Manipulating the genetic identity and biochemical surface properties of individual cells with electric-field-induced fusion

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A method for cell–cell and cell–liposome fusion at the single-cell level is described. Individual cells or liposomes were first selected and manipulated either by optical trapping or by adhesion to a micromanipulator-controlled ultramicroelectrode. Spatially selective fusion of the cell–cell or cell–liposome pair was achieved by the application of a highly focused electric field through a pair of 5-μm o.d. carbon-fiber ultramicroelectrodes. The ability to fuse together single cells opens new possibilities in the manipulation of the genetic and cellular makeup of individual cells in a controlled manner. In the study of cellular networks, for example, the alteration of the biochemical identity of a selected cell can have a profound effect on the behavior of the entire network. Fusion of a single liposome with a target cell allows the introduction of the liposomal content into the cell interior as well as the addition of lipids and membrane proteins onto the cell surface. This cell–liposome fusion represents an approach to the manipulation of the cytoplasmic contents and surface properties of single cells. As an example, we have introduced a membrane protein (γ-glutamyltransferase) reconstituted in liposomes into the cell plasma membrane.

single cell | electrofusion | microelectrode | heterokaryon

Cell–cell fusion occurs spontaneously in nature. Fertilization is the consequence of egg–sperm fusion, and multinucleated muscle cells are formed from fusion of myoblasts during embryonal development. *In vitro*, cell–cell fusion can be induced artificially by adding a fusiogenic agent, such as polyethylene glycol (PEG) (1), by subjecting cells in contact with each other to an electric field, which is called electrofusion (1), or by laser-based schemes (2, 3).

The application of an electrical field that reaches or exceeds the membrane breakdown potential induces formation of pores in phospholipid bilayer membranes. Pore formation within the contact zone between two cells may eventually lead to fusion, which is characterized by membrane and cytoplasmic continuity (1).

Electrofusion has developed into an efficient method for the fusion of mammalian cells, mainly because of its general applicability and mild conditions, which result in a high number of viable fusion products (4). This technique has been used in a wide variety of biological experiments, from the creation of hybridomas and new cell lines (4) to *in vitro* fertilization (5) and the production of cloned offspring, like the sheep Dolly and her equals (6).

Electric-field-induced fusion is widely used on a population of cells in suspension (with densities of about 10^5 cells/ml). Cells are first brought into contact by dielectrophoresis through the application of a low-amplitude high-frequency ac field, and subsequently a fraction of the cells are fused by a short high-voltage dc pulse. Bulk electrofusion of large quantities of cells is useful for creating new cell lines. One of the drawbacks of bulk electrofusions, however, is that it is impossible to select the individual cells to be fused. This feature leads to unwanted fusions between cells of the same cell type, and product-selection protocols need to be implemented. A simple way to obtain only the desired fusion product is to work with individual cells. The ability to fuse together single cells in a controlled manner represents a technique by which the long-term genetic identity and behavior of a select cell can be precisely manipulated. In combination with powerful measurement and imaging techniques, the genetic and biochemical nature of single cells can be controlled and studied in detail. Areas where single-cell fusion techniques would be preferable include, for example, cloning, *in vitro* fertilization, and studies of cell heterogeneity effects.

To address the challenge of working with individual cells and to overcome the shortcomings of bulk electrofusion, we have developed a single cell-pair electrofusion technique that also can be used for fusion between individual vesicles and proteoliposomes with cells. This method differs from previous electrofusion protocols (7, 8) in the use of carbon-fiber microelectrodes to produce tightly focused inhomogenous electric fields. The small dimensions of the electrodes (5-μm o.d.) and their precise positioning with high graduation micromanipulators allows for fusion of cells and vesicles to targeted cells in cellular networks growing on a substratum.

By fusing together single selected cells, the genetic and cellular makeup of individual cells can be manipulated in a controlled manner. The ability to selectively modify the membrane composition and contents of single cells by cell–vesicle fusion is anticipated to have useful biological applications, such as the insertion of membrane-bound receptors and the introduction of dyes, pharmaceutical compounds, and genes to the cytoplasm.

