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Nanoengineered Structures for Holding and Manipulating Liposomes and Cells

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We describe the fabrication of nanoengineered holding pipets with concave seating surfaces and fine pressure control. These pipets were shown to exhibit exceptional stability in capturing, transporting, and releasing single cells and liposomes 1−12 μm in diameter, which opens previously inaccessible avenues of research. Three specific examples demonstrated the utility and versatility of this manipulation system. In the first, carboxyrhodamine was selectively incorporated into individual cells by electroporation, after which nearly all the medium (hundreds of microliters) surrounding the docked and tagged cells was rapidly exchanged (in seconds) and the cells were subsequently probed by laser-induced fluorescence (LIF). In the second study, a single liposome containing carboxyrhodamine was transported to a dye-free solution using a transfer pipet, docked to a holding pipet, and held firmly during physical agitation and interrogation by LIF. In the third study, pairs of liposomes were positioned between two microelectrodes, held in contact, and selectively electrofused and the resulting liposomes undocked intact.

Manipulation and measurement of individual cells[1−5] and organelles[6−9] have lead to the realization that biological components can exhibit a large distribution in content and activity. In work by Gilman and Ewing,[1] for example, it was shown that among six rat pheochromocytoma cells, all contained similar levels of dopamine but only one contained norepinephrine. In work by Chiu et al.[6] and Lillard et al.,[7] individual vesicles from the atrial gland of the gastropod mollusk *Aplysia californica* were shown to contain one of two compounds (one taurine, the other unidentified) but not both. It had previously been assumed that among such populations of cells[1] and organelles[6,7] the contents were uniform. Experiments have also been successfully performed with biomimetic liposomes serving as femtoliter chemical containers.[10−12] Liposomes have been manipulated in solution, their chemical contents have been controlled both during and after their formation, and they have been fused to other liposomes[11] or cells[12] with the potential of serving as a chemical delivery system.

Technology currently utilized to manipulate single cells, organelles, and liposomes employs the use of contact methods (e.g., pipets) and noncontact methods (e.g., optical traps and dielectrophoresis). Noncontact methods allow for facile trapping and releasing on demand but rarely provide the force necessary to hold cells and liposomes in flowing media. Additionally, extended exposure to the electric fields required for optical trapping can often lead to cell damage.[13] In our previous work, manipulations were done using optical trapping and/or adhesion (to slide surfaces and carbon fiber electrodes) but not, in general, using pipets.

Pipets are excellent tools for biological manipulations because of their fine control of docking strength by pressure and their simple spatial manipulation. Micropipets with pressure control were first used to hold microorganisms[14,15] in 1904 and have since become widely used to manipulate or perform measurements on single cells for a variety of biological applications (e.g., microinjection,[16−18] patch clamp,[19−21] and cell or liposome aspiration experiments[22−27]). In recent work, the first demonstration of docking and transport of single micrometer-scale organelles by micropipets was performed by Sweedler and co-workers, in which

