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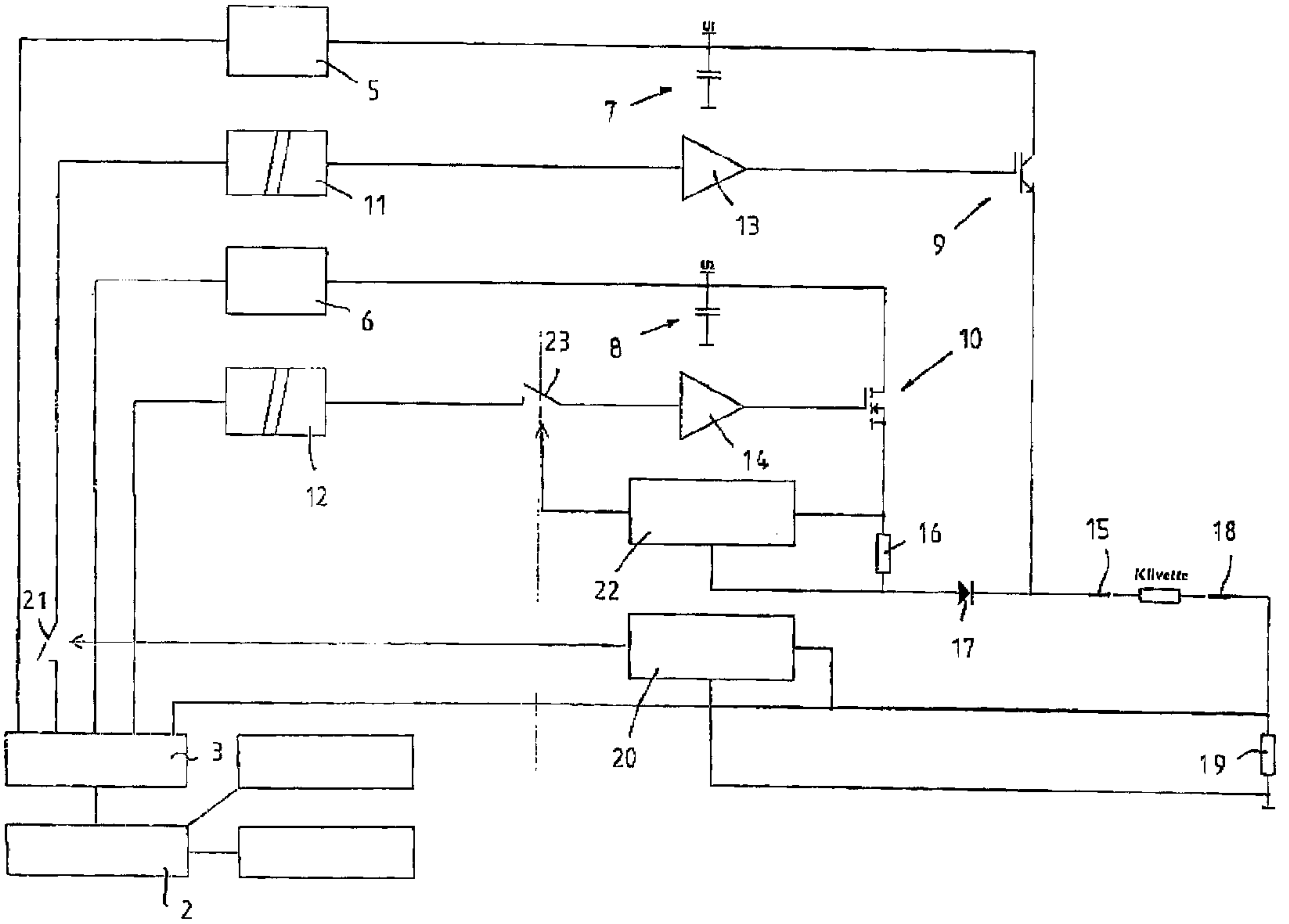

(57) Abrégé/Abstract:
The invention relates to a novel circuit arrangement for electrotransfection or electrofusion, which enables the transportation of DNA and/or other biologically active molecules to the nucleus of higher eucaryontic cells or the fusion of cells, independent of cell division and with reduced cell mortality.

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Abstract

The invention relates to a novel circuit arrangement for electrotransfection or electrofusion, which enables the transportation of DNA and/or other biologically active molecules to the nucleus of higher eukaryotic cells or the fusion of cells, independent of cell division and with reduced cell mortality.

CIRCUIT ARRANGEMENT FOR INJECTING NUCLEIC ACIDS AND OTHER BIOLOGICALLY ACTIVE MOLECULES INTO THE NUCLEUS OF HIGHER EUCARYONTIC CELLS USING ELECTRICAL CURRENT

The invention relates to a circuit arrangement for introducing nucleic acids, peptides, proteins and/or other biologically active molecules into the cell nucleus of eukaryotic cells by means of electric current, or for the treatment of cells, cell derivatives, subcellular particles and/or vesicles with electric current, consisting of at least two storage devices for quantities of electric charge, each supplied by a high-voltage power supply which each have at least one power semiconductor for transferring the quantities of charge present in the storage devices into a suspension in a cuvette and at least one monitoring device for controlling the power semiconductor.

Background of the invention

Since the place of action of eukaryotic DNA is the cell nucleus, DNA supplied from outside must enter the nucleus in order to be read out. Conventional transfection methods only bring about transport of DNA through the cell membrane into the cytoplasm. It is only because the nuclear membrane is temporarily dissolved during the cell division of higher eukaryotes that the DNA can passively enter the nucleus so that proteins encoded by it can be expressed. Only very small DNA molecules (oligonucleotides) can diffuse freely through the pores of the nuclear membrane. For the effective transfection of quiescent or weakly dividing cells it is thus necessary to create conditions which have the result that larger DNA molecules enter the nucleus through the nuclear membrane in sufficient quantity. The circuit arrangement described here makes this possible in higher eukaryotic cells.

State of the art

It has been known for some time that DNA from a buffer can be introduced into cells with the aid of electric current. However, the circuit arrangements for electroporation described so far are based on the transport of DNA into the cytoplasm of higher eukaryotic cells so that the expression of transfected DNA remains dependent on the dissolution of the nuclear membrane during the cell division. None of the circuit arrangements for electroporation known so far is concerned with bringing DNA electrically specifically into the nucleus of higher eukaryotic cells. Thus, a circuit arrangement for electrotransfection optimised for electrical nucleus transport is not known.

US Patent 4,750,100 from Bio-Rad Laboratories, Richmond, USA (1986), describes a specific equipment structure which can provide a maximum of 3000 V at a maximum of 125 A by capacitor discharge.

US Patent 5,869,326 (Genetronics, Inc., San Diego, USA, 1996) describes a specific equipment structure by which means two, three or a plurality of pulses can be generated using two separate current sources. However it is not claimed or shown that these pulses have an effect which goes beyond the transport of DNA into the cytoplasm.

US Patent 6,008,038 and the European Patent Application EP 0 866 123 A1 (Eppendorf-Netheler-Hinz GmbH, Hamburg, 1998) describe a device with which short pulses of 10–500 µs and a maximum of 1.5 kV can be generated but again give no indication that certain conditions could lead to conveying DNA into the nucleus.

None of the circuit arrangements known so far is optimised to make it possible for DNA and/or other biologically active molecules to be effectively transported into the cell nucleus with low cell mortality.

The invention relates to a circuit arrangement which makes it possible for DNA and/or biologically active molecules to be transported effectively into the cell nucleus with low cell mortality.

Description of the invention

The invention provides that the first storage device is charged with the preset voltage (U_1) as a parameter and the second storage device is charged with a voltage $U_2 = R \times I_2 \times K_2$, wherein R is the resistance of the cuvette and the suspension contained therein, I_2 is the desired current and K_2 is a correction value which takes into account the cuvette properties and wherein at least one first pulse with the capacitor voltage (U_1) of the storage device can be transferred to the cell for a preset time (T_1) by controlling a power semiconductor.

In a development of the invention it is provided that without interruption at least one second pulse with the capacitor voltage (U_2) of the storage device can also be applied to the cuvette by controlling a power semiconductor, wherein the delivered quantity of charge in at least one selectable time interval can be measured by the monitoring device, wherein the preset desired quantity of charge is compared with the actual delivered quantity of charge and on reaching or exceeding the desired quantity of charge, the power semiconductor is blocked.

In addition to the possibility of determining the delivered quantity of charge using the current flowing from the storage device, alternatively the preset desired quantity of charge is compared with the actual delivered quantity of charge in an interval of time and on reaching or exceeding the desired quantity of charge, the power semiconductor is blocked. On this occasion, depending on the pulse shape used and the number of pulses, the time interval which can be selected for the determination can be individually predefined in order, for example, to determine the delivered quantity of charge during the first or each subsequent pulse. The delivered quantity of charge can be determined by determining the difference between the original charge at least of one of the storage devices and the residual charge. In this case it is possible that according to the number of pulses used, more than one of the at least two storage devices is used in a circuit fashion wherein each storage device is assigned at least one high-voltage power supply, a monitoring device and a power semiconductor to transfer the quantity of charge to the cuvette containing the cell suspension. For the pulse transfer it is provided that the first power semiconductor transfers a pulse of 2-10 kV/cm having a duration of 10 – 100 μs and a current density of at least 2 A•cm^{-2} and, without interruption, the second power semiconductor transfers a pulse having a current density of 2 – 14 A•cm^{2} and a maximum duration of 100 ms. The time interval for determining the delivered quantity of charge can consequently be specified with the delivery of a first and/or preferably a second or each further pulse.

The delivered quantity of charge of the second pulse is preferably monitored wherein the switch-on time (T_2) of the second pulse can be specified by comparing the desired quantity of charge with the actual quantity of charge delivered by the measurement time and ends when the desired quantity of charge is reached and wherein a measurement cycle of 1 msec is provided to determine the actual quantity of charge, wherein during the time (T_2) the capacitor voltage decreases exponentially and the power semiconductor can be blocked on reaching the specified quantity of charge (Q_2).

Alternatively it is possible that after at least one predetermined time interval after triggering a first and/or second pulse, the flowing current is measured and if this exceeds or falls below a desired value, the pulse duration can be readjusted in order to keep the delivered quantity of charge constant. In another alternative it is possible that after at least one predetermined time interval after triggering a first and/or second pulse, the flowing current is measured and if this exceeds or falls below a desired value, an error message is generated to give a warning to the user of the device. It is furthermore possible that after at least one predetermined time interval after triggering a first and/or second pulse, the flowing current is measured and if this exceeds or falls below a desired value, the desired value is readjusted.

In order to determine any necessary constants, especially of the cuvette used with the cell suspension, it can be provided that a preliminary measurement of the resistance of the cuvette with the cell suspension is made. The other necessary pulse parameters are preferably pre-selected manually or if necessary specified by entering a code. It is thus also possible to use retrievable data via a card reader. The card reader can also be used at the same time to store the time profile of the voltage applied to the cuvette or the current flowing through the cuvette for documentation purposes for one or a plurality of pulse delivery processes on a commercially available memory card. This memory card is preferably used at the same time for storing the pulse parameters to be set.

As a result of the circuit regulation of the pulse delivery, the transfer of the envisaged quantity of charge is thus monitored in a reliable and advantageous fashion at least for one pulse and a controlled and sample-dependent transfer of a preset quantity of charge as well as a controlled monitoring to avoid any damage to the cells located in the sample can be achieved.
For further safety of the user and the samples used it is provided that an overcurrent cutoff is provided for the first and each subsequent pulse. The overcurrent cutoff thus allows the high-voltage pulse to be interrupted at any time in the event that preset limiting values are exceeded.

The high-voltage pulse of 2 – 10 kV/cm described is suitable for creating conditions such that DNA can enter the cell nucleus independently of the cell division. In order to keep cell damage low, this pulse is limited to between 10 and a maximum of 200 µs, preferably 10 – 50 µs. This is sufficient to achieve transfection independent of cell division. For example, such a short single high-voltage pulse was found to be optimum for the transfection of endothelial cells from the human umbilical vein. Another current pulse of lower field intensity or lower current strength or current density but of longer duration, following without interruption influences the efficiency of the transfection. As a result of the significantly lower current density, this pulse can persist significantly longer with little cell damage. An optimum current density or duration of the second pulse is obtained depending on the cell type and sensitivity of the cell. Such combined pulses are found to be optimal, for example, for primary human dermal fibroblasts or melanocytes or various human blood cells. In experiments using different cell lines and expression systems, the following was shown: the higher the current density of the second pulse, the stronger its influence on the transfection rate, i.e. the percentage of transfected cells. The lower the current density, the more the second pulse causes pure DNA transport into cells already transfected by the first pulse. The expression level of the transfected cells increases with increasing pulse duration but not the fraction of transfected cells. In order to maintain a precise cell-specific control of the transfection rate, the expression level and the cell vitality, the pulse duration and current density of the second pulse must therefore be controlled.

In order to achieve precise control of the pulse actually delivered to the cell suspension, in a preferred embodiment the delivered quantity of charge is controlled. In order to control the current strength or current density by a selectable capacitor voltage of the storage unit, the resistance of the cuvette and the cell suspension contained therein must be predefined initially. It was found that the resistance of the cuvettes when using aluminium electrodes varies during the pulse as a result of electrochemical processes. This variation is taken into account by a pulse-specific predefined correction value. Thus, precise pulse shapes for the second pulse can be predetermined using $U_2 = R \times I_2 \times K_2$ by controlling the charge, where U_2 is the capacitor voltage with which the storage device is charged, R is the resistance of the cuvette and the cell suspension contained therein, I_2 is the desired current and K_2 is the pulse-specific correction value.

In one embodiment of the invention the ohmic cuvette resistance R can be measured directly before the beginning of pulse delivery by applying a test voltage and taken into account accordingly in the calculation of the voltage U_2. Since the resistance measured before pulse delivery is subjected to larger fluctuations than the resistance during pulse delivery, presumably as a result of electrochemical processes, it is found to be advantageous to fixedly predefine the resistance R to calculate the capacitor voltage U_2 as a parameter. In a preferred embodiment of the invention the resistance of the cuvette is measured before the commencement of pulse delivery regardless of this in order to determine whether this lies within a predefined resistance window. If the measured resistance lies outside this window, there is a fault and the pulse delivery is not released.

For every cell type optimum conditions can be established for transfection rate, transfection intensity and cell vitality. In a preferred embodiment of the circuit arrangement the field intensity and duration of the first pulse and initial current intensity or current density and empirical duration of the second pulse can be selected and optimum conditions can simply be established for various cell types via a code.

The circuit arrangement can be used in an advantageous fashion for the transfection of quiescent or dividing eukaryotic cells. In the same way the circuit arrangement is also suitable for the transfection of primary cells such as human blood cells, pluripotent precursor cells from human blood, primary human fibroblasts, endothelial cells, muscle cells or melanocytes and can be used for diagnostic purposes or for the manufacture of a medicinal product for ex-vivo gene therapy.

The circuit arrangement according to the invention is furthermore also suitable, for example, for electrofusion, i.e., methods for the fusion of cells, cell derivatives, subcellular particles and/or vesicles by means of electric current, wherein, for examples the cells, cell derivatives, subcellular particles and/or vesicles are initially suspended in a suitable density in an aqueous solution, the suspension is then transferred to a cuvette and finally an electric voltage is applied to the electrodes of the cuvette and a current flow is generated through the suspension. Alternatively, for example, adherent cells, cell derivatives, subcellular particles and/or vesicles or however, also adherent cells with suspended cells, cell derivatives, subcellular particles or vesicles can be fused.

The circuit arrangement described here generates very high field intensities of 2 to 10 kV/cm which have the effect that DNA and/or biologically active molecules can enter the nucleus independently of the cell division. These field intensities are far above those normally used for electroporation and far beyond those sufficient for efficient opening of the pores in the cell membrane (on average 1 kV/cm according to Lurquin, 1997, Mol. Biotechnol. 7, 5).

The subject matter of the invention is thus a circuit arrangement for implementing a method for introducing nucleic acids, peptides, proteins and/or other biologically active molecules into the cell nucleus of higher eukaryotic cells using electric current wherein the introduction into the nucleus is achieved by a pulse having a field intensity 2-10 times that sufficient for opening the pores in the cell membrane and a duration of at least 10 µs and a current density of at least 2 $A \cdot cm^{-2}$.

The introduction of nucleic acids, peptides, proteins and/or other biologically active molecules into the cell nucleus can be achieved by a pulse of 2-10 kV/cm, preferably 3-8 kV/cm, wherein the pulse is a maximum of 200 µs long.