Materials and Methods

Optical Trapping, Microscopy, and Fluorescence Imaging. The optical trapping and fluorescence imaging systems (Fig. 1) were built in-house. The optical trap was formed by sending the output from a single-mode MOPA laser diode (model SDL-5762-A6, SDL, San Jose, CA) through a spatial filter (model 900, Newport, Fountain Valley, CA) and a dichroic mirror. The laser light is reflected from a polychroic mirror (Chroma Technology, Brattleboro, VT) placed inside the microscope (Nikon Diaphot or Leica DM IRB, Wetzlar, Germany). It is then focused to a diffraction-limited spot with a high numerical-aperture (n.a.) objective ($\times$100, n.a. 1.4, Nikon and $\times$100, n.a. 1.3, Leica, respectively). Fluorescence excitation was achieved by sending the 488-nm output of an argon ion laser (2025–05, Spectra-Physics) through a spinning disc. The spinning disc scatters the

Abbreviations: PEG, polyethylene glycol; CCD, charge-coupled device; γ-GT, γ-glutamyltransferase.

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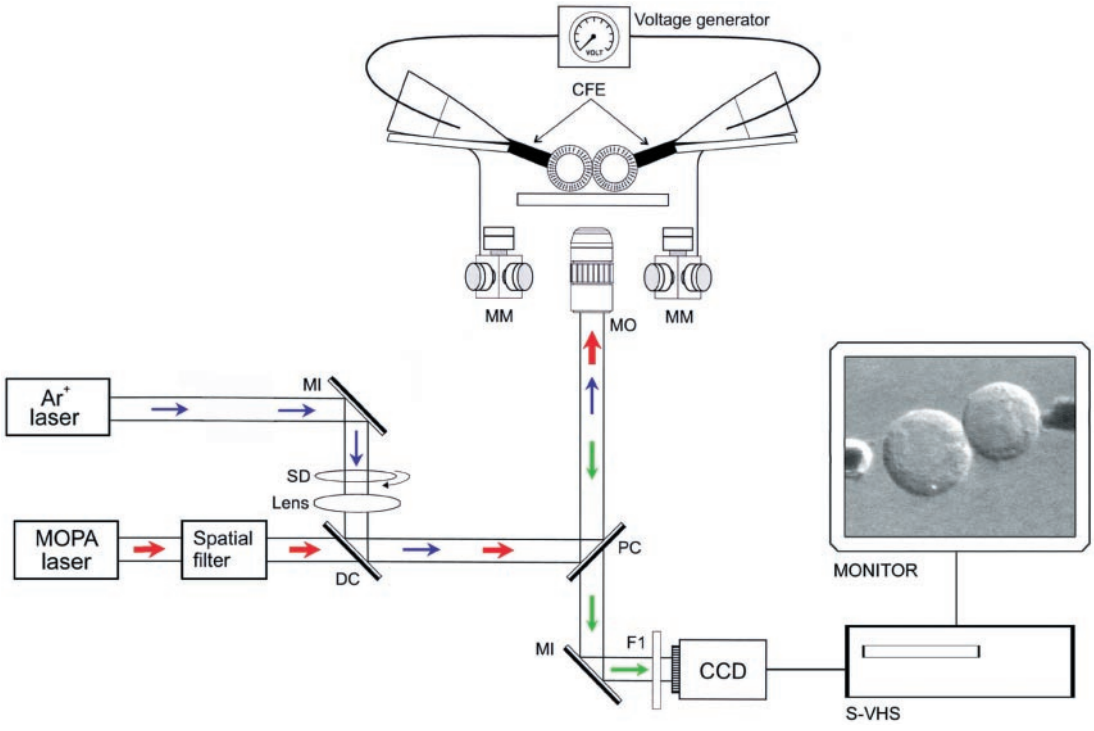

