Mechanically Facilitated Cell-Cell Electrofusion

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ABSTRACT Apparatus and methods were developed to enable mechanically facilitated cell-cell electrofusion to be performed. The apparatus and methods mechanically place cells in contact before fusion. The key component of this fusion system was a newly developed fusion chamber. The chamber was composed of two functionally identical electrodes that were housed in a multi-layer structure. The layers functioned as support for the electrodes. They also allowed adjustment of the distance between opposing electrode faces. The electrodes were constructed in a manner that allowed cells to be deposited, by vacuum, onto each face. Electrode faces were positioned at a predetermined distance from each other to mechanically force cell-cell contact between the deposited cells. Fusion was induced by delivering direct current pulses to the juxtaposed cells. Fusion products were detected and quantitated by flow cytometry. Details of the chamber design and a protocol for using the fusion chamber are given. Mechanically facilitated cell-cell electrofusion was demonstrated by using the chamber to produce fusion products from like fusion partners. The practical applicability of the chamber was demonstrated by fusing unlike cell types. Mechanically facilitated cell-cell electrofusion is not specific to the cells used in this study; the chamber can be adapted for use with other cell types.

INTRODUCTION

The first published observation of cell-cell electrofusion was by Senda et al. (1979). Since then, a number of practical and research applications of electrofusion have been realized. Cell-cell electrofusion (CCE) has been used for the production of monoclonal antibodies (Foung and Perkins, 1989; Lo et al., 1984), transfer of membrane components (Grasso et al., 1989; Heller and Grasso, 1990; Heller and Gilbert, 1992), and the production of hybrid cells (Glass, 1988; Hewish and Werkmeister, 1989; Wojchowski and Sytkowski, 1986). In addition, membrane interactions and the phenomena of electrofusion (Abidor and Sowers, 1992; Sowers, 1988; Lojewska, 1989) have been investigated.

Most methods for performing CCE involve delivering direct current pulses to fusion partners that have been forced into close contact. Exponentially decaying DC pulses (Sowers, 1986, 1989) and rectangular pulses (Heller and Grasso, 1990; Teissie et al., 1982) have both been successfully used. The differences between published methods lie primarily in the means of forcing cell-cell contact. Dielectrophoresis is one method that has been widely used to induce prefusion contact. Adherence, chemicals, and centrifugation have also been used to achieve contact.

Dielectrophoresis is the most commonly used method for achieving contact (Zimmerman, 1982; Dimitrov et al., 1990; Marszalek et al., 1990; Pohl and Crane, 1971). It involves passing alternating current (AC) through suspended cells before DC pulse delivery. Alternating current causes cells to align into chains with regions of contact between adjacent cells. Electrical conditions that yield chain formation are specific to the cell types under investigation. Heating caused by AC (joule heating) is a potential problem with dielectrophoresis. A common remedy for joule heating is to conduct fusion in a low electrolyte media. These types of media may alter cellular integrity because they are not physiologically balanced.

Adherence methods exploit the regions of contact between cells cultured to confluence. Plated cells consisting of the same (Teissie et al., 1982; Teissie and Blangero, 1984) and different cell types (Finaz et al., 1984; Sukharev et al., 1990) have been used. The cell type(s) used for this contact method must be adherent. Chemical methods have included the use of an avidin-biotin complex (Lo et al. 1984; Hewish and Werkmeister, 1989; Wojchowski and Sytkowski, 1986; Bakker Schut et al., 1993) to juxtapose fusion partners. These methods produce highly specific contact, but they also introduce molecules on cell surfaces that may influence fusion. Centrifugation methods reverse the order of contact and pulse delivery. Suspended cells are pulsed and then centrifuged to force contact (Teissie and Rols, 1986; Montane et al., 1990). These methods can be used with adherent and nonadherent cell lines. Centrifugation must be conducted in a manner that will not damage the electrically treated cells.

All of the current methods for achieving cell-cell contact have advantages as well as limitations. None of the methods are applicable for all situations. Mechanically facilitated cell contact could provide a universal prefusion contact procedure. Such a contact method would also provide an alternative that would curtail the criteria of adherence, avoid the use of chemicals, and allow the use of physiologically balanced fusion media. Therefore, a study was initiated to develop a device that utilized mechanical force to facilitate cell-cell electrofusion. To demonstrate mechanically facilitated CCE, the study was also directed toward establishing procedures for using the device to produce heterohybrids.