The circuit arrangement is designed so that the first pulse can be followed without interruption by a current flow having a current density of 2 $A \cdot cm^{-2}$ up to a maximum of 14 $A \cdot cm^{-2}$, preferably up to 5 $A \cdot cm^{-2}$, of 1 ms up to a maximum of 100 ms, preferably up to 50 ms in length.

Since the circuit arrangement makes transfection possible regardless of the cell division, in addition to dividing cells, quiescent or weakly dividing primary cells can also be transfected.

In other preferred embodiments the higher eukaryotic cells comprise primary human fibroblasts, endothelial cells and melanocytes.

The eukaryotic cells transfected using the circuit arrangement according to the invention can also be used for diagnostic and analytic purposes to produce a pharmaceutical product for ex-vivo gene therapy.

The circuit arrangement according to the invention makes it possible to achieve transfection independent of cell division and thus to considerably speed up transfection experiments. In transfection experiments using expression vectors, an analysis according to promoter and expressed protein can be made even a few hours after the transfection.

The concept "biologically active molecules" means peptides, proteins, polysaccharides, lipids or combinations or derivatives of these molecules as long as they develop a biological affinity in the cell.

Electroporation buffers having a high ionic strength and high buffer capacity are especially suitable for use with the circuit arrangement according to the invention.

The following protocol can be used to introduce nucleic acids into the cell nucleus of eukaryotic cells: $1 \times 10^5 - 1 \times 10^7$ cells and up to 10 µg DNA are incubated in 100 µl electroporation buffer in a cuvette having a 2 mm interelectrode gap for 10 min at room temperature and then transfected according to the conditions according to the invention. Immediately afterwards the cells are washed out of the cuvette with 400 µl of cell culture medium and incubated for 10 min at 37°C. The cells are then plated out in 37°C warm cell culture medium.

Suitable cuvettes are commercially available cuvettes for the electroporation of prokaryotes having an interelectrode gap or 2 mm or 1 mm, for example.

Evidence that the nucleic acids enter the cell nucleus independently of cell division can be furnished by analysing the cells which have not divided between transfection and analysis. This is achieved on the one hand by the transfection of non-dividing cells, such as for example cells of peripheral human blood and on the other hand for dividing cells by an analysis a few hours after transfection at a time when at most a fraction of the cells can have divided

The following abbreviations are used in addition to those in general use:

FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin

Examples

The following examples illustrate the invention but should not be regarded as restrictive.

Example 1

Transfection of cytotoxic T cells from human blood

Freshly prepared unstimulated (non-dividing) mononuclear cells from peripheral human blood (PBMC) were transfected with a vector which codes for the heavy chain of the mouse MHC class 1 protein H-2K^k. 5 x 10^6 cells together with 5 µg of vector DNA in a buffer having a high buffer capacity (48 mM x pH^{-1}) and high ionic strength (280 mM) were placed at room temperature in a cuvette having a 2 mm interelectrode gap and transfected by a 1000 V pulse of 100 µs duration, followed by a current flow having a current density of 5 $A \cdot cm^{-2}$ and 40 ms. Immediately afterwards, the cells were washed from the cuvette using 400 µl of culture medium, incubated for 10 minutes at 37°C and then transferred to a culture dish with pre-heated medium. After incubating for 24 h, the cells were successively incubated with digoxigenin-coupled anti-H-2K^k-antibody and Cy5-coupled anti-digoxigenin-antibody, as well as with a PerCP-coupled anti-CD8-antibody to identify human cytotoxic T cells and analysed using a flow cytometer (FACScalibur, Becton Dickinson). The number of dead cells was determined by staining with propidium iodide. As shown in Figure 1, 74.3% of the living cells express the H-2K^k antigen which corresponds to a very high transfection efficiency.

Example 2

Transfection of human haematopoeitic stem cells (CD34)

CD34-positive cells were pre-enriched from freshly prepared PBMC described as in Example 1 by magnetic cell sorting. Respectively 1 x 10^4 CD34-positive cells were then mixed with 1 x 10^6 PBMCs, placed together with 5 µg H-$2K^k$-expression vector DNA in a buffer having a high buffer capacity (54 mM x pH^{-1}) and high ionic strength (260 mM) at room temperature in a cuvette having a 2 mm interelectrode gap and transfected by a 1000 V pulse of 100 µs duration, followed by a current flow having a current density of 4 $A\cdot cm^{-2}$ and 20 ms duration. Immediately afterwards, the cells were washed from the cuvette using 400 µl of culture medium, incubated for 10 minutes at 37°C and then transferred to a culture dish with pre-heated medium. After incubating for 16 h, the cells were successively incubated with phycoerythrin-coupled anti-H-$2K^k$-antibody, as well as with an APC-coupled anti-CD34 antibody to identify human haematopoietic stem cells and analysed using a flow cytometer (FACScalibur, Becton Dickinson). The number of dead cells was determined by staining with propidium iodide. As shown in Figure 2, 66.7% of the cells express the H-$2K^k$ antigen which corresponds to a high transfection efficiency.

Example 3

Transfection of human neonatal dermal fibroblasts (NHDF-Neo)

Human neonatal dermal fibroblasts (5 x 10^5 cells) together with 5 µg H-$2K^k$-expression vector DNA were placed in a buffer having a high buffer capacity (67 mM x pH^{-1}) and high ionic strength (380 mM) at room temperature in a cuvette having a 2 mm interelectrode gap and transfected by a 1000 V pulse of 100 µs duration, followed by a current flow having a current density of 6 $A\cdot cm^{-2}$ and of 33 ms duration. Immediately afterwards, the cells were washed from the cuvette using 400 µl of culture medium, incubated for 10 minutes at 37°C and then transferred to a culture dish with pre-heated medium. After incubating for 5 h, the cells were incubated with a Cy5-coupled anti-H-$2K^k$-antibody and analysed using a flow cytometer (FACScalibur, Becton Dickinson). The number of dead cells was determined by staining with propidium iodide. As shown in

Figure 3, 93% of the cells express the H-2K^k antigen which corresponds to a very high transfection efficiency.

Example 4

Transfection of human neonatal melanocytes

Human neonatal melanocytes (2.5 x 10^5 cells) together with 5 µg H-2K^k-expression vector DNA were placed in a buffer having a high buffer capacity (54 mM x pH^{-1}) and high ionic strength (260 mM) at room temperature in a cuvette having a 2 mm interelectrode gap and transfected by a 1000 V pulse of 100 µs duration, followed by a current flow having a current density of 6 $A \cdot cm^{-2}$ and 33 ms duration. Immediately afterwards, the cells were washed from the cuvette using 400 µl of culture medium, incubated for 10 minutes at 37°C and then transferred to a culture dish with pre-heated medium. After incubating for 5 h, the cells were incubated with a Cy5-coupled anti-H-2K^k-antibody and analysed using a flow cytometer (FACScalibur, Becton Dickinson). The number of dead cells was determined by staining with propidium iodide. As shown in Figure 4, 75.1% of the cells express the H-2K^k antigen which corresponds to a very high transfection efficiency.

Example 5

Transfection of human endothelial cells from the umbilical vein (HUVEC)

Endothelial cells from the human umbilical vein (1 x 10^6 cells) together with 5 µg H-2K^k-expression vector DNA were placed in a buffer having a high buffer capacity (67 mM x pH^{-1}) and high ionic strength (378 mM) at room temperature in a cuvette having a 2 mm interelectrode gap and transfected by a 1000 V pulse of 100 µs duration. Immediately afterwards, the cells were washed from the cuvette using 400 µl of culture medium, incubated for 10 minutes at 37°C and then transferred to a culture dish with pre-heated medium. After incubating for 5 h, the cells were incubated with a Cy5-coupled anti-H-2K^k-antibody and analysed using a flow cytometer (FACScalibur, Becton Dickinson). The number of dead cells was determined by staining with propidium iodide. As shown in Figure 5, 49.7% of the cells express the H-2K^k antigen which corresponds to a high transfection efficiency.

Example 6

Transfection of the human cell line K562

K562 cells (1 x 10^6 cells) together with 5 µg H-$2K^k$-expression vector DNA were placed in a buffer having a high buffer capacity (24 mM x pH^{-1}) and high ionic strength (254 mM) at room temperature in a cuvette having a 2 mm interelectrode gap and transfected by a 1000 V pulse of 100 µs duration, followed by a current flow having a current density of 8 $A \cdot cm^{-2}$ and 10 ms duration. Immediately afterwards, the cells were washed from the cuvette using 400 µl of culture medium, incubated for 10 minutes at 37°C and then transferred to a culture dish with pre-heated medium. After incubating for 4 h, the cells were incubated with a Cy5-coupled anti-H-$2K^k$-antibody and analysed using a flow cytometer (FACScalibur, Becton Dickinson). The number of dead cells was determined by staining with propidium iodide. As shown in Figure 6, 69.5% of the cells express the H-$2K^k$ antigen which corresponds to a very high transfection efficiency.

Example 7

Transfection efficiency and average fluorescence intensity of Cycle3-GFP-transfected CHO cells

In order to investigate the transfection efficiency and the average fluorescence intensity of transfected cells as a function of the quantity of charge flowing in the second pulse, respectively 7 x 10^5 CHO cells together with 5 µg Cycle3-GFP-vector-DNA were placed in electroporation buffer in a cuvette having an interelectrode gap of 2 mm and transfected by a 1000 V, 10 µs pulse and subsequent second pulses differing in the variation of the current intensity or current density and pulse time. After cultivation for 5 hours, the cells were analysed using a flow cytometer. Figure 7 shows the transfection efficiency determined as a function of the integral of the current over the pulse time (the quantity of charge Q). It is found that the transfection efficiency can be increased with increasing current intensity (open circles). An increase in the pulse time for the same current intensity on the other hand results in no appreciable increase in efficiency (closed circles). The fluorescence intensity (brightness) of the transfected cells increases with increasing quantity of charge Q, with saturation being reached for high Q. No major differences are found whether the increase in Q was achieved by increasing the current intensity (open circles) or increasing the pulse length (closed circles).

Example 8

Transfection efficiency and average fluorescence intensity of Cycle3-GFP-transfected Jurkat cells

In order to investigate the transfection efficiency and the average fluorescence intensity of transfected cells as a function of the quantity of charge flowing in the second pulse, respectively 4 x 10^5 Jurkat cells together with 5 µg Cycle3-GFP-vector-DNA were placed in electroporation buffer in a cuvette having an interelectrode gap of 2 mm and transfected by a 1000 V, 10 µs pulse and subsequent second pulses differing in the variation of the current intensity or current density and pulse time. After cultivation for 5 hours, the cells were analysed using a flow cytometer. Figure 8 shows the transfection efficiency determined as a function of the integral of the current over the pulse time (the quantity of charge Q). As when using CHO cells, it is found that the transfection efficiency can be increased with increasing current intensity (open circles). An increase in the pulse time for the same current intensity on the other hand results in no appreciable increase in efficiency (closed circles). The fluorescence intensity (brightness) of the transfected cells increases with increasing quantity of charge Q, with saturation being reached for high Q. No major differences are found whether the increase in Q was achieved by increasing the current intensity (open circles) or increasing the pulse length (closed circles).

Example 9

Transfection efficiency and average fluorescence intensity of H-2K^k-transfected Jurkat cells

In order to investigate the transfection efficiency and the average fluorescence intensity of transfected cells as a function of the quantity of charge flowing in the second pulse, respectively 1 x 10^6 Jurkat cells together with 2 µg of H2K^k-expression vector DNA were placed in electroporation buffer in a cuvette having an interelectrode gap of 2 mm and transfected by a 1000 V, 10 μs pulse and subsequent second pulses differing in the variation of the current intensity or current density and pulse time. After cultivation for 3.5 hours, the cells were incubated with Cy5-coupled anti-H2K^k and analysed using a flow cytometer. Figure 9 shows the transfection efficiency determined as a function of the integral of the current over the pulse time (the quantity of charge Q). It is found that the transfection efficiency can be increased with increasing current intensity (open circles). An increase in the pulse time for the same current intensity on the other hand results in no appreciable increase in efficiency (closed circles). The fluorescence intensity (brightness) of the transfected cells increases with increasing quantity of charge Q, with saturation being reached for high Q. No major differences are found whether the increase in Q was achieved by increasing the current intensity (open circles) or increasing the pulse length (closed circles).

The invention is explained further with reference to the following figures.

In the figures

Figure 1 shows a transfection of cytotoxic T cells from human blood,

Figure 2 shows a transfection of pluripotent precursor cells from human blood,

Figure 3 shows a transfection of human neonatal dermal fibroblasts,

Figure 4 shows a transfection of human neonatal dermal melanocytes,

Figure 5 shows a transfection of human endothelial cells from the umbilical cord,

Figure 6 shows a transfection of the cell line K562 (analysis 4 h after transfection),

Figure 7 shows an investigation of the transfection efficiency as a function of the current intensity, pulse time and quantity of charge and of the expression intensity as a function of the quantity of charge, experiment using the CHO cell line,

Figure 8 shows an investigation of the transfection efficiency as a function of the current intensity, pulse time and quantity of charge and of the expression intensity as a function of the quantity of charge, experiment using the Jurkat cell line,

Figure 9 shows an investigation of the transfection efficiency as a function of the current intensity, pulse time and quantity of charge and of the expression intensity as a function of the quantity of charge, experiment using the Jurkat cell line and the surface marker protein H-2K^k,

Figure 10 shows a block diagram of an electroporator circuit,

Figure 11 shows a circuit diagram of a control panel,

Figure 12 shows a circuit diagram of a card reader,

Figure 13 shows a circuit diagram of a supply unit,

Figure 14 shows a circuit diagram of a HV power supply,

Figure 15 shows a circuit diagram of an HV switch,

Figure 16 shows a circuit diagram of a current regulating system,

Figure 17 shows a circuit diagram of a flash recognition system,

Figure 18 shows a circuit diagram of a control system, and

Figure 19 is a flow diagram to explain the sequence of the pulse delivery processes.

Figure 1 shows the flow cytometric analysis of PBMC which had been transfected with H-$2K^k$-expression vector. The cells were successively incubated with digoxigenin-coupled anti-H-$2K^k$-antibody and Cy5-coupled anti-digoxigenin-antibody, as well as with a PerCP-coupled anti-CD8-antibody to identify human cytotoxic T cells and were analysed using a flow cytometer (FACScalibur, Becton Dickinson). (FL-2, FL-3 = fluorescence channel 2, 3; SSC = sideward scatter, FSC = forward scatter, PerCP = peridinin chlorophyll protein, CD = cluster of differentiation).

Figure 2 shows the flow cytometric analysis of CD34-positive stem cells enriched from PBMC which had been transfected with H-$2K^k$-expression vector. The cells were successively incubated with phycoerythin-coupled anti-H-$2K^k$-antibody, as well as with a APC-coupled anti-CD34-antibody to identify human CD34 positive haematopoietic stem cells and were analysed using a flow cytometer (FACScalibur, Becton Dickinson). (FL-1, FL-3 = fluorescence channel 1, 3; SSC = sideward scatter, FSC = forward scatter, PE = phycoerythrin, APC = allophycocyanin, CD = cluster of differentiation).

Figure 3 shows the flow cytometric analysis of human neonatal dermal fibroblasts (NHDF-Neo), which had been transfected with H-$2K^k$-expression vector. The cells were incubated with Cy5-coupled anti-H-$2K^k$ and analysed using a flow cytometer (FACScalibur, Becton Dickinson). (FL-1, FL-2, FL-3 = fluorescence channel 1, 2, 3; SSC = sideward scatter, FSC = forward scatter).

Figure 4 shows the flow cytometric analysis of human neonatal melanocytes (NHEM-Neo), which had been transfected with H-$2K^k$-expression vector. The cells were incubated with Cy5-coupled anti-H-$2K^k$ and analysed using a flow cytometer (FACScalibur, Becton Dickinson). (FL-1, FL-2, FL-3 = fluorescence channel 1, 2, 3; SSC = sideward scatter, FSC = forward scatter).

Figure 5 shows the flow cytometric analysis of endothelial cells from human umbilical cord (HUVEC), which had been transfected with H-2K^k-expression vector. The cells were incubated with Cy5-coupled anti-H-2K^k and analysed using a flow cytometer (FACScalibur, Becton Dickinson). (FL-1, FL-2, FL-3 = fluorescence channel 1, 2, 3; SSC = sideward scatter, FSC = forward scatter).

Figure 6 shows the flow cytometric analysis of the human cell line K562 which had been transfected with H-2K^k-expression vector. The cells were incubated with Cy5-coupled anti-H-2K^k and analysed using a flow cytometer (FACScalibur, Becton Dickinson). (FL-1, FL-2, FL-3 = fluorescence channel 1, 2, 3; SSC = sideward scatter, FSC = forward scatter).