Fig. 1. A schematic drawing showing the experimental setup. Cells were added from suspension to microscope coverslips mounted in a polycarbonate holder. The cells were either prealigned by using optical trapping (MOPA laser) or simply by being pushed together with carbon-fiber electrodes (CFE) controlled by high-graduation micromanipulators (MM). The fusion pulse was applied through the CFEs (5-μm o.d.). For fluorescence imaging and optical trapping, two collinear laser beams were sent into the microscope objective (MO): (*i*) the 488-nm line of the argon ion laser (blue arrows) was used to excite fluorescein and FITC-labeled γ-GT, and (*ii*) the 992-nm output of the MOPA diode laser (red arrows) was used for optical trapping. The resulting fluorescence (green arrows) and bright-field images were directed to a CCD camera, recorded with a super-VHS video recorder (S-VHS), and continuously monitored (MONITOR). MI, mirror; DC, dichroic beamsplitter; PC, polychroic beamsplitter; F1, filter; SD, spinning disc.

laser light so that uniform illumination is achieved for fluorescence imaging. The scattered laser light from the disk was collected by a lens and reflected from a dichroic mirror (Chroma). This reflected light was sent into the microscope and was reflected by a polychroic mirror. Fluorescence and bright-field imaging was performed by a three-chip color charge-coupled device (CCD) camera (Hamamatsu, Kista, Sweden) and recorded by a Super VHS recorder (Panasonic). The CCD images were digitized from tape and were processed for presentation. RGB (red green blue) files were converted to CMYK (cyan magenta yellow black) file format for printing.

Electrofusion Instrumentation. For electrofusion experiments, cell dishes were mounted in a circular polycarbonate holder and transferred to the stage of an inverted microscope. Two carbon-fiber microelectrodes (Dagan Instruments, Minneapolis) with an o.d. of 5 μm were positioned on each side of the respective fusion partner by high-graduation micromanipulators (Narishige MWH-3, Tokyo). The two carbon-fiber electrode tips (anode and cathode) were positioned at an angle of 0–20° and 160–180° with respect to the object plane. Cells were fused with multiple 1-ms pulses by using a pulse generator (Digitimer Stimulator DS9A, Welwyn Garden City, U.K.) or a home-built variant. For dielectrophoresis, the alternating field was produced by a sweep function generator (model 9205, Hung Chang, Taiwan). All fusions were performed at 20°C. The voltages and field strengths reported are nominal and not corrected for electrochemical reactions.

Fusion Media. The electrofusion buffer for Jurkat cells was a Hanks' Hepes solution at 300 mosM. It consists of 137 mM NaCl/5.4 mM KCl/0.41 mM $MgSO_4$/0.4 mM $MgCl_2$/1.26 mM $CaCl_2$/0.64 mM KH_2PO_4/3.0 mM $NaHCO_3$/5.5 mM D-glucose/20 mM Hepes, pH adjusted to 7.4 with NaOH. For fusion of Cos 7 cells and vesicle–cell fusions, a 300-mosM Hepes saline buffer (140 mM NaCl/5 mM KCL/10 mM D-glucose/1 mM $MgCl_2$/1 mM $CaCl_2$/10 mM Hepes) was used. NG 108–15 cells were fused in a 200-mosM buffer, consisting of Hepes saline solution diluted with deionized water. In PC 12 and PC 12-NG 108–15 fusions, a 75-mosM electrofusion medium was used (0.07 M sorbitol/0.1 mM calcium acetate/0.5 mM magnesium acetate).

For the NG 108–15 and Cos 7 cell–cell fusion experiments, 5% PEG 4,000 was added to the fusion media, and in the cell-vesicle fusions 1.25% DMSO was added.

Cell Culture. Jurkat, NG 108–15, PC 12, and Cos 7 cells were cultured according to standard procedures. For homokaryon and cell–vesicle fusions, NG 108–15 cells were treated with 2 mg/ml protease (from *Aspergillus oryzae*) for 5–30 min in an incubator (37.25°C, 90% humidity and 5% CO_2 atmosphere) to establish close membrane contacts.

