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Electrofusion of Cells

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INTRODUCTION

Since the first applications of electric pulses from a Volta pile by Johann Wilhelm Ritter (1776–1810) on tissues of his own body (1), many studies with nerves and muscles have been done. The first evidence for protoplast fusion by electric field pulses was presented in 1979 and involved plant protoplasts of *Raowolfia* and *Hordeum* (2), and diauxotrophic yeast mutants (3-6). In the first case, needle electrodes in glass capillaries controlled by a micromanipulator were used (Technique A), whereas for the second case a macrochamber with disk electrodes, an exponential pulse, and agglutinating polyethylene glycol (PEG) was utilized for fusion and subsequent formation of prototrophic colonies (Technique B). Later, a valuable technique was introduced consisting of a microchamber with parallel wire electrodes; it used dielectrophoresis for cell collection and rectangular pulses for electrofusion under microscopic control (Technique C) (7,8). In all three cases, close contact between the cells to diminish adsorbed water layers and the repulsion of surface charges is the most important prerequisite for successful electrofusion. The initial applications of the three pulse techniques are shown in Table 1 (Figure 1).

Parallel to these pulse techniques, the influence of an alternating current on human red blood cells (dielectrophoresis) was studied, and the agglutination of cells, but not their actual electrofusion, was first described in 1976 (9-11).

For electroincorporation, the substances (DNA, proteins, drugs) for penetration are mostly adsorbed at the cell membrane prior to the electric field pulse application (12-16). Such studies (electroporation) were performed in the early 1970's (17). Two remarkable examples should be mentioned for red blood cells: the incorporation of DNA from SV 40 or RNA (18), and of cancerostatic methotrexate (19).

TABLE 1. Initial Applications of the Three Techniques for Electrofusion and Electrotransformation

TECHNIQUE	OBJECT	EVIDENCE	GROUP	REFERENCE	YEAR
Micromanipulator chamber	Plant protoplasts (*Raowolfia* + *Hordeum*)	Morphological	Senda	(2)	1979
	Mouse blastomeres	Morphological	Berg	(20)	1982
	L 1212 Ascites cells	Morphological	Berg	(20)	1982
Macrochamber	Yeast protoplast mutants	Genetic	Berg	(3-6)	1979
	Dictyostelium discoid.	Morphological	Neumann	(21)	1980
	Mouse fibroblasts	Physiological	Tsong	(22)	1982
	H. s-virus plasmid into mouse lyoma cells (TK–)	Genetic	Neumann	(14)	1982
	B. thuringiensis plasmid into *B. cereus protoplasm*	Genetic	Berg	(16)	1983
Microchamber (Dielectrophoresis)	Plant protoplasts (A. sativa)	Morphological	Zimmermann	(8)	1980
	Sea urchin eggs	Physiological	Zimmermann	(7)	1981
	Yeast protoplast mutants	Genetic	Zimmermann	(23)	1982
	Hybridomas, human cells	Physiological	Zimmermann	(24)	1982
	Plant protoplasts (tobacco, barley)	Morphological	Berg, Senda	(25)	1981
	Hybridomas, mouse cells	Morphological	Berg	(26)	1982