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(1) Gilman, S. D.; Ewing, A. G. *Anal. Chem.* **1995**, *67*, 58−64.
(2) Triller, A.; Sur, C.; Korn, H. *J. Comp. Neurol.* **1993**, *338*, 83−96.
(3) Sladek, T. L.; Laffin, J.; Lehman, J. M.; Jacobberger, J. W. *Cell Prolif.* **2000**, *33*, 115−25.
(4) Kennedy, R. T.; Oates, M. D.; Cooper, B. R., Nickerson, B.; Jorgenson, J. W. *Science* **1989**, *246*, 57−63.
(5) Szücs, S.; Vámosi, G.; Póka, R.; Sárváry, A.; Bárdos, H.; Balázs, M.; Kappelmayer, J.; Tóth, L.; Szöllősi, J.; Adány, R. *Cytometry* **1998**, *33*, 19−31.
(6) Chiu, D. T.; Lillard, S. J.; Scheller, R. H.; Zare, R. N.; Rodríguez-Cruz, S. E.; Williams, E. R.; Orwar, O.; Sandberg, M.; Lundqvist, J. A. *Science* **1998**, *279*, 1190−1193.
(7) Lillard, S. J.; Chiu, D. T.; Scheller, R. H.; Zare, R. N.; Rodríguez-Cruz, S. E.; Williams, E. R.; Orwar, O.; Sandberg, M.; Lundqvist, J. A. *Anal. Chem.* **1998**, *70*, 3517−3524.
(8) Glavinović, M. I.; Vitale, M. L.; Trifaró, J.-M. *Neuroscience* **1998**, *85*, 957−968.
(9) Prior, C.; Tian, L. *Pharmacol. Res.* **1995**, *32*, 345−353.
(10) Chiu, D. T.; Wilson, C. F.; Karlsson, A.; Danielsson, A.; Lundqvist, A.; Strömberg, A.; Ryttsén, F.; Davidson, M.; Nordholm, S.; Orwar, O.; Zare, R. N. *Chem. Phys.* **1999**, *247*, 133−139.
(11) Chiu, D. T.; Wilson, C. F.; Ryttsén, F.; Strömberg, A.; Farre, C.; Karlsson, A.; Nordholm, S.; Gaggar, A.; Modi, B. P.; Moscho, A.; Garza-López, R. A.; Orwar, O.; Zare, R. N. *Science* **1999**, *283*, 1892−1895.
(12) Strömberg, A.; Ryttsén, F.; Chiu, D. T.; Davidson, M.; Eriksson, P. S.; Wilson, C. F.; Orwar, O.; Zare, R. N. *Proc. Natl. Acad.Sci. U.S.A.* **2000**, *97*, 7−11.
(13) Neuman, K. C.; Chadd, E. H.; Liou, G. F.; Bergman, K.; Block, S. M. *Biophys. J.* **1999**, *77*, 2856−2863.
(14) Terreros, D. A.; Grantham, J. J. *Am. J. Physiol.* **1982**, *242*, F293−F296.
(15) Barber, M. A. *J. Kansas Med. Soc.* **1904**, *4*, 489−494.

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atrial gland vesicles from *A. californica* were held by surface adhesion to a micropipet tip and transported to a more adhesive target surface.[28] Despite our previous success and that of Sweedler and co-workers, several factors continue to limit the applicability of pipet manipulation at small size scales (e.g., reversibility of docking and the need for a strongly adhesive target surface). For example, our previous attempts to use standard pipets were complicated by low stability in attachment and by poor reproducibility.

Our goal has been to facilitate more complex manipulations requiring repeated docking and undocking of cells, organelles, or liposomes using pressure control. The forces required for robust manipulation, however, often lead to significant cell or liposome deformation. While such deformation has been used to advantage in aspiration experiments, it can potentially compromise the integrity of liposomes and cells, affecting subsequent measurements of their contents. To combine the advantages of noncontact methods with the control and power of contact methods, a new pipet system using a concave seating surface has been developed. The pipet's concave tip is nanoengineered to hold liposomes, cells, or organelles as small as 1 μm in diameter or less. Larger holding pipets can be made with the same design to improve the manipulative control of cells or liposomes as large as 10 μm in diameter or more. The new approach to cell and liposome manipulation with a nanoengineered holding pipet is described, along with three experiments that demonstrate its utility.

EXPERIMENTAL SECTION

The experimental apparatus generally consisted of an inverted microscope (Nikon Diaphot, Technical Instrument Co., San Francisco, CA) with up to five micromanipulators coupled to an infrared optical trapping laser and an argon ion laser excitation source. For optical trapping, the output of a MOPA diode laser (985 nm, model SDL-5762-A6, SDL, Inc., San Jose, CA) was directed into a high-numerical aperture microscope objective (100×, NA 1.4, Nikon model 85025, Technical Instrument Co.) with a polychroic mirror (Chroma Technology Corp., Brattleboro, VT). For fluorescence imaging, the 488-nm line of an argon ion laser (model 95, Lexel Corp, Fremont, CA) was reflected into the objective with a polychroic mirror. The resulting fluorescence was collected through the same objective and passed through the polychroic mirror and a band-pass filter (535DF35EM, Omega Optical, Inc., Brattleboro, VT) to a high-sensitivity SIT camera (model Hamamatsu C2400-08, Technical Instrument Co.). Images were displayed on a video screen and recorded by a VCR (Sony SVO-9500MD, Technical Instrument Co.). Image analysis was performed using ScionImage software (an analog of NIH Image).

