for example as the applied field changes direction and affects different portions of the membrane.

The invention may also advantageously improve control of the size of pores formed during membrane breakdown. This is a consequence of three factors. One is the use of a multi-polar device to produce a more uniform distribution of pore sizes than in the prior art. The second is the ability to use low electric-field strengths while achieving an adequate degree of electroporation. The third is the use of square pulses of applied electric

field, which may allow close control of the electroporation field applied to any portion of a cell wall while reducing collateral damage to the cell. These factors, taken individually or preferably in combination, may advantageously enable discrimination between molecules of differing sizes, to differentially control their entry across the cellular membrane, and/or to control the longevity of pores.

The ability to generate smaller pores than in the prior art may also advantageously shorten the membrane re-sealing time, reduce collateral damage and substantially improve the prospects for cell viability.

When multipolar pulsed electric fields are applied to cells according to one aspect of the invention, the pulses may advantageously be spaced in time so that, between the pulses, no electric field is applied. This may further improve control of electroporation conditions because pores created by a pulse may persist for some time after the electric field has been removed, and if this means that no electric field need be applied during parts of the electropermeabilisation process, cell damage may advantageously be decreased by switching off the electric field at such times.

Adherent cells, which are usually non-spherical, provide the worst-case scenario for electropermeabilisation. However, with the method or apparatus according to the invention, the chances of permeabilising irregularly-shaped cells may advantageously be greatly improved.

Aspects of the invention may find particular advantage in medical applications and in vivo applications. For example there may be advantage in administering drugs to

cells in vivo in a selective manner such that the size and number of ions or molecules admitted to cells is controlled by controlling the electropermeabilisation conditions.

One aspect of the invention relates to such a medical use, providing an apparatus comprising three or more electrodes for contacting or penetrating body tissue surrounding cells for electropermeabilisation, and administering desired ions, chemicals, drugs or the like to those cells. An example of such a use may be to treat melanoma.

Specific embodiments of the invention will now be described by way of example, with reference to the drawings, in which:

Figure 1 is a diagram of potential vs. angle, in polar coordinates, in a theoretical model of a spherical cell, with no applied electrical field;

Figure 2 is a diagram of potential vs. angle, in polar coordinates, in a theoretical model of a spherical cell, with applied uniform electrical field E, as in the prior art;

Figures 3 to 8 are diagrams of potential vs. angle, in polar coordinates, in a theoretical model of a spherical cell, with applied fields in successively rotated orientations, according to a first embodiment of the invention;

Figure 9 illustrates the electropermeabilised region of an ideal spherical cell in an applied unipolar field, as in the prior art;

Figure 10 illustrates the electropermeabilised regions of an ideal spherical cell in a rotating or moving field according to the embodiment of Figures 3 to 8.

Figure 11 is a block diagram of an electropermeabilisation apparatus according to the embodiment of Figures 3 to 8;

Figure 12 is a schematic plan view of the chamber of the apparatus of Figure 11;

Figure 13 is a schematic cross-section on A-A of the chamber of Figure 12;

Figures 14 to 18 illustrate various sequences for applying voltage pulses to the electrodes of the embodiments of figures 11, 12, 13, 19, 20 and 21;

Figure 19 is a perspective view of an apparatus embodying the invention for electroporation of skin cells;

Figure 20 is a cross-section of the embodiment of figure 19 in use;

Figure 21 is a schematic view of a second apparatus embodying the invention for electroporation of skin cells; and

Figure 22 is a plot of voltage vs. time showing two bipolar square voltage pulses output by the apparatus of figure 11.

Figures 1 and 2 have been described above and will not be discussed in detail again here.

In a method according to a first embodiment of the invention, the direction of the electric field is switched through a succession of six directions, which are coplanar and separated by 60°. Figures 3 to 8 are similar to Figures 1 and 2 but illustrate the voltage distribution 10 in the cell wall as each of the six successive field directions is applied. In each orientation of the field, a sufficient field strength is applied to cause permeabilisation in the cell surface nearest the anode. Thus, each time the field switches to a new direction, a new area of permeabilisation is correspondingly induced.

$\phi=0°$ is maintained in the same direction, horizontally to the right, in each of figures 3 to 8. $\phi=0°$ therefore is not always the direction of the electric field as in figures 1 and 2.

As described above, electropermeabilisation generally causes a highly uneven permeabilisation effect between the two sides of a spherical cell; membrane disruption will always tend to be higher at the side of the cell facing towards the anode. This condition is illustrated in Figure 9 for a cell in a uniform field (as in the prior art), such as the cell illustrated in Figure 2. The electroporated region 12 of the cell 14 is shown in black and covers approximately 60° of the circumference of the cell at the side nearest the anode. This corresponds to an applied field strength exceeding the threshold for electropermeabilisation by about 15%, as described above. Figure 10 shows the corresponding degree of electroporation when the cell is exposed to the rotating electric field illustrated in Figures 3 to 8. In each of the 6 field directions, a portion 16 of the cell wall is permeabilised and therefore, as the field rotates, a

region around substantially the entire circumference of the cell in the plane of exposure is permeabilised.

In all of the examples in Figures 2 to 10, no permeabilisation occurs at right angles to the applied field.

As clearly illustrated in Figures 9 and 10, the method of the first embodiment of the invention allows permeabilisation of a very much larger area of the cell membrane than in the prior art with substantially no increase in the strength of the applied field. By contrast, to increase the area of the porated region in a unipolar field, as in the prior art, would require a very significant increase in field strength, which would be very likely to prejudice cell viability.

As described above, when the intensity of the applied field exceeds the permeabilisation level by 15%, permeabilisation will occur on approximately 17% of the membrane facing the anode. This area subtends an angle of approximately 60° measured from the cell centre. Rotating the field in steps of 60°, as illustrated in Figure 10, therefore permeabilises most of the cell membrane whereas to double the permeabilised membrane area (from about 17% to about 34% for example) with a uniform applied field as in the prior art, the field strength has to be increased by at least 200%.

The electric field in the first embodiment can be advantageously switched at high frequencies, for example at 100kHz or 200kHz, to achieve a quasi-continuous electropermeabilisation around the entire cell circumference, although frequencies as low as 1 kHz or 10 kHz may be used to achieve quasi-continuous poration if

the resealing time is long. There may also be advantage in rotating/moving the field direction at much higher frequencies than this, so that in any one direction only a very short (e.g. <1 microsecond) pulse is applied at a time, but the pulse is repeated frequently. This rapidly repeated application of low-energy short pulses to each area of the cell surface may generate pores more gradually giving better cell viability. Pores created under low energy conditions may reseal better than pores created under high energy conditions.

General Principle of the Invention

When an electric field is applied to a cell membrane, the exposure time required to cause electroporation varies with a number of parameters, including the applied field strength, the conductivity of the medium surrounding the cell, and the response time of the cell membrane itself. However, if an electric field only slightly greater than the electroporation threshold is applied, it is expected that the field must be applied for a few microseconds (for example 1μs to 10μs).

The extent of electroporation depends on the amount by which the electroporation threshold is exceeded and the orientation of the cell membrane. Referring to the spherical cell model described above, if the field exceeds the threshold by about 15% for the portion of the cell membrane perpendicular to the applied field ($\phi=180°$ in figure 2) then the permeabilised area extends over an area of the cell membrane subtending about 60° measured from the cell centre (from $\phi=150°$ to $\phi=210°$). This is, in the theoretical model, determined solely by the orientation of the cell membrane with respect to the applied field, so that at the edge of the permeabilised area the component of the applied field perpendicular to the cell membrane is

equal to or only slightly greater than the electropermeabilisation threshold. The permeabilised area is therefore a function of cos ϕ, as expressed in equation (1). Because of the shape of the cosine function, this means that if the applied field is increased from an initial value below the electropermeabilisation threshold then as the applied field starts to exceed the electropermeabilisation threshold at $\phi=180°$, the permeabilised area increase rapidly but as the field rises further (for example to double the electropermeabilisation threshold at $\phi=180°$) the permeabilised area increases much more slowly. This is one reason that increasing the strength of a unipolar field is not an effective way to increase the area of electropermeabilisation.

A further problem arising from simply increasing the field strength is as follows. The electropermeabilised area is made up of many, smaller pores and the size and other properties of each pore may vary depending on the applied field strength perpendicular to the cell membrane at the location of each pore. The pores at the centre of the electropermeabilised area, where the effective field strength is greatest, will be larger than those at the edge. Therefore, as the applied field strength of a unipolar field is increased in order to increase the electropermeabilised are, the field strength and the pore size at the centre of the area will become very large. Such pores are much more likely to prejudice cell viability than smaller pores created by smaller applied fields.

There is therefore a significant benefit in using the smallest possible field for electropermeabilisation.

Compared with the permeabilisation time of a few microseconds described above, the resealing time of the pores generated in conventional electropermeabilisation is usually much longer, of the order of milliseconds, and can be even longer, of the order of seconds or even minutes. This will vary with the type of the cell membrane as well the pore size, which is related to the applied field strength as described above.