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MATERIALS AND METHODS

Cell lines and culture methods

Murine WEHI7.1 lymphoma cells (ATCC TIB 53; American Type Culture Collection, Rockville, MD) were used for the majority of the experimental work done for this study. The cell line was grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Washington, D.C.) supplemented with 9% heat-inactivated (56°C, 30 min) fetal bovine serum (Hyclone, Logan, UT) and 90 μg/ml gentamicin (Gibco, Grand Island, NY). Cultures were seeded with 3.5×10^5 viable cells/ml and were harvested approximately 48 h after seeding. Cell densities were approximately 1.9×10^6 cells/ml at the time of harvest and typically had 96% viability or greater. The mean diameter of WEHI7.1 cells at the time of harvest was 13.5 μm. All cultures were grown in plastic tissue culture flasks (Costar, Cambridge, MA) at 37°C in a humidified air containing 5% CO_2.

Human HL60 promyelocytic leukemia (ATCC CCL 240) cells were also used for this study. This second cell line was grown in the same mixture of medium, fetal bovine serum, and gentamicin that was used for WEHI7.1 cultures. They were also grown in the same flasks under identical environmental conditions as WEHI7.1 cultures. Human HL60 cultures were seeded with viable cell concentrations of 6×10^5 cells/ml and harvested 96 h after seeding. At the time of harvest, the mean diameter of HL60 cells was 17.5 μm.

Fusion product detection and quantitation

Fusion product detection and quantitation procedures utilized two different vital fluorescent dyes in conjunction with a flow cytometer. These dyes were 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR) and 5-(and 6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR; Molecular Probes). Equal numbers of CMFDA-stained and CMTMR-stained cells were fused. Flow cytometry was used to detect and quantitate dual fluorescing hybrids that resulted from DC pulse delivery. Cytometry was also used to detect and quantitate unfused CMFDA- and CMTMR-stained cells that remained mixed with the hybrids after fusion.

Vital fluorescent dyes

The vital fluorescent dyes used for this study, CMFDA and CMTMR, were both supplied in 1-mg aliquots by the manufacturer. Each dye was used as a 5 mM stock solution in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO). Stock solutions of CMFDA (M_r = 465) were mixed by dissolving the supplied 1-mg aliquot in DMSO to yield a final volume of 430 μl. Similarly, CMTMR (M_r = 554) stock solutions were made by adding the supplied 1-mg aliquot of CMTMR in DMSO to yield a final volume of 360 μl. Both dyes were easily dissolved in DMSO at room temperature. Stock solutions were stored at 4°C and yielded consistent results if used within several months.

Cell staining protocol

Before fusion, one fusion partner was stained with CMFDA and the other with CMTMR. The staining procedure used was a modification of the manufacturer's recommendations. Briefly, cell density and viability were determined at the time of harvest using a hemacytometer and trypan blue exclusion dye. All staining was conducted at cell densities of 1.5×10^6 cells/ml in growth medium. Murine WEHI7.1 cells were stained in 0.30 μM CMFDA and in 6.0 μM CMTMR. Human HL60 cells were stained in 4.5 μM CMTMR. Cells were incubated in the presence of their respective dyes at 37°C for 30 min, washed once with DMEM, resuspended in DMEM, and then incubated for 1 h at 37°C. After the second incubation period, the stained cells were washed 3 times with phosphate-buffered saline (PBS; Mediatech) by centrifugation ($225 \times g$). Cell density and viability were determined using a hemacytometer and trypan blue exclusion dye after the final PBS wash (26).

Flow cytometry

Fusion products were detected and quantitated using a flow cytometer (Becton Dickinson Model FACStar Plus, Becton Dickinson, San Jose, CA). An 80 mW argon laser tuned to a 488 nm excitation wavelength was used. The FL1 (green, 530 ± 15 nm) cytometer channel was used to detect CMFDA emission; CMTMR emission was detected in the FL2 (red, 585 ± 21 nm) channel. All samples included acquisition of data from 20,000 events. Forward light scatter (FSC) and side light scatter (SSC) data were collected for each sample in addition to fluorescent data. Events were triggered on the FSC signal.

Analysis of cytometric data from a set of CCE samples was a multistep process that utilized FSC, SSC, FL1, and FL2 information acquired from each post-fusion sample. All sample sets included fusion negative control samples. These cells did not receive fusogenic DC pulses. However, they were processed in a manner identical to samples that received DC pulses to induce cell-cell fusion.

Fig. 1 shows a typical FSC versus SSC dot plot from a fusion negative control sample. The sample was from the fusion of CMFDA-stained WEHI7.1 cells to CMTMR-stained WEHI7.1 cells. A region (R1) containing the major population was identified. Typically, the region contained greater than 95% of all the events acquired. Fluorescent information from the cells contained in the region (R1) was then displayed in the form of FL1 versus FL2 surface plots. Fig. 2 illustrates two major fluorescent populations that were present in fusion negative control samples. The surface near the FL1 axis represents the CMFDA-stained WEHI7.1 cells, and the surface located near the FL2 axis indicates unfused CMTMR-stained WEHI7.1 cells.