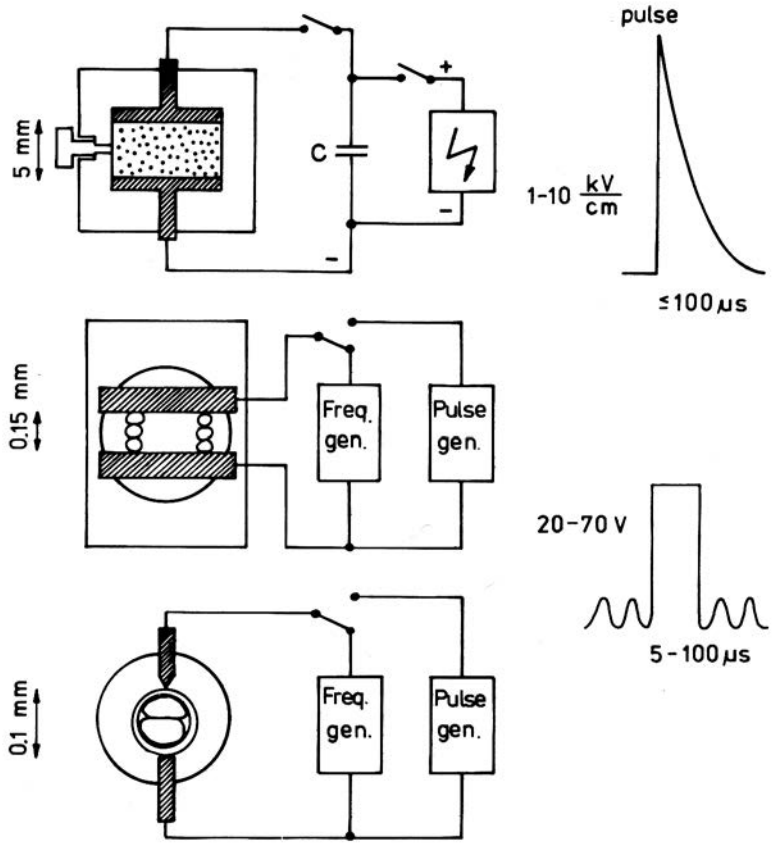

FIGURE 1. Three main techniques for electrofusion, described in the text as (from top to bottom) B, C, and A.

In each case, enhancement of membrane pores and/or destabilization (reversible or irreversible breakdown) of the entire membrane structure occurs, usually followed by resealing, depending on the electrical pulse parameters, the preparation of the cells or protoplasts, and the composition of the solution. For each kind of cell a suitable pulse characteristic must be determined to get an optimum yield of fused cells. This brief review is devoted mainly to applications of electric-field pulses as a tool in modern genetics of prokaryotic and eukaryotic cells. In-depth treatments of the broad field are given elsewhere (1,2).

THEORETICAL AND EXPERIMENTAL PRINCIPLES

Successful electrofusion requires cell preparation, cell contact, appropriate equipment, and cultivation of the fused cells. Only some of these factors will be discussed in the following sections (3).

METHODS FOR CONTACTING CELLS

The yield of electrofusion depends strongly on the preparation of the membranes and their contact. For Technique A, a slight mechanical pressure against both cells by the electrode tips forming a contact is enough for starting fusion after the pulse or the dielectrophoretic alignment. For Technique B, agglutination by an agent such as PEG (molecular weight 4000-6000) or dextran (concentration greater than 10%) is most effective. The water layer is squeezed out from the membrane surface, bridges of dextran molecules are for med, and the electrostatic repulsion is decreased. For Technique C, dielectrophoresis (4) is a rather universal method for collecting particles or cells oriented in the direction of electric field lines as a "pearl-chain" formation (Figure 7). This process is brought about by an inhomogeneous alternating electric field which causes the cells to become polarized by dipole induction. The net force causes a motion (4,5) towards the region of highest field intensity at the electrodes. The cells move along the field lines and approach one another. The dielectric force on a spherical cell of radius r in a field of strength E is proportional r^3 and to the divergence of E^2 (modified by the relative dielectric constants of the medium, $\varepsilon 1$, and the cell, $\varepsilon 2$) (4). The force increases with the square of the applied voltage U, and for cells having small r or for small differences in the dielectric constants ($\varepsilon 1 > \varepsilon 2$) the voltage must be stronger for effective pearl-chain formation. Electrostatic repulsion due to surface charges and water layers on membranes may be overcome by dielectrophoresis in the MHz range. On the other hand, voltage, heat production, and collection time must be controlled such that both breakdown and membrane stabilization are avoided. For cells having radii of 5 μm, the time constant for pearl-chain formation is 1–10 sec in an electric field of 10 kV/m. Along with pearl-chain formation, a remarkable effect occasionally has been observed since 1960 (4,5), namely the spinning of particles and cells at certain resonant frequencies of the electric field. For electrofusion, this rotation is a disturbing event that hinders tight contact, and it must be avoided by changing the frequency of dielectrophoresis. On the other hand, cell rotation and the influence on it by drugs and other substances in solution is now a valuable tool in cell research (5-7).

Besides dielectrophoresis, there are other physical possibilities for collection of cells, including centrifugal forces (8) and ultrasonics (9).