Figure 7 shows a graphical representation of the transfection efficiency of CHO cells and the average fluorescence intensity (brightness) of the positive cells as a function of the quantity of charge Q which has flowed. The CHO cells were transfected with Cycle3-GFP expression vector and analysed after five hours using a flow cytometer (FACScalibur, Becton Dickinson). Closed circles correspond to a gradual increase in the pulse time for the same current intensity (2 A) or current density (4 $A \cdot cm^{-2}$), open circles correspond to an increase in current intensity.

Figure 8 shows a graphical representation of the transfection efficiency of Jurkat cells and the average fluorescence intensity (brightness) of the positive cells as a function of the quantity of charge Q which has flowed. The Jurkat cells were transfected with Cycle3-GFP expression vector and analysed after five hours using a flow cytometer (FACScalibur, Becton Dickinson). Closed circles correspond to a gradual increase in the pulse time for the same current intensity (2 A) or current density (4 $A \cdot cm^{-2}$), open circles correspond to an increase in current intensity.

Figure 9 shows a graphical representation of the transfection efficiency of Jurkat cells and the average fluorescence intensity (brightness) of the positive cells as a function of the quantity of charge Q which has flowed. The Jurkat cells were transfected with H-2K^k expression vector and incubated after 3.5 hours with a Cy5-coupled anti-H-2K^k and analysed using a flow cytometer (FACScalibur, Becton Dickinson). Closed circles correspond to a gradual increase in the pulse time for the same current intensity (2 A) or current density (4 A$\cdot$cm^{-2}), open circles correspond to an increase in current intensity.

Figure 10 shows a block diagram of the electroporator 1 with the necessary individual components. These comprise an adjusting unit 2, a control unit 3 to which a voltage supply unit 4 is connected as well as at least two HV power supplies 5, 6 with following storage devices 7, 8 and two power semiconductors 9, 10 provided for pulse delivery. The power semiconductors 9, 10 are controlled via a potential divider stage 11, 12 by the control unit 3 by means of an HV switch 13 and a regulating unit 14. The storage devices 7, 8 are directly connected to the inputs of the power semiconductors 9, 10, wherein the storage devices 7, 8 can consist of one or a plurality of capacitors depending on the field strength and the pulse duration. The power semiconductor 9 can for example consist of an IGBT and the power semiconductor 10 can consist of a MOSFET. However, the term "power semiconductor" should comprise all other electronic components or component assemblies by which means the voltages and currents to be switched within the scope of the invention can be switched with the required switching times. The output of the IGBT is directly connected to the cuvette connection 15 whereas the output of the MOSFET 10 is connected via a resistance 16 and a diode 17 to the cuvette connection 15 so that no pulse can flow back via the second power semiconductor 10 if both power semiconductors 9, 10 are controlled simultaneously. For this purpose the diode 17 is connected to the cuvette connection 15 on the cathode side. The second cuvette connection 18 is connected to earth via a resistance 19. The resistance 19 comprises a measuring shunt to measure the voltage drop and supply to an overcurrent switching stage 20. The overcurrent switching stage 20 can interrupt the pulse delivery by means of a switch 21 via the potential divider stage 11 and the HV switch 13 whereas a second overcurrent switching stage 22 interrupts a control system of the regulating unit 14 for the MOSFET

10 via a switch 23. The voltage applied via the resistance 16 is fed to the overcurrent switching stage 22 in order to bring about a current switchoff in the event that the maximum current is exceeded. Since the resistance 16 is located directly in the high-voltage circuit, the switch 23 is located after the potential divider stage 12 so that no high-voltage pulses can enter the control unit 3 and the operating staff are not endangered. In the case of the overcurrent switching stage 20, the low-resistance measuring resistance 19 lies behind the cuvette connections 15, 18 and is connected to earth so that the transmission of high-voltage pulses can be eliminated. Depending on the intended usage of the electroporator 1, one or a plurality of high-voltage power supplies 5, 6 with the relevant storage devices 7, 8 and the necessary potential divider stages 11, 12 and HV switch 13 or regulating unit 14 to control the power semiconductors 9, 10 can be used. The storage devices 7, 8 are equipped with one or a plurality of capacitors of the required capacity and breakdown voltage so that a suitably high quantity of charge can be stored and transferred to the cuvette connection 15.

The following Figures 11 to 18 shows the circuit diagrams of the individual components in the block diagram.

Figure 11 shows a circuit diagram of the control panel for entering the parameter signals to be set wherein these can be preselected via a pushbutton switch 30 and checked visually using display elements 31. LEDs 32 shows when the equipment is ready for operation. The necessary parameters are prepared in the circuit and transmitted via a connector 33 to the control system in accordance with Figure 14.

Figure 12 shows a circuit diagram of a card reader 34 via which preset parameters for certain biological substances are read in and transmitted to the control unit as shown in Figure 14.

Figure 13 shows a circuit diagram of the supply unit which substantially consists of a 150/230 Volt changeover switch 35, a transformer stage 36 with primary-side wiring and voltage lead and secondary regulating stages to produce the necessary operating voltages. For this purpose a plurality of voltage regulators 38 are inserted after the rectifier 37.

Figure 14 shows a circuit diagram of the two HV power supplies 5, 6 which can be identified from the block diagram. Both HV power supplies 5, 6 are acted upon by the voltage U_1 from the supply unit, wherein each regulating stage 39 receives a control signal U3on, U5on from the control system and the applied voltage U_1 charges the storage device 7, 8 consisting of a plurality of capacitors, in pulsed mode via a transformer stage 40. The desired voltage reached is transmitted via output signals U3sense, U5sense of the control system as shown in Figure 15. The voltage U_5 of the storage device 7 is fed to an HV switch 13 as shown in Figure 15 and the voltage U_3 of the storage device 8 is fed to a current regulating stage as shown in Figure 16.

Figure 15 shows a circuit diagram of the HV switch 13. The HV switch 13 receives the signal HIN generated by the pulse monitoring stage as shown in Figure 16 to control the first power semiconductor 9. This transmits the applied voltage U_5 to a solder pad 41 for the HV cable for connecting the cuvette which is then connected to earth via a second solder pad 42 via a low-resistance measuring resistance. An overcurrent cutoff stage 20 delivers a control signal for the control unit as shown in Figure 18 for switching off the power semiconductor 9 in the event of a preset maximum current rise being exceeded. The first solder pad 41 is further connected to the voltage output U_4 of the current regulating stage from Figure 12 in order that a controlled current flow into the cuvette to deliver a specific quantity of charge can be achieved following the high-voltage pulse. The current regulating stage from Figure 16 receives the control signals from the control unit from Figure 18 via a potential divider stage and regulates the voltage U_3 applied to the storage device 8 to the voltage U_4 delivered via the solder pad 41. In this case, according to the invention, Q regulation or current regulation is used whereby the charge in the storage device 8 is determined at predefined time intervals of, for example, one millisecond and the delivered quantity of charge is determined taking into account the original charge.

Figure 18 shows a circuit diagram of the control unit 3 which either takes into account the preset manual values or the values entered via a card reader 34 and controls the current regulating unit 14 as shown in Figure 16 on the basis of further monitoring signals. The HV switch 13 as shown in Figure 15 however is controlled after manually triggering the high-voltage pulse via a pulse monitoring stage 43 as shown in **Figure 17** so that after the HV pulse has been delivered, the quantity of charge can be monitored via the current regulating unit 14 as shown in Figure 16.

The pulse parameters can thus on the one hand be preset manually and on the other hand via a card reader so that when a pulse is triggered manually via the existing regulating electronics, a high-voltage pulse with or without monitoring of the flowing current and if necessary, a continuous current signal with monitoring of the quantity of charge can be delivered via a second HV power supply.

Figure 19 shows a schematic flow diagram of the operating sequence of a pulse delivery process controlled by the control unit 3 (see Figure 10) according to a preferred embodiment of the invention. First, the required pulse parameters are predefined manually or by reading out a memory card (not shown). After starting the process (e.g. by actuating a corresponding trigger button), the ohmic resistance of the cuvette is first measured by briefly applying a low voltage (e.g. 12 V) to the cuvette connections 15, 18 and a subsequent current measurement (e.g. for 2 ms) in step 44. As part of the interrogation 45 it is checked whether this resistance lies within a predefined window. If not, the subsequent process is interrupted. The measured resistance is not used subsequently to calculate the charging voltage U_2 in the present embodiment of the invention. If the resistance lies in order within the predefined window, the storage devices 7, 8 are charged to the predefined voltages U_1 and U_2 in step 46. When the desired charging voltages are achieved, the charging by the HV power supplies 5, 6 is switched off. During the following pulse delivery, no recharging of the storage devices takes place. The pulse delivery for the high-voltage pulse then begins in step 47 by closing the semiconductor switch 9. As a result, a relatively high current flows through the cell. An excessively steep current rise is recognised by the overcurrent cutoff stage 20 and results in immediate opening of the switch 9 for safety reasons and interrupts the routine. In the present embodiment the high-voltage pulse is terminated after a predefined time of a few microseconds whereupon the second pulse follows immediately and without interruption. For this purpose in step 48 the second semiconductor switch 10 is already closed a short time before opening the first semiconductor switch 9 so that there is an interruption-free transition between the two pulses. In the short time interval in which both high-voltage switches 9, 10 are closed simultaneously, the diode 17 prevents any higher voltage from being able to flow from the storage device 7 into the storage device 8. The semiconductor switch 10 then remains open (provided that the maximum current is not exceeded by an overcurrent cutoff stage 22) until the predefined charge Q has flowed through the cuvette. For this purpose in step 49 the current flowing through the cuvette is measured and integrated in predefined time intervals (e.g. 1 ms). As soon as the predefined charge has been reached (see interrogation 50), the switch 10 is opened and the routine is terminated. The capacity of the storage device 8 is selected so that the voltage decreases gradually or slowly during the duration of the second pulse. If as a result of a fault, the predefined desired charge is still not yet achieved even when the storage device is almost completely discharged, the process will also be interrupted after a suitably selected time limit has been exceeded.

Reference list

1	Electroporator
2	Adjusting unit
3	Control unit
4	Voltage supply unit
5	HV power supply
6	HV power supply
7	Storage device
8	Storage device
9	Power semiconductor
10	Power semiconductor
11	Potential divider stage
12	Potential divider stage
13	HV switch
14	Regulating unit
15	Cuvette connection
16	Resistance
17	Diode
18	Cuvette connection
19	Resistance
20	Overcurrent cutoff stage
21	Switch
22	Overcurrent cutoff stage
23	Switch
30	Push-button switch
31	Display element
32	LED
33	Connector
34	Card reader
35	Changeover switch
36	Transformer
37	Rectifier
38	Voltage regulator
39	Regulating stage
40	Transformer stage

41	Solder pad
42	Solder pad
43	Pulse monitoring stage
44	to 51 steps

Claims

1. A circuit arrangement for introducing a biologically active molecule into the cell nucleus of eukaryotic cells by means of electric current, the circuit arrangement comprising at least two storage devices for quantities of electrical charge, each supplied by a high-voltage power supply, each having at least one power semiconductor for transferring the quantities of charge present in to the at least two storage devices into a suspension in a cuvette; and at least one control device for controlling the at least one power semiconductor, wherein a first of the at least two storage devices is charged with a preset voltage (U_1) as a parameter and a second of the at least two storage devices is charged with a voltage $U_2 = R \times I_2 \times K_2$, wherein R is the resistance of the cuvette and the suspension contained therein, I_2 is the desired current and K_2 is a correction value which takes into account the properties of the cuvette, and wherein the control device is designed to control the at least one power semiconductor in a way that at least one first pulse with the capacitor voltage (U_1) of the first storage device can be transferred to the cuvette for a preset time (T_1) by controlling the at least one power semiconductor, wherein the delivered quantity of charge of the at least one pulse in at least one selectable time interval can be measured by a monitoring device, and wherein a preset desired quantity of charge is compared with the delivered actual quantity of charge and on reaching or exceeding the desired quantity of charge, the at least one power semiconductor is blocked, wherein, the biologically active molecule is a nucleic acid, a peptide, a protein, a polysaccharide, a lipid, a derivative thereof, or a combination thereof.

2. The circuit arrangement according to claim 1, wherein without interruption at least one second pulse with the capacitor voltage (U_2) of the second storage device can also be transferred to the cuvette by controlling a second power semiconductor.

3. The circuit arrangement according to claim 1 or 2, wherein the delivered quantity of charge is determined from the difference between the original charge of the corresponding storage device and the residual charge.

4. The circuit arrangement according to claim 1, wherein a first power semiconductor transfers a pulse of 2 – 10 kV/cm having a duration of 10 – 100 μs and a current density of at least 2 $A \cdot cm^{-2}$ to the cuvette and a second power semiconductor transfers a pulse having a current density of 2 – 14 $A \cdot cm^{-2}$ and a maximum duration of 100 ms without interruption.

5. The circuit arrangement according to any one of claims 1 – 4, wherein the time interval for determining the delivered quantity of charge can be specified as synchronous with the delivery of the charge of the first and/or a second or each further pulse.

6. The circuit arrangement according to any one of claims 1 – 5, wherein a switch-on time (T_2) of the second pulse can be specified by comparing the desired quantity of charge with the actual quantity of charge delivered by the measurement time and ends when the desired quantity of charge is reached.

7. The circuit arrangement according to any one of claims 1 – 6, wherein in order to determine the actual quantity of charge there is provided a measuring cycle of 1 msec, wherein during the time (T_2) the capacitor voltage decreases exponentially and on reaching a specified quantity of charge (Q_2) the power semiconductor can be blocked.

8. The circuit arrangement according to any one of claims 1 – 7, wherein after at least one pre-determined time interval after delivering one first and/or second pulse the flowing current is measured and if this exceeds or falls below a desired value the duration of the pulse can be readjusted in order to keep the delivered quantity of charge constant.

9. The circuit arrangement according to any one of claims 1 – 7, wherein after at least one pre-determined time interval after delivering one first and/or second pulse the flowing current is measured and if this exceeds or falls below a desired value, an error message is given.

10. The circuit arrangement according to any one of claims 1 – 7, wherein after at least one pre-determined time interval after delivering one first and/or second pulse the flowing current is measured and if this exceeds or falls below the desired value, the desired value is readjusted.

11. The circuit arrangement according to any one of claims 1 – 10, wherein the pre-selected setting parameters of the pulse (U_1, T_1, I_2, T_2, K_2) can be input manually or by entering a code.

12. The circuit arrangement according to any one of claims 1 – 11, wherein an overcurrrent cutoff is provided for the first and second pulse.

13. The circuit arrangement according to any one of claims 1 – 12, wherein the resistance R of the cuvette used to calculate U_2 can be measured by a resistance measurement before controlling a power semiconductor.

14. The circuit arrangement according to any one of claims 1 – 12, wherein the resistance R of the cuvette used to calculate U_2 is predetermined.

15. The circuit arrangement according to any one of claims 1 – 14, wherein pre-selected setting parameters of the pulse (U_1, T_1, I_2, T_2, K_2, R) can be read in via a memory card.

16. The circuit arrangement according to any one of claims 1 – 15, wherein a device is provided for measuring and recording a time profile of the voltage applied to the cuvette and/or the current flowing through the cuvette and that the measured time profile of the voltage and/or current can be stored on a memory card.

17. Use of the circuit arrangement according to any one of claims 1 – 16 for the transfection of quiescent or dividing eukaryotic cells.

18. Use of the circuit arrangement according to any one of claims 1 –16 for the transfection of primary cells.

19. Use of the circuit arrangement according to any one of claims 1 –16 for the transfection of human blood cells.

20. Use of the circuit arrangement according to any one of claims 1 – 16 for the transfection of pluripotent precursor cells of human blood.

21. Use of the circuit arrangement according to any one of claims 1 – 16 for the transfection of primary human fibroblasts, endothelial cells, muscle cells or melanocytes.

22. A method for introducing nucleic acids, peptides, proteins, polysaccharides, lipids, derivatives thereof and/or combinations thereof into the cell nucleus of eukaryotic cells by means of electric current or for the *in vitro* treatment of cells, cell derivatives, subcellular particles and/or vesicles with electric current for fusion of the cells, cell derivatives, subcellular particles and/or vesicles, said method comprising:

    supplying at least one voltage pulse to the cells,

    measuring the quantity of charge supplied by the voltage pulse in at least one selectable time interval, wherein a preset desired quantity of charge is compared with the actually supplied quantity of charge, and

    terminating the voltage pulse on reaching or exceeding the desired quantity of charge.

23. The method of claim 22, wherein the supplied quantity of charge is determined from the difference between the original charge of a corresponding storage device and the residual charge of this storage device.