Preparation of Vesicles and Proteoliposomes. Vesicles from L-α-phosphatidylcholine in chloroform were obtained in high yield by using a rotary evaporation method (9). Fluorescently labeled γ-glutamyltransferase (γ-GT) was prepared by reacting γ-GT with 2.3 mM FITC. Unreacted FITC was removed by running the γ-GT-FITC solution through an Econopac 10DG column (Bio-Rad, with a 6,000 D cutoff. Labeled γ-GT was then incorporated into the vesicles during their formation process.

Preparation of Poly-L-Lysine-Coated Coverslips. Coverslips (borosilicate, no. 1, Kebo, Göteborg, Sweden) were washed in 70%

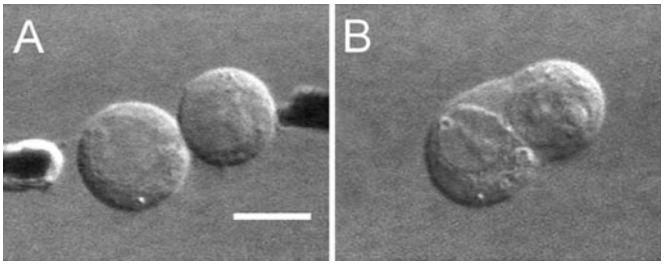

Fig. 2. Creation of a PC-12 homokaryon. The cells were originally located at a distance of about 100 μm from each other. By using the microelectrodes, they were placed next to each other. The cells were pretreated with ac dielectrophoresis (0.3–3 kV/cm, 1 MHz for 1 min), in hypoosmotic sorbitol fusion medium. *A* shows cells immediately after dielectrophoresis, and a close contact is visualized by the flattened contact zone between the cells. After dielectrophoresis for 30 s, five dc fusion pulses were applied (3 kV/cm, 1-ms duration for each pulse, at approximately 1 Hz), after which an immediate broadening of the contact zone between the cells could be visualized. Within 1 min, a cytoplasmic continuity was observed, and after 2 min, the shape had stabilized, as shown in *B*. Bar = 10 μm.

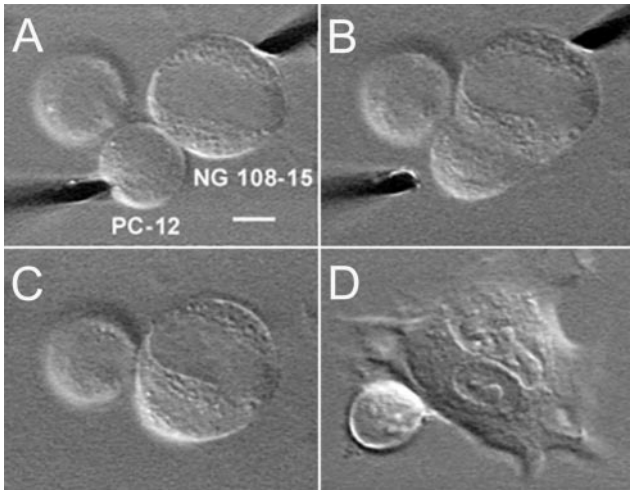

Fig. 3. PC 12 x NG 108–15 heterokaryon formation. The NG 108–15 cells are growing on the glass surface (processes out of focus), and PC 12 cells were added from suspension. In *A*, the cells are shown during ac dielectrophoresis (1–1.8 kV/cm, 1 MHz, for 1 min). Dielectrophoresis and electrofusion were performed in a hypoosmotic sorbitol fusion medium. (*B*) Twenty seconds after the dc fusion pulse (1.8 kV/cm, 1 ms), the PC 12 cell appears to be fused to the NG 108–15 cell, but the resulting membrane of the heterokaryon is not fully reorganized. After about 40 s, the fusion of the two cells appears to be complete (*C*). After growth in cell-culture media for 20 h in incubator (37°C, 90% humidity and 5% CO_2 atmosphere), the heterokaryon developed new processes and grows on the glass surface (*D*). The third cell (also a PC 12 cell) (*Upper Left*), which is attached to the PC 12 cell, remains unaffected throughout the fusion process. In the first three panels, it is slightly swollen from the hypoosmotic fusion media. Bar = 10 μm.

ethanol/water solution (vol/vol) followed by Milli-Q-water. The glasses were placed in a poly-L-lysine/Milli-Q-water solution [0.1% (wt/vol)]. Coverslips were mounted in a circular polycarbonate holder. One milliliter of vesicle solution was added. After 30 min, a sufficient number of vesicles were immobilized, and the outside solution could be changed and cells added.

