Homokaryons from Animal and Plant Cells Generated by Electrofusion

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Abstract. A new apparatus was constructed which enables the use of the electrofusion method to obtain polynuclear cells of various mammalian cell lines, erythrocytes and plant protoplasts. This technique was applied to both suspensions and monolayers. Electrical and other physical parameters were monitored to find optimal conditions for mutual contact of cells (dielectrophoresis) and subsequent fusion. In the suspension technique, dielectrophoresis of mouse erythrocytes occurred at a field frequency of 20 kHz and a strength of 500 $V.cm^{-1}$, whereas cultured mammalian cells and plant protoplasts required a frequency of 1 — 1.4 MHz and a strength of 250—800 $V.cm^{-1}$. Fusion of cells was induced after the application of 1 to 10 high-voltage pulses of 1—5 $kV.cm^{-1}$, 10—36 μs duration. After these high-voltage pulses were applied to the monolayer of mouse L cells, about 12 % viable homokaryons were obtained.

Key words: Electrofusion — Permanent cell lines — Nicotiana protoplasts — Homokaryons

Introduction

Recently cell hybridization has become a method frequently used in various biological laboratories. The technique of fusion of complete cells or cell fragments (e. g. minicells, cytoplasts) permits production of hybrid cells, especially hybridomas, cybrids and reconstituted cells, which are of great importance not only for the understanding of fundamental questions of gene expression in cells, but also for practical applications in medicine and agriculture (see Ringertz and Savage 1976; Köhler and Milstein 1975; Zimmermann et al. 1984). The most frequently used fusion inducers are chemical agents (polyethylene glycol, lysolecithin) or virus particles. Over the last years a new fusion technique based upon the application of very brief electrical pulses to cells in contact was developed

(Zimmermann et al. 1985). This procedure has the following advantages: easy microscopic control of the fusion process, exactly defined fusion conditions, and fusion efficiency exceeding that of chemical or viral induction.

Electrofusion in suspension consists of two steps: (i) dielectrophoresis, when in an alternating field chains of cells in intimate contact are formed ("pearl chains"); (ii) controlled electrical breakdown of the adjacent membranes as a result of high-voltage direct current pulse (Zimmermann 1982; Zimmermann et al. 1985).

It is also possible to induce electrical breakdown of the membranes in monolayer cultured cells without prior dielectrophoresis (Teissié et al. 1982; Finaz et al. 1984; Orgambide et al. 1985). Some authors enhance the fusion efficiency using proteolytic enzymes (pronase or dispase), which are presumed to protect cells against field pulses of high intensity (Zimmermann 1982; Zimmermann et al. 1982a).

In the present paper we describe electrofusion experiments performed with the aid of an apparatus constructed in our institute. The simple power supply proved suitable for dielectrophoresis and fusion of suspensions of both mammalian and plant cells. The viability of fusion products was not influenced. In the monolayer technique about 12 % of polykaryons were scored after 24 h cultivation.

Materials and Methods

Cell types. Mouse erythrocytes were obtained from peripheral blood of C 57 Bl strain mice, washed with phosphate balanced saline solution (PBS) and sodium citrate (0.01 mol . l^{-1}). Mouse lymphosarcoma cells LS/BL were propagated in vivo in the peritoneal cavity of females of host mice. After removal, the cells were washed in PBS. Permanent cell lines of mouse L libroblasts, Chinese hamster V 79 cells and human HeLa cells were cultivated in vitro in minimal Eagle's medium (MEM) supplemented with 10 % fetal calf serum (FCS). Cells were trypsinized and washed in PBS. Protoplasts of *Nicotiana plumbaginifolia* were isolated from mesophyl leaf tissue by enzymatic digestion and resuspended in 0.4 mol . l^{-1} mannitol (Saunders and Gillespie 1984).

In the case of fusion in monolayer the L cells were plated in plastic culture dishes containing MEM supplemented with 10 % FCS. The 24-hour-culture was washed with PBS before use.