24. The method of claim 22, wherein a first pulse with a capacitor voltage (U_1) is supplied to the cells and subsequently without interruption at least one second pulse with a capacitor voltage (U_2) is also supplied to the cells.

25. The method of claim 24, wherein the first pulse having a field strength of 2 – 10 kV/cm, a duration of 10 – 100 µs and a current density of at least 2 $A \cdot cm^{-2}$ is applied to the cells, and subsequently without interruption the second pulse having a current density of 2 – 14 $A \cdot cm^{-2}$ and a maximum duration of 100 ms is also supplied to the cells.

26. The method of claim 22, wherein the time interval for determining the supplied charge is specified simultaneously with the supply of the charge of a first and/or a second or each further pulse.

27. The method of claim 24, wherein a switch-on time (T_2) of the second pulse is specified by comparing the desired quantity of charge with the actual quantity of charge supplied by the measurement time and terminated when the desired quantity of charge is reached.

28. The method of claim 22, wherein in order to determine the actual quantity of charge a measuring cycle of 1 msec is selected, wherein during a time (T_2) the capacitor voltage decreases exponentially and the pulse is terminated on reaching a specified quantity of charge (Q_2).

29. The method of claim 22, wherein after at least one pre-determined time interval after triggering one first and/or second pulse the flowing current is measured and, if said current exceeds or falls below a desired value, the duration of the pulse is readjusted in order to keep the supplied quantity of charge constant.

30. The method of claim 22, wherein after at least one pre-determined time interval after triggering one first and/or second pulse the flowing current is measured and, if said current exceeds or falls below a desired value, an error message is given.

31. The method of claim 22, wherein after at least one pre-determined time interval after triggering one first and/or second pulse the flowing current is measured and, if said current exceeds or falls below the desired value, the desired value is readjusted.

32. The method of claim 22, wherein a pre-selected setting parameter of the pulse (I_2, T_2, K_2) is inputted manually or by entering a code, wherein I_2 is the desired current, T_2 is the switch-on time of the voltage pulse, and K_2 is a correction value which takes into account properties of a cuvette.

33. The method of claim 24, wherein an overcurrent cutoff for the first and second pulse is accomplished by interrupting the pulse at any time in the event that preset limiting values are exceeded.

34. The method of claim 24, wherein a resistance R of a cuvette used to calculate U_2 is determined by a resistance measurement before triggering a power semiconductor.

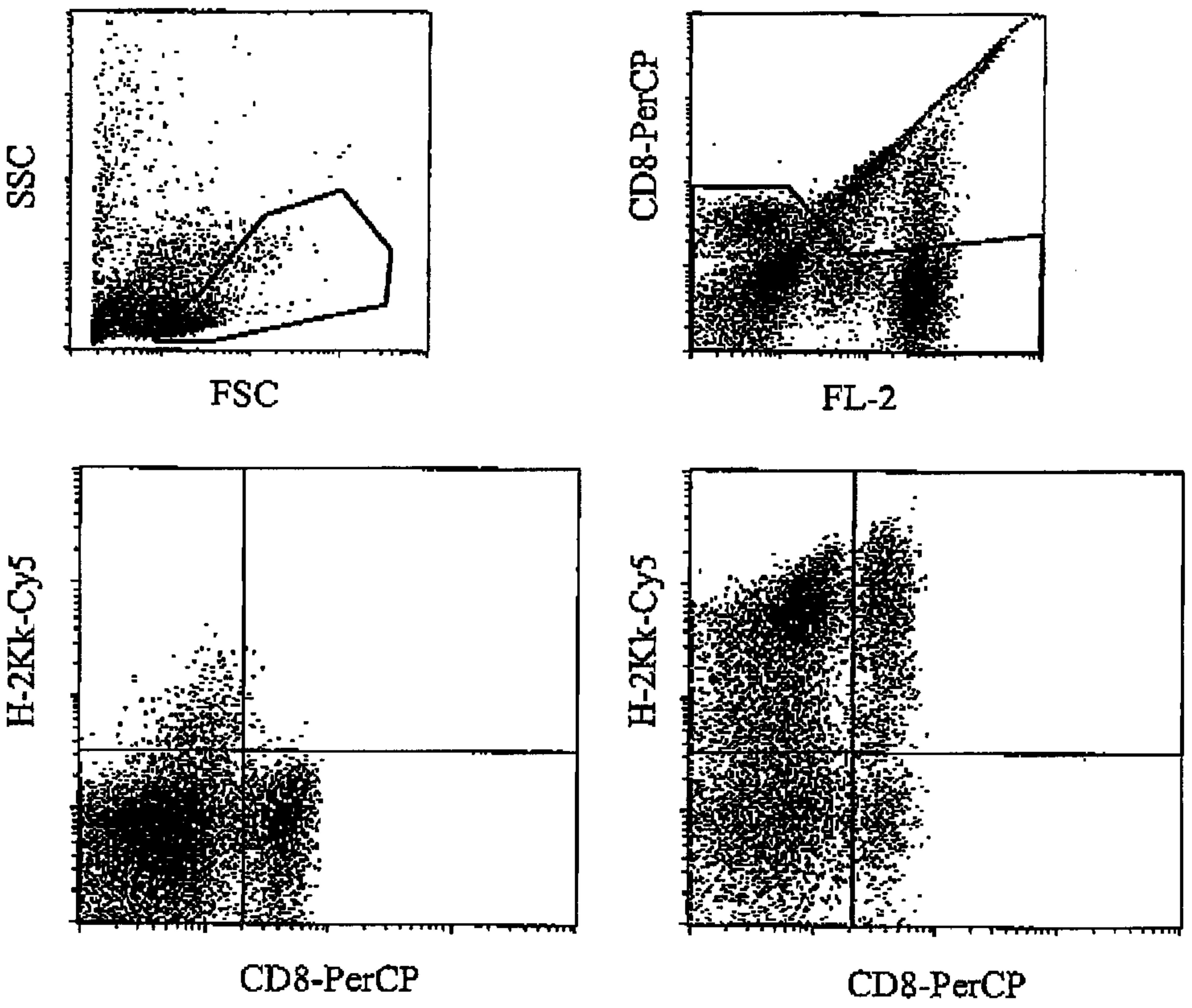
35. The method of claim 24, wherein a resistance R of a cuvette used to calculate U_2 is predetermined.

36. The method of claim 24, wherein a pre-selected setting parameter of the pulse (U_1, T_1, I_2, T_2, K_2, R) is read in via a memory card, wherein T_1 is the switch-on time of the first pulse, I_2 is the desired current, T_2 is the switch-on time of the second pulse, K_2 is a correction value which takes into account properties of a cuvette and R is a resistance of the cuvette.

Fig. 1

CD8-positive cytotoxic T-cells from human blood

(74,3 % H-2Kk-positive)

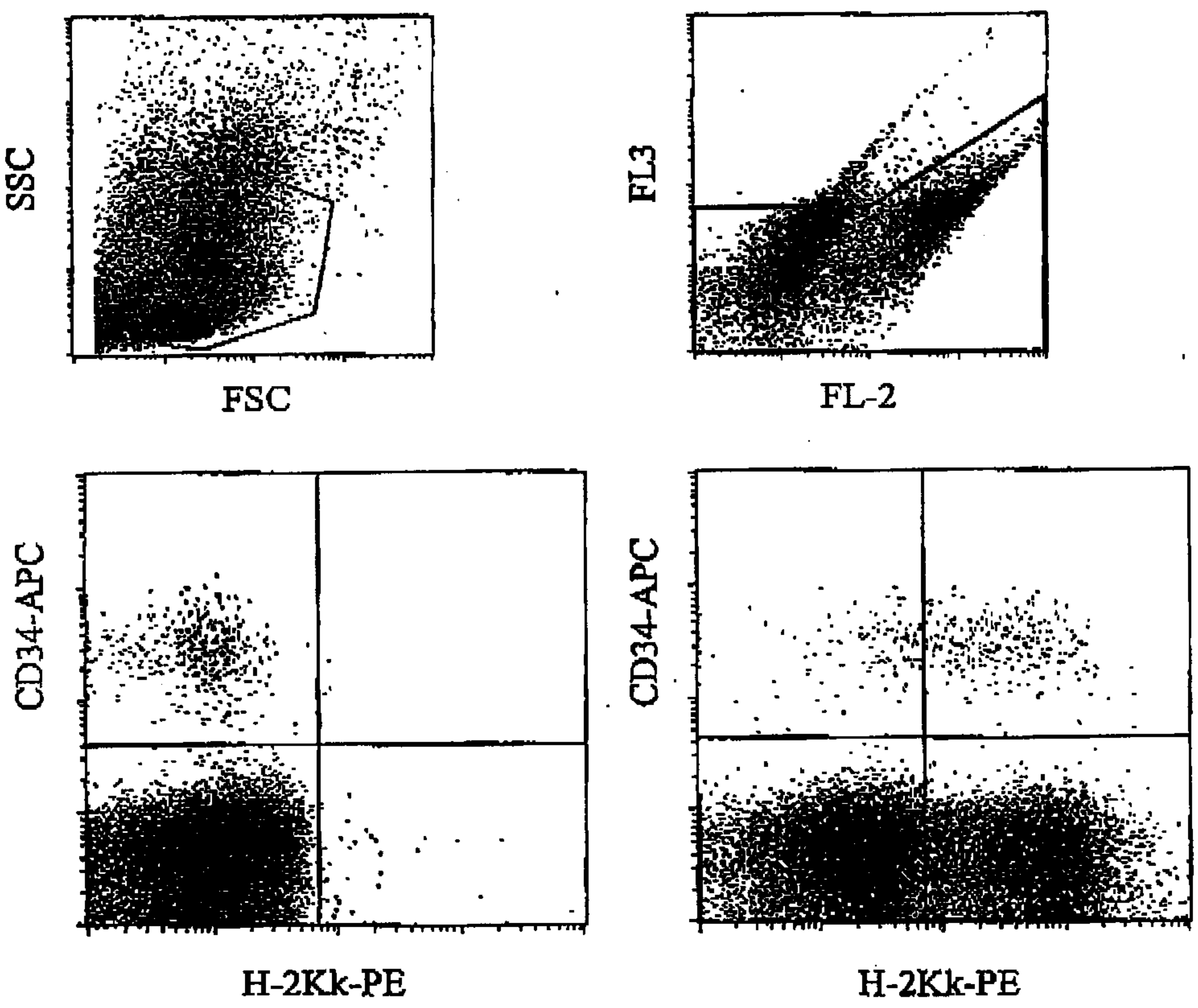


negative control **transfected**

Fig. 2

CD34-positive cells from human blood

(66,7 % H-2Kk-positive)



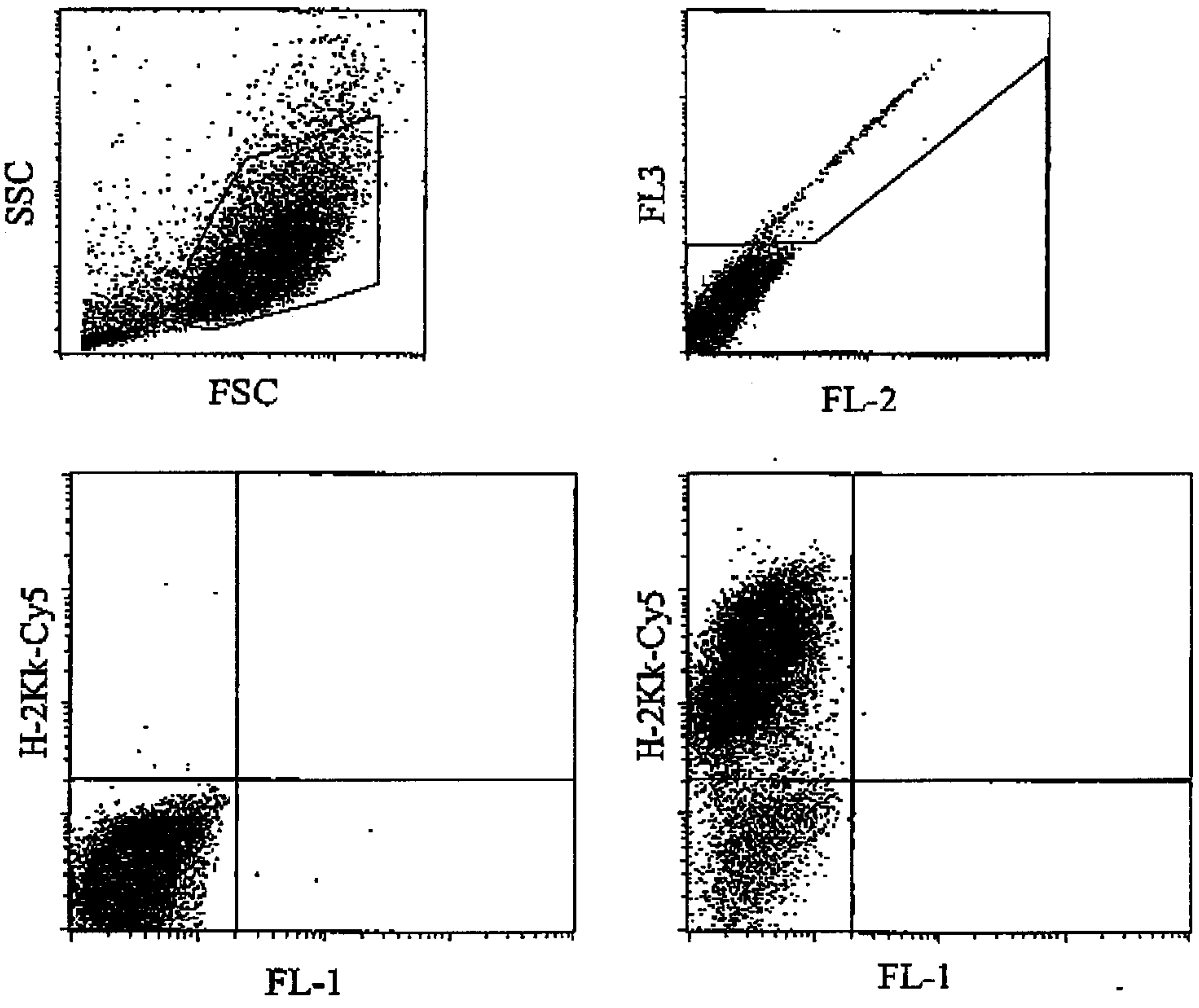
negative control

transfected

Fig. 3

Human neonatal dermal fibroblasts

(93 % H-2Kk-positive)



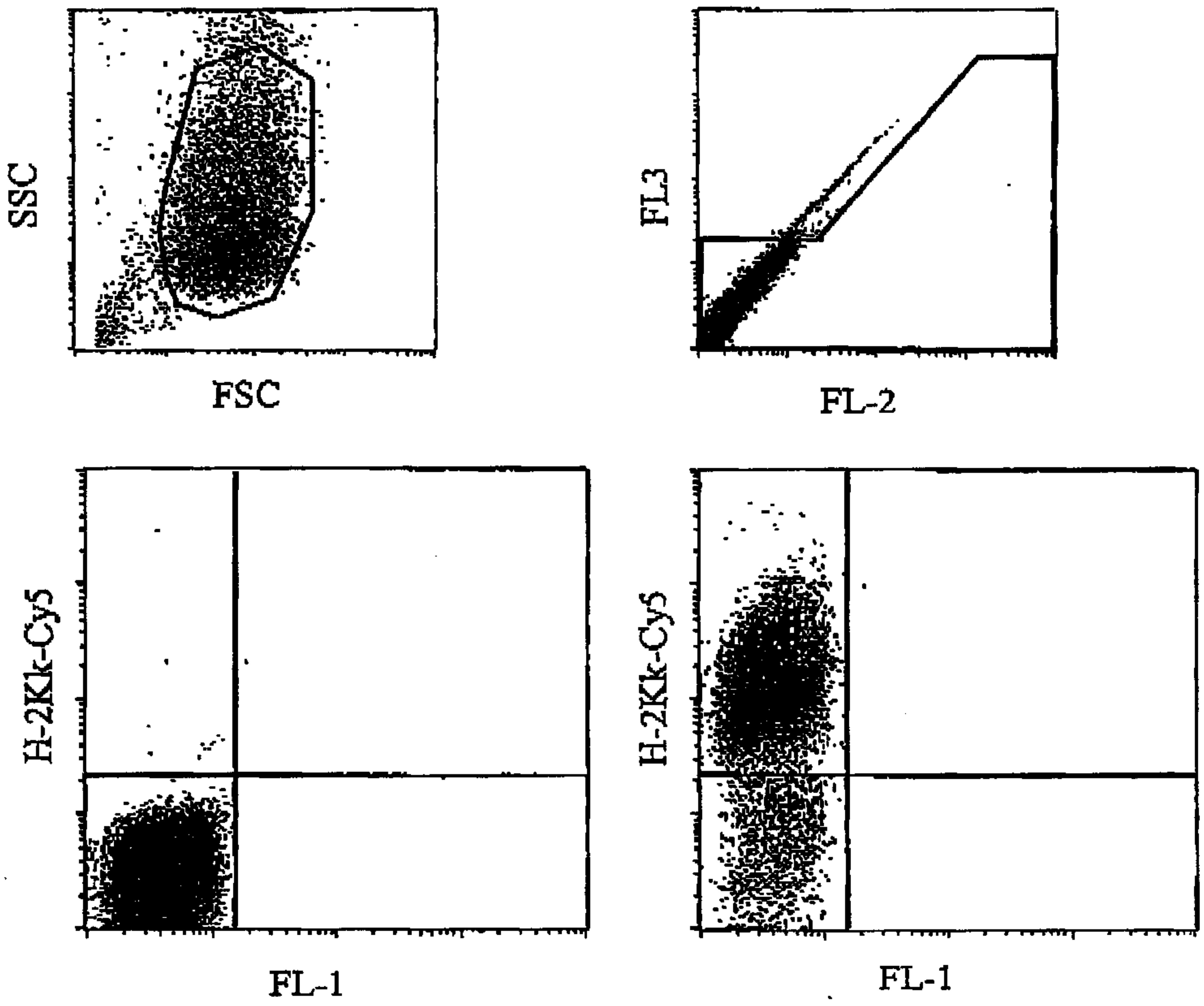
negative control

transfected

Fig. 4

Human neonatal dermal melanocytes

(75,1 % H-2Kk-positive)

