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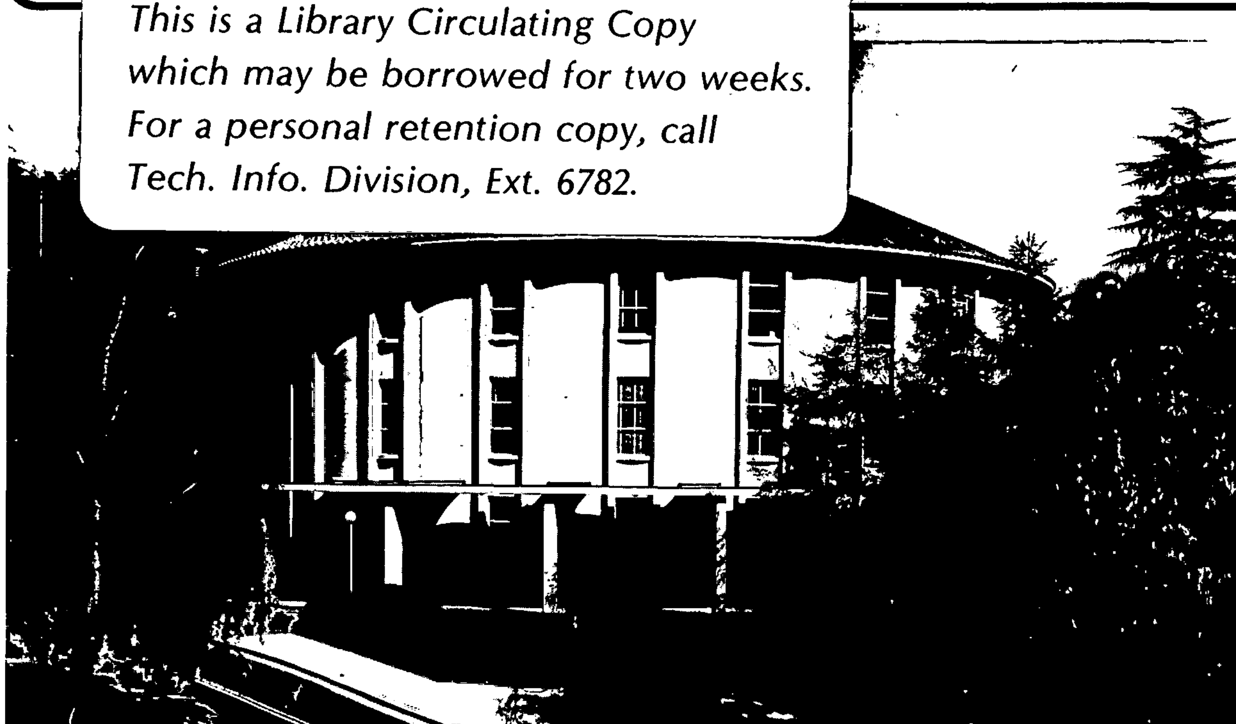

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CHARACTERIZATION AND SEPARATION OF PLANT PROTOPLASTS VIA FLOW CYTOMETRY AND CELL SORTING

Keith Redenbaugh [1], Steven Ruzin [2], James Bartholomew and James A. Bassham

Lawrence Berkeley Laboratory, Laboratory of Chemical Biodynamics,
University of California, Berkeley, CA 94720

[1]Present address: Plant Genetics, INC., 1930 Fifth Street, Davis, CA 95616

[2]To whom reprint requests should be addressed.

Key Words

Euphorbia, Nicotiana, Petunia, heterokaryons, protoplast fusion, protoplast selection, somatic cell genetics, FITC, RITC

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Summary

Large populations of protoplasts from five species: Euphorbia lathyris, Nicotiana glauca, N. langsdorfii, Petunia parodii and P. inflata (albino) have been characterized by flow cytometry on the basis of laser light scatter and chlorophyll fluorescence or exogenously-added stains, fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC). Stain concentrations were 2.2 to 3.6ul FITC and 7.2ul RITC stock solutions per ml enzyme-protoplast solution using 5mg FITC or RITC per ml absolute ethanol or acetone. Optimum conditions for FITC and RITC staining was found to be at pH 7.5 or greater and with acetone as the stain solvent Stained E. lathyris mesophyll protoplasts produced callus and regenerated shoots, indicating non-toxicity of the fluorochromes. E. lathyris protoplasts fused from two differentially-stained populations produced unique histograms when compared to mixed, but unfused populations. Further analysis and sorting of mesophyll protoplasts from these species was done with a Becton Dickinson Fluorescence-Activated Cell Sorter (FACS IV). Euphorbia and Petunia protoplasts were sorted according to various combinations of parameters: light scatter and fluorescence from chlorophyll, FITC, RITC or both stains. Up to 2000 intact protoplasts were sorted and recovered within 1 hr. Sorting can be done under sterile conditions to allow culturing of the collected protoplasts.