Fusion protocol. Fusion of cells in suspensions. The suspensions of animal cells were resuspended in 1 mmol . l^{-1} phosphate buffer (pH 7.4) containing saccharose (250 mmol . l^{-1}) and 1 mmol . l^{-1} $MgCl_2$ (slightly modified from Finaz et al. 1984). In some experiments Pronase P (Serva, 1 mg . ml^{-1}) was added and the cells were preincubated for 20—40 minutes. Immediately before fusion the cells were transferred to saccharose without pronase (250 mmol . l^{-1}), adjusted to a density of 10^4 cells per ml, and pipetted in a plastic fusion chamber with Pt electrodes (wires 200 μm in diameter, gap width 150 μm). *Nicotiana* protoplasts were transferred from mannitol solution to saccharose (300 mmol . l^{-1}) and placed in a fusion chamber. The alignment of cells by

dielectrophoresis was induced after application of non-uniform alternating (AC) field. The process of fusion was immediately induced using one or more high voltage current (DC) pulses. The field strength and pulse duration were monitored on a Philips PM 3266 model memory scope.

Fusion in monolayers. Similarly to the method of fusion in suspensions, cells in dishes were washed in phosphate-saccharose-$MgCl_2$ buffer (pH 7.4) and then overlaied by 250 mmol.l^{-1} saccharose (0.5 ml per dish 60 mm in diameter). Stainless steel flat electrodes (2.4 mm apart) were applied to a cell monolayer and fusion was initiated by 3 to 10 high voltage pulses. MEM with 10 % FCS was added to the dishes and the cell monolayer was fixed using a mixture of glacial acetic acid and methanol (1:3), and stained with methyl-green pyronine after 24 hours.

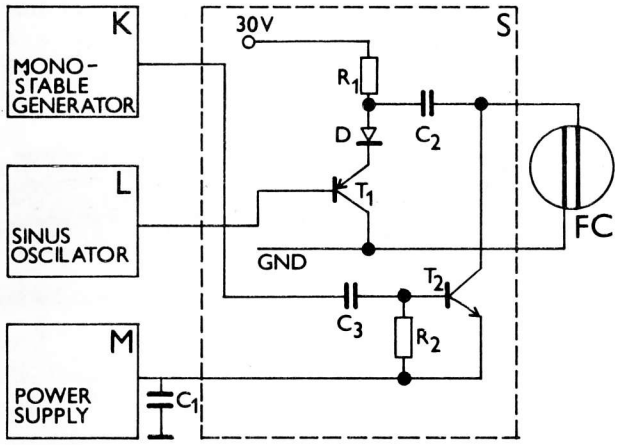

Fig. 1. Electrofusion circuit. The apparatus contains four basic electronic blocks: (K) pulse generator with monostable circuit (switched on only at the moment the pulse is generated), (L) Wien bridge oscilator with amplitude stabilization, (M) power supply and switching converter. To obtain strong energy of fusing pulse the output is connected to large capacitor C_1. (S) electronic switch allows immediate attachment of the DC pulse to the sine wave AC field. C_1, 3 × 10 μF/450 V; 390 pF/400 V; C_3, 15 nF/400 V; R_1, 470 ohms; R_2, 12 kohms; T_1, transistor type PNP; T_2, BF 459; D, KY 199. (FC) fusion chamber, (GND) ground.

Electrofusion apparatus: An AC — DC power supply of original design was developed (Fig. 1). The supply is tunable up to a frequency of 2 MHz and the output is variable over a range of 0—9 V, and it generates DC square pulses 0—240 V of 10—50 μs duration.

Results

The technique of fusing cells in suspensions makes direct investigation of dielectrophoresis and fusion itself under an optical microscope and thus determina-

tion of optimal AC and DC parameters, possible. To obtain sulfficiently high field values fusion experiments had to be carried out in isotonic media of a relatively low conductance (Zimmermann and Vienken 1982). Owing to this the experimets were performed in 250 mmol . l^{-1} saccharose for animal cells, and in 300 mmol . l^{-1} saccharose with plant protoplasts. In Table 1 optimal voltage values and frequencies of AC field for particular cell types are shown at which formation of "pearl chains" exhibiting intimate cell-to-cell contact occurred. Mouse erythrocytes formed contact even in a field with a frequency of 20 kHz and strength 500 V . cm^{-1}, while optimal conditions for mammalian permanent cell lines and plant protoplasts were 1 — 1.4 MHz and 250—800 V . cm^{-1}. In the case of dielectrophoresis of two cell types with various requirements for frequency and field strength, the higher ones were also optimal for the mixture of cells (Fig. 2).