Micromanipulation of Vesicles and Cells by Using Microelectrodes. For translation of vesicles to different locations on both uncoated and poly-L-lysine-coated borosilicate surfaces, carbon-fiber microelectrodes controlled by high-graduation *xyz*-micromanipulators (0.2-μm resolution) were used to loosen the vesicles from the surface by a combined pushing/scraping movement. After the vesicles were detached from the surface, they adhered to the electrode tip and could be moved over long distances before being placed close to a cellular target. To determine whether significant loss of the vesicle content is a result of this handling, vesicles were primed with fluorescein (10 μM), attached to the coverslips, and washed thoroughly so the fluorescence background was significantly reduced. Comparison between the intravesicular fluorescence intensity before and after moving the vesicles did not show signs of substantial loss of content (data not shown).

Chemicals and Materials. Hepes (>99%), sodium chloride, potassium chloride, and sodium hydroxide (all Suprapur), calcium dichloride, magnesium dichloride, magnesium sulfate, potassium dihydrogen phosphate, PEG 4000, and sodium hydrocarbonate, were purchased from Merck. D-glucose (AnalaR) was from BDH and L-α-phosphatidylcholine (powder from fresh egg yolk), poly-L-lysine, γ-GT, protease (type XXIII, from *Aspergillus oryzae*), fluorescein (sodium salt), and DMSO were obtained from Sigma. D-Sorbitol ("Baker" grade) and calcium acetate ("Baker analyzed" reagent) were from J. T. Baker, and magnesium acetate (analytical grade) was from Amresco, Euclid, Ohio. FITC was from Molecular Probes. Deionized water from a Milli-Q system (Millipore) was used.

Results and Discussion

Micromanipulation and Fusion. To fuse a cell or vesicle to another cell, individual manipulation of at least one of the fusion partners is required for alignment. It is well documented that individual cells, as well as other biological structures, including organelles of small dimension, can be manipulated and moved at will in solution by using optical trapping with highly focused laser beams (10–13). Here, a cell in solution was trapped by an IR laser beam and brought into contact with an adherent cell, by using the setup schematically shown in Fig. 1.

As an alternative to positioning with optical tweezers, the carbon-fiber microelectrode tips were used to place the cells or vesicles on the glass surface next to a target cell. This procedure works well for cells and vesicles newly seeded onto uncoated glass surfaces and poly-L-lysine-coated glass surfaces. The selected cell or vesicle was detached from the surface by pushing the electrode tip against the vesicle roughly parallel to the object plane. The vesicle/cell then adhered lightly to the electrode tip and could be placed close to a target cell. The movements of the electrodes were performed by using high-graduation micromanipulators. One advantage of this technique over optical trapping is the much larger force that can be applied to the vesicles and cells.

For electrofusion, the same pair of 5-μm o.d. carbon-fiber microelectrodes was used. Fig. 1 shows the geometrical arrangement of the electrodes with respect to a pair of cells prealigned for fusion. For cell–cell and cell–vesicle fusion, tightly focused rectangular-waveform electrical fields (nominally 3 kV/cm for a duration of 1 ms) were applied by using a low-voltage pulse-generator. In addition to enabling fusion of single cells grown on a substratum, the tightly focused electric field minimizes the risk for unwanted fusion or electroporation of surrounding cells.