METHODS OF PULSE APPLICATIONS FOR ELECTROFUSION

The main aim is to generate a higher transmembrane potential. This induced potential difference ΔU depends on the cell radius, the angle θ between the electric field vector and the surface element considered, and the applied external field strength E_0 , and is given (assuming the membrane conductivity is zero) for spheres by (7):

$$\Delta U = 1.5 r E 0 \cos\theta \quad (1)$$

A more general expression especially suitable for vesical membranes has been given (10).

From Equation (1) it can be seen that pulse effects are strongest at the membrane surface for $\theta = 0$, which occurs at the contact areas of the poles of the cells within the pearl chain. For larger cells the critical transmembrane breakdown voltage (on the order of 1 V) occurs at a lower external field strength. For example, for a cell of r = 5 μm, from Equation (1) we have:

$$E_0 = 1 \text{ V}/(1.5 \times 0.5 \times 10^{-3} \text{ cm}) = 1.33 \text{ kV/cm}.$$

The three main experimental systems used to apply external electric fields to cells with r values between 1–50 μm are shown in Figure 1: the micromanipulator technique with two movable needle electrodes Technique A) (11-13); the macrotechnique with disk electrodes and agglutinating polymers (Technique B) (8,14-17); and the microtechnique with parallel electrodes, in combination with dielectrophoresis (Technique C) (18-23).

Using Technique A for electrofusion of large cells under microscopic observation, the platinum needle electrodes must have a point diameter of less than 100 μm. In the special case of blastomere fusion within the zona pellucida of a mouse zygote (Figure 2) three electrode positions can be employed; 20 μm from the cell surface, a slight tangential contact at the cell surface, or a tight contact. These positions determine the optimum pulse parameters for viability of the fused cells. Small cells can be aligned between the electrode tips using dielectrophoresis only in a low conducting medium.

Using Technique B, 1–5 ml of a cell suspension can be fused in the macrochambers with stainless-steel electrodes (Figure 3). Chambers having a variable electrode distance are also possible. The electric-field pulses are caused by the discharge of a high-voltage capacitor (0.075–2 μF). The exponential time course of the field has a time constant of $\tau = RC = 20$ μsec and a heating time of RC/2 = 10 μs. The maximum field strength is of the order of 35 kV/cm. The same suspension may be subjected to several discharges. Agglutination of cells before pulse application is a prerequisite, and is sometimes accomplished by centrifugation.

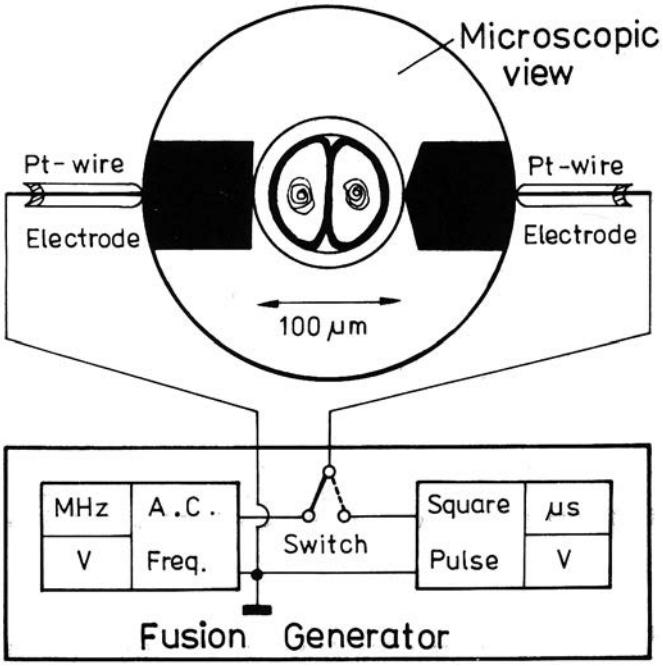

FIGURE 2. Technique A with a two-cell blastomere as the object between the needle electrodes.

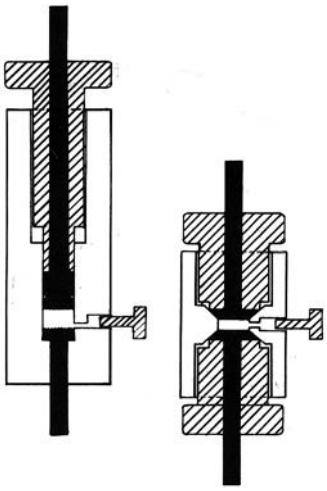

FIGURE 3. Macrochamber: left, for changing the volume continuously; right, for a fixed volume (black, stainless steel electrodes).