The microelectrodes used for the cell electroporation experiments were constructed by attaching Nanotips (No. 26 PtIr, Digital Instruments, Santa Barbara, CA) to copper wire mounted on micromanipulators. The microelectrodes used for the electrofusion experiments and the wire used to heat the holding pipet tips in the final heating step of their manufacture were made by electrochemically etching platinum wire (100 μm, 20% Ir) in 2 M $CaCl_2$ using 30 V ac. The electric field pulse shape used for both electroporation and electrofusion was produced with a function generator (Stanford Research Systems DS345, Sunnyvale, CA), a voltage regulator (Kepco Inc. ABC 30-0.3M, Flushing, NY), a power amplifier (Sola SLD-15−3030015T, Skokie, IL), and a rf amplifier built in-house.

Liposome preparation was carried out by a modified version of a protocol described previously.[29] It has recently been learned that this procedure produces primarily multilamellar liposomes (data unpublished). Approximately 0.2 mL of 6 mM phosphatidylcholine (Sigma) in chloroform was added to a round-bottom flask. Three milliliters of aqueous buffer solution containing CR6G (Molecular Probes, Eugene, OR) was then added to the same round-bottom flask. The chloroform was boiled away at 40 °C (Buchi Waterbath B-481) and low pressure (Cole-Parmer aspirator pump 7049-00, final vacuum 40 mmHg) on a rotary evaporator (Buchi R-124). The lipids spontaneously form liposomes in the aqueous solution as their solvent (choroform) is removed.

RESULTS AND DISCUSSION

Preparation of Nanoengineered Holding Pipets. The manipulation system was composed of a closed hydraulic pressure-control system coupled to holding pipets with specially engineered concave seating surfaces into which cells or liposomes were docked and undocked. Figure 1 illustrates the construction steps needed to generate the concave tip shape. A borosilicate capillary (BF120-69-15, Sutter Instrument Co., Novato, CA) was pulled to a long taper using a standard micropipet puller (Sutter Instrument Co.model P-87), and the tip was broken off with tweezers to obtain roughly the final tip size desired. The pipet and instruments used to form its tip were mounted on micromanipulators (Narishege MWH-3, Tokyo, Japan) over the 40× objective of a microscope. A flow break[18] was forged into the pipet with a circular filament 30 μm or more away from the crude break made with tweezers (Figure 1A). Heating of the filament resulted in partial melting of the glass and a localized restriction of the pipet inner diameter. The interior of the holding pipet prepared in this manner exhibited an elongated hourglass shape at the flow break (Figure 1A). Diamond knives were used to cut a new tip near the flow break's greatest restriction, exposing a concave inner tip shape. The submicrometer tip of a resistively heated etched Pt wire was used to gently "point forge" the pipet tip smooth at the inner opening (Figure 1B). The concave opening of the smoothed holding pipet tip constitutes the actual seating surface for cells and liposomes (Figure 1C). The final inner diameter (i.d.) at the pipet tip (∼1−4 μm) was 2−3 times larger than the inner diameter at the greatest