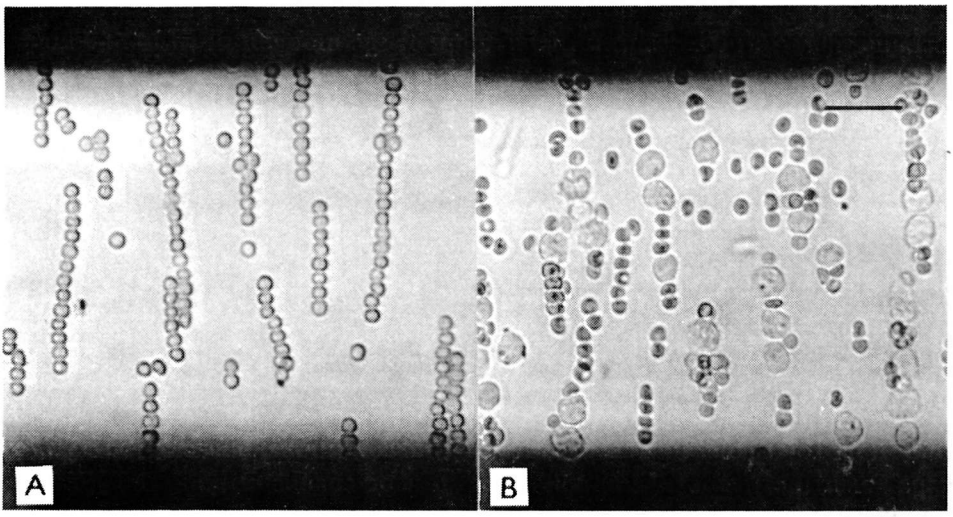

Fig. 2. Dielectrophoresis of cells in AC field. Mouse erythrocytes (*A*) formed "pearl chains" by 0.5 kV . cm^{-1} field strength and 20 kHz frequency. In a mixture with lymphosarcoma LS/BL cells (*B*) dielectrophoresis was achieved by the application of AC field 0.8 kV . cm^{-1}, 1 MHz. Cells were resuspended in 250 mmol . l^{-1} saccharose; bar : 20 μm.

Cell fusion was induced immediately after dielectrophoresis occurred using one or more pulses (1—5 kV . cm^{-1}, 10—36 μs duration). Effective values for various cell types are also presented in Table 1. Higher field strength or longer pulse duration resulted in considerable cell lysis. Repeated pulsation (up to 10,

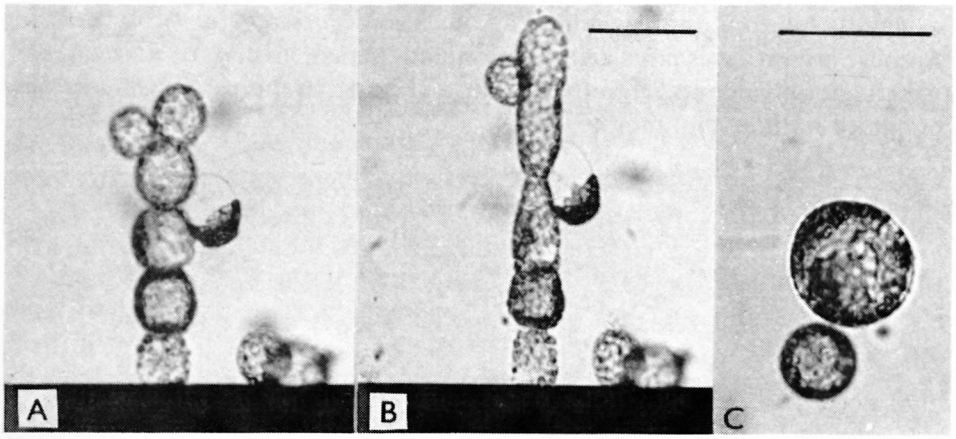

Fig. 3. Fusion of *Nicotiana* protoplasts. (*A*) Dielectrophoresis conditions: field strength 0.2 kV . cm^{-1}, frequency 1 MHz. (*B*) Cell fusion immediately after breakdown pulse application (3 pulses in 1 s intervals, intensity 1 kV . cm^{-1} and 10 μs duration). (*C*) Fused protoplast 5 min after pulse application. Bar: 50 μm.