Cell–Cell Fusion. Fig. 2 shows the formation of a homokaryon composed of two PC 12 cells. These particular cells were fused in a hypoosmolar sorbitol medium. The cells were aligned by using the microelectrodes and pretreated with dielectrophoresis (0.3–3 kV/cm, 1 MHz, 60 s). Although cells can be pushed tightly together with the electrodes, we found that pretreatment of aligned cells with dielectrophoresis significantly en-

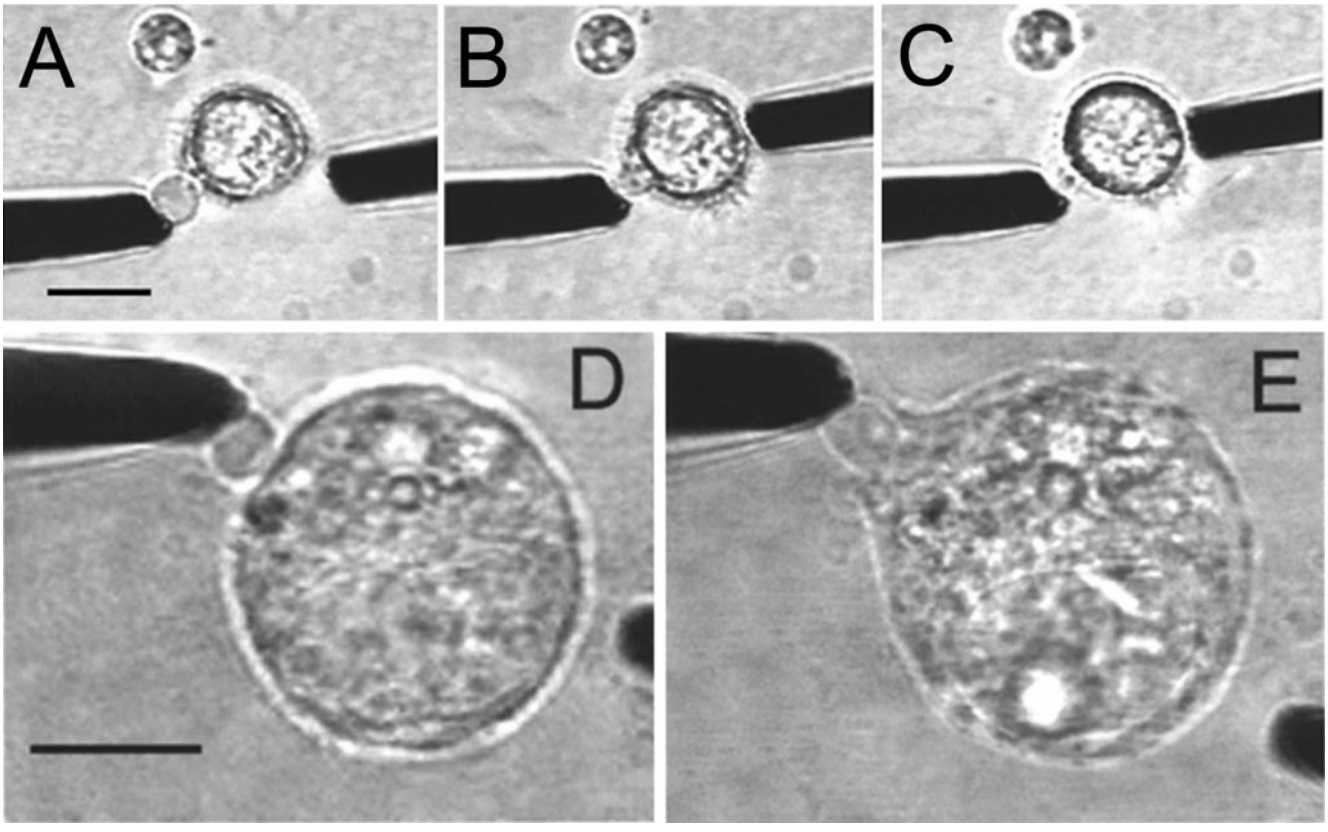

Fig. 4. Bright-field images taken (*A*) before, (*B*) during, and (*C*) after the electrofusion (≈8 kV/cm, 4 ms) of a PC vesicle (*Left*) with a Cos 7 cell (*Right*) in Hepes-buffered saline solution. To assist vesicle–cell electrofusion, 1.25% dimethyl sulfoxide and 20% Milli-Q-water was added to the external buffer solution. *D–E* show fusion or hemifusion between a NG 108–15 cell (protease-treated for 30 min) and a PC vesicle with incorporated γ-GT. The resulting fluorescence (not shown) from the vesicle, after fusion, was too weak to detect by the CCD camera used for these experiments. The fusion protocol was the same as in *A–C*. In *E*, the microelectrode is gently pulled away from the cell, and the vesicle (which is attached to the microelectrode) is stretched but does not detach from the cell. Bar = 10 μm.

hances fusion yields. This result is probably caused by the much closer membrane contact formed between the cells, as the alternating field causes cells to become dipoles. The polarity is induced in the interior of the cell as a result of ac-induced charge separation, and the attractive forces from these intracellular dipoles are higher than the repulsive forces from net surface charges (1).

Fig. 3 shows the creation of a heterokaryon between a PC 12 and a NG 108–15 cell. These cells were fused under similar conditions as the PC 12 cells above. However, the fusion success rate was generally higher fusing these two cell types, compared with the homokaryon fusions we have performed (Cos 7, NG 108–15, Jurkat and PC 12 cells). Different cell types require slightly different conditions, voltage amplitudes, pulse lengths, and media for optimal results (1). Although we did not perform detailed experiments to find optimal conditions for fusing the respective cell types, fusion yields of about 20% were accomplished for heterokaryon production by using this method.