With Techniques A and C, fusion can be observed microscopically and analyzed using an image analyzer. The microchamber, which is mounted on a slide, has two parallel platinum wires separated by > 0.1 mm (Figure 1). Sufficient membrane contact is achieved before the fusion pulse when a flattening in the membrane touching area is observed. Depending on cell diameter, the breakdown field strength is 0.3–12 kV/cm (compare Equation (1)). Both the resealing time and the breakdown strength decrease with increasing temperature. Low conductivity of the solution is the prerequisite during this procedure.

RESULTS

THE MICROMANIPULATOR TECHNIQUE

1. Electrofusion of Blastomeres of Murine and Rat Zygotes

For electrofusion of blastomeres surrounded by the zona pellucida, both electrodes are in tight contact as shown in Figure 2. Several minutes after the pulse, the membranes between the blastomeres melt and only one cell is observed; this means that a kind of dedifferentiation has taken place (11,12,24).

To preserve a high viability rate, as indicated by the ability of the fused cells to divide either within an incubator or after implantation (24), a low field strength (≤1 kV/cm) should be applied in about 0.3 msec (Figure 4). Fusion by alternating current has also been detected (24).

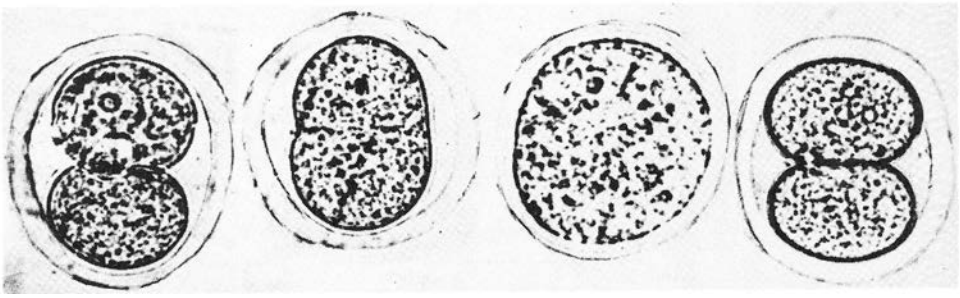

FIGURE 4. Electrofusion of rat blastomeres. Left to right, before the pulse (1 kV/300 μsec); after 2 min; fusion finished after 30 min; division after 24 hours' incubation (24).

Since the aim is to fuse different oocytes or zygotes in the early stages of embryonic development, the zona pellucida must be removed by pronase treatment. Consequently, lower pulse energies are needed and the naturally adherent membranes fuse first, followed by fusion of the outer membranes compressed by the tops of both needle electrodes (12).

THE MACROCHAMBER TECHNIQUE

Prior to application of the fusion pulse, the cells must be agglutinated by an appropriate agent such as PEG, dextrane or polylysine. Although their mechanism of action is rather complicated, the basic effects are dehydration and destabilization of membranes, osmotic effects with shrinking of the cells, and finally tight contacts (14-17).

1. Electrofusion of Protoplasts of Yeast Mutants

Treatment of a suspension of mixed strains of auxotrophic yeast protoplasts in the presence of 30–40% PEG and 10^{-3} M Ca^{2+} ions by electric field pulses strongly enhances the fusion rate as determined from analysis of the number of prototrophic colonies formed on minimal nutrition med um (Figure 5 and Table 2). For optimum intergeneric fusion, higher pulse strengths are necessary (Table 2, *Saccharomycopsis lipolytica* + *Lodderomyces elongisporus*). Isolated prototrophic colonies, especially of the intraspecific type, are stable over more than 20 passages.

2. Electrofusion of Two Protoplast Strains of *Bacillus thuringiensis*

Conventional fusion by PEG did not result in any colony formation from the kanamycin-resistant strain and the kanamycin-sensitive strain, which produces a brown pigment on the selective kanamycin medium (25). Treatment with the electric pulse technique in the presence of PEG at 14 kV/cm was not sufficient, but three subsequent 5-µsec pulses at 20 kV/cm yielded recombinants that formed colonies which were kanamycin resistant and were also able to produce the brown pigment. The electrofusion frequency (the ratio of recombinant colonies to regenerated protoplasts) was found to be 10^{-3}. They were stable even after 30 passages on selective as well as on non-selective media. Moreover they were able to form the characteristic protein crystals that are toxic against insects.