The invention aims to use these factors to increase the area of electropermeabilisation by moving the applied field so as to permeabilise different areas of a cell membrane in a rapid succession. Because the resealing time of a pore is greater than the time taken to create a pore, then the invention may allow a large area of a cell membrane to be permeabilised continuously or quasi-continuously. For example, if the invention is embodied in a method applying electric fields successively in several different directions to a cell, then pores in the portions of the cell membrane permeabilised by all the field directions will remain open continuously as long as the interval between the repeated application of the field in each direction is less than the pore resealing time.

For example, if six different field directions are used to electropermeabilise different areas of a cell membrane and the pore resealing time is 10ms, then to keep the pores open the field must be applied in each direction more often than once in every 10ms, that is at greater than 100Hz. If the field is applied at even time intervals in each direction, it must therefore be switched from one direction to the next at more than 600Hz.

In each direction, the field must be applied for long enough to electropermeabilise the membrane which, as

discussed above, may be 1μs to 10μs. This can easily be achieved as, at 600Hz, 1.67ms is available before the field must be switched to the next direction.

If the resealing time is only 1ms, then a switching frequency of 6kHz is required, but this still allows an interval of 167μs in each field direction to apply an electric field pulse of 1μs to 10μs duration.

There may be advantage in switching the field direction at higher frequency in order to minimise resealing of pores, which may be a gradual process with some pores sealing, or partially sealing, in less than 1ms.

The example discussed uses six field directions. More or less directions may be used, which would raise or lower the required minimum switching frequency accordingly if quasi-continuous poration is to be achieved. However, six coplanar field directions is a practical embodiment because it is possible to generate an electropermeabilised area covering about 60° of a cell circumference by exceeding the electropermeabilisation threshold only by about 15% as described above, and with six field directions this advantageously achieves permeabilisation around an entire circumference of a cell.

Non-coplanar field directions may be used to electropermeabilise areas of a cell membrane not permeabilised by a set of coplanar field directions. This may entail the use of more field directions and correspondingly higher switching frequencies. In principle, this may even enable quasi-continuous poration over the entire surface of a cell, even if the cell is non-spherical.

The model of the principle behind the invention described above assumes that the areas permeabilised by fields applied in different directions do not overlap. It also does not consider the option of applying a continuously-moving field. However, the necessary modifications to the model required to accommodate these factors can easily be made by the skilled person without inventive effort.

In each field direction an electric field pulse of the order of microseconds duration may be applied, for example as a square wave pulse or any other convenient pulse shape. A square wave pulse is expected to optimise permeabilisation while reducing the likehood of damaging a cell.

Although it is believed that the invention operates most advantageously on the principles described above, it is nevertheless envisaged that advantages over the prior art may be obtained even if the field direction moves at lower frequencies such that electropermeabilisation is discontinuous.

The square wave pulse shape is preferred because it reduces collateral damage to the cell. The need is to create transient pores so that substances such as a chemical, DNA or other molecule can be taken up into the cell and the cell envelope, or wall, repairs so that the cell remains viable. The greater the voltage and the greater the time (pulse length) the larger the pores and the longer they typically last. Having wave-forms with significant tailing, such as a sinusoidal or decaying pulse in which the voltage reduces relatively slowly from its maximum, can cause additional damage to the cell without aiding poration. The embodiment allows finely controlled field strengths to achieve effective

electroporation based on transient pores, minimal cell death and hence maximum cell viability post electroporation. This has been exemplified in experiments by the applicant with 99% viability in respect of electroporation with Ca^{2+} and >90% viability when molecules of 1,000 daltons were electroporated into cells.

The ability to control the pulse shape and intensity enables control of the longevity of pore formation; hence transient or longer-lasting pores may advantageously be selectively generated.

Varying the pulse parameters enables the creation of pores of different sizes. This can be used to define pore sizes and hence control the size of molecules entering a cell. In this way size-exclusion principles can be employed for selective uptake. This has been exemplified in experiments by the applicants in which molecules of different sizes were successfully selectively electroporated into a mammalian cell line. Differential field strengths were used to enable Ca^{2+} (small pore sizes) and Fura-2 or ruthenium red (1,000 daltons; medium pore sizes) to gain entry into cells. It was observed that lower field strengths were required to get Ca^{2+} into the cells as compared to Fura-2 or ruthenium red.

In tests performed by the applicant, attempts were made to promote the uptake of an 850 dalton molecule into HeLa cells. Tests using square pulses of amplitude 500V and duration 150 microseconds to generate a bipolar rotating field (in apparatus as illustrated in figure 12), failed to cause uptake of the molecules. Using the same apparatus, bipolar rotation and pulse duration but with square pulses of 750V, uptake was successful with a high degree of cell viability. It is believed that a similar

effect may be achieved with even greater viability by using a shorter pulse length. These tests clearly show the implementation of selective uptake using a preferred embodiment of the invention.

Apparatus

An apparatus for implementing the method of the first embodiment is illustrated in Figures 11, 12 and 13.

Figure 11 shows a schematic plan view of an electropermeabilisation chamber 20 containing 6 electrodes 22, 24, 26, 28 30 and 32 and associated circuitry. The electrodes are positioned at 60° intervals around a central portion of the chamber in which cells may be placed. The electrodes are thus arranged as three pairs of electrodes facing each other.

Each electrode can be coupled via a respective high voltage switch 34, 36, 38, 40, 42 and 44 either to a high positive voltage, or to earth, or to a high negative voltage. (This preferred apparatus applies square voltage pulses to the electrodes; other embodiments may apply other pulse types if required). The high positive and negative voltages are generated by respective voltage sources 46, 48 coupled to respective capacitor banks 50, 52. The outputs of each capacitor bank are connected in parallel to inputs of each high voltage switch. A PC 54 controls the high voltage sources and, via a microprocessor 56, the high voltage switches. Figure 22 illustrates the production of two successive bipolar square voltage pulses by a practical implementation of this apparatus tested by the applicant.

Advantageously, the voltage supply to each opposite pair of electrodes is switched using an H-bridge configuration

to connect each electrode either to a positive voltage or a negative voltage, enabling high voltages to be rapidly switched to the electrodes in a bipolar, biphase (push-pull) mode. For example, a field may be generated by applying a high voltage to electrode 22 and an opposite voltage to the opposite electrode 28. Figures 14 and 15 illustrate possible sequences of voltage pulses to be delivered to each electrode to implement this embodiment. The figures differ in that shorter pulses are used in figure 14, leaving quiescent periods between pulses in order to minimise cell damage. Alternatively, a field may be generated by applying voltages to four opposite electrodes at a time. Thus, a positive voltage might be applied to two adjacent electrodes 22, 24 and a negative voltage applied to the opposite two adjacent electrodes 28, 30. Figure 16 illustrates a possible sequence of voltage pulses to be delivered to each electrode to implement this embodiment. In these preferred embodiments, the apparatus may therefore be designed with electronic elements rated at half of the voltage and power capacity that can actually be generated by the apparatus.

By successively, or alternately, applying voltages to two opposite electrodes and four opposite electrodes, twelve field directions may be generated from the three pairs of electrodes. In general, this technique may allow twice as many field directions to be generated as there are electrodes provided. Figure 17 illustrates a possible sequence of voltage pulses to be delivered to each electrode to implement this embodiment. It should be noted that figure 17 illustrates an electric field rotation of only about half a revolution. Further alternatives in which voltages are applied to groups of more than two adjacent electrodes may also be useful.

For example, in a further preferred embodiment, the voltages may be applied to adjacent groups of three electrodes opposite each other.

The PC can be programmed to control various electric field exposure sequences by controlling the high voltage sources and switches appropriately. It should be noted that although figures 14 to 17 illustrate rotating fields, any desired sequence of different field directions may be used, and controlled by suitable programming of the PC. There may be advantage in not using a rotating field in some cases if it is preferred not to apply the electric field successively to adjacent cell-wall regions. This may increase cell viability.

Taking, for example, a rotating electric field, during each full rotation each pair or group of opposed electrodes is used first to apply an electric field with one polarity and then, later on, with reversed polarity. This is achieved by applying opposite voltages to the electrodes in each case. Each electrode may therefore be termed biphase, such that the electrodes operate in a push-pull mode.

Although the bipolar, biphase system of applying voltage pulses to the electrodes described above is the preferred option for generating electric fields for electroporation in the embodiment, alternatives are possible. For example, a unipolar voltage pulse could be applied to each electrode in turn around the ring of electrodes in figure 12, while each opposed electrode is grounded, or earthed. Figure 21 illustrates a possible sequence of voltage pulses to be delivered to each electrode to implement this embodiment.

This method would have the disadvantages that the voltage generator(s) for generating the pulses and the associated cables would need to be rated at twice the voltage of the voltage generators in the biphase system, making them more dangerous and more expensive, and that the electrode opposite the electrode at high voltage would need to be connected to 0V. This might be done using a 2000 Ohm resistor but this would be in series with the electroporated sample and would limit the current flow through the sample. For example with a 2000V voltage and 2000 Ohm resistor, only a maximum of 1 amp could pass through the sample.

Alternatively, one of each pair of opposite electrodes (say electrodes 22, 24, 26 in figure 12) could be permanently connected to 0V while biphase (alternately positive and negative) voltages are applied to the other of each pair of electrodes in sequence. This method would again suffer the disadvantage of needing voltage generator(s) and cables rated at twice the voltage of those in the bipolar system, and would lack flexibility in rotating the electrical field if half of the electrodes are permanently grounded. However this problem could be reduced by only connecting to 0V the electrode opposite the electrode at high or low voltage at any time.

