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Micromanipulation of Retinal Neurons by Optical Tweezers

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Micromanipulation by optical tweezers has been tested in cultures of mature isolated retinal cells to determine its potential for use in creating synaptic circuits in vitro. Rod and cone photoreceptors as well as other retinal nerve cell types could be optically trapped with a 980 nm diode laser mounted on an inverted light microscope using a 40x oil immersion objective numerical aperture of 1.3. Manipulation was done under sterile conditions using transparent culture dishes. To form cell groups, one half of a culture dish was made less adhesive by application of a thin layer of silicone elastomer. Unattached cells were trapped and relocated next to cells lying on an adhesive culture substrate. Optical trapping did not affect the ability of neurons to subsequently attach to the culture substrate. Up to 60% of trapped cells survived for 2 or more days. The pattern and rate of process outgrowth for manipulated cells was comparable to unmanipulated cells and by 2 days, cell-cell contacts were observed. Cultures were fixed at 2 and 5 days for electron microscopy. Organelle, nuclear and cytoplasmic structure of manipulated cells was completely normal and in photoreceptors, synaptic vesicles and ribbons were intact. Optical tweezers, therefore, provide a benign technique with which to micromanipulate whole neurons. The procedures also bestow increased precision to the study of cell-cell interactions by allowing the selection of potentially interacting cell types at a single cell level.

Soon after its invention, the laser was foreseen to serve a role in medicine and biology [1,2]. In medicine, the use of the laser has indeed become well established, predominantly in surgical procedures [3]. In experimental biology, the recent creation of microtools known as optical tweezers and optical scissors has provided a new use for the laser in the field of micromanipulation [4].

We have investigated the use of the optical tweezers for controlled movement of retinal cells in vitro in order to study the regeneration of synapses between photoreceptors and second and third order neurons. Optical tweezers, also known as the single beam gradient force optical trap, were first described by Ashkin in 1978 [5]. The optical trap that has proven effective for biological material consists of a highly focused infrared laser beam which immobilizes living particles at the focal point of light [6]. The use of near-infrared radiation provides a laser beam in a wavelength region which is poorly absorbed by water. Force generation can be best explained by ray optics theory for large (Mie-sized, diameter of particle larger than the wavelength of light) particles and electromagnetic theory for small (Rayleigh-sized, diameter of particle smaller than the wavelength of light) particles [7,8]. For cells, it is perhaps easiest to envision a force generated by the refraction of light. Light has momentum, and when a light ray is bent in passing through the cell, the change in the direction of the momentum requires a force. Because of the law of conservation of momentum, a force in the opposite direction must in turn react back on the cell. In a tightly focused beam where the intensity of light is greatest in the center, the net momentum exchange works to move the cell toward the center of the light. Since light focused through a microscope objective lens creates a three-dimensional light gradient, these forces act on the cell in both the horizontal and vertical planes and thereby provide a three-dimensional optical trap.

In prior studies on regeneration of retinal synapses [9], contacts between retinal neurons arose from purely random associations which occurred after cell plating. The use of micromanipulation to select the cell types that could potentially associate, holds the promise of dramatically expediting the study of specific cell-cell interactions. This report describes (1) procedures developed for whole cell micromanipulation with optical tweezers and (2) morphological data on the long term effects of optical trapping on adult neurons. The precision of movement, the lack of injury to the cells, and the maintenance of sterility made the optical tweezers a remarkably effective tool in retinal cell culture.

METHODS

The optical tweezers-microtool used here was provided by Carl Zeiss Inc. (Thornwood, NY) and Cell Robotics Inc. (Albuquerque, NM) and consisted of several components. A 1W, continuous wave diode laser of 980 nm wavelength was mounted on an Axiovert 100 inverted light microscope with a television port. The laser beam entered the microscope through the “cellar hole” and, therefore, had a straight optical path to the cells. The microscope was equipped with a motorized stage controlled by a joy stick with a minimal fine movement of 0.2 µm, a CCD camera, and a computer with a digitizing board for visualizing the cells and the location of the laser trap. Computer software was used for control of laser power, storage of stage coordinates and creation of macros for cell movement. The viewing lenses and camera were mounted on the standard trinocular head. For optical trapping, a high numerical aperture (N. A. 1.3), 40x oil immersion plan neofluor objective was used with brightfield optics.