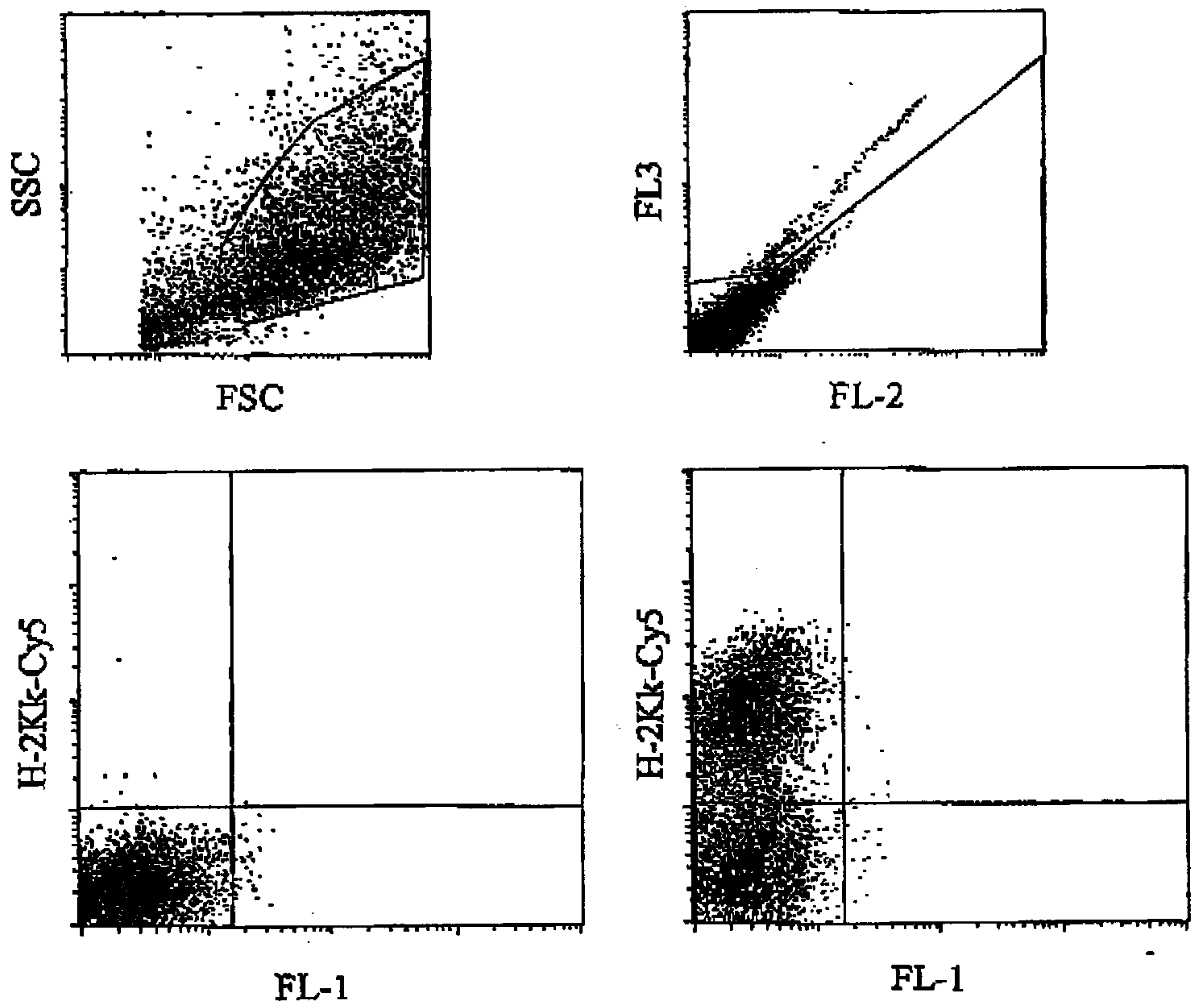


negative control **transfected**

Fig. 5

Primary endothelial cells from human umbilical cord

(49,7 % H-2Kk-positive)

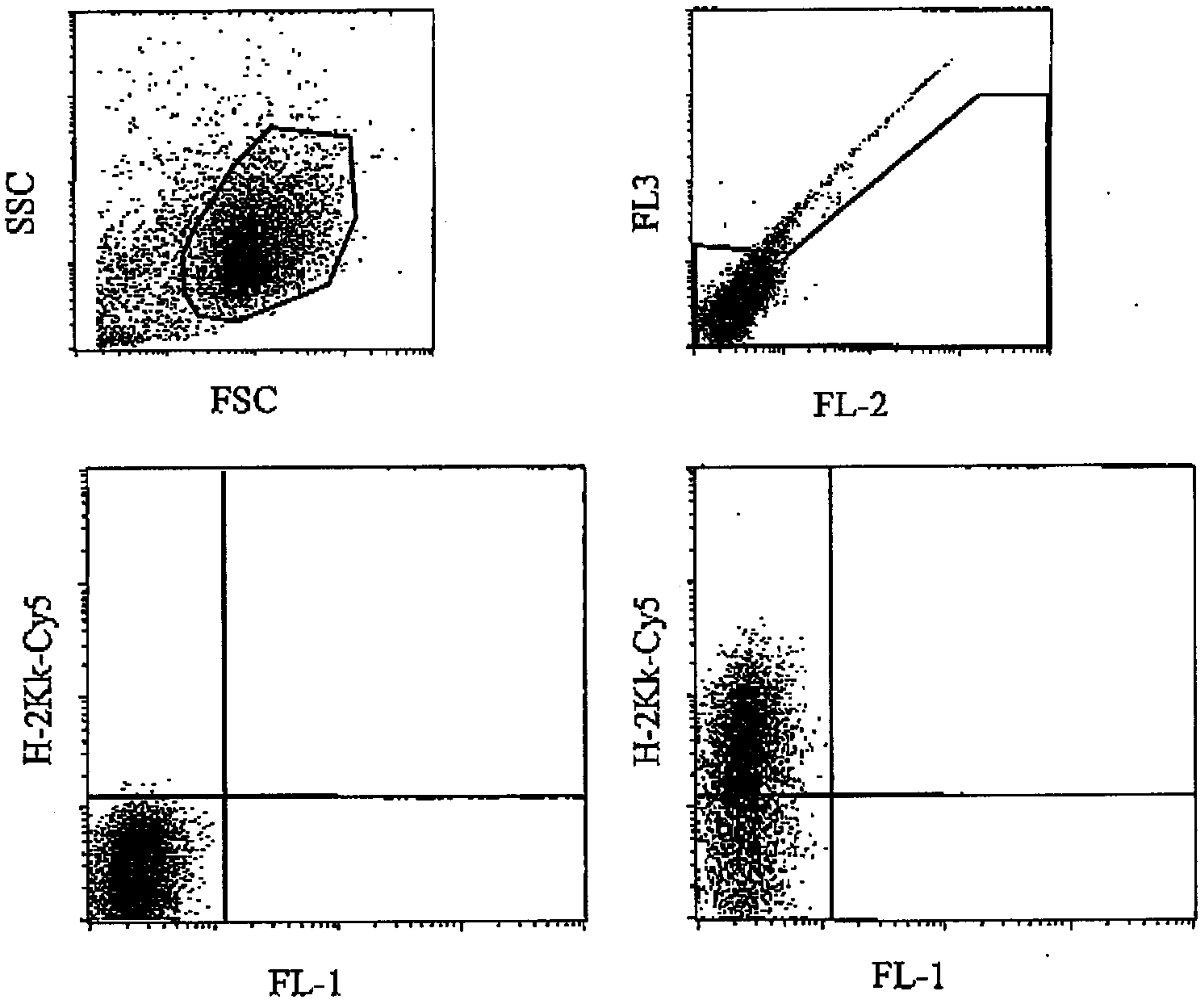


negative control **transfected**

Fig. 6

cell line K562

(69,5 % transfected)



negative control

transfected

Fig. 7

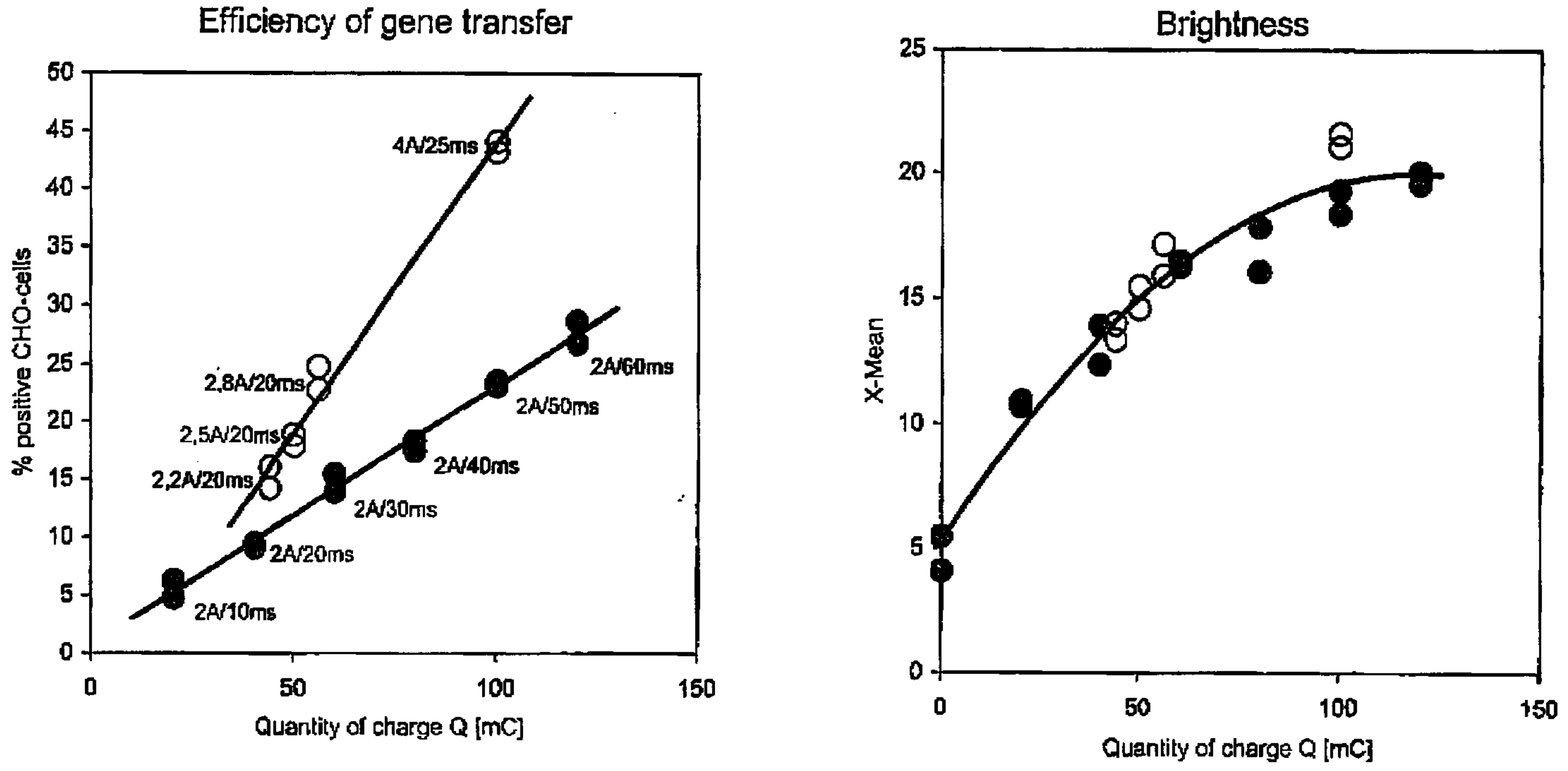

Efficiency of gene transfer
% positive CHO-cells
0
5
10
15
20
25
30
35
40
45
50
0
50
100
150
Quantity of charge Q [mC]
4A/25ms
2,8A/20ms
2,5A/20ms
2,2A/20ms
2A/60ms
2A/50ms
2A/40ms
2A/30ms
2A/20ms
2A/10ms
Brightness
X-Mean
0
5
10
15
20
25
0
50
100
150
Quantity of charge Q [mC]

Fig. 8

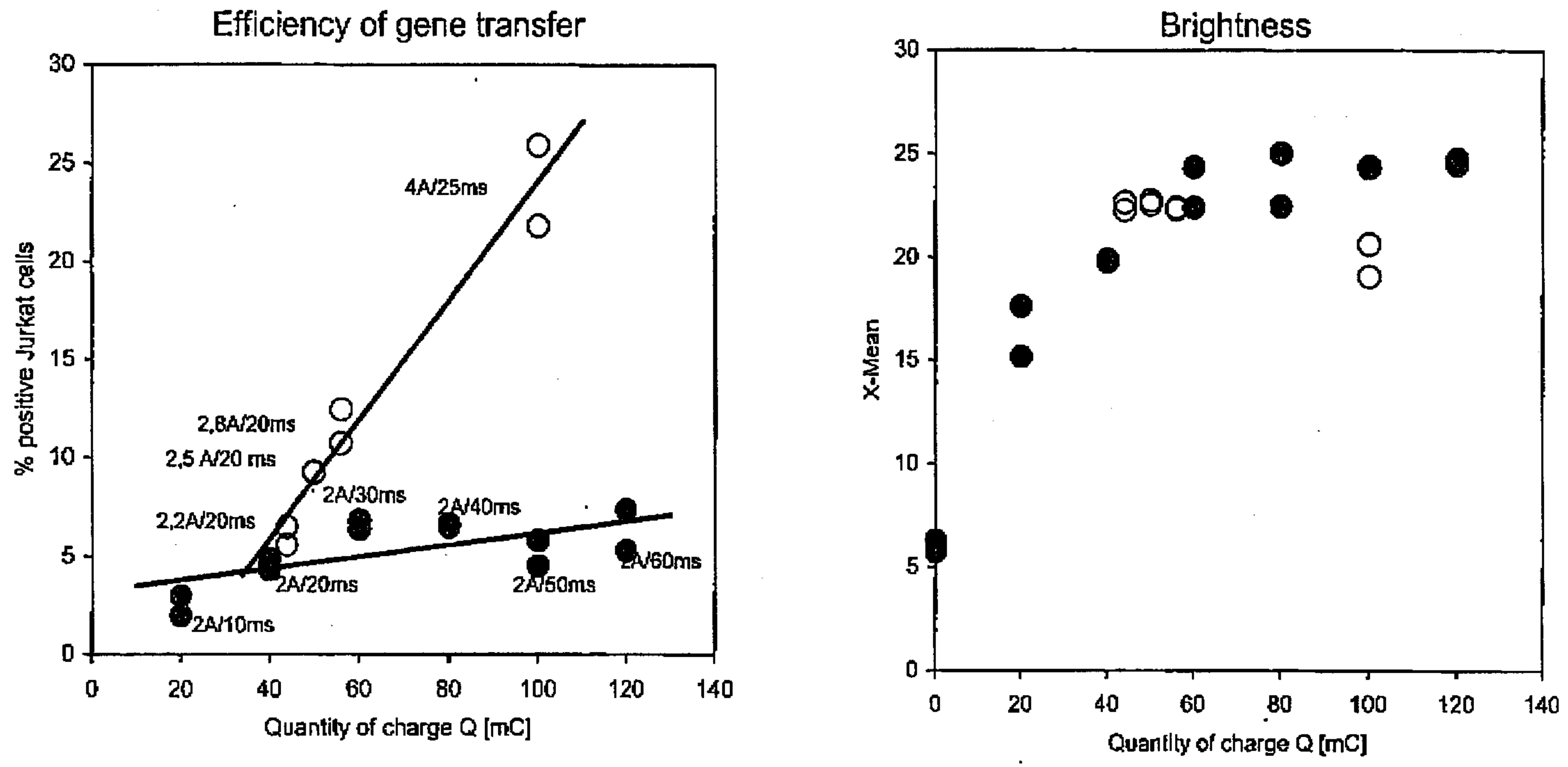

Efficiency of gene transfer
% positive Jurkat cells
0
5
10
15
20
25
30
0
20
40
60
80
100
120
140
Quantity of charge Q [mC]
4A/25ms
2,8A/20ms
2,5 A/20 ms
2,2A/20ms
2A/30ms
2A/40ms
2A/20ms
2A/50ms
2A/60ms
2A/10ms
Brightness
X-Mean
0
5
10
15
20
25
30
0
20
40
60
80
100
120
140
Quantity of charge Q [mC]