(16) Barber, M. A. *J. Infect. Dis.* **1911**, *8*, 348−360.
(17) Silver, R. B. *Proc. Natl. Acad.Sci. U.S.A.* **1986**, *83*, 4302−4306.
(18) Silver, R. B. In *Cells: A Laboratory Manual*. Spector, D. L., Goldman, R. D., Leinwand, L., Eds.; Cold Spring Harbor Laboratory Press: Plainview, NY, 1998; Chapter 83.
(19) Neher, E.; Sakmann, B. *Nature* **1976**, *260*, 799−802.
(20) Hamill, O. P.; Marty, A.; Neher, E.; Sakmann, B.; Sigworth, F. J. *Pflügers Arch.* **1981**, *391*, 85−100.
(21) Sakmann, B.; Neher, E. In *Single Channel Recording*, 2nd ed.; Sakmann, B., Neher, E., Eds.; Plenum Press: New York, 1995; pp 637−650.
(22) Mitchison, J. M.; Swann, M. M. *J. Exp. Biol.* **1954**, *31*, 443−460.
(23) Mitchison, J. M.; Swann, M. M. *J. Exp. Biol.* **1954**, *31*, 461−472.
(24) Rand, R. P.; Burton, A. C. *Biophys. J.* **1964**, *4*, 115−135.
(25) Evans, E. A. *Biophys. J.* **1973**, *13*, 941−954.
(26) Evans, E.; Needham, D. *J. Phys. Chem.* **1987**, *91*, 4219−4228.
(27) Zhelev, D. V.; Needham, D.; Hochmuth, R. M. *Biophys. J.* **1994**, *67*, 696−705.
(28) Rubakhin, S. S.; Garden, R. W.; Fuller, R. R.; Sweedler, J. V. *Nature Biotechnol.* **2000**, *18*, 172−175.
(29) Moscho, A.; Orwar, O.; Chiu, D. T.; Modi, B. P.; Zare, R. N. *Proc. Natl. Acad.Sci. U.S.A.* **1996**, *93*, 11443−11447.

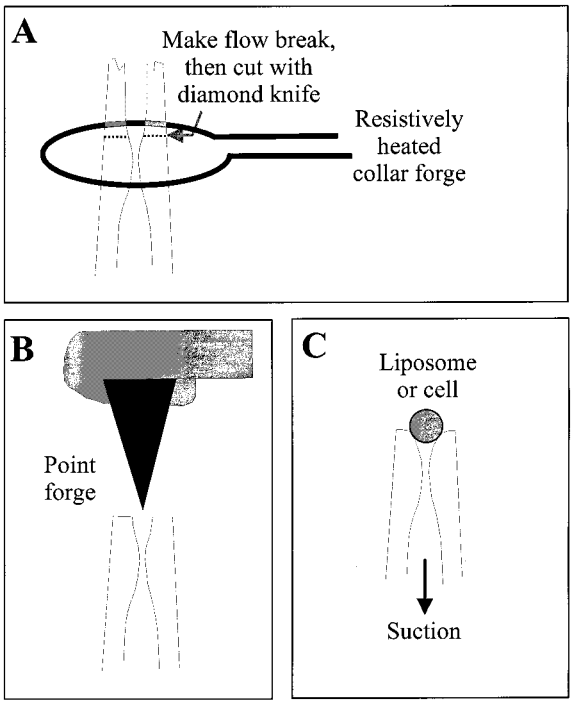

Figure 1. Key steps in the fabrication of nanoengineered holding pipets: (A) formation of a flow break to generate an extended hourglass region in the pipet, followed by diamond knife cutting to expose a concave shape at the tip, and (B) use of a point forge to smooth the inner seating surface. A representation of the pressure-controlled seating is shown in (C).