Analysis procedures for samples that received fusogenic pulses were similar to those of the negative control samples. However, plots from pulsed samples contained an additional population. This population contained fusion products that were comprised of CMFDA- and CMTMR-stained cells. These dual fluorescing hybrids were evident in region H shown in Fig. 3. The figure shows a plot that resulted from fusion using WEHI7.1 cells as both partners and is typical of fusion samples from like as well as unlike cell types. Quantitation of the hybrid population (H) was the basis for rating fusion success. Hybrid yields were expressed as a percentage of the total number of events analyzed minus the percentage of dual fluorescing cell aggregates that was present in fusion negative control samples (Jaroszeski et al., 1994).

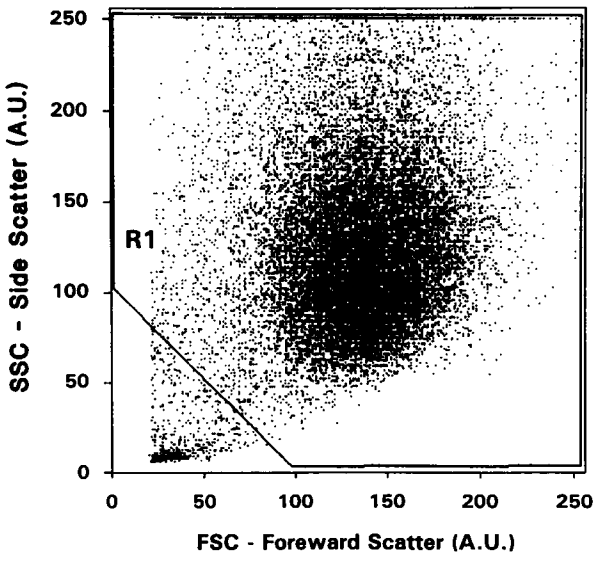

FIGURE 1 Typical FSC versus SSC dot plot from a fusion negative control (not pulsed) sample. The region R1 indicates the major population used for fluorescent analysis. The sample shown contained CMFDA-stained and CMTMR-stained WEHI7.1 cells as fusion partners. FSC and SSC data are expressed in arbitrary units (A.U.).

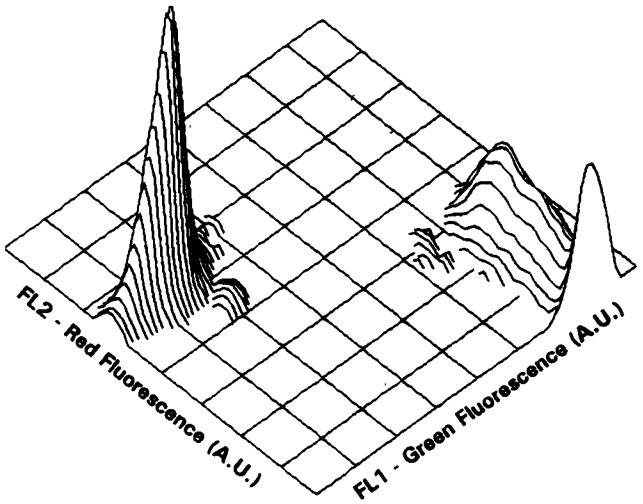

FIGURE 2 FL1 versus FL2 surface plot for a typical fusion negative control sample. The surface near the FL1 axis was CMFDA-stained WEHI7.1 cells, and the surface near the FL2 axis was CMTMR-stained WEHI7.1 cells. The relative number of cells is indicated by surface height. Fluorescence is expressed in arbitrary units (A.U.).

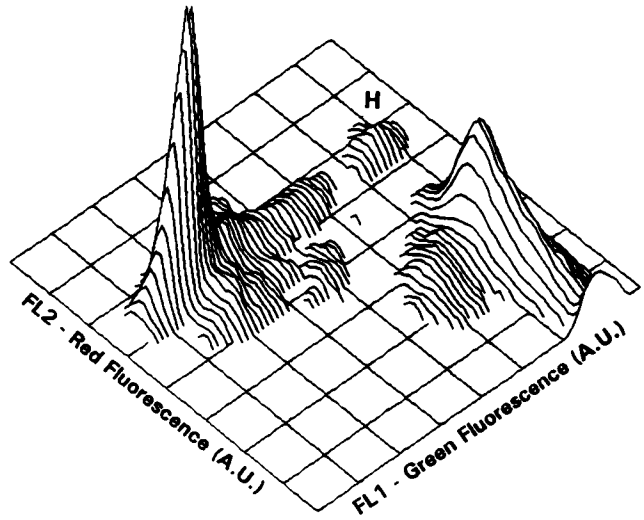

FIGURE 3 FL1 versus FL2 surface plot from a typical electrofusion sample. Dual fluorescing hybrid cells were present and quantitated as the surface labeled H. The relative number of cells is indicated by surface height. Fluorescence was expressed in arbitrary units (A.U.). The fusion sample shown contained CMFDA-stained WEHI7.1 cells and CMTMR-stained WEHI7.1 cells as fusion partners. Fusion was induced using 10 rectangular direct current pulses with a duty cycle of 1 s, a 50 μs pulse width, and an electric field strength of 1500 V/cm. Cell-cell contact was mechanically facilitated using a custom-built fusion chamber an electrode spacing calibrated to 120 μm.