separated by one-second intervals) resulted in a partially higher yield of multinuclear cells, but a further increase also induced cell lysis. Cell fusion was observed immediately after the application of breakdown pulses and was usually completed within few minutes (Fig. 3). Binuclear cells were observed most

Table 1. Electrofusion parameters optimal for various cell types

Cells	Dielectrophoresis		Electrical pulse	
	Field strength (V/cm)	Frequency (MHz)	Field strength (kV/cm)	Duration (μs)
Mouse erythrocytes	500	0.02	5	20—36
Mouse fibroblasts L	500	1—1.4	1	10
Mouse lymphosarcoma LS/BL cells	500—800	1	2	36
Chinese hamster V 79 cells	800	1	1	20
Human HeLa cells	250—500	1	1—2	20
N. plumbaginifolia protoplasts	250	1	1	10

Fusions were performed in suspensions in the medium described in Materials an Methods. The distance between electrodes was 150 μm. One pulse was applied to cells immediately after dielectrophoresis.

frequently, but multinuclear homokaryons also occurred (Fig. 4). After fusion of mouse erythrocytes large cells were often obtained, arising from several cells aligned not only in parallel to the field lines (Fig. 5). In this case, fusion was not completed until several minutes later.

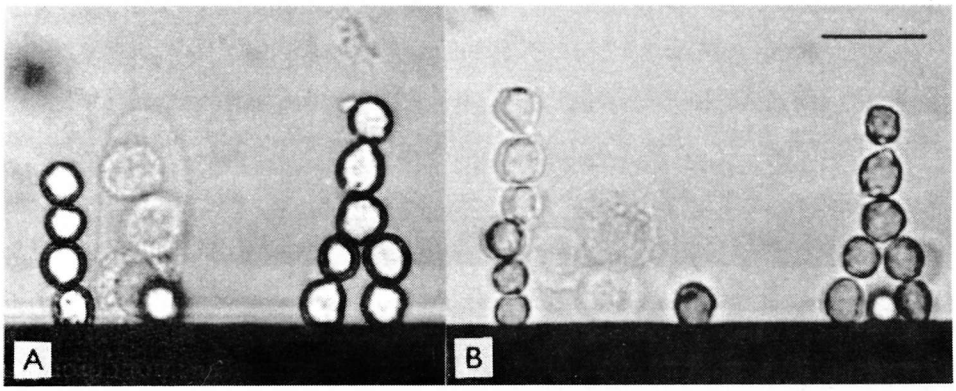

Fig. 4. Electrofusion of Chinese hamster V 79 cells. Dielectrophoresis: 0.8 kV . cm^{-1}, 1 MHz. Fusion: one pulse 1 kV . cm^{-1}, 20 μs duration. Enlargement of the diameter of fusing cells occurred immediately after the pulse was applied. (*A*) homokaryon 3 s after pulse application, (*B*) rounding up after 15 s. Bar: 30 μm.

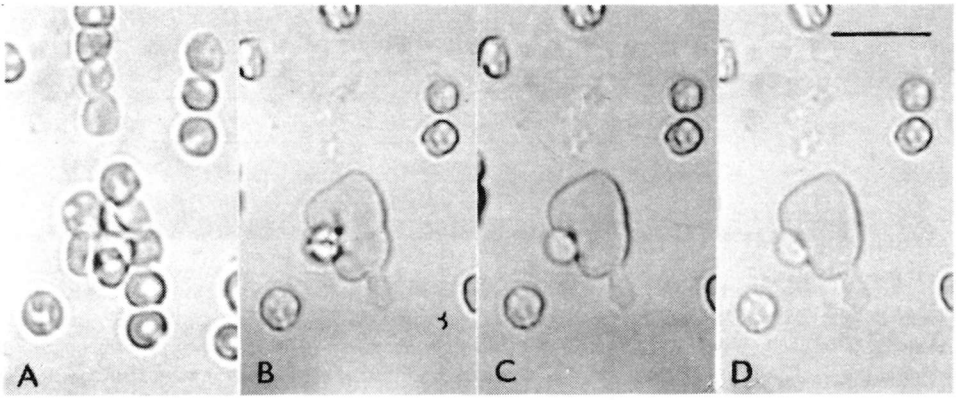

Fig. 5. Electrofusion of mouse erythrocytes. (*A*) Dielectrophoresis 0.5 kV . cm^{-1}, 20 kHz. (*B*—*D*) Fusion after breakdown pulse of 5 kV . cm^{-1}, 20 μs duration. Photographs were taken 20 s (*B*), 40 s (*C*) and 60 s (*D*) after pulse application. Bar: 10 μm.