Isolated retinal neurons were obtained from the adult, aquatic-phase tiger salamander, *Ambystoma tigrinum*, by

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enzymatic digestion and mechanical trituration as previously described [10]. Animal care was consistent with the guidelines of the Institute for Laboratory Animal Research. Cells were maintained in suspension in a simple Ringer's solution [9].

To test the feasibility of trapping photoreceptors, as well as second and third order retinal neurons, aliquots of the cell suspension were plated into shallow wells which were created in 35 mm culture dishes by drilling a hole, 1 cm in diameter, in the center of the dish and affixing a #1 glass coverslip to the bottom of the hole [11]. The culture dishes were filled, prior to cell plating, with the Ringer's solution and were maintained at room temperature.

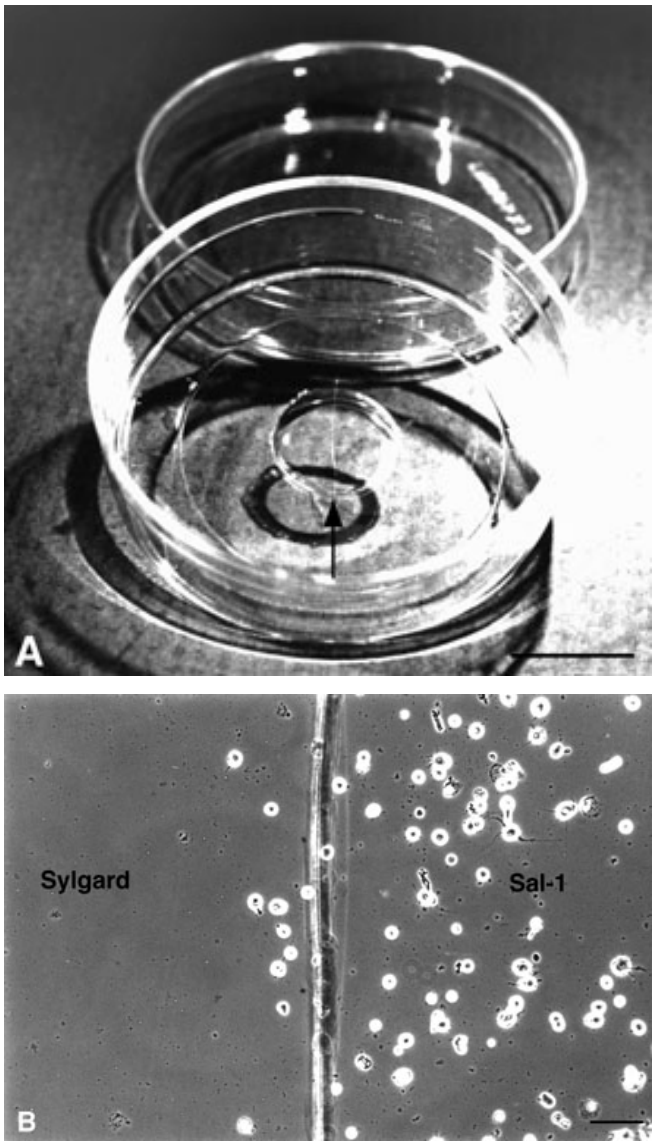

Figure 1. Culture dish preparation. (A) A well was created in a 35 mm plastic dish by drilling a hole and affixing a glass coverslip to the dish [11]. One side of the well was coated with an adherent substrate consisting of a monoclonal antibody, Sal-1 [12]; the other side was made less adherent by applying a thin coat of the elastomer Sylgard (Dow Corning Corp.). The juncture of the two surfaces was marked with an etched line on the back surface of the glass coverslip (arrow). Bar = 1 cm. (B) After 8 days in culture the difference in cell adhesion was apparent with growing cells attached mainly on the surface coated with Sal-1. Bar = 100 µm.