Fig. 9

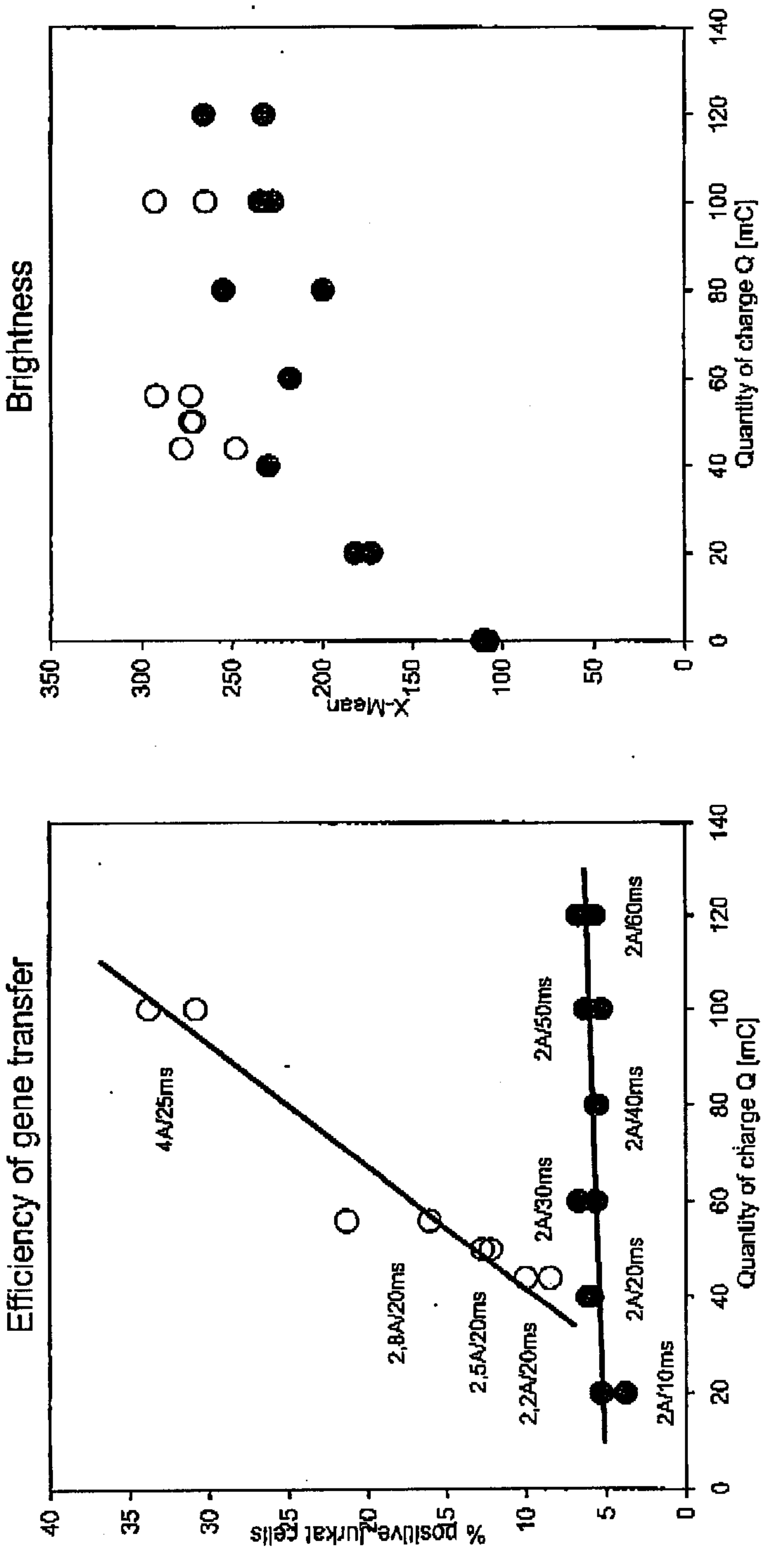

Efficiency of gene transfer
% positive Jurkat cells
0
5
10
15
20
25
30
35
40
Quantity of charge Q [mC]
0
20
40
60
80
100
120
140
4A/25ms
2,8A/20ms
2,5A/20ms
2,2A/20ms
2A/10ms
2A/20ms
2A/30ms
2A/40ms
2A/50ms
2A/60ms
Brightness
X-Mean
0
50
100
150
200
250
300
350
Quantity of charge Q [mC]
0
20
40
60
80
100
120
140

Fig. 10

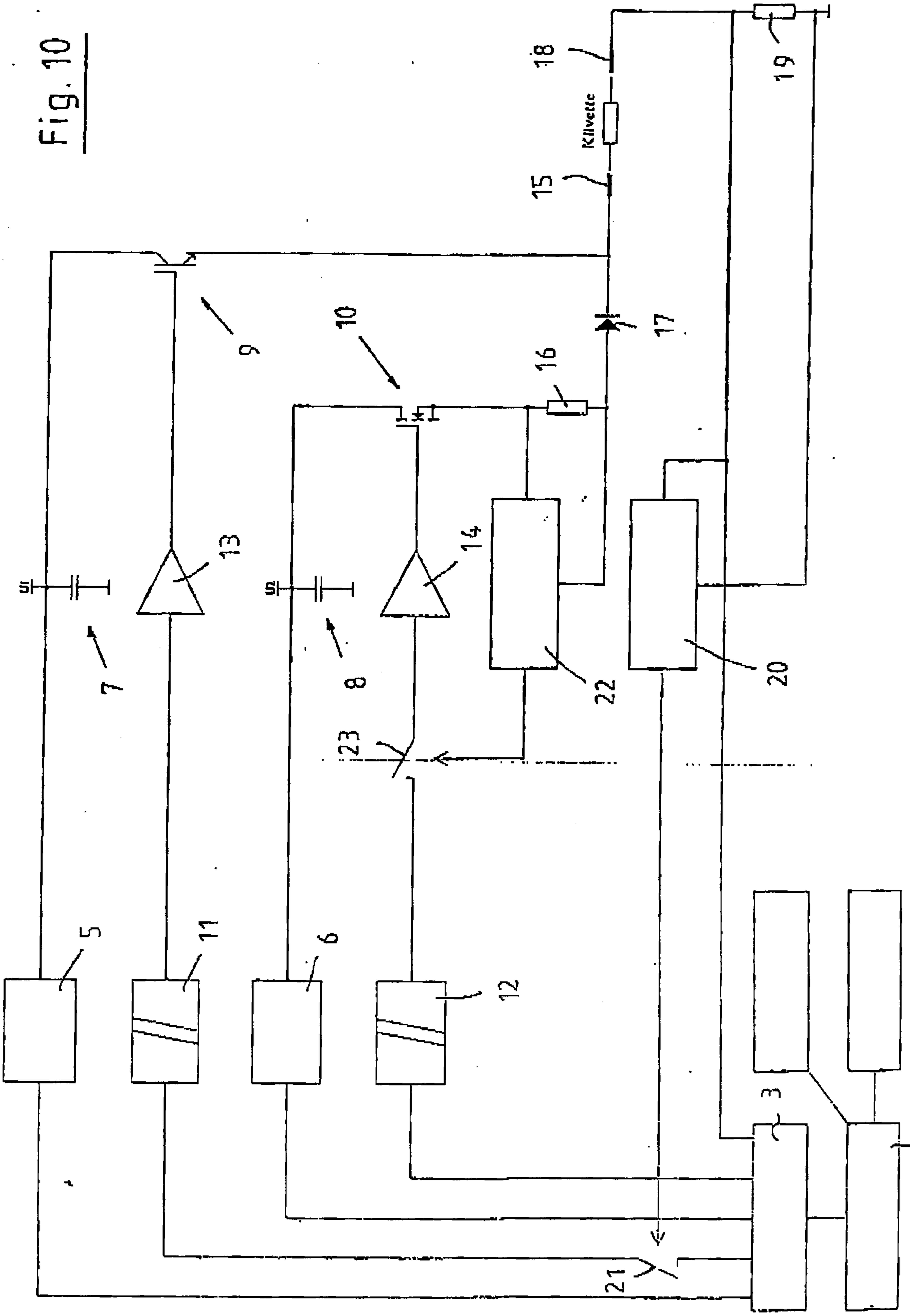
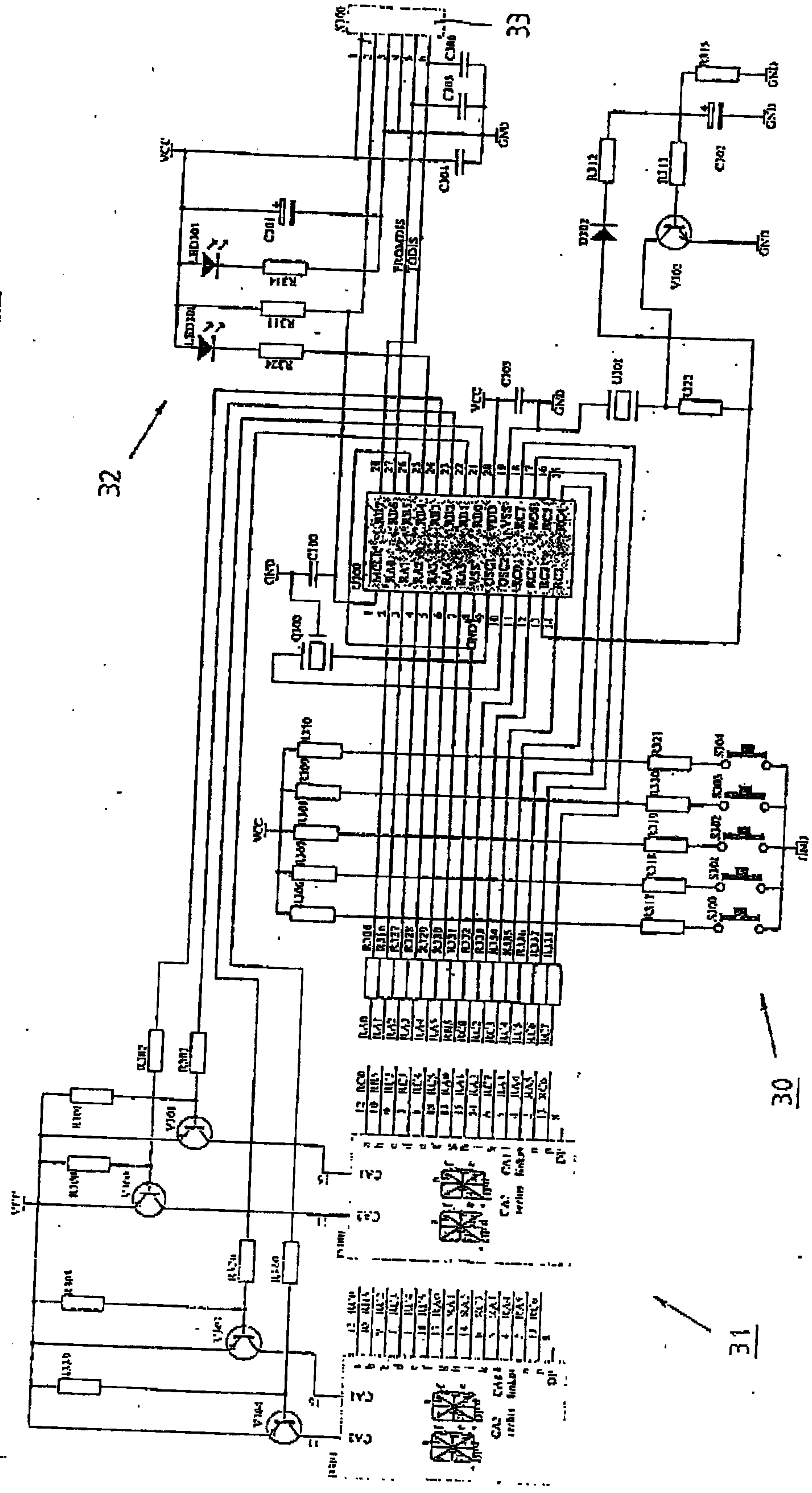


Fig.11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32
30
31
VCC
GND
FROMDIS
TODIS
LED301
LED302
C301
C302
C303
C304
C305
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R311
R314
R324
R312
R313
R315
R322
D302
VJ02
U300
U302
Q300
C300
J300
S300
S301
S302
S303
S304

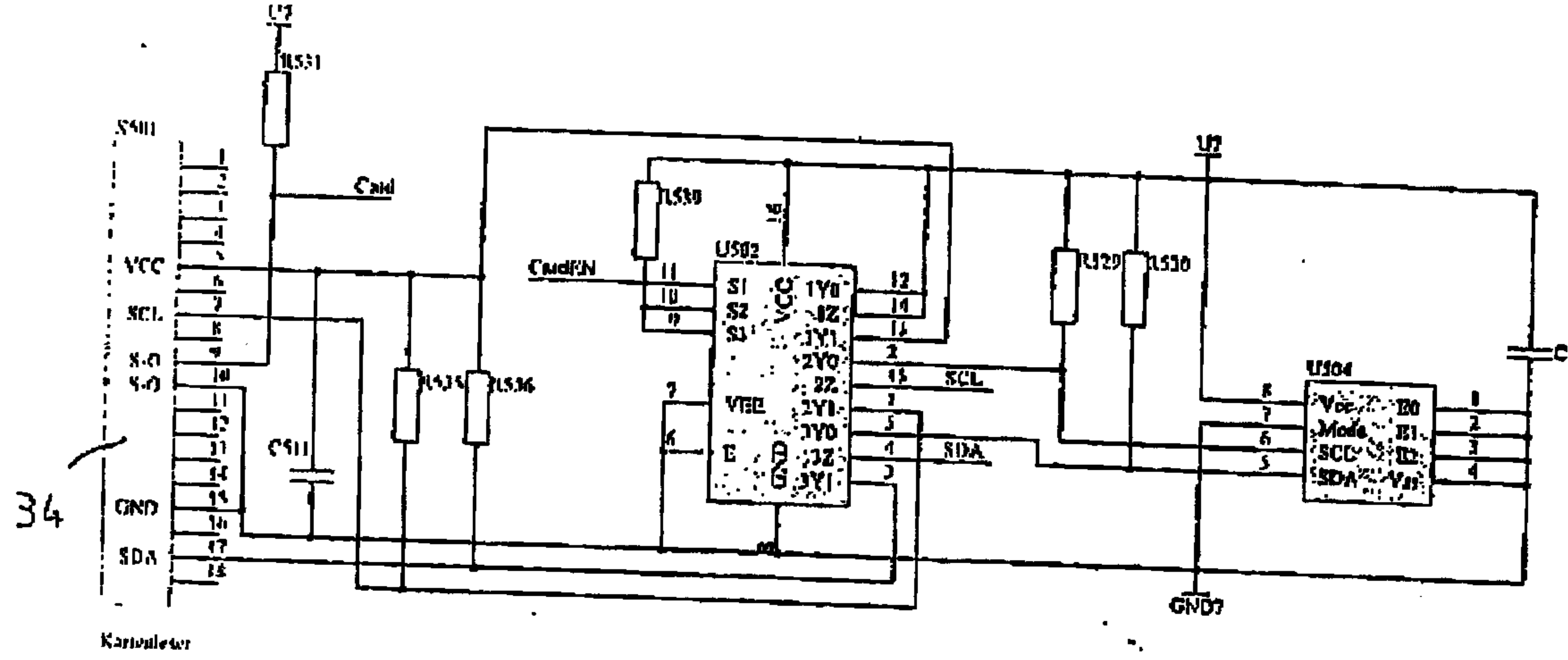

U2
R531
Card
S501
VCC
SCL
S-O
S-O
GND
SDA
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
34
Kartenleser
C511
R535
R536
CardEN
R530
U502
S1
S2
S3
VCC
VEE
E
GND
1Y0
1Z
1Y1
2Y0
2Z
2Y1
3Y0
3Z
3Y1
SCL
SDA
U2
R529
R530
U504
Vcc
Mode
SCL
SDA
E0
E1
E2
Vss
C508
GND7