The electropermeabilisation chamber 20 is illustrated in more detail in Figures 12 and 13. The body 60 of the chamber is made of a non-conducting, transparent material. The six electrodes are arranged around a compartment 62 in which cells 64 may be placed for exposure. A respective channel 66, 68, 70, 72, 74 and 76 leads radially outwards from the exposure compartment 62 past each electrode and then leads to the upper surface 78 of the chamber. A central channel 80 leads from the exposure compartment 60

to the upper surface 78 of the chamber. The channels thus provide perfusion pathways leading past each electrode and through the exposure compartment for passing fluids across cells held therein.

A respective electrical connection 82 couples each electrode via the upper surface 78 of the chamber to a respective high voltage switch.

Perfusion media may or may not be electrically conducting. If the medium is conducting, the efficiency of permeabilisation may be increased as a greater electric field and current flow may be experienced by the cell membrane.

In some experiments or procedures, fluids passed through the chamber may be electrolysed by the electrodes to produce hazardous or toxic products. The channels containing the electrodes may be filled with agar bridges to provide protection from such possible toxic products, although perfusion would then only be possible along the central channel 80. In an alternative embodiment, two central channels may be provided to enhance perfusion flow. Further optional measures to avoid electrolysis products include providing a constant flow of fluid perfused though the compartment, as well as the application of charge-balanced exposure (the application of opposite high and low voltages to opposite electrodes) and the use of inert platinum electrodes.

The cells in the chamber are cultured on glass coverslips 24mm in diameter. Such cover slips are commonly used by researchers and are readily available from commercial suppliers. The bottom of the chamber is arranged to mount such a cover slip with attached cells. The design of the

electropermeabilisation chamber is based on the typical fluid volumes and cell culture materials currently used by researchers. However, it could be modified into other arrangements as required. The chamber of Figures 12 and 13 is designed for use with adherent cells attached to a substrate. Clearly the design would need to be modified, in particular regarding the perfusion of fluid, for use with free-floating cells. Such modifications are, however, within the ability of the skilled man.

To illustrate the size of the apparatus of the embodiment, the electrodes in the chamber of figures 12 and 13 are arranged on a circle of radius approximately 20mm. Total voltages of 1kV to 2kV are typically applied between opposed electrodes and currents of between 1A and 10A may flow between opposed electrodes. These parameters may vary significantly with the application of the apparatus.

In other embodiments, a number of variations from the embodiments described above may be incorporated. For example a greater or smaller number of electrodes may be used to generate the changing electric field. Also, the electrodes need not be arranged in a plane. For example, effective electropermeabilisation using six electrodes in opposed pairs may be achieved if the pairs are arranged on three axes at right angles to each other. Alternatively, a ring of six electrodes in a plane may be supplemented by one or more electrodes above and/or below the plane.

Varying or moving electric fields may also be generated by varying the voltages applied to electrodes, either continuously or in steps, rather than the simple switching on and off of electrodes described above. For example, if four electrodes are spaced at 90° intervals around a circle, and sinusoidal alternative voltages in antiphase

are applied to one opposed pair of electrodes and cosinusoidal alternating voltages in antiphase are applied to the other opposed pair of electrodes, cells between the electrodes will experience a smoothly rotating electric field.

In a further embodiment, the electrodes or the cells being electroporated may be physically moved in order to produce a moving field.

As will be appreciated by the skilled man, techniques such as those described above may be combined with each other.

Compared to current technologies producing only static, uniform electrical fields, the embodiments of the invention may thus give a superior efficiency of electropermeabilisation. In particular, they may provide better control over pore position, size and resealing time, as well as causing minimal disturbance to the cells. This increase in efficiency of electropermeabilisation may be particularly beneficial under conditions where cell viability is paramount, and may significantly reduce the pain associated with the use of electropermeabilistation in the clinical environment. The technique can be used for introduction into cells of a variety of chemical agents including proteins, antibodies, drugs, enzymes, DNA, RNA and antisense oligonucleotides.

In the clinical environment the invention may find application in a variety of medical treatments. One example is in the treatment of skin conditions such as melanoma, in which electroporation may broaden the range of substances which can be administered into the cells of the melanoma. Two suitable apparatuses for enabling this are illustrated in figures 19, 20 and 21.

Figure 21 illustrates the use of a hexagonal ring of needles 122, 124, 126, 128, 130, 132 which are held in a support (not shown) from which the sharp ends of the needles extend in a hexagonal arrangement. The opposite ends of the needles are connected to circuitry similar to that of figure 11 or the other embodiments discussed above. In use the needles pierce the skin 134 of a patient, surrounding a melanoma 136, the substance to be introduced to the cells is placed over the melanoma and a moving or rotating electric field is applied as described herein.

Figure 19 and 20 illustrate an alternative apparatus which does not require the patient's skin to be punctured. It consists of a bowl-shaped vessel 220 of non-conducting material, such as a transparent plastic. Electrodes 222, 224, 226, 228, 230, 232 are set into the inner surface of the bowl. The electrodes are arranged at 60° intervals around the inner rim of the bowl, set back slightly from the rim. The electrodes are connected to circuitry similar to that of figure 11 or other embodiments discussed above (not shown). Two channels 234, 236 extend from the outside to the inside of the bowl and are connected in use to a source of a perfusion medium.

Figure 20 is a cross-section of the apparatus of figure 14 in use. The inverted bowl-shaped vessel 220 is placed on the skin 238 of a patient over a melanoma 240. Perfusion medium 242 is passed through the interior of the vessel via the channels 234, 236 and the pressure of the medium is controlled to be below atmospheric pressure such that the skin of the patient is drawn into the bowl and into contact with the electrodes. Electroporation can then be performed as described herein.

Although the method and apparatus of the embodiments permit significant advantages over conventional electropermeabilisation, they also retain the existing advantages of electropermeabilisation over other techniques. Thus, control of the electric field permits permeabilisation for only a short controllable period of time after exposure. Ability to manipulate the recovery time of the plasma membrane will allow introduction of a desired quantity of non-permeable molecules. For larger quantities, long-lasting pores can be created while, for introduction of smaller quantities, the electrical field can be controlled to allow formation of pores for only a short period.

The cells being exposed may be monitored directly by a microscope or microscope-based video acquisition software.

Pore size and duration may be controlled by the frequency, intensity, duration time and signal shape of an applied electric field.

The technique described herein may be suitable for broad-based use in cell physiology. In particular, it may be applicable in the plant science field where ester loading of cells is not practical and where all such work conventionally has to take place with micro-injection. It may be useful for loading non-permeant species which currently require expensive and time-consuming micro-injection. It may be useful for rapid loading of large populations of cells for biochemistry and molecular biology applications or for in situ loading of cells in tissue slices which is not possible at present.

CLAIMS

1. A method for electropermeabilising a membrane by exposing the membrane to an electric field, in which the direction of the electric field moves during exposure.

2. A method according to claim 1, in which the electric field direction moves so as to cause electropermeabilisation of membrane portions positioned at different orientations.

3. A method according to claim 1, in which the electric field direction moves discontinuously, or stepwise.

4. A method according to any claim 1, comprising the steps of:

spacing three or more electrodes around the membrane to be electropermeabilised; and,

supplying a time-variant voltage to each electrode so as to generate the electric field.

5. A method according to claim 4, in which the membrane is placed between each of two or more pairs of opposed electrodes, the pairs of electrodes being positioned on respective, angularly-spaced axes.

6. A method according to claim 4, in which the time-variant voltage comprises a sequence of square voltage pulses.

7. A method according to claim 4, comprising the steps of;

applying a set of predetermined voltages to the electrodes in a first orientation to generate an electric field therebetween in a first direction;

switching the voltages to apply them to the electrodes in a different orientation to generate an electric field in a different direction; and

repeating the last step set out above.

8. A method according to claim 1 in which the electric field direction is moved discontinuously through each of six directions, the directions being coplanar and at 60° spacings.

9. A method according to claim 1, in which the electric field direction moves at high frequency.

10. A method according to claim 1, in which the electric field direction moves at radio frequency.

11. A method according to claim 1, in which electropermeabilisation creates pores in the cell membrane which reseal in a resealing time, and in which the direction of the electric field moves so as to cause electropermeabilisation of three or more areas of the cell membrane, and returns to electropermeabilise each area again within a time equal to or less than the resealing time.

12. An apparatus for electropermeabilising a membrane, comprising;

three or more electrodes spaced around a region within which a membrane may be placed for electropermeabilisation;

a voltage supply; and

a controller for controlling the voltage supplied from the voltage supply to each electrode so that an electric field, the direction of which moves, is generated in the electropermeabilisation region.

13. An apparatus according to claim 12, in which the direction of the electric field moves discontinuously, or stepwise.

14. An apparatus according to claim 12, comprising two or more pairs of opposed electrodes, the pairs of electrodes being positioned on respective, angularly-spaced axes.

15. An apparatus according to claim 12, in which a set of predetermined voltages is supplied to the electrodes in successive, different orientations so as to generate electric fields in successive, different directions.