It is well known that electrically fused cells are viable. The viability of the resulting multinuclear cells, in our experiments, was demonstrated by the ability of these cells to adhere and grow on the surface of the coverslip for several hours after fusion. Fig. 3*D* shows a PC 12 x NG 108–15 heterokaryon 20 hr after fusion.

Because of the presence of cytoskeleton, a fused cell takes much longer to reorganize its membrane and become spherical, compared with vesicles. For example, typical vesicle–vesicle fusion, by using an identical experimental setup, is in the millisecond regime (14). For some cells with extensive cytoskeletal scaffolding, membrane reorganization can take minutes (1). It was generally observed that fusion of cells preelectroporated in a 200-mosM buffer were much faster than the fusion of cells held in an isotonic 300-mosM buffer. The fusion process from initial cytoplasmic continuity to totally fused cells in an isotonic medium took several hours. This finding is consistent with earlier accounts on the impact of the fusion medium on membrane fusion events, like exocytosis (15) and cell–cell fusion (16, 17). It has been proposed that the electrofusion enhancement reached by using hypoosmotic fusion media is caused by spectrin denaturation (18, 19).

Cell–Vesicle Fusion. Previous studies of cell fusion have demonstrated that in addition to mammalian cells of other phenotypes, synthetic vesicles as well as plant protoplasts can be fused to mammalian cells (20). Synthetic vesicles have been fused with cells by using some kind of chemical treatment or such, for example PEG (21). Synthetic lipid films have been incorporated in the plasma membrane in cells by gentle contact between a lipid-coated glass micropipette and the cellular membrane (22). Fusion schemes involving synthetic vesicles can be used for purposes of transferring cell-impermeant molecules into a target cell through mixing of contents and for transfer of membrane lipids and membrane-associated structures, such as proteins, into the target cell membrane. The ability to fuse single vesicles to adherent cells grown on a substratum with this technique was explored. Fig. 4 *A–C* shows the fusion of a PC vesicle to a single adherent Cos 7 cell. To demonstrate the potential of introducing vesicle-incorporated proteins into a cellular structure, proteoliposomes with the membrane-bound protein γ-GT were fused to single NG 108–15 cells (Fig. 4 *D* and *E*).