The hybrid cell surface H, as shown in Fig. 3, was the most important difference between the cytometric results from electrofusion samples and fusion negative control samples. However, the surface plots for electrofusion samples and negative control samples consistently exhibited other differences. The shapes of the contours near the FL1 and FL2 axes were extended toward the dual fluorescing region for electrofusion (pulsed) samples. A comparison of the surface plots given as Figs. 2 and 3 reveals these differences. Based on extensive microscopic examination of sorted cells, regions of pulsed sample surfaces that were distorted when compared with unpulsed samples contained electrically damaged cells and/or cellular debris.

Fusion events of two or more cells that exhibited the same fluorescence were detected on fluorescent plots within their respective surfaces the FL1 or FL2 axes. These types of homofusion products could not be cytometrically quantitated because they exhibited the fluorescence of a single stain. Therefore, they were present in the surfaces near the axes of fluorescent plots mixed with single unfused cells. Observation of cells that were sorted from the surfaces at either axis was used to confirm the presence of single fluorescing fusion products.

Cell-cell electrofusion apparatus

The apparatus used to perform electrofusion was a collection of instruments and a fusion chamber. Instrumentation included a commercial electrofusion pulse generator (Model T800, BTX, San Diego, CA), an oscilloscope (Philips Model PM3375, Philips, Eindhoven, The Netherlands), and an IEEE-488 data acquisition system supported by a personal computer. The generator produced rectangular pulses with a duty cycle of 1 pulse per second. The number of pulses as well as their amplitude and duration could be adjusted. Pulse amplitudes could be set from 0 to 950 V. The generator could be adjusted to deliver pulses that ranged from 0 to 99 μs, and the number of pulses could be set from 0 to 99. The oscilloscope, computer, software, and IEEE-488 system provided the system with characterization capacity. The oscilloscope had the capability to digitize and temporarily store measured waveforms. A custom designed software package was written to handle data transfer, file naming, storage, graphics, and hard copy output.

The custom-built fusion chamber was composed of two electrodes that were contained in a multi-layer plexiglass housing. One of the electrodes was adjustable, and the other was in a fixed position. Electrode spacing was calibrated by installing a metal disk with a known thickness onto the face of the fixed electrode. After installation, the adjustable electrode was moved toward the fixed electrode until it was stopped by the disk. A setscrew was used to hold the adjustable electrode in the calibrated position.

RESULTS AND DISCUSSION

Mechanically facilitated CCE was conducted in a multilayered fusion chamber. Fig. 4 shows the completely assembled chamber. The device consisted of three plexiglass layers (*a–c*). The layers functioned as support and to align two functionally identical electrodes (*d* and *e*). The three-layer design also allowed the chamber to be disassembled to make the space between the electrodes accessible for loading and unloading cells. The position of the upper electrode could be fixed by tightening the setscrew (*f*) after electrode spacing adjustment.

Fig. 5 is a view of the disassembled fusion chamber. Both of the functionally identical electrodes were composed of a hollow stainless steel body (*a*) with a connection for vacuum at one end (*b*). The opposing ends of each electrode contained porous stainless steel frits (*c*). Both frits were 5.5 mm in diameter and had particle retention sizes of 20 μm (Model 707325, Altech Associates, Inc., Deerfield, IL). A porous polycarbonate track etch membrane (PCTE; Poretics Corporation, Livermore, CA) was placed on the end of each electrode to cover the frits. Membranes were cut so that their diameters covered only the electrode ends. Membranes with a pore diameter of 0.6 μm, pore density of 3×10^7 pores/cm^2, and thickness of 15 μm were used exclusively. The membranes served as a matrix for forming and mechanically contacting cell layers.

Before mechanically facilitated CCE could be demonstrated, a protocol for using the chamber had to be developed.