16. An apparatus according to claim 12, in which the electric field direction moves at high frequency.

17. An apparatus according to claims 12, in which the electric field direction moves at radio frequency.

18. An apparatus according to claim 12, in which electropermeabilisation creates pores in the cell membrane which reseal in a resealing time, and in which the direction of the electric field moves so as to cause electropermeabilisation of three or more areas of the cell membrane, and returns to electropermeabilise each area again within a time equal to or less than the resealing time.

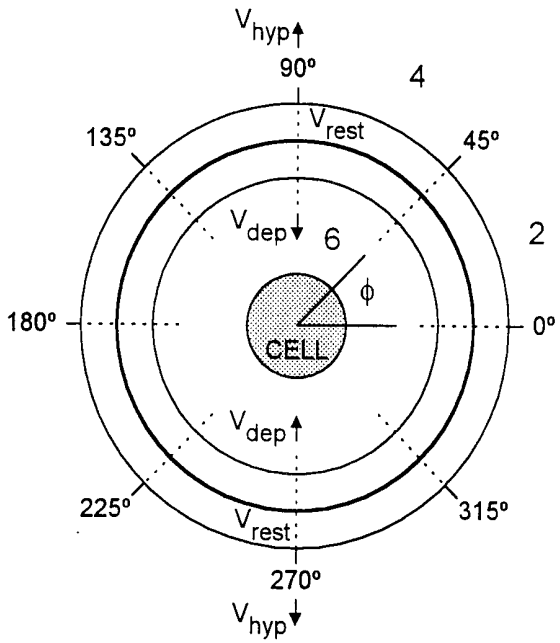

FIG. 1

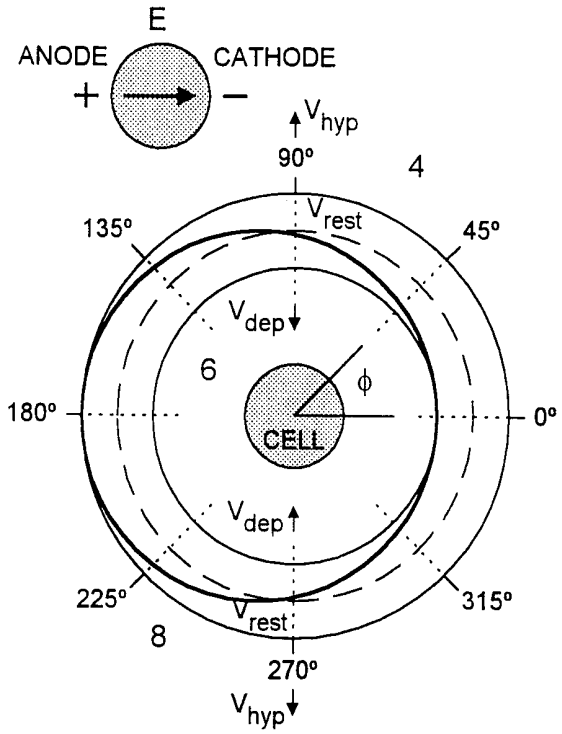