To test the feasibility of forming small groups of neurons for subsequent cell culture, culture dishes fitted with glass coverslips were prepared so that they contained surfaces of differing adhesiveness (Figure 1). A thin coat of Sylgard (Dow Corning Corp., Midland, MI), a nontoxic silicone elastomer, was applied to one half of the glass culture surface. After the elastomer had polymerized, the uncoated side was treated with an antibody to salamander cell membranes (Sal-1) [12] using procedures routinely followed for culture of adult salamander retinal neurons [13]. Finally, the junction between the adherent (antibody-coated) and nonadherent (Sylgard-coated) sides was marked by an etched line on the bottom surface of the culture dish. After completion of retinal dissociation, the cell suspension was maintained at 10 ^{O}C for up to 4 hr. 100 µl of the suspension was plated into a culture dish filled with a defined culture medium [9]. The dish was covered to maintain sterility. After 1-3 min, when cells on the adherent side of the dish had attached to the antibody substrate, the dish was moved to the microscope. Cultures were prepared one at a time and only immediately before use to optimize the number of floating and unattached cells on the nonadherent side of the dish.

To create cell groups, the adherent side of the dish was first examined to locate a second or third order neuron and the cell's x and y stage coordinates were noted. Then a photoreceptor was found on the nonadherent side of the dish and optically trapped. The etched line placed between adherent and nonadherent surfaces was visible in the light microscope and ensured that cells from the appropriate side were chosen. Although trapping could be achieved over a broad range of power levels (from 3-100% laser power), for our micromanipulations we routinely used the laser at 75% power. The light intensity actually reaching the cells was not determined. While holding the trapped cell, the stage was

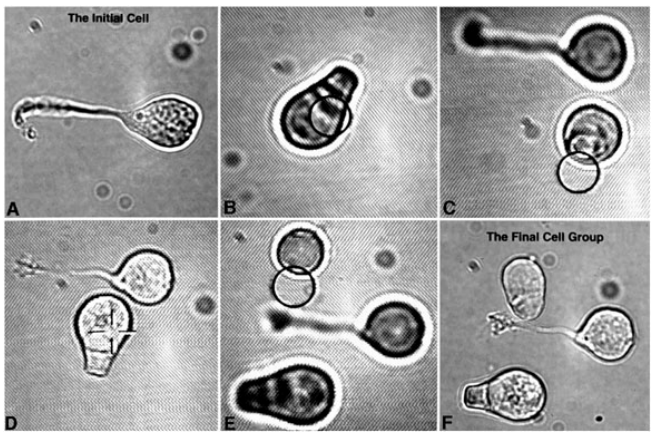

Figure 2. Formation of cell groups. Using a sealed culture dish, an adherent cell was chosen from the antibody-coated side of the dish; nonadherent cells were selected from the opposite side, and placed next to the chosen cell. Here, the adherent cell was a multipolar neuron (A); then a nonadherent rod cell (B-D) and a cone cell (E-F) were optically trapped and moved to it. Circles indicate the approximate location of the optical trap; cross indicates laser off. A and F, images obtained with CCD camera mounted on the microscope; B-E, images obtained from video tape of monitor as cells were being moved.

lowered so that the trapped neuron was well above the surface of the culture dish and any attached neurons. The stage was then moved to bring the cell to the location of the identified adherent cell. Stage movement was 4-8 μm/sec. Finally, the stage was raised to place the trapped cell next to the adherent cell (Figure 2). For each manipulation, digitized images of the adherent cell before and after formation of a cell group were obtained and video movies were taken of the trapping and movement. Cells were maintained in the defined culture medium at 10 ^{O}C in air [13]. Each newly formed group was monitored on a daily basis and rephotographed. Images taken at low (10x) magnification were critical for relocation of the groups over time.