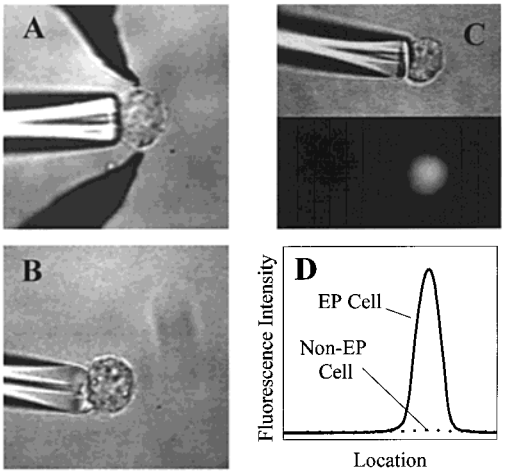

Figure 2. Single-cell electroporation and LIF investigations: (A) electroporation of a docked cell (12-μm diameter) in fluorescent medium, (B) rapid exchange to a dye-free medium, (C) alignment (top) and LIF response (bottom) of an electroporated cell, and (D) comparison of the fluorescence responses for electroporated (EP) and nonelectroporated cells. The horizontal scale in (D) is identical to that in (C).

flow restriction near the tip and 2−3 times smaller than the outer diameter at the tip. Using this procedure, pipet-to-pipet reproducibility in tip shape is excellent for larger pipets (near unity yield) and good for smaller diameter tips (70−80% yield). Prior to use, pipet surfaces were vapor-coated with hexamethyldisilazane.[30] Failure to surface-coat the pipet with this hydrophobic layer resulted in strong surface attachment of liposomes and poor pressure control. Currently, the lowest yield step in pipet manufacture is the surface coating, in which 20−50% of the pipets can become blocked. The rear of the holding pipet was inserted into Tygon tubing connected to the pressure-control system. Fine and coarse pressure controls were respectively delivered by 10- and 100-μL syringes. The plungers of both pressure-control syringes were operated by 127 thread/in. screws, which provided volume control as fine as 100 pL.

Single-Cell Electroporation and Laser-Induced Fluorescence (LIF). A fluorescent tag (6-carboxyrhodamine 6G, CR6G) was selectively incorporated into individual cells by electroporation, and the tagged cells were probed by LIF. Figure 2 contains magnified images representative of several of the key steps in the experimental procedure. In a typical experiment, a droplet (200 μL) of medium containing cells from a human monocyte cell line (U937) was placed on a glass slide on a microscope stage, and a single cell was immobilized in an optical trap. The trapped cell was then docked onto a holding pipet and removed from the trap. The entire droplet of cell medium was then removed with an Eppendorf pipet, leaving only a thin aqueous film near the slide surface containing the docked cell and a small population of cells that had attached to the slide surface. A droplet (200 μL) of 1 mM CR6G in buffer solution was added, and two microelectrodes were positioned on opposing sides of the docked cell, as shown in Figure 2A. Electroporation was achieved by applying 10 pulses across the microelectrodes with 20 s between pulses. Each 50-μs pulse consisted of superimposed 30 V dc and 5 V ac (200 kHz) potentials applied across 12 μm. The superposition of dc and ac fields has been shown to increase cell electrofusion efficiency and cell viability.[31] Following electroporation, the entire accessible volume of dye solution was removed with an Eppendorf pipet, and the docked and tagged cell was rinsed several times by the rapid addition and subsequent removal (seconds) of 0.5-mL aliquots of dye-free buffer solution. The cell under investigation remained docked to the holding pipet throughout the entire solution-transfer sequence (Figure 2B). After rinsing, the docked cell was positioned at the focal point of an Ar ion laser (Figure 2C, top panel) and probed by LIF (Figure 2C, bottom panel). For comparison, the fluorescence response of a nonelectroporated cell that had attached to the slide surface was measured.

Representative fluorescence profiles for an electroporated and a nonelectroporated cell are respectively given by the upper and lower curves in Figure 2D. The fluorescence data clearly indicate highly selective incorporation of rhodamine dye into the electroporated cell, with negligible dye incorporation into cells treated similarly but not electroporated. This experiment was repeated six times, each time with success.

Certain aspects of Figure 2B are of particular interest. The blurred figure to the right of the docked cell and holding pipet is the image of a cell suspended in the ambient medium during solution removal with an Eppendorf pipet. The cell in motion was moving too quickly to be resolved in the 0.03-s acquisition time of the SIT camera. While much milder agitation of the surrounding medium is typically sufficient to remove a cell or liposome from

(30) DePamphilis, M. L.; Herman, S. A.; Martínez-Salas, E.; Chalifour, L. E.; Wirak, D. O.; Cupo, D. Y.; Miranda, M. *BioTechniques* **1988**, *6*, 662−680.