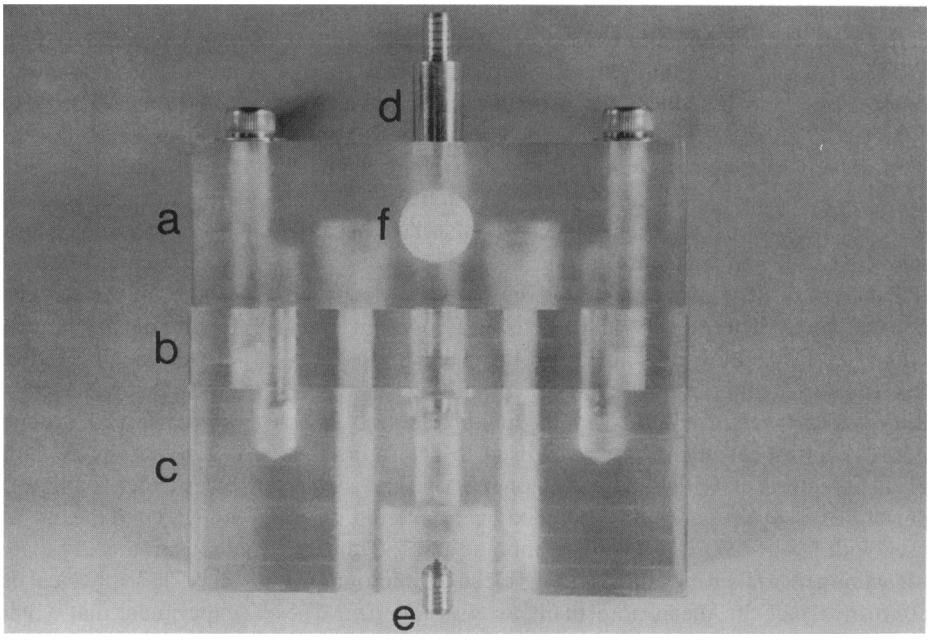

FIGURE 4 Completely assembled fusion chamber. (*a–c*) Plexiglass layers. (*d* and *e*) Functionally identical electrodes. (*f*) Setscrew.

The protocol involved installing PCTE membranes at each electrode face while the layers of the device were completely disassembled. Next, CMFDA- and CMTMR-stained cells were placed onto each membrane. This was achieved by placing 1×10^6 cells suspended in a small volume of PBS onto each membrane. Vacuum was applied to each electrode (150 mmHg) to draw and hold the cells against the membranes. After the cells were deposited onto the membranes, the chamber was completely assembled. Then, vacuum was removed from both electrodes. Finally, fusion was induced by delivering one or more DC pulses to the electrodes. Fusion products were unloaded from the chamber 2 min after pulse delivery by gently removing the membranes and disbursing the cells in PBS.

Fig. 6 illustrates some of the geometric details near the electrode faces during fusion and emphasizes the mechanical nature of the induced cell-cell contact. For clarity, only the ends of each electrode, PCTE membranes, and cell layers are shown. Note that the cell layers are held in contact with each other by the mechanical force of the electrodes. Also note that there are multiple cell layers between the membranes. Deposition onto each membrane contributed one-half of the cells between the electrodes.

Two different options for forming cell layers were used in this study. The first option utilized CMFDA-stained WEHI7.1 cells on one of the membranes and CMTMR-stained WEHI7.1 cells on the other. This type of deposition would result in contact of oppositely stained cells in the middle two cell layers between the membranes. Thus, production of dual fluorescing hybrid cells, and the maximum hybrid yield, would be limited to the juxtaposed cells in these two layers. The second option was to deposit a mixture of CMFDA- and CMTMR-stained WEHI7.1 cells onto each membrane. The mixtures contained equal fractions of both stained cell types. This type of deposition insured contact