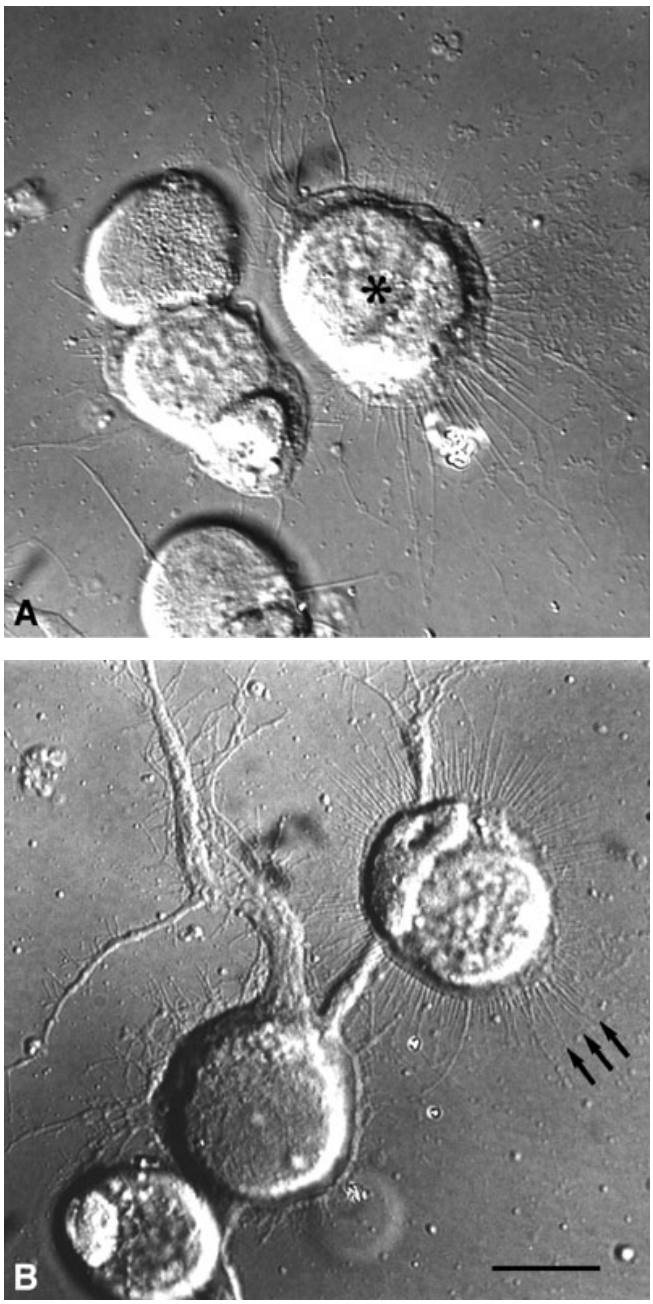

Figure 3. Trapped cells exhibit normal growth. (A) Cells which had been trapped for many minutes, attached to the culture substrate and exhibited normal process outgrowth. Asterisk (*) indicates a rod cell which had been trapped and positioned next to two other cells. The culture was fixed after 2 days in vitro. (B) Rod cells, from the same culture, which had not been optically trapped and repositioned showed very similar growth patterns to moved cells. In this cell, arrows indicate the presence of multiple filopodial processes after 2 days; compare with trapped cell in A. Bar = 15 μm.

In order to evaluate cell morphology, cultures were fixed with 2.5% glutaraldehyde, 2.5% paraformaldehyde, and 0.05% picric acid in 0.1 M cacodylate buffer after 2 and 5 days in vitro and selected cell groups were photographed with Nomarski optics. Cultures were subsequently postfixed, embedded, and sectioned for electron microscopy following previously described procedures [14]. Serial thin sections were examined in a JEOL CXII electron microscope (JEOL USA, Peabody, MA).

RESULTS & DISCUSSION

There have been prior reports of cell trapping by optical tweezers, mostly using small cells. Viruses, bacteria, yeast, sperm, lymphocytes, hydra epithelial cells, and Chinese Hamster Ovary (CHO) cells all survive in the traps [15-17]. Trapping of neurons has not been described although near-infrared lasers have been used to investigate mechanical membrane properties of the neuronal growth cone [18]. The cells that we were primarily interested in moving, the rod and cone cells of the tiger salamander, presented two challenges. First, salamanders have the largest known vertebrate cells. Photoreceptors, for instance, measure approximately 15 μm in diameter and 30 μm in length excluding the outer segment and synaptic terminal. The outer segment of rod cells adds an additional 40 μm of length and the terminal extends another 10 μm making an intact rod cell approximately 80 μm long. Second, photoreceptors are photon-absorbing cells and, therefore, might be susceptible to damage from the laser light. The salamander retina contains several rod and cone photoreceptor types with five different visual pigment absorbance spectra. Two rod populations have been identified with maximal absorbance at 433 nm and 502-523 nm [19,20]. Cone cell types have maximal absorbances at approximately 610 nm, 444 nm, and 366 nm [21-23]. With our procedures and equipment, however, neither size nor absorbance of light prevented rod and cone cells from being optically trapped and moved through the cell suspension. Retinal dissociations produce photoreceptors in several morphological states: they can be completely intact or missing their axon or outer segment or both. Photoreceptors missing the outer segment can still be identified unequivocally by the presence of the ellipsoid, an organelle unique to photoreceptors. Optical trapping was possible with all types of photoreceptors (Figure 2). Additionally, multipolar cells (ganglion and amacrine cells), some with processes more than 100 μm long, and bipolar neurons were successfully trapped. In contrast to the relatively transparent neurons, retinal pigmented epithelial cells exploded when entering the laser trap presumably due to photon absorption by melanin.