(31) Chang, D. C. *Biophys. J.* **1989**, *56*, 641−652.

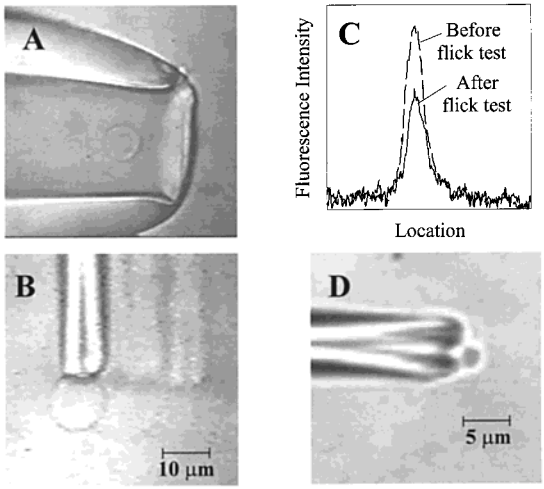

Figure 3. Liposome manipulation and LIF: (A) optical trapping of an individual CR6G-doped liposome (6.5 μm) immediately following transfer to a dye-free solution, (B) flick test demonstrating the strength of the docking attachment, (C) fluorescence profiles acquired before (dashed line) and after (solid line) performance of several flick tests (peak widths ~7 μm), and (D) a 1.6-μm-diameter liposome docked to a holding pipet.

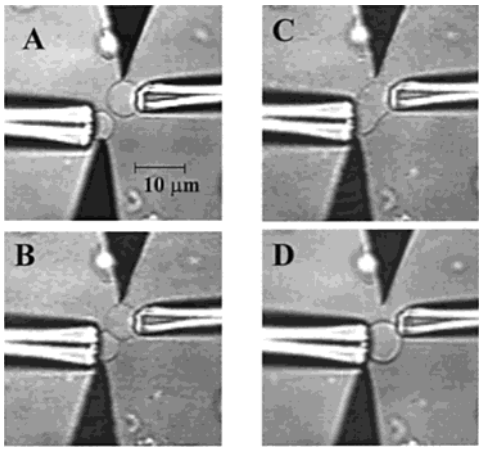

Figure 4. Electrofusion of liposome pairs. The positioning of liposomes pair and microelectrodes prior to electrofusion is shown in (A). Panels B and C are adjacent frames (separated by 0.03 s) acquired during electrofusion, demonstrating first the formation of a planar contact between liposomes (B) followed by fusion to a single a spherical liposome (C). The final fusion product is shown in (D).

an optical trap, the holding pipet system retains the docked cell during the rather harsh treatment of solution replacement.

Orwar and co-workers[32] in 1998 were the first to selectively introduce material into single cells by electroporation. The procedure used was similar to that described here except that the cells electroporated were selected from those plated onto treated glass slides. The relative ease with which single cells may be manipulated using the nanoengineered holding pipets in experiments requiring multiple treatment and measurement steps yields more flexibility than with the previous methodology. Multiple treatment and measurement steps might include the delivery of chemical or genetic material to a cell or organelle followed by the transport of that cell or organelle to another location for analysis. For example, work is currently in progress to introduce a protein tag into individual live cells, followed by capillary electrophoretic separation with the goal of evaluating inhomogeneities in cellular composition.