FIG. 2

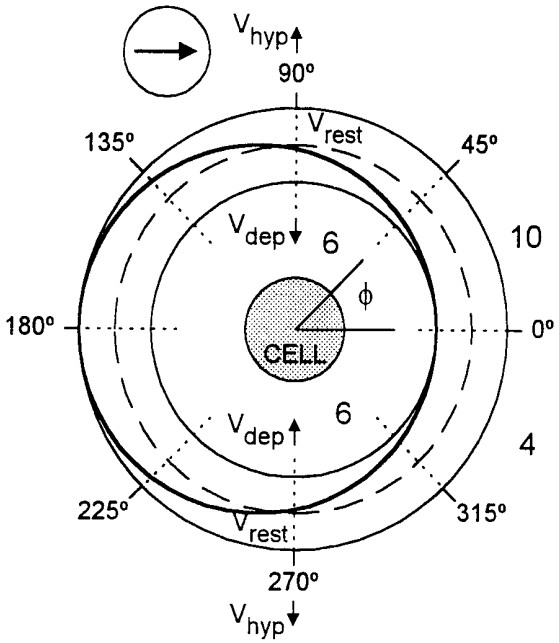

FIG. 3

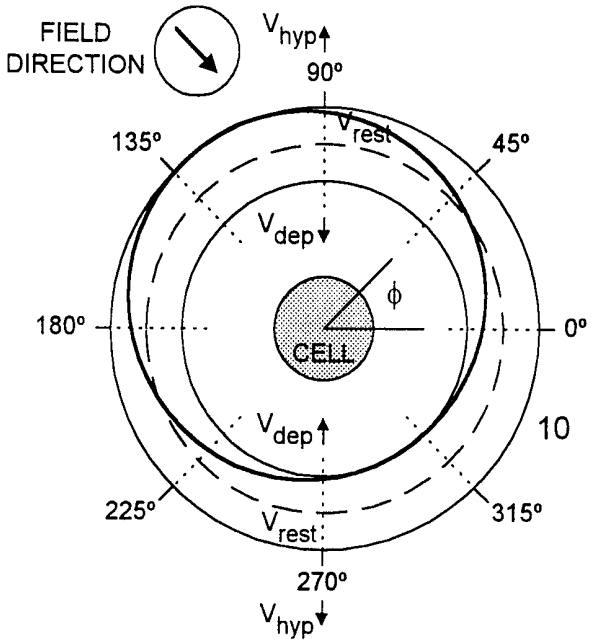

FIG. 4

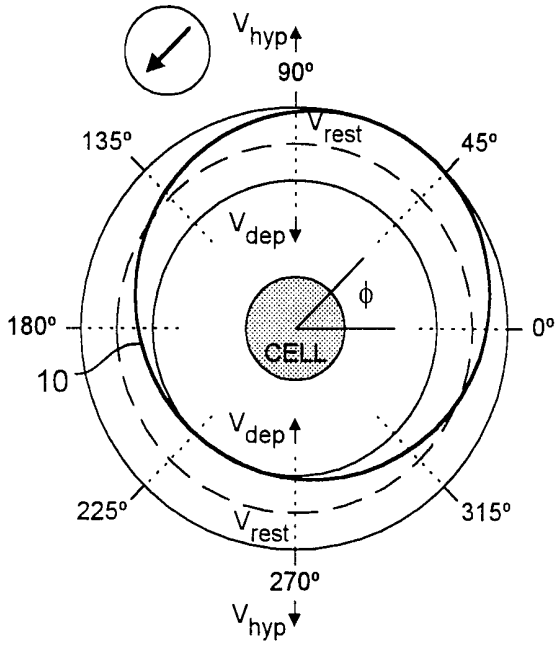

FIG. 5

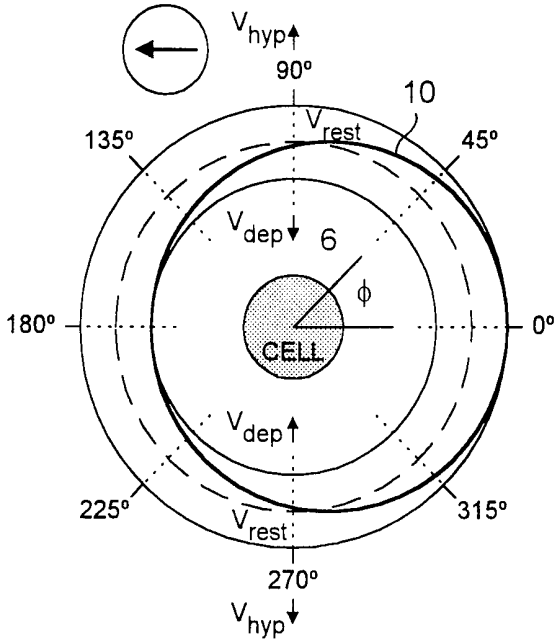

FIG. 6

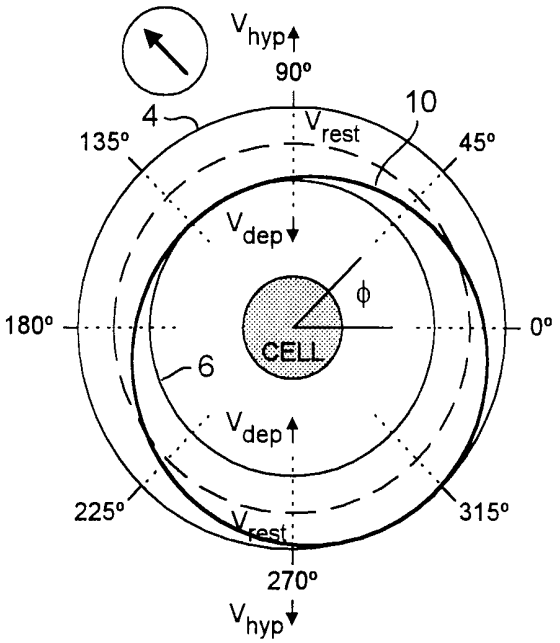

FIG. 7

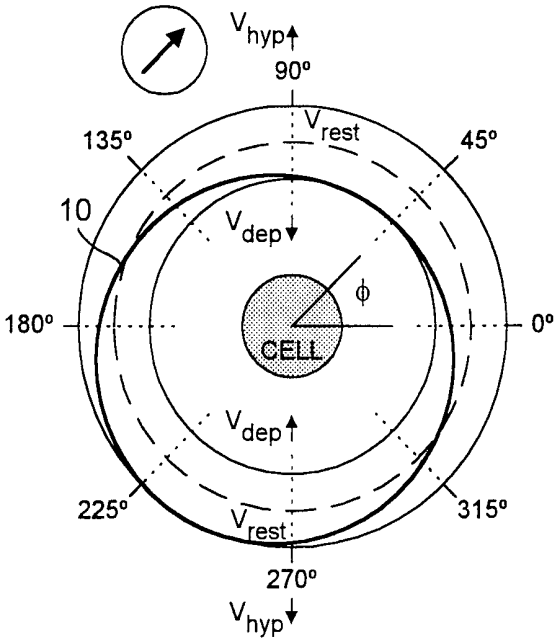

FIG. 8

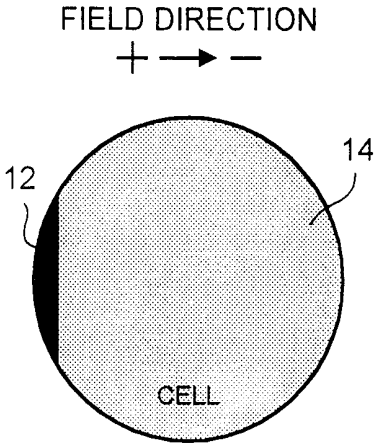

FIG. 9

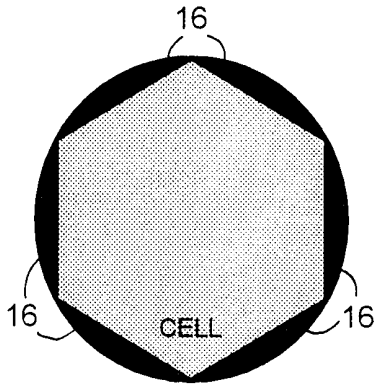

FIG. 10

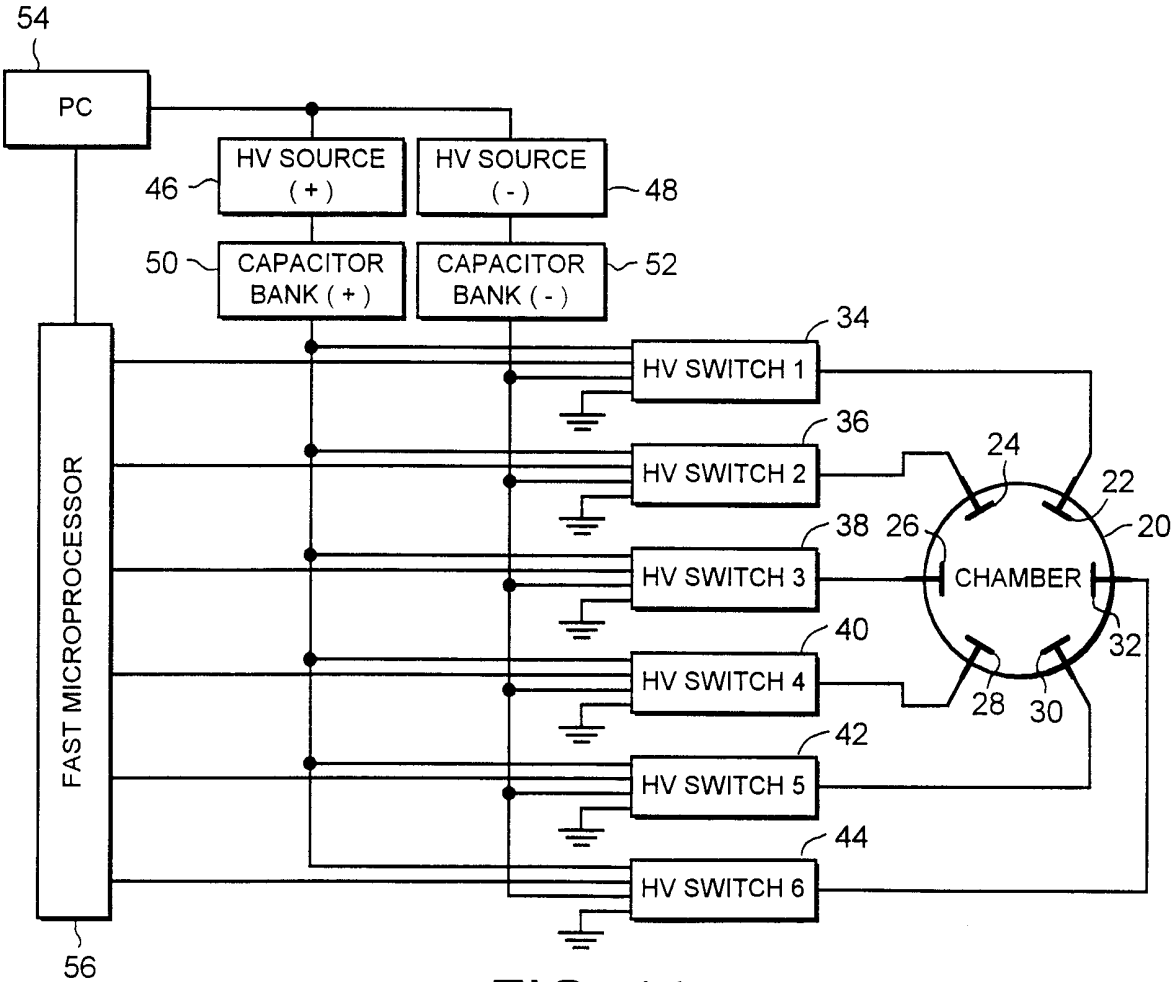

FIG. 11

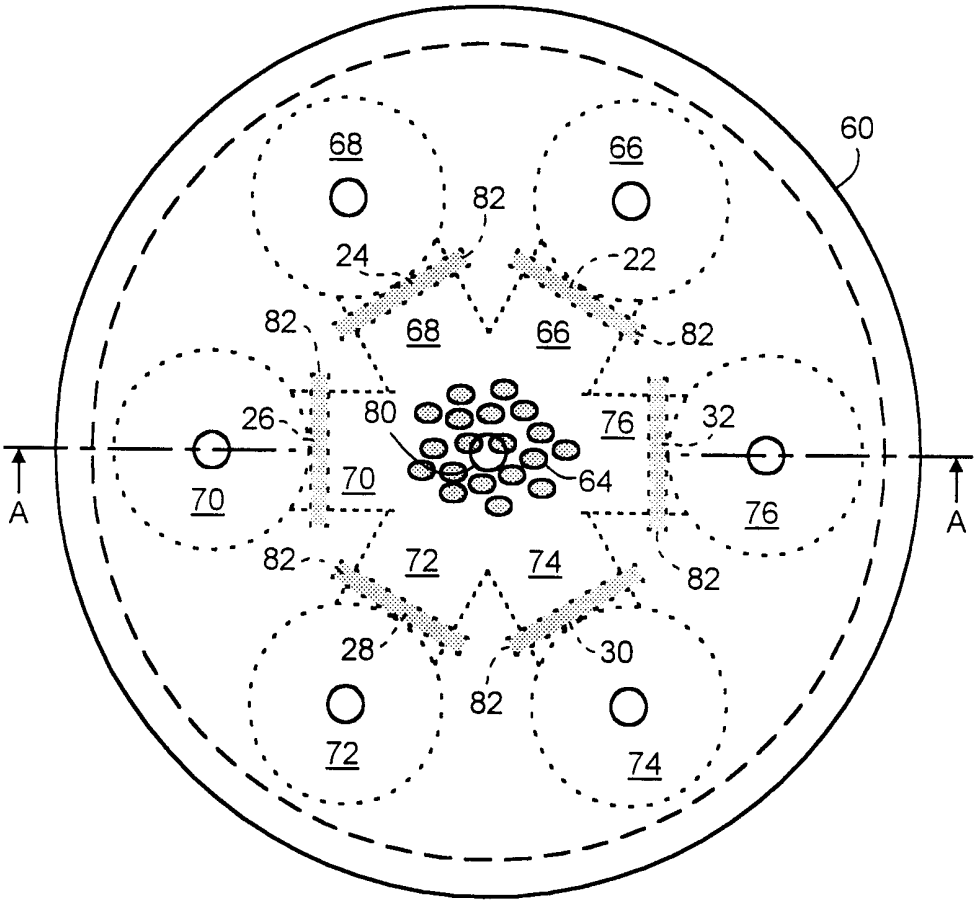
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FIG. 12

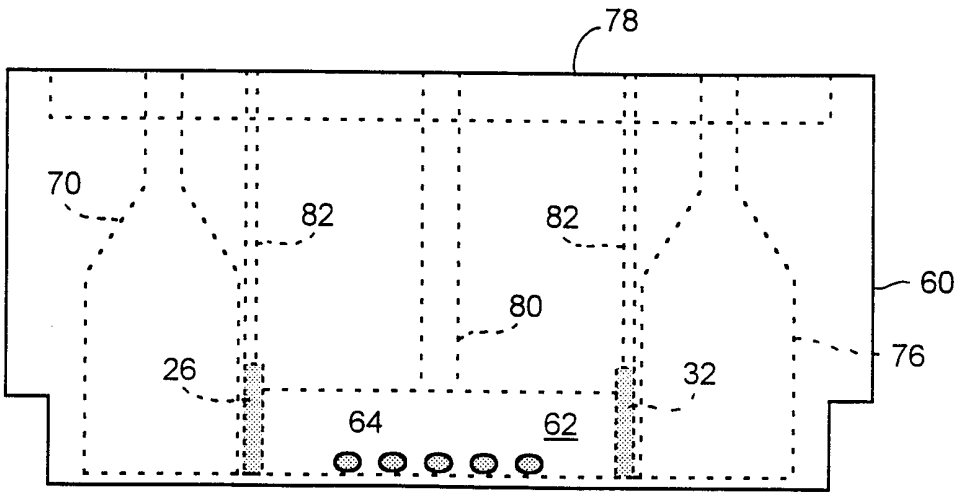
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FIG. 13