To induce fusion of plant protoplasts after dielectrophoresis DC pulses of 1 kV . cm^{-1}, 10 μs duration were used. Contrary to the mammalian cells, protoplasts elongate at the moment of the pulse application (Fig. 3*b*). This elongation

is oriented parallel to the field lines and disappears immediately after the AC field is switched off; rounding up of the protoplasts is completed within few minutes (Fig. 3*c*).

The stimulation effect of pronase treatment on the fusion of mammalian cells was studied in the presence of Pronase P in fusion mixture or after 20—40 minutes of pretreatment and subsequent enzyme removal. The presence of pronase during the fusion process decreased "pearl chain" formation as well as the efficiency of fusion; on the other hand twenty minutes' pretreatment slightly increased the yield of fusion compared to control experiments without pronase. Pretreatment prolongation caused cell lysis.

Table 2. Fusion indices after fusion of mouse L fibroblasts in monolayers

	Number of polykaryons					
	2 nuclei	3 nuclei	4 nuclei	5 or more nuclei	Total	%
Control	10	1	1	0	12	0.6
3 pulses	195	36	9	3	243	12.5
10 pulses	187	25	0	4	216	10.8

Pulses (1 kV . cm^{-1}, 36 μs duration) were repeated at 1 s intervals. As controls, non-pulsed cells were scored. In each case 2000 cells were evaluated.

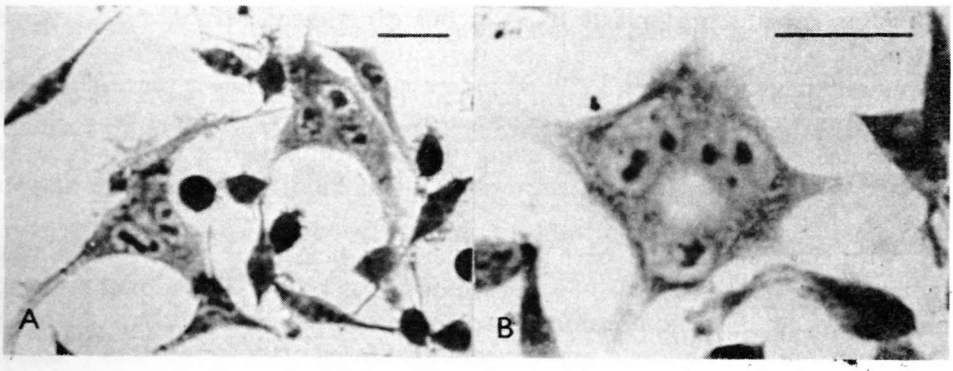

Fig. 6. Electrofusion of mouse L fibroblasts in monolayer culture. Cells were fused by 3 pulses of 0.9 kV . cm^{-1}, 36 μs duration, repeated at 1 s intervals. Multinuclear cells were fixed 24 h after fusion. Bar:20 μm.

The fusion of cells in monolayers was performed by setting stainless steel electrodes to the cells attached at the bottom of Petri dishes. After 3—10 pulses (1 kV . cm^{-1}, 1-s intervals), no visible morphological changes and/or damage to cells were noted in light microscope. The extent of fusion of mouse L cells was

determined after 24 hour-cultivation in fixed preparations. The frequency (in %) of multinuclear cells is presented in Table 2. As compared to control (non-pulsed) cells an increase in bi- and trinuclear homokaryons was noted (Fig. 6). Moreover, cells having five or more nuclei were scored; similar cells were not observed in controls. Data in Table 2 show that increasing the number of pulses from 3 to 10 did not improve fusion frequency; on the other hand, no adverse effects on cell morphology were seen after repeated pulsation.