A critical factor in successful whole-cell trapping was the absence of adhesion to the culture substrate; cells which had even small focal adhesions to the substrate could not be lifted by the optical tweezers. To obtain cells without cell-substrate

attachments two approaches were used. First, optical trapping was done soon after cell plating to increase the chances of finding cells which had not reached the surface of the culture dish. Second, substrates were used that would repel or slow cell-surface attachment. The elastomer Sylgard was effectively repellent for tens of minutes. It also was easy to apply to the culture dishes and was inert in the culture medium. Unfortunately, however, even this substrate became adhesive to some cells over time.

After achieving reliable trapping of neurons, the effects of cell movement with the optical tweezers on cell survival and cell growth in vitro were determined. We had already shown that isolated retinal neurons remain structurally and functionally intact [10] and are capable of reforming synaptic connections under defined culture conditions [9,13]. There was no evidence of cell damage during trapping at the light microscopic level. This was significant because cells were often trapped for many minutes (20 min maximum) and moved several millimeters to create cell groups. After placement on the adherent side of the culture dish, all trapped cells adhered to the substrate; laser trapping, therefore, did not alter cell adhesion. An average of 10 cells were successfully trapped and relocated per retinal dissociation; 1-2 cell groups were formed per culture dish. With practice, a 60% survival of trapped cells after 48 hr was achieved. Cell growth patterns also were not altered (Figure 3 and Figure 4A). For rod cells