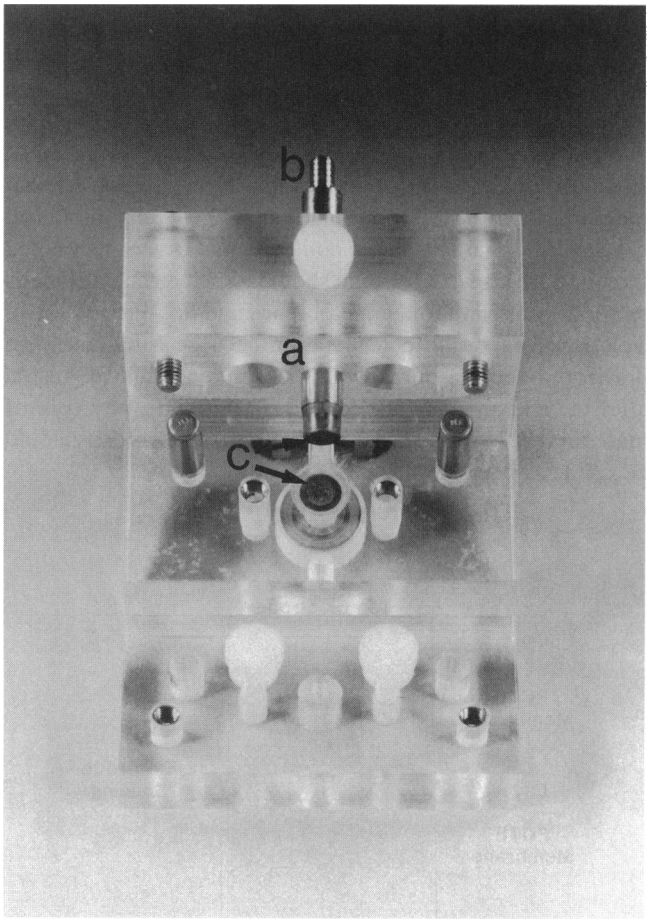

FIGURE 5 Disassembled fusion chamber. (*a*) Hollow stainless steel body. (*b*) Connection for vacuum. (*c*) Stainless steel frits.

between CMFDA- and CMTMR-stained cells throughout all layers between the membranes.

Membrane type, vacuum, and the number of cells to deposit were three variables that had to be manipulated to achieve the geometry shown in Fig. 6. These three variables were closely related to each other and to the size of the cells under investigation. Polycarbonate track etch membranes were selected for use as a renewable fusion surface because they had very flat and uniform surfaces. They were also transparent. This allowed stained cells that were deposited onto the membranes to be visualized using fluorescence microscopy. Deposition of stained WEHI7.1 cells was conducted using membranes with a range of pore sizes and pore densities that were available from the manufacturer as stock items. Fluorescent microscopy was used to determine that PCTE membranes with a pore size of 0.6 μm and a pore density of 3×10^7 pores/cm^2 provided even layering when used with 1×10^6 WEHI7.1 cells/membrane and 150 mmHg. Measurements taken from a scanning electron micrographs confirmed that the mean pore diameter was 0.6 μm. The mean distance between pore centers was determined to be 1.7 μm based on measurements from micrographs.

The combination of PCTE membrane type, vacuum, and number of cells used for this study was judged as best for WEHI7.1 cells. The combination also worked well for HL60 cells that were close in diameter to the WEHI7.1 cells. Similar combinations provided adequate cell layering. It was noted that a compromise between membrane type, vacuum, and number of cells must be made to achieve the physical situation depicted in Fig. 6. For example, it was observed that membranes with higher pore densities generally produced cell layering that was more homogenous. It was also noted that the pore size and vacuum should be selected so that cells would be drawn onto the membranes and not through them. This is an important consideration because cells that were drawn through the pores would not be available for fusion after the chamber was assembled. In principle, any type of material that has a uniform surface and high porosity could be used for deposition.

After developing the chamber and protocol, experimentation was focused on variables that would influence fusion and an investigation of practical applicability. Experimentation focused on determining electrode spacings that yielded contact for fusion, and establishing that fusion yield is a function of DC-pulsed characteristics. The production of heterohybrids was addressed to assess practical utility.

Based on the geometry shown in Fig. 6, cell-cell contact is a function of electrode spacing. This spacing is directly related to the thickness of the cell layers formed on each membrane during cell deposition. Microscopic examination of stained WEHI7.1 cells revealed that deposition resulted in layers that were approximately five cells thick. Cell layering was observed in an area that was slightly larger than the surface of the membranes that were adjacent to the frit surfaces (refer to Fig. 6). Layering extended approximately 0.5 mm beyond the outside diameter of the frits. This resulted in deposition areas that were approximately 6.5 mm in diameter. These physical data were used to design an electrofusion experiment that would identify electrode spacings that provided contact for fusing WEHI7.1 cells.

Estimations of electrode spacings for use in the experiment were computed based on the thickness of 10 WEHI7.1 cell layers between the membranes. Five of the layers were from deposition at each electrode face. Additional space of 30 μm had to be factored into the determination to account for the thickness of both membranes. If perfect packing of cellular volume was assumed, then an electrode spacing of 107 μm would provide contact. This represented a conservative electrode spacing estimate. An unconservative electrode spacing was estimated by assuming that each cell layer would occupy a distance of one cell diameter (13.5 μm for WEHI7.1 cells). The assumption resulted in a 167-μm estimate of the electrode spacing that would provide contact. Electrofusion was conducted in the chamber using electrode spacings ranging from 60 to 200 μm to overlap these two spacing limits.

Table 1 shows the mean hybrid yield for eight different electrode spacings in the range of 60–200 μm. Fusion was induced using ten 50-μs pulses with an electric field strength

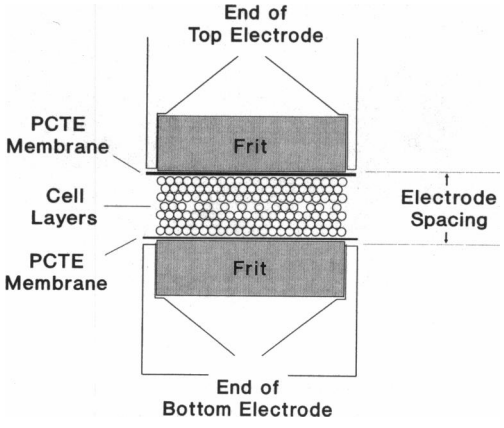

FIGURE 6 Geometric details near the electrode faces during fusion. Cell size is greatly exaggerated for clarity.