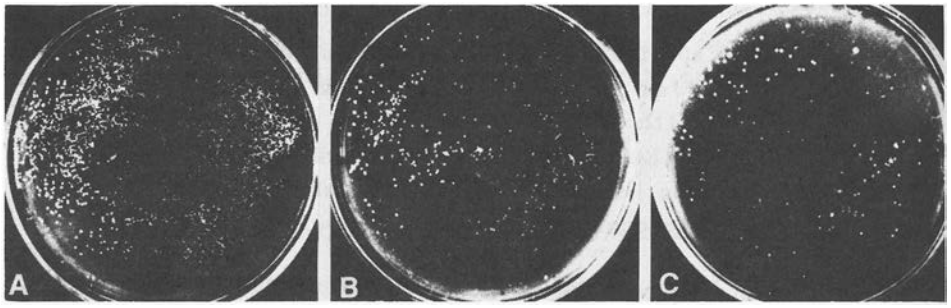

FIGURE 5. Phototrophic yeast colonies formation as a function of pulse intensity (see also the last column of Table 2). A, 10 kV/cm; B, 15 kV/cm; C, 20 kV/cm. Fusion products, 622, 160, 66 respectively (control, 8) (courtesy H. Weber, Jena).

TABLE 2. Relative Number of Prototrophic Cell Colonies on Minimal Medium from Electrofusion of Auxotrophic Yeast Protoplast Mutants in Dependence of Field Strength

E/kV cm^{-1}	*S. cereus*	*S. lipolytica*	*S. lipolytica* + *L. elongisporus*
0 (control	1	1	1
1.25	122	1	–
2.5	233	8	–
3.75	50	36	–
5.0	30	10	–
10.0	–	–	78
15.0	–	–	20
20.0	–	–	8

3. Electrotransformation of Bacillus cereus by the Plasmid-DNA of *Bacillus thuringiensis*

B. cereus is suitable for biotechnological cultivation and therefore was combined with properties of *B. thuringiensis* by electrotransformation with the useful plasmid pUB 110. The stability of the protoplasts of *B. cereus* was tested, and no inactivation of cells up to 20 kV/cm was seen. Applying 14 kV/cm three times, a tenfold higher transformation frequency on selective medium occurred as compared to the control. These kanamycin-resistant colonies were stable after 30 passages, but the toxic protein crystals were not found microscopically.

THE MICROCHAMBER TECHNIQUE

1. Electrofusion of Plant Protoplasts

During the fusion process it can be seen that the membrane material from the breakdown area disappears into the cell body, resulting in a sphere from two protoplasts. Twenty-six percent of the original membranes are lost (Figure 6). Further examples and conditions are shown in Table 3 and are described in detail elsewhere (1,26-31).

TABLE 3. Electrofusion of Microorganisms and Plant Protoplasts (Different Cells)

OBJECT	ELECTRIC FIELD (kV/cm)	PULSE WIDTH (µsec)	AUTHOR	REFERENCE	YEAR
R. serpent. + *H. vulgare*	(12 µa)	5000	Senda	(13)	1979
Yeast mutants: *Saccharomycopsis* strains S113+26–10	3.75	100	Weber	(14-17)	1981
S. lipolitica + *L. elongisporus*	10	100			
B. thurintiengis + mutants	14	100	Shivarova	(25)	1983
*V. faba**	1.65	50	Scheurich	(32)	1981
*K. daigrem.** mesophyll + vacuole	0.5	50	Vienken	(33)	1981
*A. sativa**	0.6	15	Zimmerman	(34)	1981
Sacc. c. respir. defect + *Sacch. c.*	7.9	40	Halfmann	(21)	1982
N. tabacum 63 + *N. tabacum* 68	1.5	20	Kohn	(35)	1984
*B. napus**	0.96=5	15	Zachrisson	(36)	1984
B. campestris + *P. acaulis*	0.48	15	Zachrisson	(36)	1984
L. esc. + *L. peruvianum*	2.2	20	Siegemund	(37)	1984
A. sativa + *Z. mays*	0.7	50	Bates	(38)	1983
N. plumbaginifolia + *D. carota*	0.8	50	Bates	(39)	1984
N. glauca + *N. langsdorffi*	1.0	200	Morikawa	(40)	1986