Introduction

Protoplast isolation and fusion techniques are now being used for the production of hybrids that are not possible using traditional sexual hybridization, even in conjunction with tissue culture techniques such as embryo rescue. These techniques are radically different from sexual hybridization since somatic cells, rather than germ cells, are fused. Hybridization barriers, both pre- and post-zygotic, can be breached using protoplast fusion. The opportunities for transfer of genetic information from one species to another are certainly very great and have been adequately discussed elsewhere (Schieder and Vasil 1980). Protoplast fusion with subsequent plant regeneration and verification of hybrid nature was first reported by Carlson et al. (1972). Since then, many other researchers have demonstrated hybrid plant production using protoplast fusion, usually with sexually-compatible species. One early report (Gleba et al. 1975) demonstrated production of a cytoplasmic hybrid tobacco that was sexually unattainable. Melchers et al. (1978) reported the first plant production from protoplast fusion of incombatible species: _Solanum tubersom_ and _Lycopersicum esculentum_. Others have since reported on unique hybrid plant production (Schieder and Vasil 1980).

One of the main barriers in wide-scale use of protoplast fusion for plant hybridization is the identification and the physical selection of the hybrid away from the non-hybrid protoplasts or cells. In order to minimize the number of non-hybrid cells that must be maintained, the optimum selection time should be just after protoplast fusion has occured. Several selection methods have been developed with possibly the easiest technique being visually to select the hybrid on the basis of morphological characteristics of the protoplasts and then to isolate the fused

protoplasts as individual units in a Cuprak dish (Kao 1977) or with a micromanipulator (Gleba and Hoffman 1978; Patnaik et al 1982). This technique is limited by the number of fused protoplasts an operator can identify and sort, which is approximately 100 to 200 a day (Patnaik et al. 1982).

Four other selection methods have been used which have resulted in the production of regenerated hybrid plants from fused protoplasts. The most common method to date, albino mutant complementation (Dudits et al. 1977), requires finding and characterizing mutant genotypes (cell lines or plants) that are complementary for albinism. Albinos may be relatively common in some species but extremely rare or absent in others, particularly tetraploids or plants of higher ploidy levels. Fusion of two complementary albino parents will result in the production of green functional chloroplasts and hence green callus and plants. A third selection method uses differential drug resistance so that only the fused, hybrid protoplasts will grow on the antibiotics- containing medium, while unfused protoplasts or homokaryons will not grow (Power et al. 1976). In a similiar manner, hybrid protoplasts can be selected using biochemical complementation (Maliga et al. 1977) such as differential resistance to amino acid analogs (Harms et al. 1981). A fifth method uses differential growth responses of the various populations of protoplasts on specific media. In this case, only the hybrid protoplasts or cell lines can grow into callus or regenerated plants (Smith et al. 1976). This method has been useful in conjunction with albino complementation, differential drug resistance or biochemical complementation selection methods.

These selection methods have major limitations. The visual selection method is very labor intensive and can only provide a small number of fused

hybrid protoplasts per person-hour. The other methods require selection of plant or cell lines with specific properties. The complementation methods are particularly limited since mutants must be identified and characterized. Pre-selection for any of these traits greatly decreases the amount of genetic variability available for subsequent plant breeding and may include undesirable gene combinations. A more universal protoplast selection method is needed so that any plant species or plant material can be used for protoplast fusion

Flow cytometry and cell sorting techniques offer such a system for characterizing, identifying, and selecting plant protoplasts regardless of their origin. Individual particles or cells can be analyzed at a very high rate by passing them in a liquid stream through a laser beam. A particular cell type or cell condition can be characterized according to the emission spectrum for an endogenous or introduced fluorochrome. A cell or cells identified to contain fluorochromes from both parental populations indicates a fused hybrid. Cell sorting is a method whereby any identified population of cells can be separated from a large heterogeneous population by applying an electrostatic charge to the desired fused protoplasts or cells. There are no reports in the literature of flow cytometric analysis and/or sorting of plant protoplasts, except for a brief mention of flow cytometric analysis of *Euphorbia* leaf protoplasts (Redenbaugh et al. 1981). Melamed et al. (1979) gave an excellant review of the techniques and application of flow cytometry and cell sorting.