TABLE 1 Quantitation of WEHI7.1-WEHI7.1 Samples fused using different electrode spacings

Electrode spacing (μm)	Mean hybrid percentage (SD)*
60	0.73 (0.36)
80	2.42 (1.18)
100	3.92 (0.53)
120	3.10 (0.59)
140	1.55 (0.48)
160	0.44 (0.16)
180	0.49 (0.08)
200	0.55 (0.01)

* Computed by subtracting the mean percentage of aggregates detected in fusion negative control samples (0.54%) from the mean percentage of hybrids and aggregates detected for the samples at each table entry. Three replicate samples contributed to each mean. All samples received ten 50-μs pulses at an electric field strength of 2000 V/cm. CMFDA-stained cells were deposited onto one electrode, and CMTMR-stained cells were deposited onto the opposite electrode.

of 2000 V/cm for all electrode spacings. These electrical conditions were known to be fusogenic for WEHI7.1 cells based on preliminary experimentation. Murine WEHI7.1 cells were used as both fusion partners to create dual fluorescing homohybrids. Fusion was conducted by depositing CMFDA-stained cells onto one electrode and CMTMR-stained cells onto the other, as described above. Deposition was conducted in this manner to limit production of dual fluorescing fusion products to electrode spacings that produced contact between the cell layers on opposing membranes. Electrode spacings from 160 to 200 μm resulted in very low hybrid production. This indicated that the electrode spacing was too wide to produce contact between the layers on opposing membranes. Fusion yields for spacings that ranged from 80 to 140 μm were much higher, indicating that contact had been achieved. A maximum hybrid percentage was obtained at electrode spacing of 100 μm.

The data presented in Table 1 investigated the manipulation of electrode spacing keeping DC pulse characteristics constant. One physical consequence of changing the electrode spacing is that the amount of free space between cells would be reduced as the electrode spacing is decreased. Cells would aslo be deformed as the spacing is decreased. Deformation would result in cells that were wider in the dimension normal to the electric field lines and narrower in the direction parallel to the field lines. This deformation may have an advantage because it produces wider areas of cell-cell contact in the regions where fusion is induced. However, stronger electric fields would be required to induce fusion as the cell dimension parallel to the field lines is decreased.

Hybrid yield is a function of the DC pulse characteristics used to induce fusion regardless of the electrofusion method used. Also, the electrical conditions that result in fusion vary depending on the cell-cell system under investigation. The data presented in Table 2 confirm the influence of pulse characteristics on mechanically facilitated CCE. The table shows five different sets of electrical conditions used to induce fusion and the respective yields. All fusion indicated in the table was conducted using an electrode spacing of 120 μm. Murine WEHI7.1 cells were used as both fusion partners. A mixture of 50% CMFDA-stained and 50% CMTMR-stained cells was deposited onto each electrode during the fusion procedure. Therefore, contact between CMFDA- and CMTMR-stained fusion partners was obtained throughout all of the cell layers.

The data presented in Tables 1 and 2 demonstrated that mechanically facilitated cell-cell electrofusion can be used to fuse WEHI7.1 cells to WEHI7.1 cells. The use of WEHI7.1 cells as both fusion partners was a simple system that was used for establishing electrode spacings that yielded contact throughout all cell layers between the membranes. The system was also used for demonstrating that fusion yield is dependent on electrical conditions used to induce fusion. To establish the practical utility of mechanically facilitated fusion, the chamber was used for the production of WEHI7.1-HL60 heterohybrids.

TABLE 2 Quantitation of WEHI7.1-WEHI7.1 Samples fused using different electrical conditions

Electrical conditions	Mean hybrid percentage (SD)*
12 pulses 50 μs/pulse 1000 V/cm	1.63 (0.03)
10 pulses 50 μs/pulse 2000 V/cm	9.64 (1.52)
8 pulses 50 μs/pulse 3000 V/cm	6.94 (1.76)
6 pulses 50 μs/pulse 4000 V/cm	6.55 (1.55)
4 pulses 50 μs/pulse 6000 V/cm	3.44 (0.98)

* Computed by subtracting the mean percentage of aggregates detected in fusion negative control samples (0.47%) from the mean percentage of hybrids and aggregates detected for the samples at each table entry. Three replicate samples contributed to each mean. A mixture containing 50% CMFDA-stained cells and 50% CMTMR-stained cells was deposited at each electrode. An electrode spacing of 120 μm was used for all samples.