Discussion

Cells in suspensions subjected to non-uniform alternating electric field behave like neutral or charged particles and move toward regions of highest field intensities. If the frequency of the field ranges between kHz and MHz and if field strengths of the order of $100\,V\,.\,cm^{-1}$ are applied, the cells come into intimate contact, forming "pearl chains" (Zimmermann et al. 1985). Frequency values for optimal dielectrophoresis depend on cell type and cell diameter, e. g. mouse erythrocytes formed "pearl chains" at lower frequencies (20 kHz), whereas for permanent cell lines a frequency of 1 to 1.4 MHz was required. It is important to determine optimal conditions for dielectrophoresis, because at lower frequencies the cells rotate, which prevents their fusion. Another important condition for dielectrophoresis to occur is AC field strength. The optimum for mammalian cell lines lies between 250—$800\,V\,.\,cm^{-1}$, whereas at field strengths exceeding $250\,V\,.\,cm^{-1}$ plant protoplasts lysed in our experiments. If AC field voltage reaches breakdown voltage or exceeds it, it causes cell deformation or even lysis (Zimmermann and Vienken 1982; Saunders et al. 1986). During the application of pulses inducing fusion, some deformation of cells also occurs. One example of such deformation is protoplast elongation (Fig. 3*b*) also observed by Bates et al. (1983) in Petunia and Zea protoplasts. This kind of deformation has no adverse effect on cell fusion.

After cell contact had been established by dielectrophoresis, fusion was induced by 1—10 pulses (10—36 μs duration, 1—$5\,kV\,.\,cm^{-1}$). A lower voltage, about $1\,kV\,.\,cm^{-1}$, induced fusion of two or three cells, oriented in parallel to the field lines, but at $5\,kV\,.\,cm^{-1}$ erythrocytes also fused at various angles (Fig. 5). The formation of giant cells arising from thousands of human erythrocytes was also decribed by Zimmermann et al. (1982b) and Zimmermann and Vienken (1982) who applied $6\,kV\,.\,cm^{-1}$ and stimulated fusion with pronase.

High fusion efficiency of erythrocytes has been also described by other authors working with human erythrocytes (Scheurich and Zimmermann 1981; Zimmermann 1982). Fusion efficiency of erythrocytes can be increased due to long-lived fusogenic state of the membranes as shown by Sowers (1986) in erythrocyte ghosts after stimulation by electrical pulses. It is also known that

differences in the fusion capacity exist between malignant and non-malignant cells when the fusion is stimulated by viral or chemical inducers. A no less important role is played by cell origin, culture conditions, phase of the cell cycle etc. (Poste 1972; Ringertz and Savage 1976). In our experiments no differences in fusion capacity were found after electrical stimulation between cells derived from tumours (HeLa, LS/BL) and permanent cell lines of non-malignant origin (L, V 79).

The yield of fusion products has been reported to be enhanced by pronase in some works (Zimmermann et al. 1982a; Zimmermann and Vienken 1982; Berg et al. 1983). This phenomenon is explained by a protective effect of pronase against the higher pulse voltages, which cause cell disruption. Such a stimulating effect of pronase was not observed in our experiments either for mouse erythrocytes, or for fusion of permanent cell lines. The influence of pronase is probably not the same for all cell types; moreover, it has been postulated that enzymes have to be present in the medium at the moment of fusion (Zimmermann et al. 1982a). It was not possible to arrange experiments in the same way, because pronase partially enhanced medium conductance and consequently decreased pulse voltage and fusion efficiency. The presence of pronase seems to be important only for some cell types and experimental conditions because high fusion efficiency for LM mouse line ID clones without pronase treatment has been reported by Finaz et al. (1984).

Employing a modified method of pulsing the cells in monolayer in close contact omitting dielectrophoresis, we obtained multinuclear mouse L cells ($1\,kV \cdot cm^{-1}$, 3—10 pulses, 36 μs duration). Their viability was confirmed after 24-h cultivation. The fusion frequency was higher than 10%, which is in accordance with the results of Teissié et al. (1982), who fused mouse 3T3 cells under similar conditions. We did not reach 90 % fusion efficiency, as did Finaz et al. (1984) for LM mouse line, but the frequency was higher than those observed after PEG (3—10%) or Sendai virus (1—4%) induced fusion in similar cell systems. In particular, the number of multinuclear cells was higher than after treatment with PEG or Sendai. Moreover, a varying fusion efficiency was obtained after these inducers since the fusion conditions are not exactly reproducible. On the other hand, electrical parameters for electrofusion can be well determined and adjusted. In addition, fusogenic compounds and viruses can also affect the viability of fusion products because of uncontrollable interactions with the membrane surface (Zimmermann and Vienken 1982).