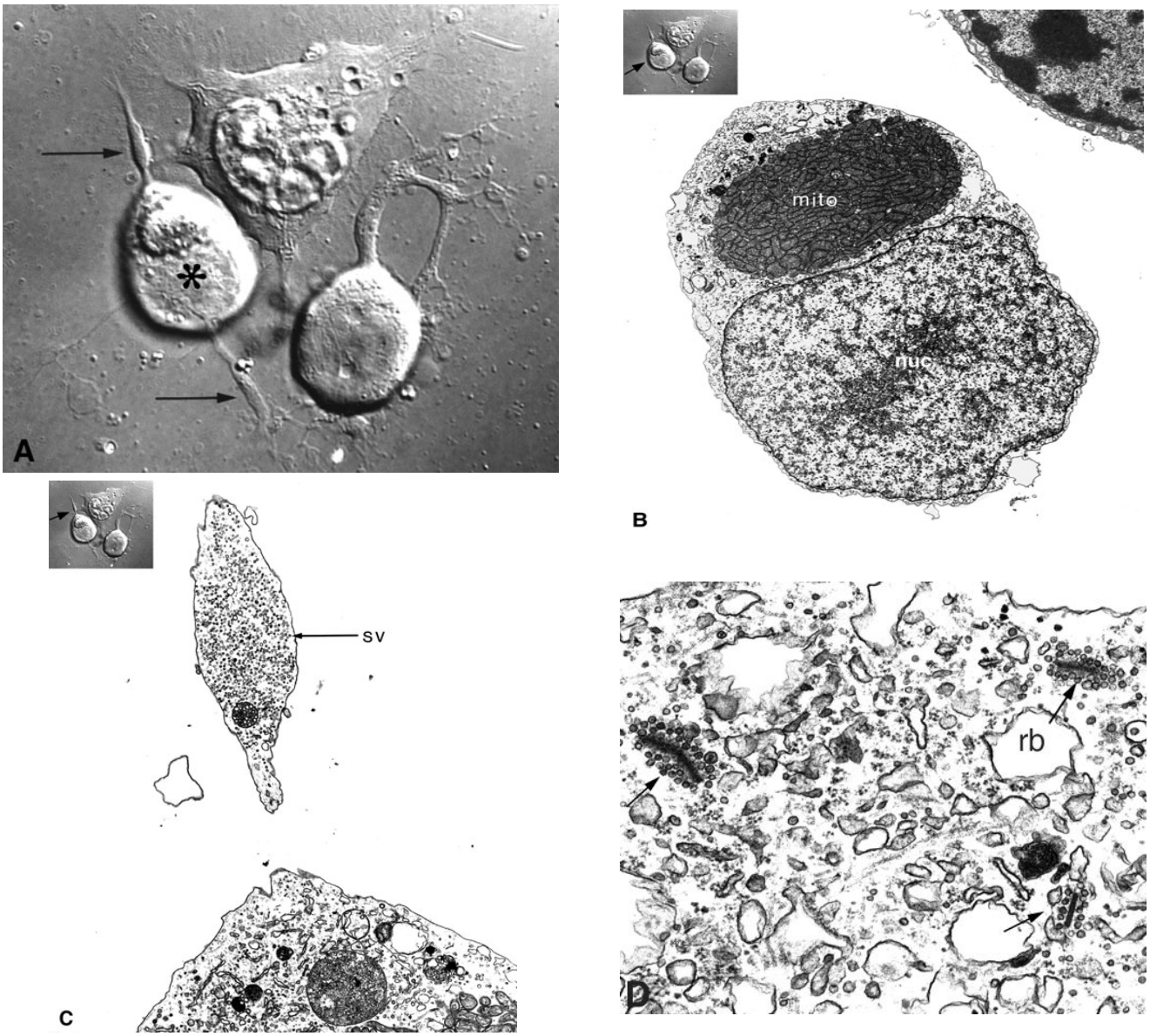

Figure 4. Fine structure of trapped cells is normal. (A) An optically trapped and repositioned cone cell (*) showing process outgrowth and formation of synaptic-like varicosities (arrows) after 2 days in vitro. (B) Electron microscopy demonstrated that the cell soma contained the characteristic complement of cell organelles. Note the normal appearance of mitochondria (mito) and the nucleus (nuc). Inset, arrow indicates location of the electron micrograph. (C) Presynaptic varicosities were filled with synaptic vesicles (sv). A multivesicular body is also present in this varicosity. Inset, arrow indicates location of the electron micrograph. (D) Synaptic ribbons (rb, arrows) were present in the cell body. Presumably, they moved from the axon into the cell soma after cell isolation and as the cell began to regenerate new neuritic processes. They remain surrounded by a halo of synaptic vesicles.

which have lost their outer segment, cell growth consists of the production of filopodia and lamellipodia during the first 24 hr in culture and subsequent development of one or more neurites containing varicosities filled with synaptic vesicles between 2-5 days [14]. The same sequence and timing of process outgrowth was observed in trapped and relocated photoreceptors followed for 5 days. Additionally, the amount of outgrowth was similar in manipulated and unmanipulated cells from the same culture. Although synapses were not observed in the cell groups formed by micromanipulation (functional synapses do not appear until after 10 days in vitro, [13]), there were contacts between processes (Figure 5). Contacts were defined as overlapping or juxtaposed processes in the light microscope and plasma membranes separated by an intercellular space of 20 nm or less in the electron microscope.

At the fine structural level, cells examined 2 or 5 days after trapping (3 photoreceptors; 1 multipolar cell) showed no abnormalities in the distribution or appearance of their organelles: the mitochondria were intact; the endoplasmic reticulum was not dilated; and the Golgi apparatus organization was normal (Figure 4B). Moreover, the cytoplasm, nucleoplasm, and cytoskeleton all appeared normal. The fine structure of newly formed presynaptic varicosities made by optically manipulated cells was also normal; the varicosities were filled with small clear vesicles (Figure 4C). Synaptic ribbons surrounded by synaptic vesicles were present in the cell body of one trapped cell (Figure 4D). This, however, is a frequent observation in cultured photoreceptors (personal observations) and is due to the ability of ribbon complexes to detach from the membrane and move within the cell [24]. Thus, morphological observation indicated that optical trapping does not deleteriously affect either the culture as a whole or trapped cells in particular.

Finally, groups of cells were formed in established cultures to test the possibility of introducing new cells to neurons with preexisting neurites. Freshly isolated retinal cells were plated into 7 day old cultures. The dishes had been prepared with an adhesive and a nonadhesive side as described above. Cells with newly formed processes were chosen from the adherent side of the dish and photoreceptors from the opposite side of the dish were trapped and relocated (Figure 6). Although examination was not as rigorous as for fresh cultures, cell-substrate adhesion, cell survival, and process outgrowth of trapped and untrapped cells seemed unaffected by the optical tweezers.