Table 3 shows the results of heterofusion for four different sets of DC pulse conditions. A mixture of WEHI7.1 cells and HL60 cells was deposited onto the membrane at each electrode. A 120 μm electrode spacing was used for all samples. This spacing was selected for two reasons. First, it provided contact when used with WEHI7.1 cells as both fusion partners. And second, one-half of the cells between the membranes would be HL60 cells that were larger in diameter than WEHI7.1 cells. Thus, the cellular volume in the electrode gap would be greater compared with the volume from the use WEHI7.1 as both fusion partners. This larger cellular volume within a 120 μm electrode spacing ensured contact when a mixture of WEHI7.1 and HL60 cells was deposited. Electrical conditions were selected based on the data from WEHI7.1-WEHI7.1 fusion. Hybrid yields were comparable with those given in Tables 1 and 2. The data demonstrate that mechanically facilitated cell-cell electrofusion can be used to make heterohybrids. Fluorescent microscopy was used to confirm that sorted hybrids were composed of at least one CMFDA-stained WEHI7.1 cell and one CMTMR-stained HL60 cell.

In summary, the developed fusion chamber utilized two functionally identical electrodes and vacuum to deposit cells into layers on PCTE membranes. The device was constructed so that mechanical force induced contact between fusion partners present in the layers at each electrode. The utility of mechanical force was demonstrated by producing WEHI7.1-WEHI7.1 fusion products as well as WEHI7.1-HL60 hybrid cells. The importance of electrode spacing for achieving contact for fusion was identified, and the dependence of hybrid yield on DC pulse conditions used to induce fusion was demonstrated.

TABLE 3 Quantitation of WEHI7.1-HL60 hybrid cells

Electrical conditions	Mean hybrid percentage (SD)*
8 pulses 50 μs/pulse 1000 V/cm	1.50 (0.39)
8 pulses 50 μs/pulse 2000 V/cm	5.45 (1.04)
8 pulses 50 μs/pulse 3000 V/cm	2.86 (0.52)
2 pulses 99 μs/pulse 6000 V/cm	1.38 (0.63)

* Computed by subtracting the mean percentage of aggregates in fusion negative control samples (0.34%) from the mean percentage of hybrids and aggregates detected for the samples at each table entry. A mixture containing 50% CMFDA-stained WEHI7.1 cells and 50% CMTMR-stained HL60 cells was deposited at each electrode. Five replicate samples contributed to each mean, and an electrode spacing of 120 μm was used for all samples.

The use of mechanical force to achieve cell-cell contact has several advantages that make it a suitable alternative to existing contact methods. First, it is widely applicable. Mechanically facilitated fusion was demonstrated with data from nonadherent cell lines. However, adherent cells have also been fused using the chamber (human fibroblasts and rat sertoli cells from primary culture). The methods for fusing adherent cells were identical to those used for nonadherent lines except that the cells were detached before staining and subsequent fusion. So, mechanical force is applicable to both adherent and nonadherent cell types. Second, the use of mechanical force does not require chemicals. This eliminates the influence of molecules that bind to cell surface markers. And finally, the use of mechanical force does not require AC. Therefore, joule heating and the use of low ionic strength media are not potential problems. In addition, the instrumentation required to perform mechanically facilitated CCE is simpler and less costly because only a DC generator is required. Dielectrophoresis methods require both AC and DC generators.

The advantages of using the chamber also include a degree of specificity between fusion partners. This degree is dependent on the manner that cells are deposited onto the membranes. If CMFDA-stained cells are deposited onto one electrode and CMTMR-stained cells on the other, contact between oppositely stained partners occurs only in the two cell layers that are in the middle of the electrode gap. Like-stained cells are in contact with each other in the layers away from the center. This increases the possibility of obtaining single fluorescing homofusion products. If a mixture of stained cell types is deposited onto each membrane, then contact is randomly achieved throughout the cells in the electrode gap. In general, higher fusion yields were obtained when mixtures of stained fusion partners were used for deposition. Sorted dual fluorescing hybrids were found to be composed of at least one fusion partner of each type. This included hybrids that contained from 2 to 10 cells, regardless of the way that cells were deposited. Fusion products composed of like-stained cells, homofusion events and polykaryons, were observed by fluorescence microscopy.

This technology is currently being used to produce hybrids for cell transplantation work. It is also being applied to continue in vitro cell-tissue electrofusion research. Modified electrode designs are under investigation. These modifications are directed toward achieving better contact specificity by producing monolayers of opposing fusion partners on each membrane. Physical principles for forcing contact are also being used to advance existing electrodes for in vivo cell-tissue electrofusion.

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