We note that fusion of vesicles with cells was much more

difficult to accomplish than cell–cell fusions. Both addition of DMSO (≈2%) to 300-mosM fusion media and use of 200-mosM fusion media were found to facilitate fusion. DMSO has been shown to facilitate the uptake of DNA by using electroporation and to increase the yield of fusion. Similarly, the use of hypoosmolar medium is known to increase the fusion yield of cells. Still, in our vesicle–cell fusion experiments, the yield was low, about 2–3%, and rarely did the vesicles reorganize completely with the cell membrane. An explanation for this low yield could be the multilamellar nature of the vesicles, so that the presence of the inner membrane layers inhibits fusion.

Conclusions

This paper presents, to our knowledge, the first selective electrofusion of cells. This method offers the advantage of cell-selective fusion to adherent cells with a high spatial resolution, which offers the possibility of creating complex cellular networks. Although demonstrations of single-cell fusion by using highly focused UV laser beams exist (1, 2), the technique described here is based on a well-documented and highly efficient physical principle for hybridoma production. Furthermore, it is inexpensive and does not rely on the use of damaging UV radiation. The method might become useful for cloning on the single-cell level and for *in vitro* fertilization. In particular, the present technique overcomes a major shortcoming of bulk electroporation for cloning in that it offers complete control over cell selection and the fusion process.

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1. Neil, G. A. & Zimmermann, U. (1993) *Methods Enzymol.* **220,** 174–196.
2. Steubing, R. W., Cheng, S., Wright, W. H., Numajiri, Y. & Berns, M. W. (1991) *Cytometry* **12,** 505–510.
3. Weber, G. & Greulich, K. O. (1992) *Int. Rev. Cytol.* **133,** 1–41.
4. White, K. L. (1995) *Methods Mol. Biol.* **48,** 283–293.
5. Ogura, A. & Yanagimachi, R. (1995) *Reprod. Fertil. Dev.* **7,** 155–159.
6. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. S. (1997) *Nature (London)* **385,** 810–813.
7. Vienken J., Zimmermann, U., Fouchard, M. & Zagury, D. (1983) *FEBS Lett.* **163,** 54–56.
8. Schweiger, H.-G., Dirk, J., Koop, H.-U., Kranz, E., Neuhaus, G., Spangenberg, G. & Wolff, D. (1987) *Theor. Appl. Genet.* **73,** 769–783.
9. Moscho, A., Orwar, O., Chiu, D. T., Modi, B. P. & Zare, R. N. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11443–11445.
10. Ashkin, A. (1970) *Phys. Rev. Lett.* **24,** 156–160.
11. Uchida, M., Sato-Maeda, M. & Tashiro, H. (1995) *Curr. Biol.* **5,** 380–382.
12. Chiu, D. T., Hsiao, A., Gaggar, A., Garza-Lopez, R. A., Orwar, O. & Zare, R. N. (1997) *Anal. Chem.* **69,** 1801–1807.
13. Chiu, D. T., Lillard, S. J., Scheller, R. H., Zare, R. N., Rodriguez-Cruz, S. E., Williams, E. R., Orwar, O., Sandberg, M. & Lundqvist, J. A. (1998) *Science* **279,** 1190–1193.
14. Chiu, D. T., Wilson, C. F. W., Ryttsén, F., Strömberg, A., Farre, C., Karlsson, A., Nordholm, S., Gaggar, A., Biren, P. M., Moschp, A., *et al.* (1999) *Science* **283,** 1892–1895.
15. Zimmermann, U., Gessner, P., Schnettler, R., Perkins, S. & Foung, S. K. H. (1990) *J. Immunol. Methods* **134,** 43–50.
16. Chernomordik, L. V. & Sowers, A. E. (1991) *Biophys. J.* **60,** 1026–1037.
17. Sowers, A. E. (1995) *Biophys. J.* **69,** 2507–2516.
18. Weldon Jones, C., Mastrangelo, I. A., Smith, H. H., Liu, H. Z. & Meck, R. A. (1976) *Science* **193,** 401–403.
19. Seibicke, S., Zimmermann, H.-P. & Haeffner, E. W. (1988) *Biochim. Biophys. Acta* **944,** 487–496.
20. Laffafian, I. & Hallett, M. B. (1998) *Biophys. J.* **75,** 2558–2563.