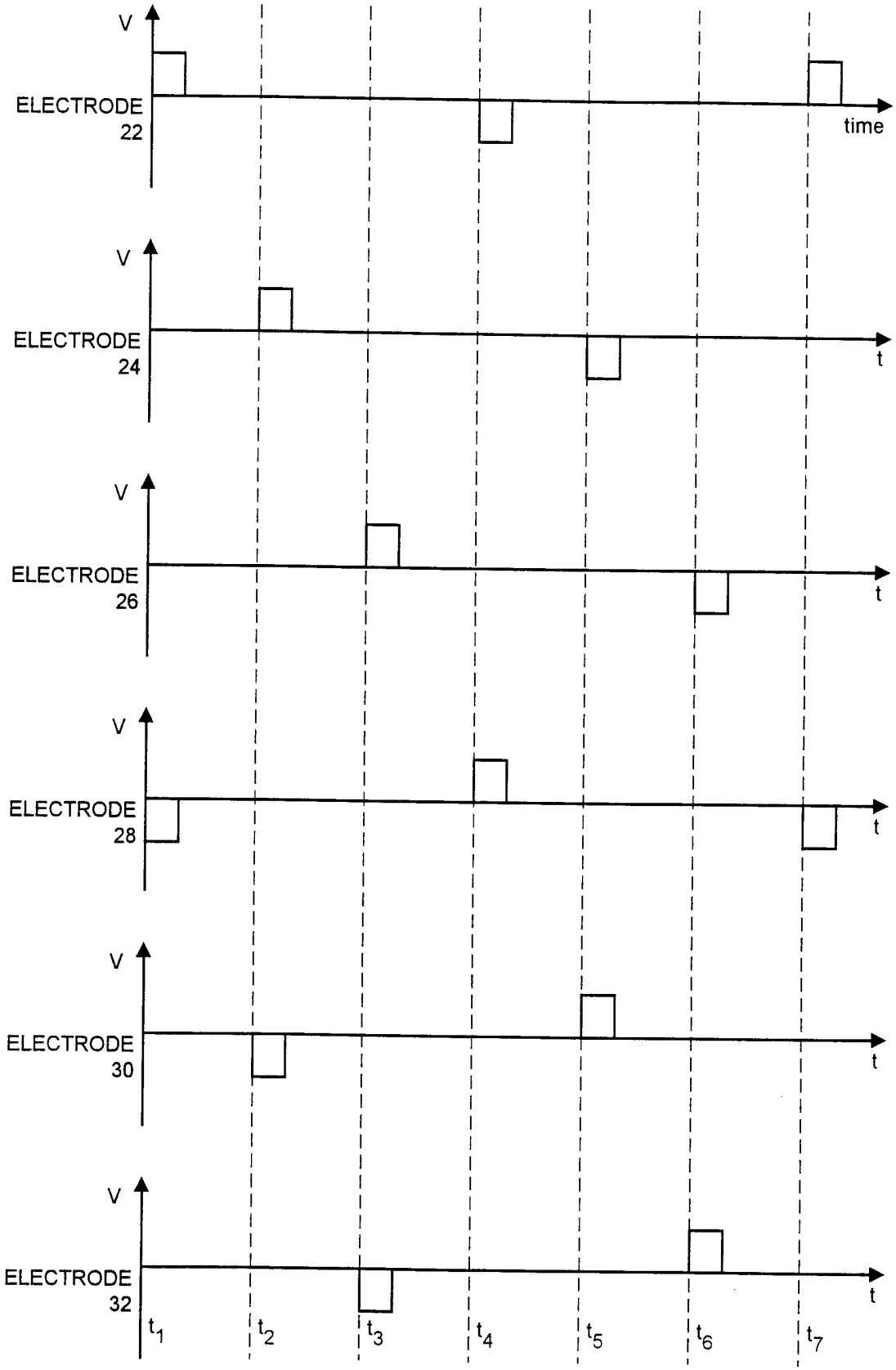
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FIG. 14

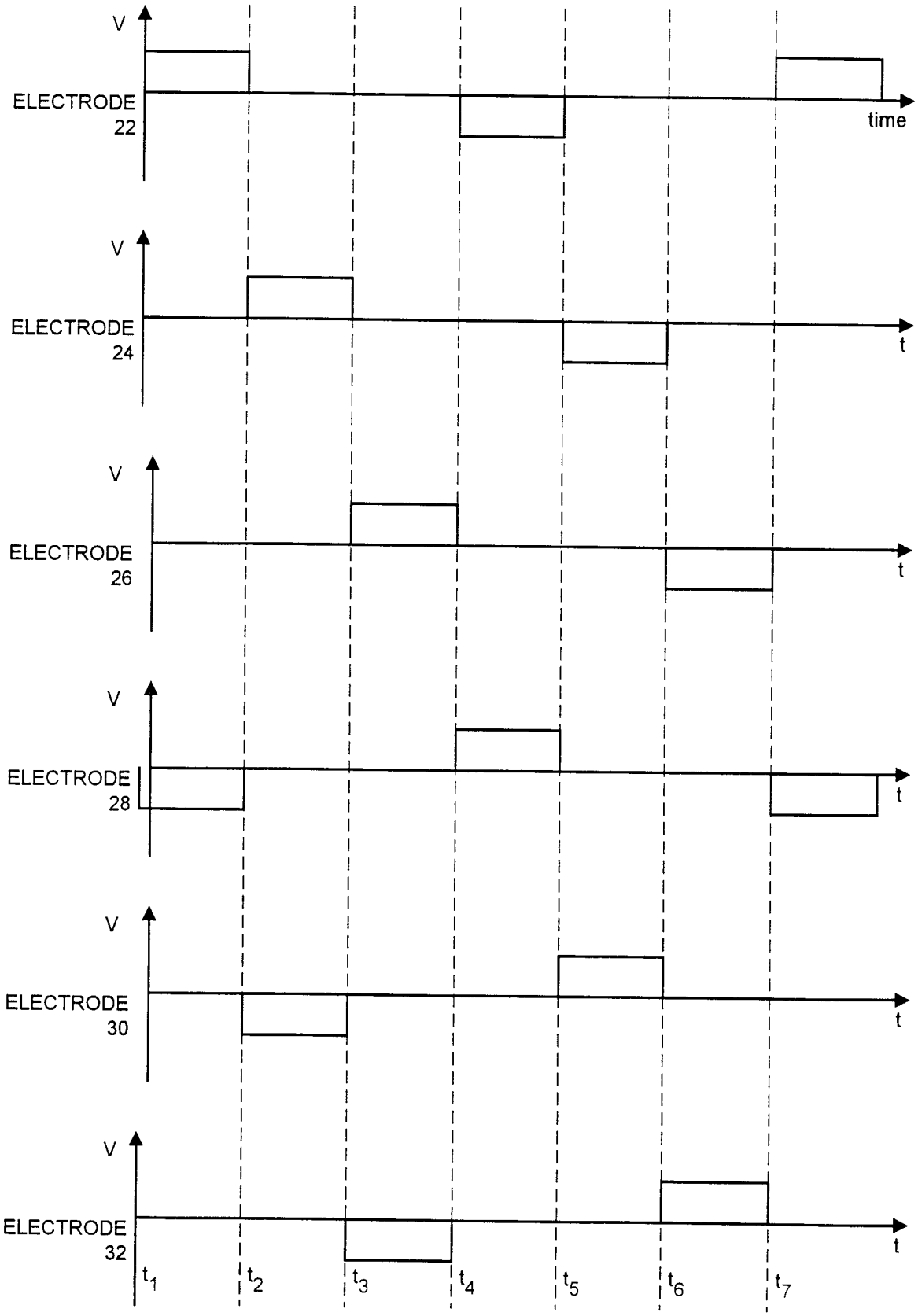
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FIG. 15