This paper presents results from experiments in which protoplasts (fused or unfused) were characterized and sorted for five species: *Euphorbia lathyris*, *Nicotiana glauca*, *N. langsdorfii*, *Petunia parodii*, and *P. inflata* using flow cytometry and cell sorting techniques.

Materials and Methods

Preparation of Protoplasts

Protoplasts were isolated from fully expanded leaves of three to six month old, greenhouse-grown plants of Euphorbia lathryis, Nicotiana glauca, N. langsdorfii, and Petunia parodii and from leaf callus of the cytoplasmic albino P. inflata grown on BGS medium (Power and Davey 1979). Leaves were surface sterilized for 15 minutes in 0.6% sodium hypochlorite containing a drop of Tween 20 and rinsed with sterile distilled water. The leaves were sliced into small pieces using a five-blade scalpel, and incubated in darkness overnight in 14ml of a solution of CPW21S (Power and Davey 1979) with 2.5% Cellulase "Onozuka" R-10 and 0.5% Macerozyme R-10 (Yakult Honsha Co. Ltd., Japan). P. inflata albino callus was also finely chopped and placed in the same enzyme solution. After 12-16 hours the enzyme solution was pipetted out and fresh CPW21S solution was added. Protoplasts were released from the leaf pieces (or callus) by gently pressing the leaves (or callus) against the sides of the petri dish with forceps. The protoplasts were filtered through a 74 um stainless steel filter (stainless steel mesh disc melted to an autoclavable plastic bottle) and centrifuged at 100g for 15 minutes. Intact protoplasts which floated to the top of the solution were collected, resuspended in CPW21S solution minus enzyme, and centrifuged a second time. The protoplasts were then resuspended in M/SP1-9 solution (Power and Davey 1979) at a concentration of 10^6 protoplasts per ml.

Protoplast Staining

Protoplast staining followed the methods of Galbraith and Mauch (1980). Solutions of fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) (Sigma Chemical Co.) were dissolved in absolute ethanol at a

concentration of 5 mg/ml. The solutions were stored in darkness at 4 C and were used within one month of preparation. Two methods of protoplast staining were used. In one, fluorochrome solutions were added directly to the enzyme solutions at the beginning of protoplast isolation. After 12 to 16 h incubation with the fluorochromes, protoplasts were isolated as described above. Alternatively, the fluorochromes were added to a suspension of the isolated, purified protoplasts. After 30 minutes incubation, excess stain was washed out by repeated centrifugation (100g for 10 min) in fresh M/SP1-9 medium. This latter method did not provide sufficient staining efficiency and was not used widely. Some protoplast populations were stained with both FITC and RITC to mimic fusion of differentially-stained populations. Quantity of the stain solutions per ml enzyme-protoplast solution varied from 1 to 36ul with the majority of experiments using 3.6ul FITC and/or 7.2ul RITC. A control population of protoplasts was left unstained.

Emission spectra of the fluorochrome solutions and of the four protoplast populations were determined at various excitation wavelengths using a Perkin-Elmer MPF2A fluorescence spectrophotometer. The wavelengths used (457, 488, 497 and 502nm) matched those available on the argon-ion laser and were within the published range of excitation wavelengths for FITC and RITC. Protoplast density for all determinations was approximately 10^6/ml.

Protoplast Fusion

Two populations of *E. lathyris* protoplasts, one stained with 3.6ul FITC/ml enzyme-protoplast solution and the other with 7.2ul RITC/ml were fused using PEG (MW 6000) following the methods of Power and Davey (1979). As a control, two additional populations of FITC- and RITC-stained

protoplasts were prepared and treated in the same manner as the fused population but without the addition of the PEG. A doubly-stained population was used as an additional control.