Fig. 12

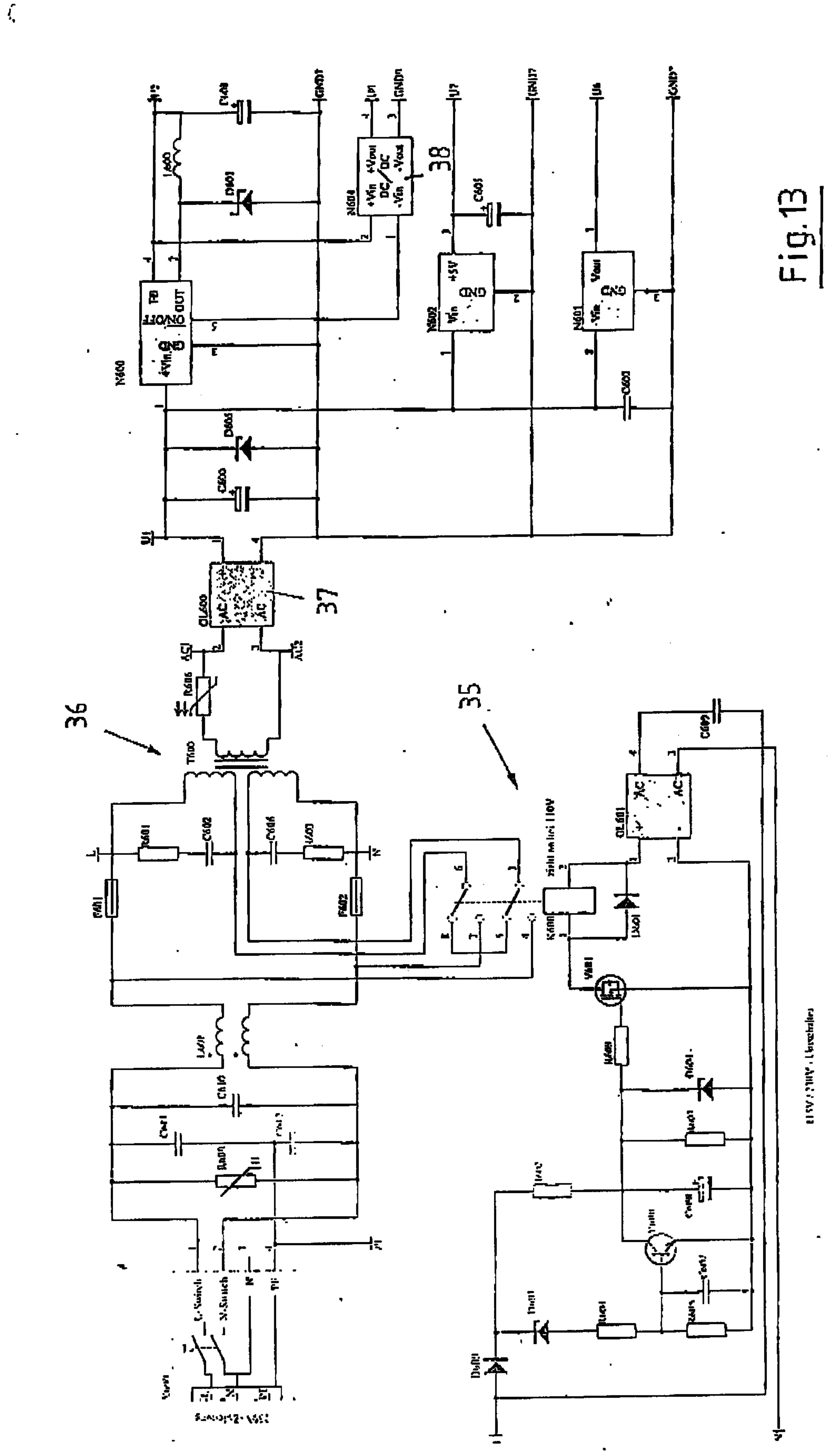

36
35
37
38
N600
+Vin
GND
ON/OFF
OUT
FB
L600
D603
N604
+Vin
-Vin
+Vout
-Vout
DC/DC
N602
Vin
+5V
GND
N601
Vin
Vout
GND
C605
C603
C600
D605
U1
GND1
OL600
AC
T600
F601
F602
OL601
K600
V601
C609

Fig.13

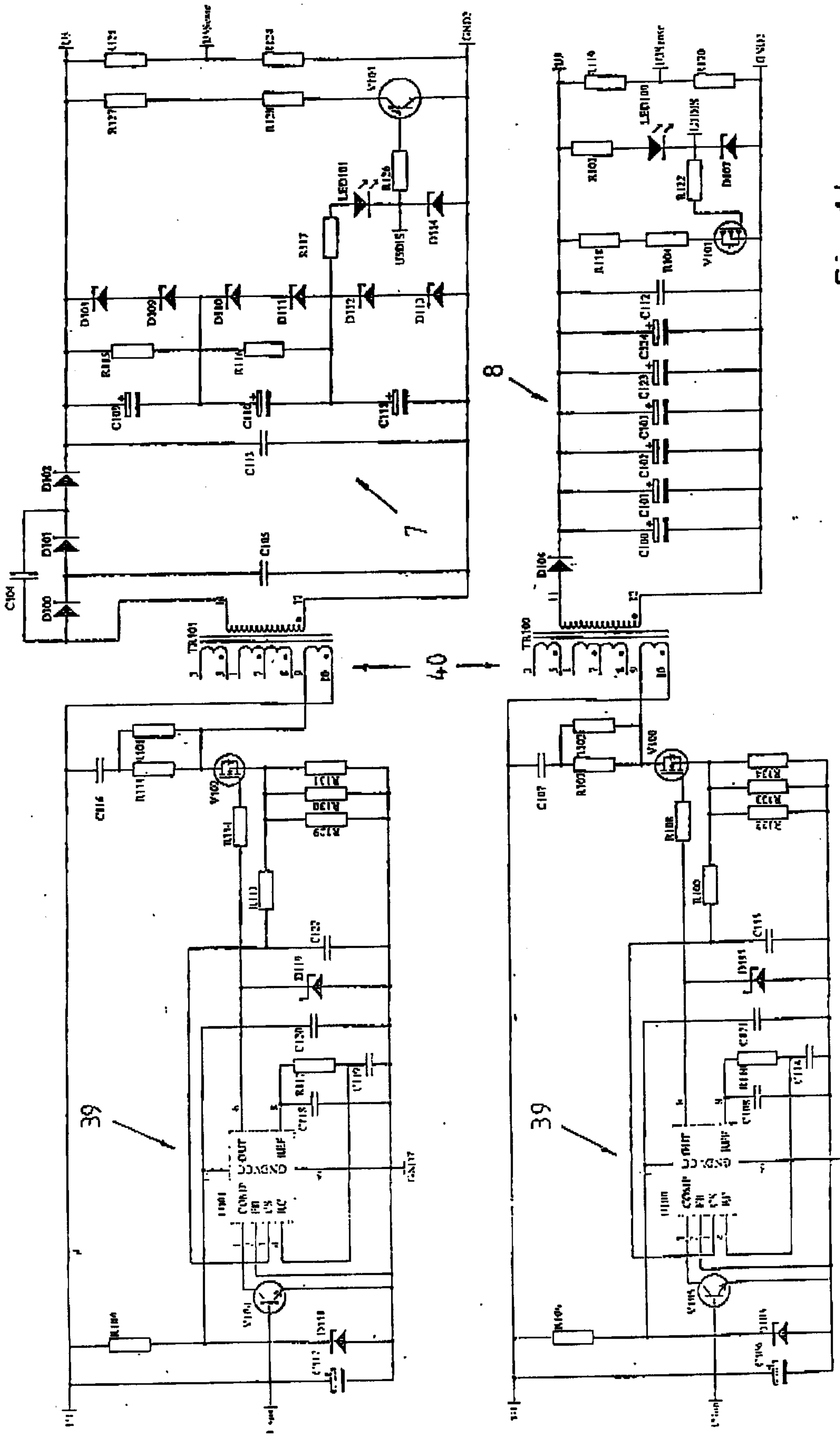

7
8
39
39
40
TR101
TR100
D100
D101
D102
D104
D106
C104
C105
C115
C100
C101
C102
C103
C123
C124
C112
R115
R116
R117
R118
R122
R126
R127
R128
R104
LED101
LED100
V101
V100
V102
D112
D113
D114
GND2
OUT
REF
GND
COMP
FB
CS
RC

Fig.14

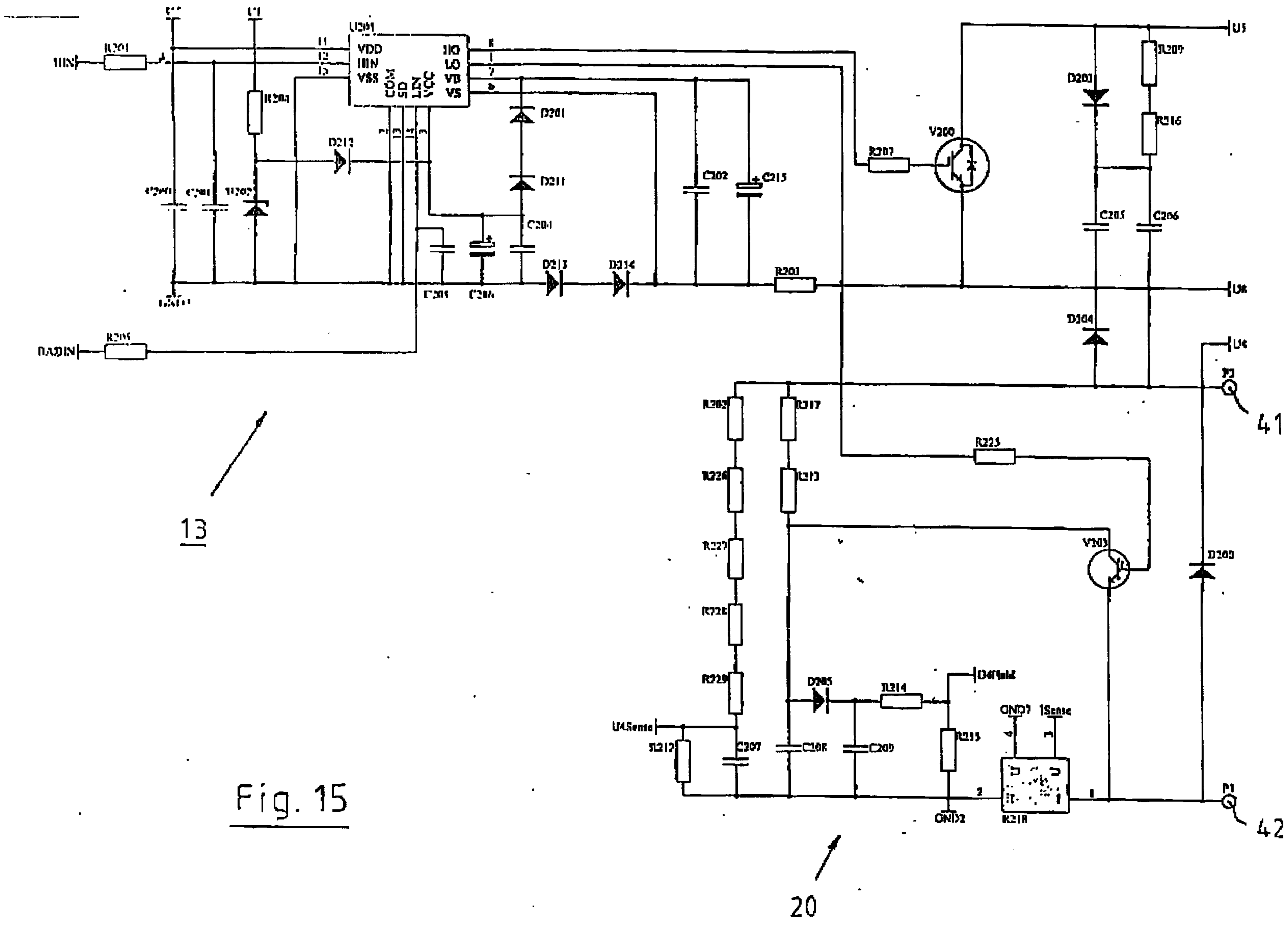

IIN
R201
U201
VDD
IIN
VSS
COM
SD
LIN
VCC
HO
LO
VB
VS
R204
D212
C201
D201
D211
C204
C203
C206
D213
D314
C202
C215
R201
R205
DADIN
R207
V200
D203
R209
R216
C205
C206
U3
U6
D304
U4
41
42
R202
R226
R227
R228
R229
R317
R213
R225
V203
D300
D205
R214
U4Feld
R215
C307
C308
C209
U4Sense
R212
GND2
ISense
R218
13
20

Fig. 15

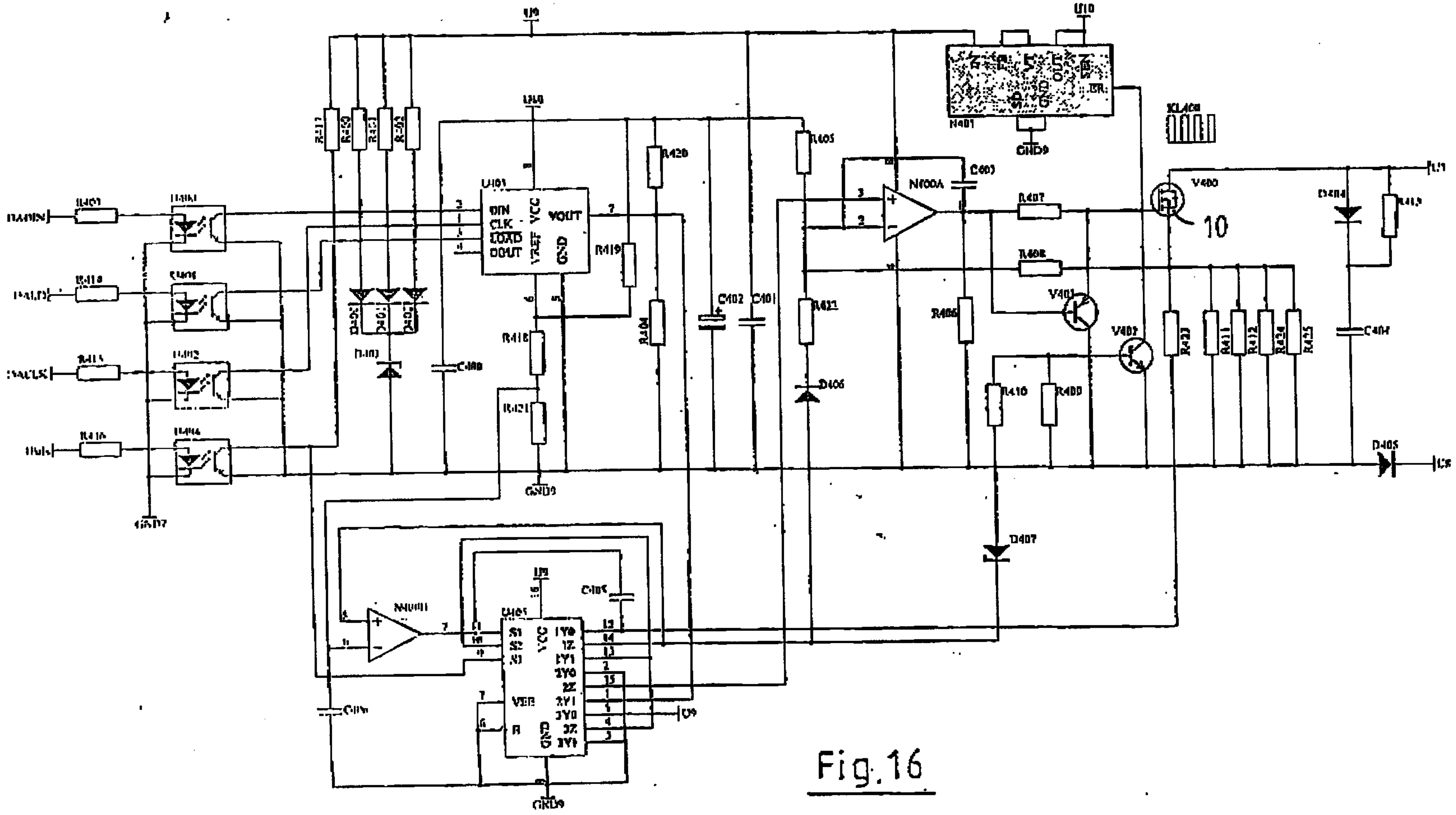

U9
U10
N401
GND9
KL400
U1
V400
10
D404
R413
C404
N100A
C403
R407
R408
V401
V402
R406
R410
R400
R422
R411
R412
R424
R425
D405
D407
R405
R421
D406
C401
C402
R420
R404
R419
U103
DIN
CLK
LOAD
DOUT
VCC
VREF
GND
VOUT
R418
GND9
C400
R401
R409
R415
R416
GND7
N400B
U405
S1
S2
S3
VCC
VEE
1Y0
1Z
1Y1
2Y0
2Z
2Y1
3Y0
3Z
3Y1
C405
C406
GND9