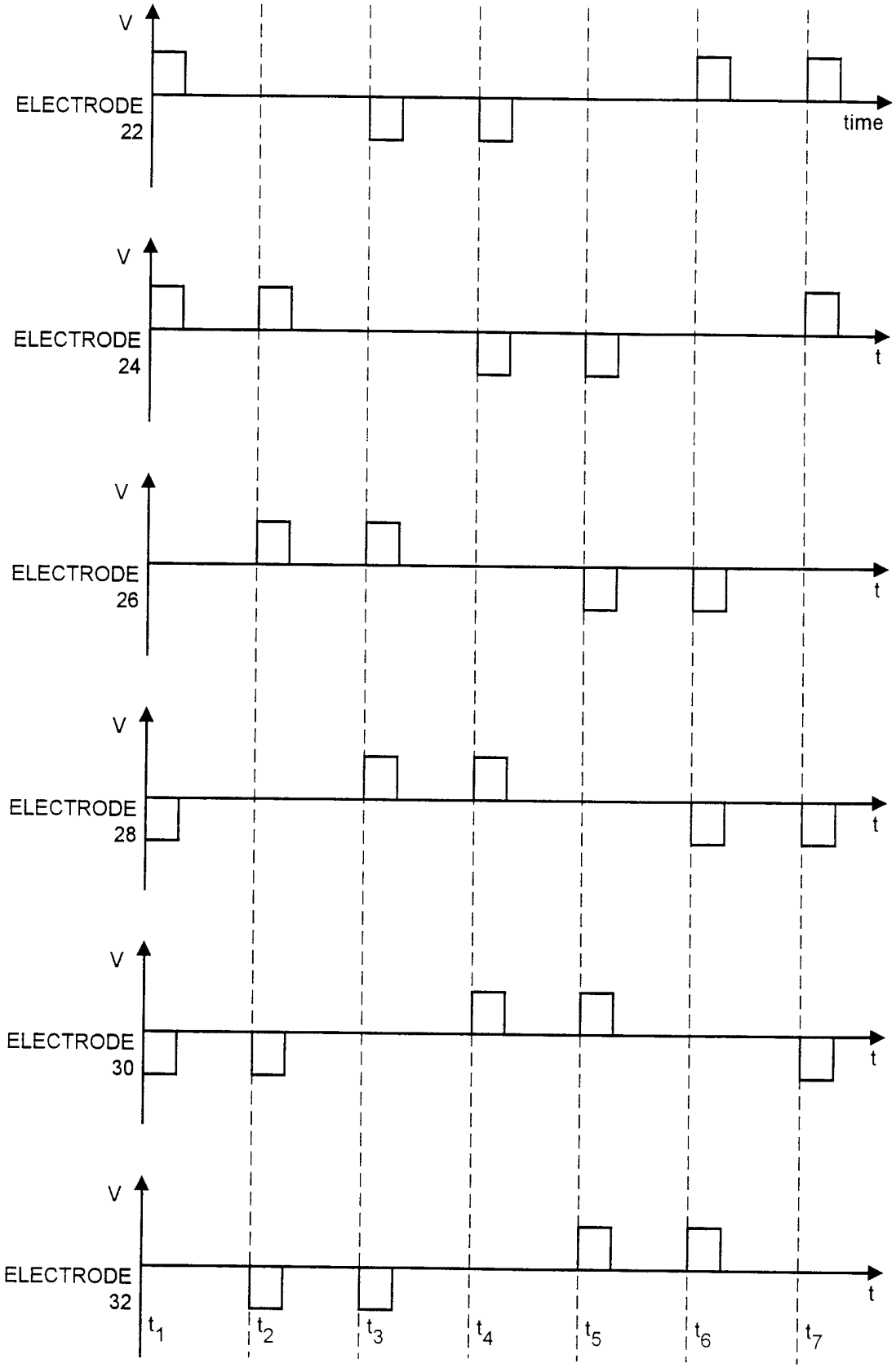
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FIG. 16

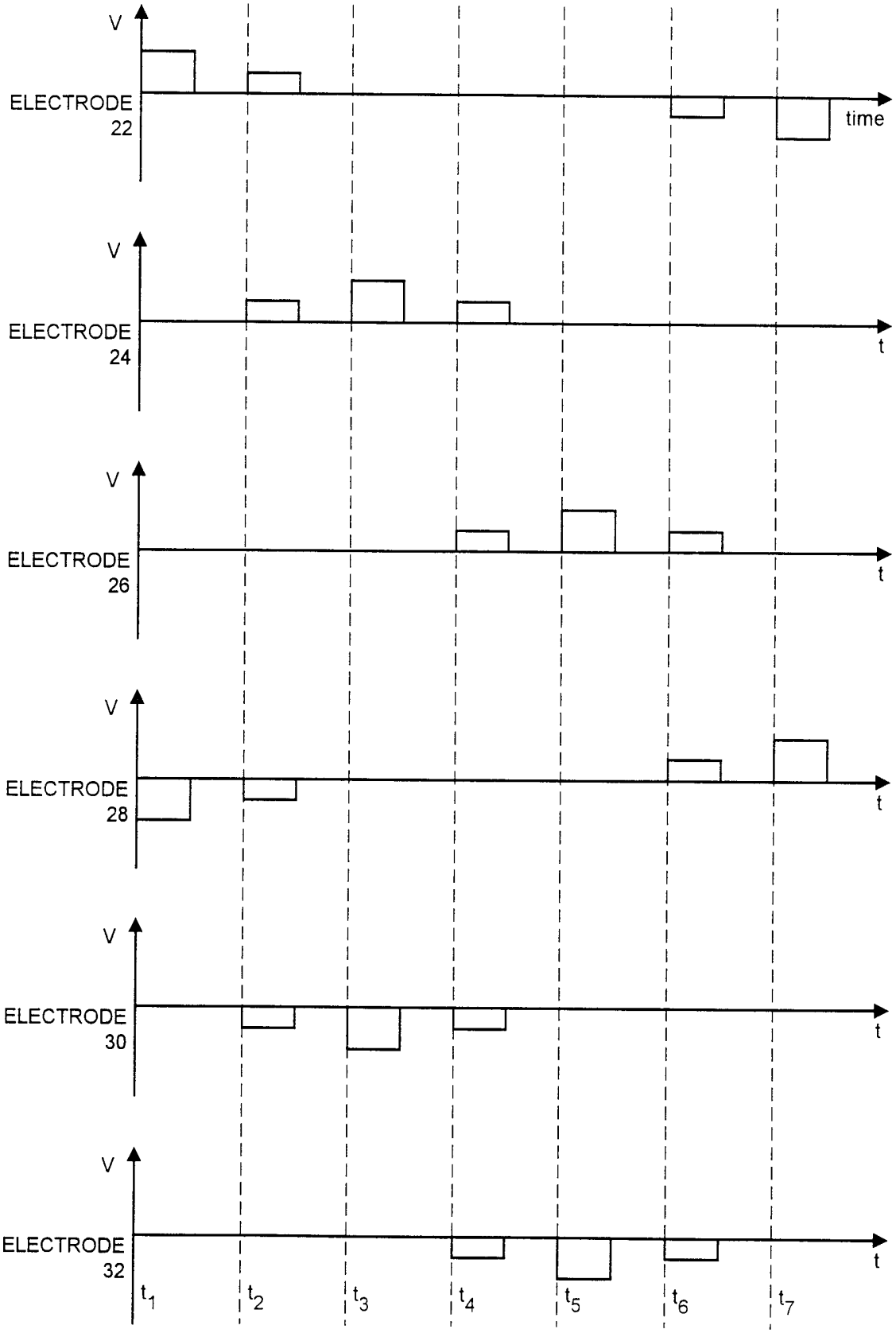
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FIG. 17

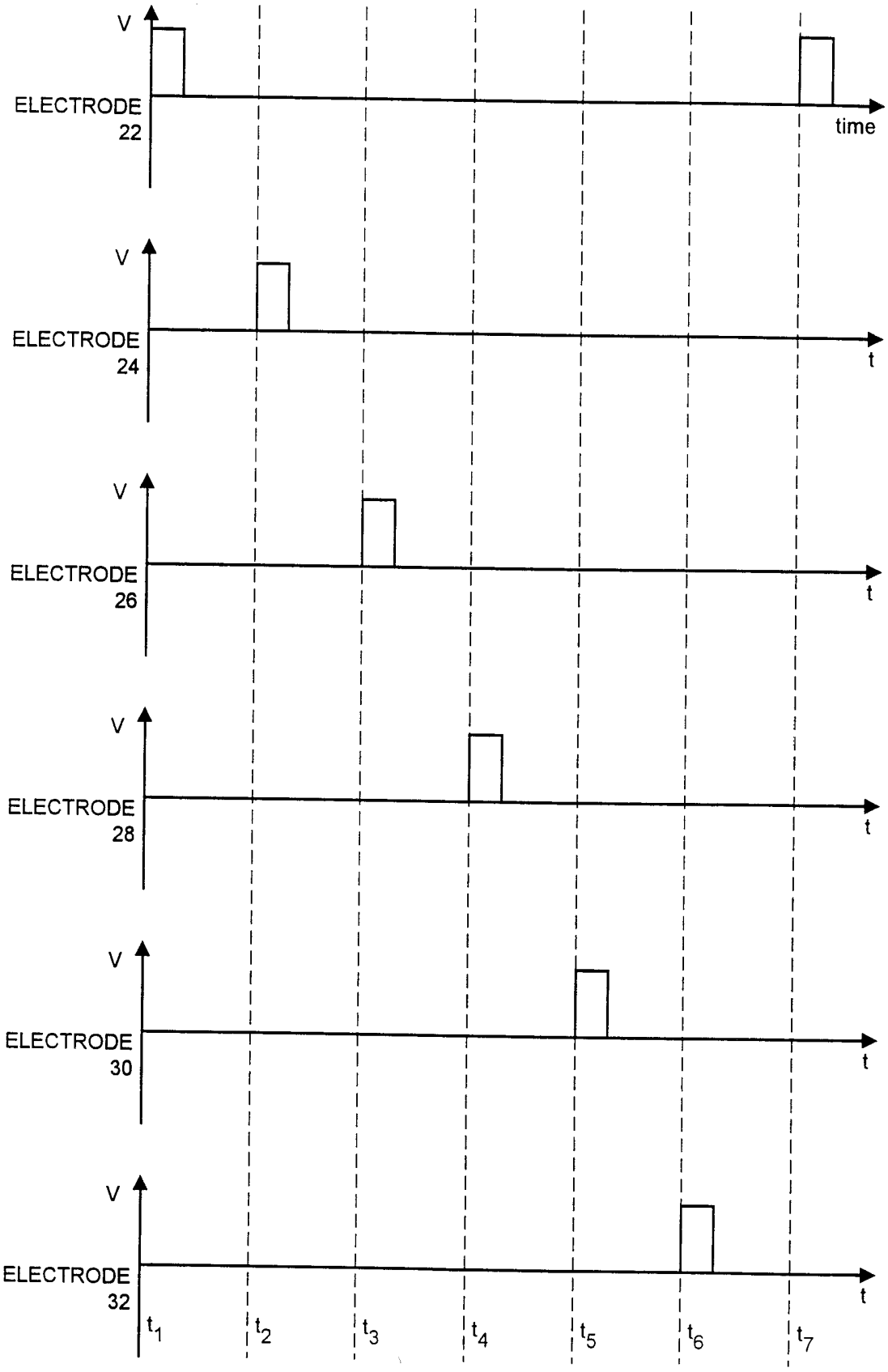
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FIG. 18