The ability to form pairs or groups of neurons using preselected cells bestows an exciting new level of control to experiments concerned with neuron-neuron interactions and synaptogenesis. In adult salamander retinal cultures, all nerve cell types including the photosensitive rod and cone cells could be trapped with a 980 nm laser without apparent short or long term injury. In other cell systems, effects of the optical trap on survival were wavelength dependent [25,26] but near-infrared lasers were also relatively benign. For CHO cells, the band of near-infrared wavelengths which gave optimal results was relatively narrow; cell proliferation was maximal at wavelengths of 950-990 nm and dramatically reduced at 900 and 1064 nm [26]. Moreover, the improvement in cell survival and proliferation with 950-990 nm over other wavelengths was more pronounced when laser power was increased. In agreement with these data, use of a 1064 nm wavelength laser at high (>200 mW) power has been reported to damage neurons [27]. It is reasonable to expect that in the future the use of carefully selected infrared optical tweezers will allow pursuit of a variety of questions about cell-cell communication between neurons, as well as between other cell types.

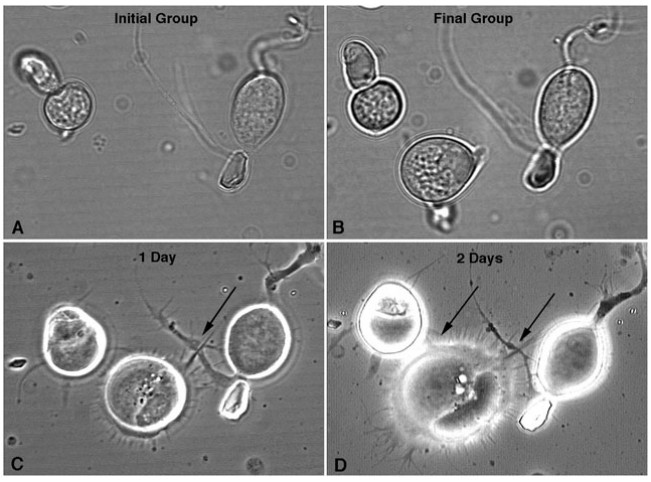

Figure 5. Cell contacts form after optical trapping. Cell groups which were formed by optical tweezers micromanipulation were followed over time to observe the initial stages of contact formation. Here, the initial group contained a cone cell (left) and a multipolar neuron (right). A rod cell was placed in this group. Prominent growth is exhibited by the moved rod cell. A large process is directed toward the multipolar neuron. Arrows, first contacts at 1 and 2 days in vitro.

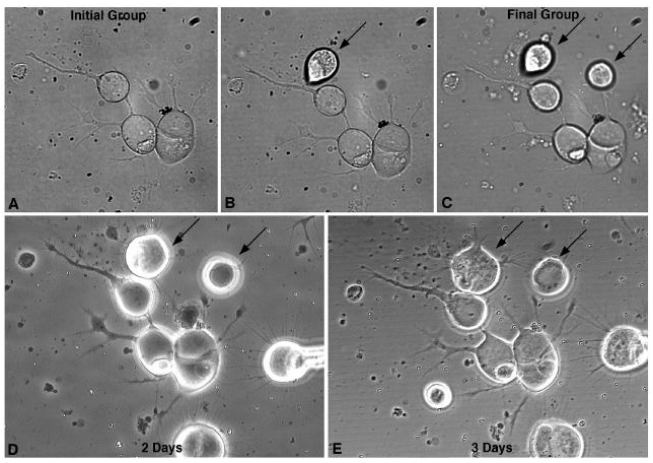

Figure 6. Freshly isolated retinal cells can be trapped and positioned in established cultures. In this example, two cells (arrows) were placed into a group which contained a multipolar neuron and two cone cells. The trapped cells successfully attached and grew processes. The established culture was 7 days old at the time of new cell plating and optical trapping.

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REFERENCES

1. Townes CH. Optical masers and their possible applications to biology. Biophys J 1962; 2:325-329.

2. Malt RA, Townes CH. Medical progress. Optical masers in biology and medicine. N Engl J Med 1963; 269:1417-1421.

3. Lasers in medicine and surgery. Council on Scientific Affairs. JAMA 1986; 256:900-907.

4. Monajembashi S, Hoyer C, Greulich KO. Microbeams and optical tweezers convert the microscope into a versatile microtool. USA Microscopy and Analysis 1997; January:15-17.