*different protoplasts of the same plant

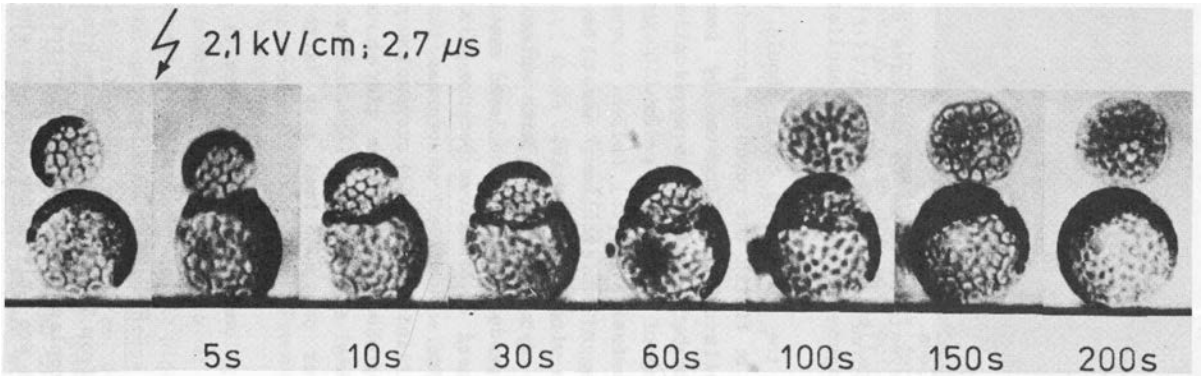

FIGURE 6. Electrofusion of two barley protoplasts (*H. volgare*) by a pulse of 2.1 kV, 2.7 µsec using Technique C (courtesy H.-E. Jacob, Jena).

2. Electrofusion of Animal Cells

The same basic events occur as in the case of protoplasts, but the cells have smaller diameters (Figure 7), and the necessary pulses are stronger (Table 4). Some peculiarities must be taken into account:

— Shortly after the pulse, the cells should be transferred to a nutrient medium to finish the rounding procedure.

— Red blood cells must be treated by neuraminidase to remove the glycocalix before the pulse application (41). Then giant cells consisting of about 1000 cells (100 μm in diameter) are formed by lateral fusion (23).

— Sea urchin eggs (unfertilized) must be treated with pronase to remove the vitellin layer (22).

— Electrofusion of myeloma cells with spleen cells to get hybridomas for the production of monoclonal antibodies causes difficulties with regard to breakdown because of the 3:1 difference in cell diameters. Addition of pronase (1 mg/ml) to the glucose solution containing the cell suspension may be helpful (20). The fusion products should be placed in to wells for cultivation as quickly as possible (18). The ideal is 1:1 electrofusion, however one sometimes gets large multicellular bodies (Figure 8).