Fig.16

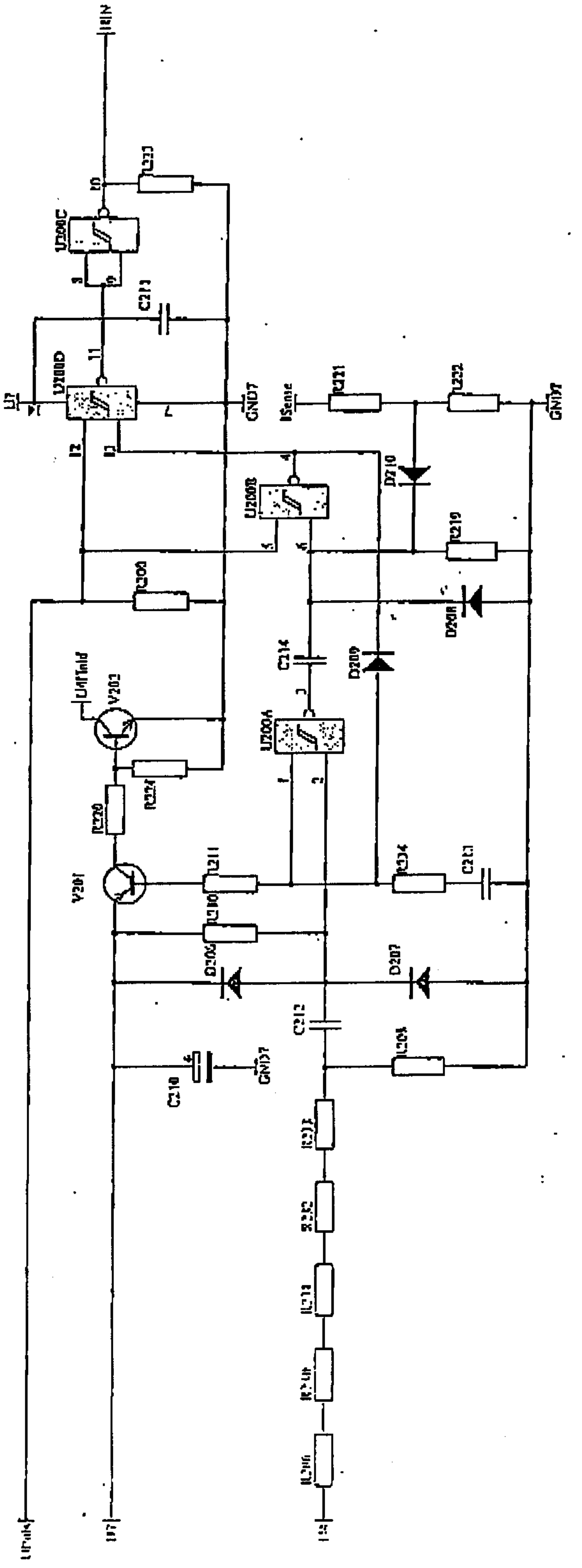

U200C
U200D
U200B
U200A
C211
C214
C212
C210
C213
R233
R221
R222
R219
R200
R220
R224
R211
R234
R204
D210
D208
D209
D206
D207
V201
V202
GND7
ISense
43

Fig. 17

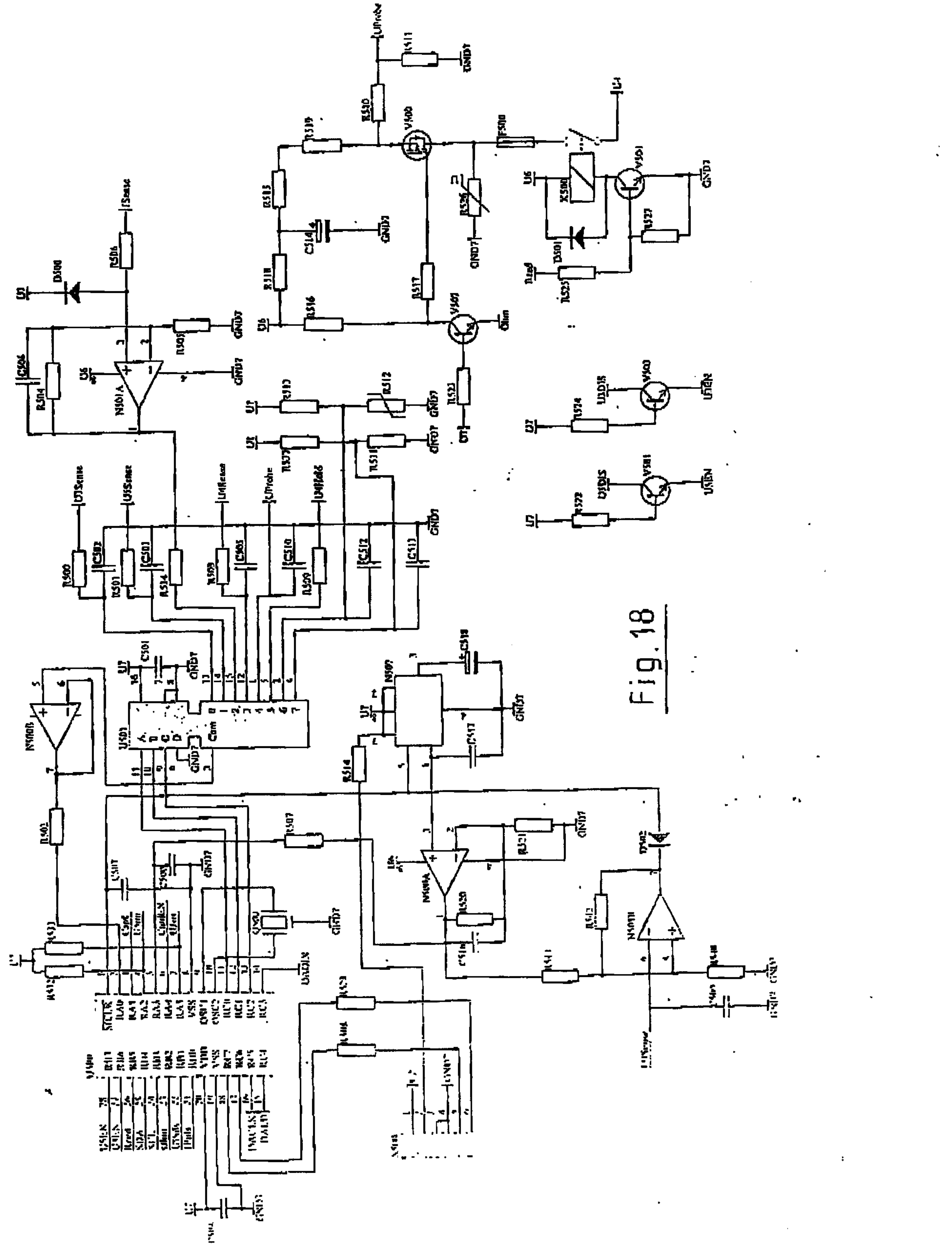

V500
V501
V502
V503
N501A
U502
K500
F500
D500
D501
R510
R511
C514
C512
GND7

Fig.18

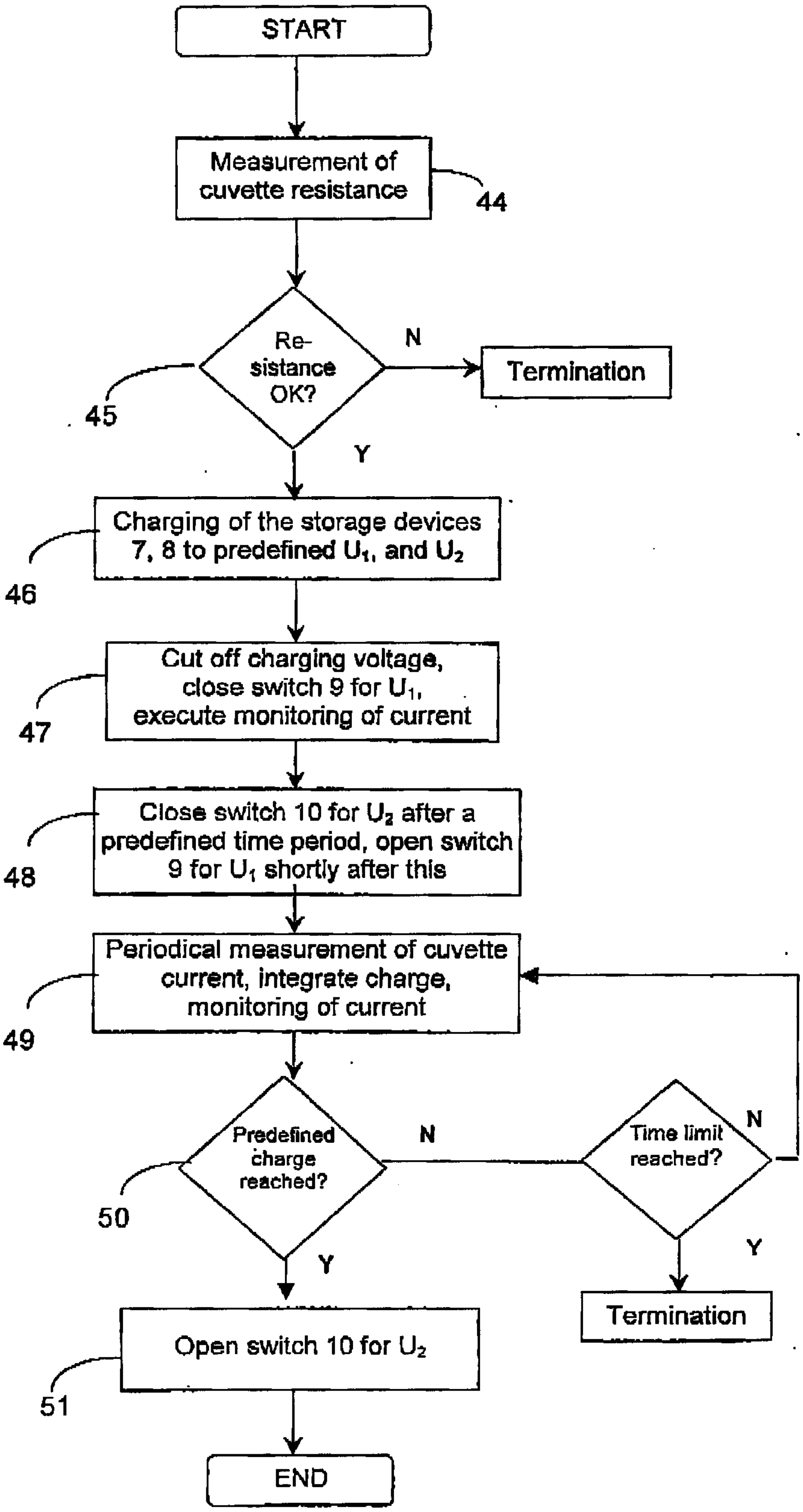
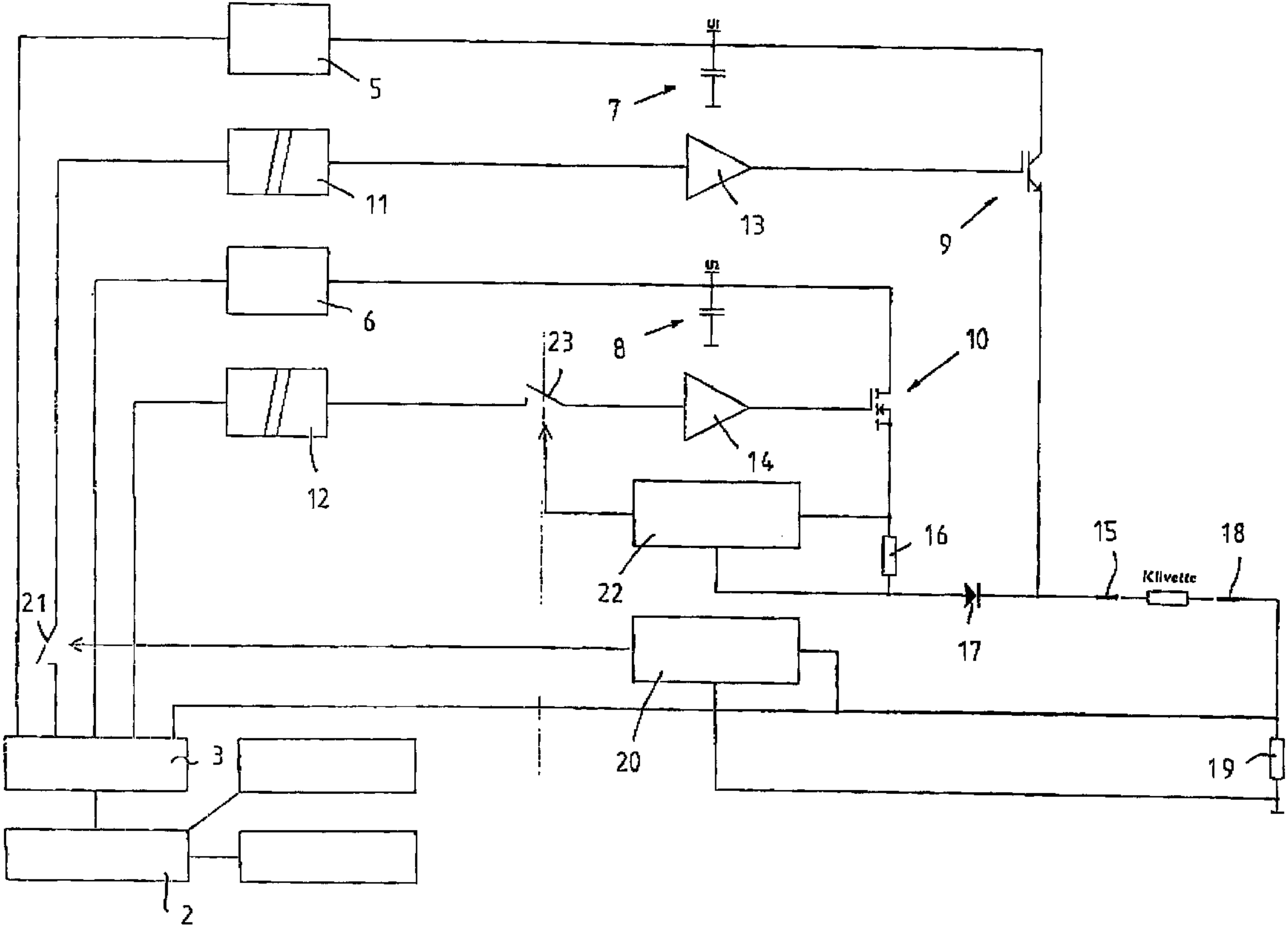

START
Measurement of cuvette resistance
44
Re-sistance OK?
45
N
Termination
Y
Charging of the storage devices 7, 8 to predefined U₁, and U₂
46
Cut off charging voltage, close switch 9 for U₁, execute monitoring of current
47
Close switch 10 for U₂ after a predefined time period, open switch 9 for U₁ shortly after this
48
Periodical measurement of cuvette current, integrate charge, monitoring of current
49
Predefined charge reached?
50
N
Time limit reached?
N
Y
Termination
Y
Open switch 10 for U₂
51
END

Fig. 19


U1
5
7
11
13
9
U2
6
8
23
10
12
14
22
16
15
Küvette
18
17
21
20
3
19
2