Contrary to the report by Teissié et al. (1982), the fusion frequency was not influenced by multiplication of pulses to ten. Increased pulse numbers (over 10) also led to massive cell destruction or detachment from the substrate. Preliminary results obtained after fusion of mixed cell populations give good prospects for isolation of hybrid cells.

It can be assumed that the method of electrofusion, especially in monolayers, is very successful, and acceptable for many laboratories. The equipment is now commercially available, but it presents no problems to construct not too complicated apparatus suitable for electrofusion. It should be mentioned that at a lower output only non-conductive media have to be used and the electrode distance should permit a pulse strength of at least 1 to 6 kV . cm^{-1}. Owing to the advantages mentioned above, electrofusion is becoming an attractive technique in studying the transport of genetic material and its expression.

References

Bates G. W., Gaynor J. J., Shekhawat N. S. (1983): Fusion of plant protoplasts by electric fields. Plant Physiol. **72**, 1110—1113

Berg D., Schumann I., Stelzner A. (1983): Electrically stimulated fusion between myeloma cells and spleen cells. Stud. Biophys. **94**, 101—102

Finaz C., Lefevre A., Teissié J. (1984): Electrofusion. A new, highly efficient technique for generating somatic cell hybrids. Exp. Cell Res. **150**, 477—482

Köhler G., Milstein C. (1975): Continuous cultures of fused cells secreting antibody of predefined specificity. Nature **256**, 495—497

Orgambide G., Blangero C., Teissié J. (1985): Electrofusion of Chinese hamster ovary cells after ethanol incubation. Biochim. Biophys. Acta **820**, 58—62

Poste G. (1972): Mechanisms of virus-induced cell fusion. Int. Rev. Cytol. **33**, 157—252

Ringertz N. R., Savage R. E. (1976): Cell Hybrids. Academic Press, Inc., London

Saunders J. A., Gillespie J. M. (1984): Localization and substrate specificity of glycosidases in vacuoles of *Nicotiana rustica*. Plant Physiol. **76**, 885—888

Saunders J. A., Roskos L. A., Mischke S., Aly M. A. M., Owens L. D. (1986): Behavior and viability of tobacco protoplasts in response to electrofusion parameters. Plant Physiol. **80**, 117—121

Scheurich P., Zimmermann U. (1981): Giant human erythrocytes by electric-field-induced cell-to-cell fusion. Naturwissenschaften **68**, 45—46

Sowers A. E. (1986): A long-lived fusogenic state is induced in erythrocyte ghosts by electric pulses. J. Cell Biol. **102**, 1358—1362

Teissié J., Knutson V. P., Tsong T. Y., Lane M. D. (1982): Electric pulse-induced fusion of 3T3 cells in monolayer culture. Science **216**, 537—538

Zimmermann U. (1982): Electric field-mediated fusion and related electrical phenomena. Biochim. Biophys. Acta **694**, 227—277

Zimmermann U., Vienken J. (1982): Electric field-induced cell-to-cell fusion. J. Membrane Biol. **67**, 165—182

Zimmermann U., Pilwat G., Pohl H. A. (1982a): Electric field-mediated cell fusion. J. Biol. Phys. **10**, 43—50

Zimmermann U., Vienken J., Halfmann J., Emeis C. C. (1985): Electrofusion: A novel hybridization technique. Adv. Biotechnol. Processes **4**, 79—150

Zimmermann U., Vienken J., Pilwat G. (1982b): Electric-field-induced fusion of cells. Stud. Biophys. **90**, 177—184

Zimmermann U., Vienken J., Pilwat G., Arnold W. M. (1984): Electrofusion of cells: principles and potentials for the future. In: Cell Fusion. Ciba Foundation Symposium **103** (Eds. D. Evered, J. Whelan), pp. 60—85, Pitman Books, London

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