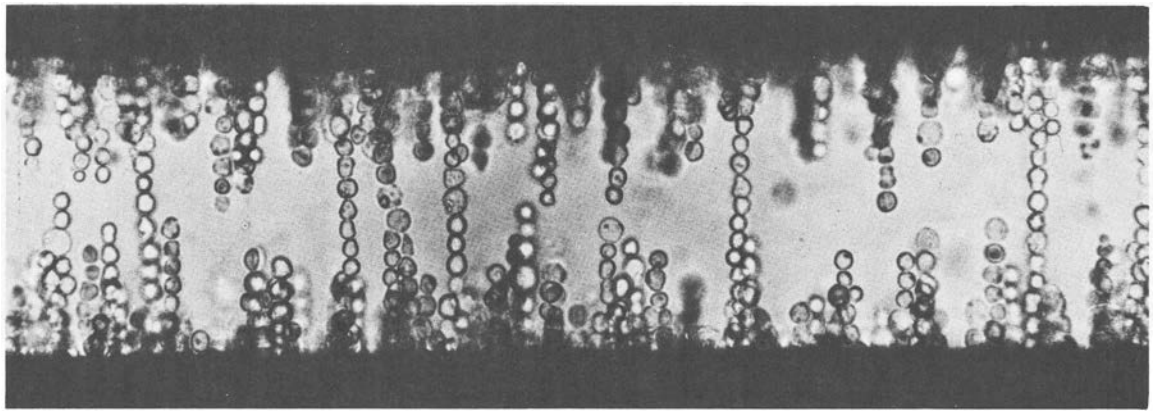

FIGURE 7. Pearl-chain formation of cancer cells L1210 by dielectrophoresis (courtesy H.-E. Jacob, Jena).

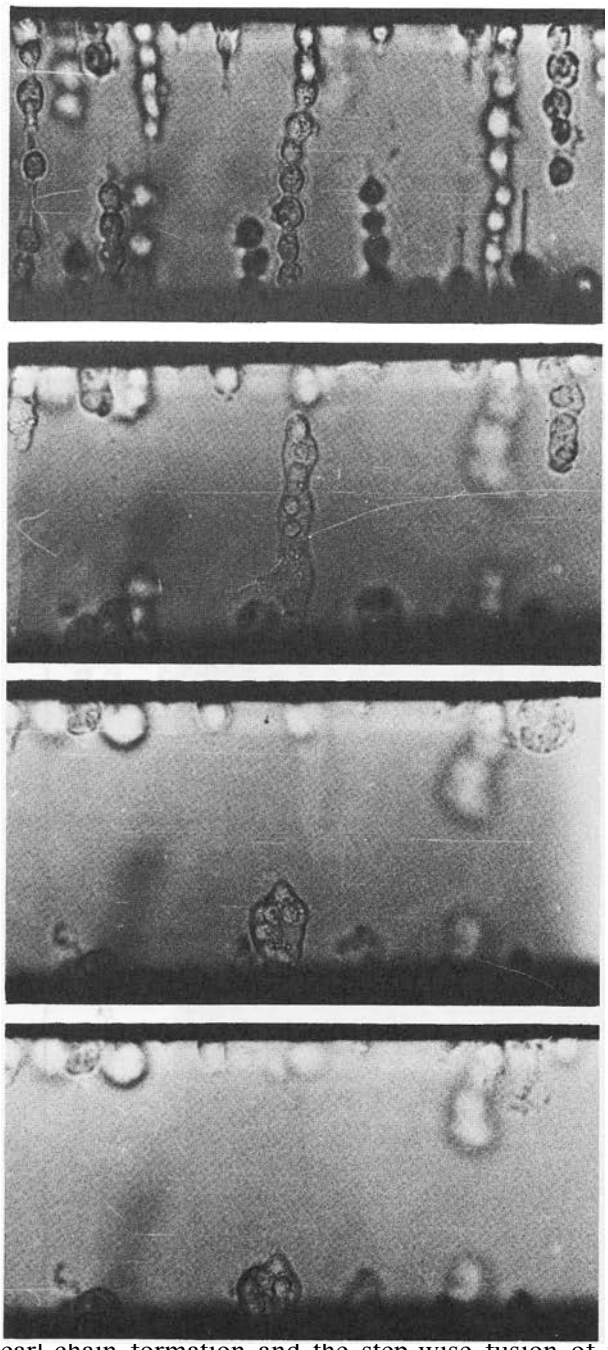

FIGURE 8. Pearl-chain formation and the step-wise fusion of myeloma cells (courtesy D. Berg, Jena).

DISCUSSION

FUSION PRODUCTS

As can be seen from Tables 3 and 4, more kinds of plant and microbiological protoplasts have been electrofused than have animal cells. There are only a few examples

where cultivation of fusion products performed and unambiguous tests were made of the stability and viability of the fused structures; this was done for microorganisms (14-17,21,25), plants (40,42), zygotes (24), and oocytes (22). In the future, cultivation will be necessary for comparison with other fusion methods or for industrial use (43).