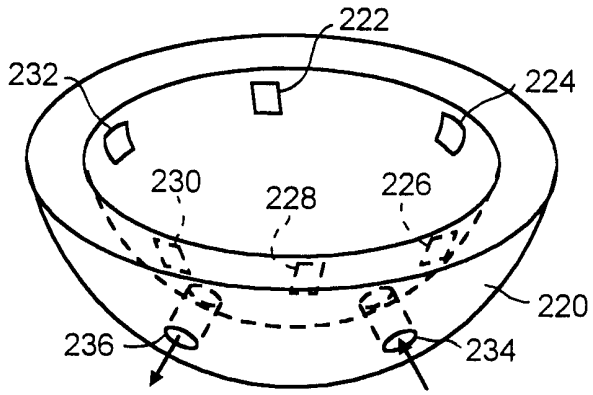


FIG. 19

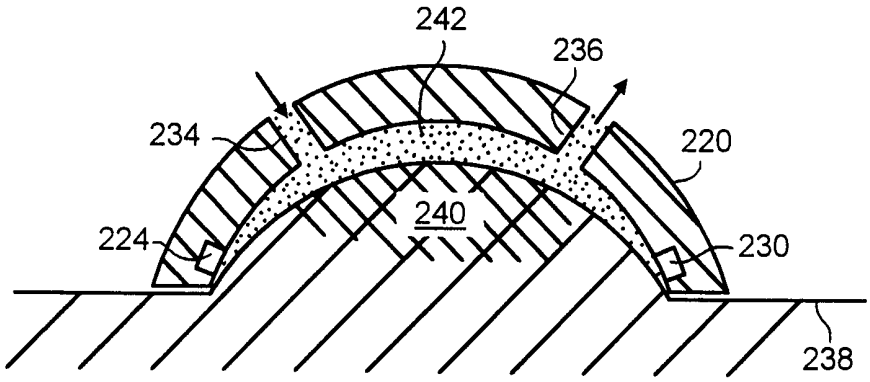


FIG. 20

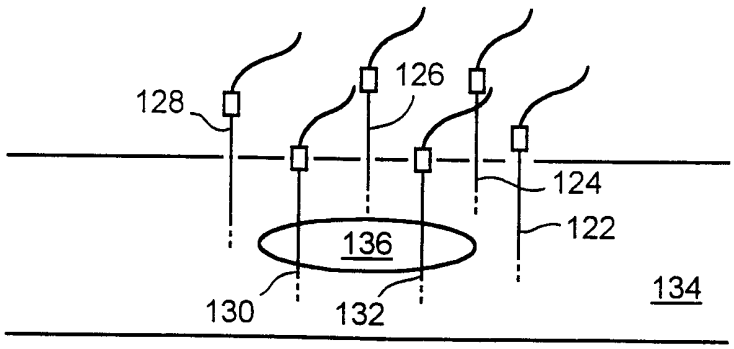
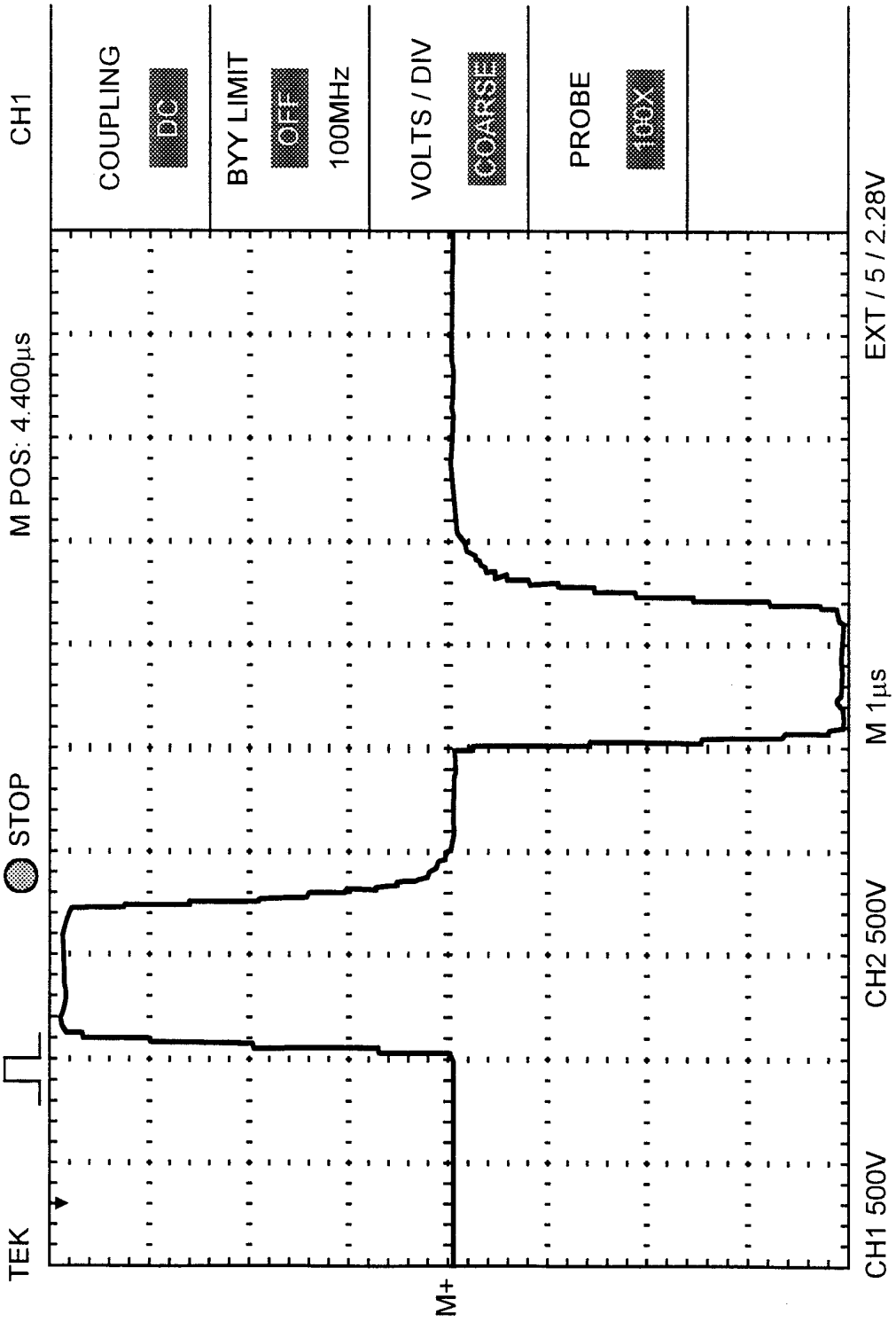


FIG. 21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VOLTS / DIV
COARSE
PROBE
100X
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STOP
TEK
M+
CH1 500V
CH2 500V
M 1µs
EXT / 5 / 2.28V

FIG. 22

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 99/00613

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12M3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12M C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 56893 A (WALTERS RICHARD E ;KING ALAN D (US); WALTERS DERIN C (US)) 17 December 1998 (1998-12-17) claims; figures 9-13	1-5,7
X	PATENT ABSTRACTS OF JAPAN vol. 012, no. 270 (C-515), 27 July 1988 (1988-07-27) & JP 63 049071 A (SHIMADZU CORP), 1 March 1988 (1988-03-01) abstract	1-5
Y	EP 0 126 389 A (KERNFORSCHUNGSANLAGE JUELICH) 28 November 1984 (1984-11-28) claims; figures	1-3

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"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 when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
9 July 1999	16/07/1999

Name and mailing address of the ISA	Authorized officer
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1

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