Liposome Transfer and LIF. Mechanical suction was used to load the liposome (6-μm diameter) into a transfer pipet (18-μm i.d.). The liposome was transported into a dye-free solution and delivered into the optical trap. The liposome was then docked onto a holding pipet and removed from the optical trap. To simulate the strong agitation possible during experimentation, "flick tests" were performed. The micromanipulator controlling the holding pipet was flicked (literally with a finger) so that the pipet tip with the liposome docked onto it was physically jolted. For the 6-μm liposome shown in Figure 3, each treatment induced a maximum displacement of 25 μm, which damped out in 2 s. The displacement frequency was measured by light scattering to be ~125 Hz. Two dozen flick tests were performed consecutively. An example of one portion of the flick tests (over a 0.03-s image-capture time) is shown in Figure 3B. The CR6G fluorescence in the liposome was evident both before and after the flick test (see Figure 3C). Over the course of 10 min, a loss in fluorescence intensity was observed as the liposome was intermittently exposed to the LIF excitation source. We have observed the slow (tens of minutes) leakage of fluorescent contents from liposomes free-floating in solution in the past, as well as rapid (less than 1 s) nearly complete loss of absorption dyes from liposomes when they adhere to the surface of untreated glass slides. A fluorescence loss with time was therefore expected for a liposome docked to a holding pipet and was similar to the loss rates for free-floating liposomes. The loss in fluorescence before and after we performed the flick tests is consistent with that observed when no flick tests were performed (within the experimental error of our measurements), indicating that the tests did not produce any additional losses.

Manipulations using the holding pipet system have been performed with liposomes as small as 1 μm in diameter. An image of a 1.6-μm liposome docked to a nanoengineered holding-pipet tip is shown in Figure 3D. In all cases, the liposomes could be undocked, optically trapped, and redocked to the holding pipet tip repeatedly, with flick tests performed between each undocking and redocking sequence.

An estimate of the force F applied during a flick test can be made using Stoke's law,[33] in which $F = 6\pi\eta rv$, where η is viscosity of the surrounding solution, r is the liposome or cell radius, and v is the liposome or cell velocity. Displacements of over 150 μm were generated for a 4-μm liposome, corresponding to forces in excess of 2000 pN. For comparison, the strength of the optical trap in the system described here for a similar liposome is ~3 pN. Assuming syringe suction was the dominant contributor to the pipet-docking force, a pressure differential greater than 2500 Pa was applied to the liposome. In an analogous experiment using a pipet without a concave tip, greater liposome deformation was observed but with negligible manipulative force.

The use of a transfer pipet provides an alternative method of changing the medium surrounding individual cells or liposomes

(32) Lundqvist, J. A.; Sahlin, F.; Åberg, M. A. I.; Strömberg, A.; Eriksson, P. S.; Orwar, O. *Proc. Natl. Acad.Sci. U.S.A.* **1998**, *95*, 10356−10360.

(33) Svoboda, K.; Block, S. M. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 247−285.

(compared with complete removal and replacement as described in the electroporation experiment). Although experimentally more complicated than the earlier approach, use of a transfer pipet reduced the physical stress placed on the liposomes. Because the liposomes were more fragile than the cells used, this transfer method was preferred.

Liposome Pair Electrofusion. Two liposomes were docked onto separate holding pipets and held in contact while microelectrodes were used to initiate their electrofusion. The two lipsomes were held such that the field generated by the microelectrodes passed through the liposomes perpendicularly at the liposome−liposome point of contact (Figure 4A). The 100-μs electrofusion pulse consisted of superimposed 10 V dc and 3 V ac (100 kHz) potentials applied across 10 μm. Figure 4 shows the electrofusion process. Shown in panels B and C of Figure 4 are adjacent frames (separated by 67 ms) acquired immediately following application of the electric field pulse. The final fusion product is shown in Figure 4D.

In previous work from our laboratory, individual liposome pairs[11] and combinations of liposomes and cells[12] were positioned and immobilized on glass slides and/or electrodes prior to being electrofused. However, potential complications associated with leakage upon surface attachment, discussed in the previous section, can make quantitative treatments difficult. The use of the new manipulation system should allow for more reliable quantification in the delivery and mixing of chemicals within biological (cellular and organellar) and biomimetic (liposomal) containers, as well as within hybrid (cell−liposome) fusion product vessels.

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