5. Ashkin A. Trapping of atoms by resonance radiation pressure. Physical Review Letters 1978; 40:729-732.

6. Ashkin A, Dziedzic JM, Yamane T. Optical trapping and manipulation of single cells using infrared laser beams. Nature 1987; 330:769-771.

7. Ashkin A, Dziedzic JM. Optical trapping and manipulation of single living cells using infra-red laser beams. Berichte der Bunsen-Gesellschaft für physikalische Chemie 1989; 93:254-260.

8. Block SM. Making light work with optical tweezers. Nature 1992; 360:493-495.

9. Sherry DM, St Jules RS, Townes-Anderson E. Morphologic and neurochemical target selectivity of regenerating adult photoreceptors in vitro. J Comp Neurol 1996; 376:476-488.

10. Townes-Anderson E, MacLeish PR, Raviola E. Rod cells dissociated from mature salamander retina: ultrastructure and uptake of horseradish peroxidase. J Cell Biol 1985; 100:175-188.

11. Bray D. Surface movements during the growth of single explanted neurons. Proc Natl Acad Sci U S A 1970; 65:905-910.

12. MacLeish PR, Barnstable CJ, Townes-Anderson E. Use of a monoclonal antibody as a substrate for mature neurons in vitro. Proc Natl Acad Sci U S A 1983; 80:7014-7018.

13. MacLeish PR, Townes-Anderson E. Growth and synapse formation among major classes of adult salamander retinal neurons in vitro. Neuron 1988; 1:751-760.

14. Mandell JW, MacLeish PR, Townes-Anderson E. Process outgrowth and synaptic varicosity formation by adult photoreceptors in vitro. J Neurosci 1993; 13:3533-3548.

15. Weber G, Greulich KO. Manipulation of cells, organelles, and genomes by laser microbeam and optical trap. Int Rev Cytol 1992; 133:1-41.

16. Kuo SC. Optical tweezers: a practical guide. Journal of the Microscopy Society of America 1995; 1:65-74.

17. Liu Y, Sonek GJ, Berns MW, Tromberg BJ. Physiological monitoring of optically trapped cells: assessing the effects of confinement by 1064-nm laser tweezers using microfluorometry. Biophys J 1996; 71:2158-2167.

18. Kuo SC, Sheetz MP. Optical tweezers in cell biology. Trends Cell Biol 1992; 2:116-118.

19. Harosi F. Absorption spectra and linear dichroism of some amphibian photoreceptors. J Gen Physiol 1975; 66:357-382.

20. Cornwall MC, MacNichol EF, Fein A. Absorptance and spectral sensitivity measurements of rod photoreceptors of the tiger salamander, Ambystoma tigrinum. Vision Res 1984; 24:1651-1659.

21. Makino CL, Kraft TW, Mathies RA, Lugtenburg J, Miley ME van der Steen R, Baylor DA. Effects of modified chromophores on the spectral sensitivity of salamander, squirrel and macaque cones. J Physiol (Lond) 1990; 424:545-560.

22. Perry RJ, McNaughton PA. Response properties of cones from the retina of the tiger salamander. J Physiol (Lond) 1991; 433:561-587.

23. Makino CL, Dodd RL. Multiple visual pigments in a photoreceptor of the salamander retina. J Gen Physiol 1996; 108:27-34.

24. Reme CE, Young RW. The effects of hibernation on cone visual cells in the ground squirrel. Invest Ophthalmol Vis Sci 1977; 16:815-840.

25. Konig K, Tadir Y, Patrizio P, Berns MW, Tromberg BJ. Effects of ultraviolet exposure and near infrared laser tweezers on human spermatozoa. Hum Reprod 1996; 11:2162-2164.

26. Liang H, Vu KT, Krishnan P, Trang TC, Shin D, Kimel S, Berns MW. Wavelength dependence of cell cloning efficiency after optical trapping. Biophys J 1996; 70:1529-1533.

27. Dai J, Sheetz MP. Mechanical properties of neuronal growth cone membranes studied by tether formation with laser optical tweezers. Biophys J 1995; 68:988-996.

28. Townes-Anderson E, St Jules RS, Sherry DM, Lictenberger J, Hassanain M. Micromanipulation of retinal cells by optical tweezers. Invest Ophthalmol Vis Sci 1997; 38:S29.