TABLE 4. Electrofusion of Animal Cells

OBJECT	ELECTRIC FIELD (kV/cm)	PULSE WIDTH (µsec)	AUTHOR	REFERENCE	YEAR
Mouse blastomeres, rat blastomeres, mouse blastocytes (without and with zona pellucida)	0.75–5	100–300	Berg	(11) (12,24)	1982 1983
Mouse fibroblasts 3T3	1–2	100	Teissie	(44)	1982
Macrophages	5	5	Berg	(3)	1984
Human erythrocytes	2	3 (3x)	Scheurich	(23)	1981
Mouse L 1210	3.6	25	Berg	(19)	1981
Friend cells	2.7	20	Pilwat	(41)	1981
Sea urchin eggs	<1	≤50	Richter	(22)	1981
Human myeloma + B lymphocytes	3.5	7	Bischoff	(20)	1982
Mouse fibroblasts 3T3	7	50	Zimmerman	(45)	1982
Mouse myeloma + spleen cells	3	50	Berg	(18)	1982
Mouse erythroleukaemic erythroblasts	2.7	20	Zimmermann	(46)	1984
Mouse myeloma + B lymphocytes	4 (4x)	5	Lo	(47)	1984
Erythrocyte ghosts	5	200	Sowers	(48)	1986

MODELS FOR PULSE EFFECTS ON MEMBRANES

The various theories are listed in Table 5 and explained elsewhere (27). The induced dipole repulsion model is schematically shown in Figure 9. The main idea is similar to that of electrodichroism. Membrane proteins can be oriented suddenly by the field pulse into the field direction, thereby causing destruction of the bilayer and intermingling of the adjacent parts of two cells thus starting the fusion.

TABLE 5. Models for Pore Formation and Breakdown

TYPE	AUTHOR	REFERENCE
Electromechanical	Crowley	(49)
Pore enhancement	Chernomordik	(50)
Pore enhancement	Weaver	(51)
Induced dipole repulsion	Berg	(26)
Periodic block-lipids	Sugar	(52)

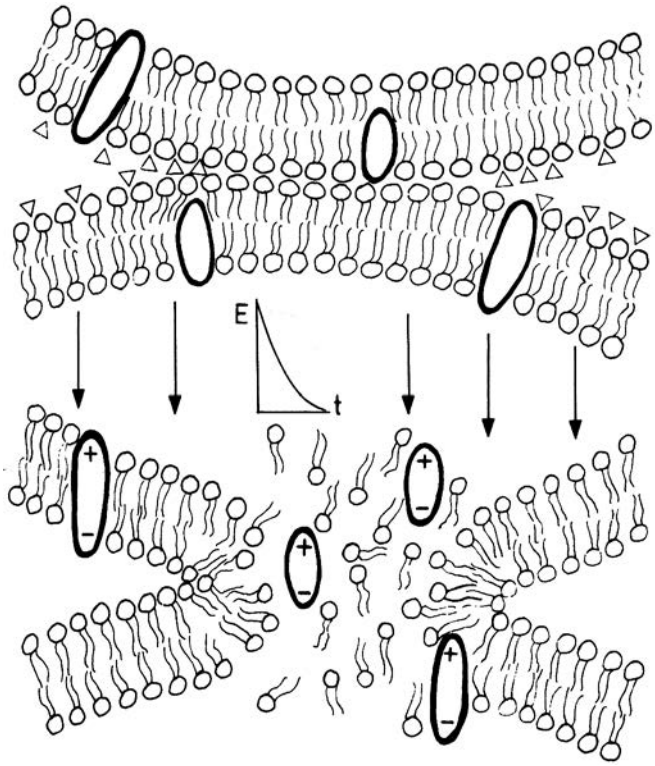

FIGURE 9. Scheme for breakdown fusion after a pulse caused by induced dipole formation and rectification.

SOME TRENDS

Using the three main techniques discussed (and some sophisticated modifications) one can assume that all fusion problems can be solved including inter-kingdom fusion between plant protoplasts and animal cells.

There are two main difficulties: (1) the validity of Equation (1) for cells of the same diameter and different membranes; and (2) the correct electrical window to guarantee the viability of the fusion products. Viability may be increased by using chemicals that stabilize or labilize the membranes. The combination of electrical and chemical techniques may yield a synergistic breakdown effect that decreases the applied electrical

energy and increases viability. In the future, quasi-continuous electrofusion using pulsating electromagnetic induced currents may be possible. In any event, it is clear that electrofusion is a powerful tool in genetics and bioprocessing.

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