Flow Cytometry

Protoplast characterization was done using a flow cytometer made specifically for the Laboratory of Chemical Biodynamics (Pearlman 1978) and is equivalent to the Los Alamos Scientific Laboratory Flow System II described by Holm and Cram (1973). Protoplasts are introduced into a flow system via vacuum uptake (2 PSI) and are focused by a sheath liquid into a thin laminar flow stream which passes through a 250u diameter aperture at a flow velocity of 4-5 M/sec. This separates and aligns the cells so that they pass singly through the area of illumination in a specially designed flow cell. Fluorochromes within the protoplasts are excited by a laser beam (Spectra Physics Model 171 argon-ion laser, 1 watt intensity) tuned to 488nm with an eliptical cross-section of 9 X 75um. Other wavelengths (457.9, 465.8, 472.7, 476.5, 496.5, 501.7, and 514.5 nm) were found to be suboptimal for simultaneous maximization of both FITC and RITC emission. The emitted light is split by a dichroic mirror (Fig. 1) through which passes light of wavelengths shorter than 540nm and impinge on one photomultiplier tube (BLUE PMT) while light of wavelengths longer than 540nm is reflected into a second tube (RED PMT) (Hawkes and Bartholomew, 1977). In addition, the light passes through specific band-pass filters centered at either 526.0nm (BLUE PMT) or 576.8nm (RED PMT) (bandwidths @ 50% of T_{max} are 22.6 and 25.5nm, respectively). Thus, BLUE PMT receives FITC fluorescence and RED PMT receives RITC fluorescence. Other bandpass filters, 503, 514, and 603 nm, were tested but not used because of poorer light transmission for FITC and RITC fluorescence. Chlorophyll fluorescence

and light scatter are excluded by the combination of filters used. The light pulses from individual protoplasts are detected by BLUE and RED PMT, and amplified by a non-integrating, real-time preamplifier and an Ortec Model 450 research amplifier. The amplified pulses are digitized by an analog-digital converter (Northern Scientific 1024) and stored in a 4096 channel pulse-height analyzer (Northern Scientific NS-636). When one of the channels reaches a predetermined capacity (usually 500 or 1,000 digitized pulses), measurement stops and the data is transfered to a Digital VAX 11/780 computer for storage and manipulation. The flow system in our laboratory can analyze 500 to 2500 protoplasts per second and the total time required for analysis and processing a single sample is usually 1 to 15 minutes, depending on the frequency of targetted protoplasts in the population.

Cell Sorting

A Becton Dickinson FACS IV was used for sorting Euphorbia and Petunia protoplasts. It is equipped with a Spectra Physics Model 164 argon-ion laser that produces a beam of circular cross-section of 70um. protoplasts are propelled in a fluid stream by compressed N_2 through a small orifice (50, 70 and 90um nozzle tips were used) and are irradiated by the laser beam. Unlike the analyzer described above, the FACS IV in our laboratory is equipped with only one fluorescence detector; thus, FITC and RITC could not be measured simultaneously. Because of this deficiency, sorting was based on light scatter vs chlorophyll, FITC, or RITC fluorescence (519.5nm and 580.0nm bp filters, respectively, with bandwidths at 50% of T_{max} of 9.0 and 10.0nm, respectively). Light scattering properties are related, though not specifically proportional, to protoplast size.

A vibrating piezoelectric quartz crystal (23-37KHz) breaks the flow stream into small droplets just below the tip of the flow nozzle and below the point of laser impingement. Each droplet contains either one or no protoplasts depending on the flow rate. The hydrodynamics of the system are such that approximately 1 in 7 droplets contain a protoplast. After a protoplast of interest is identified by the analytical portion of the FACS IV, a time delay is triggered so that just as droplet formation occurs for a particular protoplast, that droplet plus the two bordering droplets are electrically charged. The charge can be negative or positive so two separate populations of protoplasts can be sorted simultaneously. The droplets then pass between two electrostatic plates and are deflected right or left depending on the charge of the droplet. The sort mechanism is not activated if two droplets of different charges occur adjacent to one another so that cross contamination is minimized. Solution M/SP1-9 was used for both the sample stream and surrounding sheathing solution to avoid protoplast lysis due to osmotic stress. Sterile sorting was achieved by prior sequential flushing of the hydraulic system with detergent, 70% ethanol and sterile water. A detailed description of the FACS IV is given by Fulwyler et al. (1979).

RESULTS

Protoplast Preparation and Staining

A density of protoplasts per ml CPW21S solution (protoplast isolation frequency) of approximately 1 - 2 x 10^6 was achieved for all species. *E. lathyris* protoplasts were used for much of the flow cytometric analysis and protoplast numbers were often in excess of 10^7 protoplasts per gfw of leaves.

Fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC), when dissolved in absolute ethanol, have fluorescence emission peaks at 520 and 580nm respectively (Fig. 2). Both fluorochromes can be excited at 488nm. *E. lathyris* protoplasts which were unstained, stained with either FITC or RITC, or a combination of both to mimic fusion, have different emission spectra when irradiated at 488nm (Fig. 3). FITC-stained protoplasts have a peak fluorescence emission at 520nm and RITC-stained protoplasts show an emission peak at 580nm. The doubly-stained protoplasts fluoresce at both 520 and 580nm while unstained protoplasts exhibit no fluorescence peaks in this spectral region. The 680nm peak is due to chlorophyll fluorescence which is excluded by the specific band-pass filters used for flow cytometric analysis. Stain concentrations less than 2ul/ml of enzyme-protoplast solution gave variable staining efficiencies and concentrations greater than 15ul/ml were often very destructive to protoplasts. The optimum, single excitation wavelength for FITC and RITC was determined to be 488nm. Other wavelengths tested, both with the fluorescence spectrophotometer and flow cytometer, did not sufficiently differentiate RITC from FITC on the basis of fluorescence spectra.

Flow Cytometry

Protoplast populations from *E. lathyris*, *N. glauca*, and *N. langsdorfii*, stained with FITC, RITC, or both stains, were analyzed with the flow cytometer. Only those populations which showed a staining frequency of greater than 99% were used for analysis. Protoplast counts and staining frequencies were determined on a Zeiss ICM 405 inverted microscope equipped with a UV fluorescence epi-illuminator and standard filter combinations to distinguish FITC and RITC fluorescence. Fluorescent light emitted from FITC- and RITC-stained protoplasts passes and appears only

through one or the other filter combination, while doubly-stained protoplasts fluoresced under both filter combinations (Fig 4,A-G). Fused protoplasts could be identified under the microscope by the presence of both fluorochromes within one protoplast (Fig. 4,H-J). Chlorophyll fluorescence is visible in the photomicrographs but is easily identified on color photographs. Callus has been produced from E. lathyris protoplasts stained with either or both fluorochromes and shoots regenerated from callus of both FITC- and RITC-stained protoplasts (results to be published elsewhere).

Flow cytometric analysis was able to resolve the differentially-stained protoplast populations into separate histograms. Protoplasts stained with FITC have a very strong signal through the 526 bp filter or along the 526:576 axis and a negligible signal along the other axis, while RITC-stained protoplasts have a strong signal only along the 576 or 576:526 axis. Protoplasts stained with both fluorochromes have a peak displaced along both axes. (Figs. 5-7). Stained N. glauca and N. langsdorfii protoplast populations had identical histograms and are not shown. Electronic discrimination of doubly- stained protoplasts was used to gate out lower limits of the signals and to leave a histogram of only highly stained protoplasts. A contour plot of the electronically gated doubly-stained protoplast population is shown in Figure 8 as compared to contour plots of the singly-stained protoplasts. Figure 8 illustrates separation of the fluorescence signals of singly- vs doubly-stained protoplasts. Therefore, it is theoretically possible to separate doubly-stained from singly-stained protoplasts by appropriately programming a cell sorter (equipped with dual fluorescence detection capability) on the basis of FITC and RITC staining alone.

A ratio of the analog signals from the PMT's (576:526 or 526:576) was used because of a machine requirement for a minimum signal from both of the PMT's (along the Y-axis only) before the signal would register in the multichannel pulse height analyzer. Without the ratio no signal would register along the Y-axis for a fluorochrome that has only a minimum fluorescence along the X-axis. This poses no difficulty for sorting purposes since doubly-stained or fused protoplasts appear between the two axes; but for flow cytometric analysis and display requirements it is desirable to have all three histograms available (as seen in Fig. 8). The problem can be partially overcome, without using a ratio for the two signals, if the PMT high voltage and gain are increased; however, the resulting histograms lose quality.

The net effect of using a ratio of fluorescence signals (526:576) is that the histogram derived from FITC-stained protoplasts shows fluorescence intensity values of a magnitude greater than either RITC-stained protoplasts (Fig. 5) or doubly-stained protoplasts (Fig. 7). The effect is to increase artificially the fluorescence intensity by shifting the histogram up the 526:576 axis. The shape of the histogram is not altered. The doubly-stained protoplasts have signals from both PMT's yielding a ratio value close to 1 while the FITC-stained protoplasts yield a ratio value much larger than 1 due to the very small signal detected in the 576 PMT (RITC channel). Therefore the effect is to separate further the three histograms.

Samples of fused FITC- and RITC-stained protoplasts were analyzed and compared with mixed, but unfused samples (Figs. 9-12). The unfused population had fluorescence peaks along both axes but few signals near the origin. The relatively low peak along the 576:526 axis is due to a larger

number of FITC-stained protoplasts as compared to RITC-stained protoplasts. The multichannel analyzer stops compiling when any one channel is filled, as in this case for FITC-stained protoplasts. The fused sample has a large number of signals near the origin, which compares favorably with the control histogram of the doubly-stained population (Fig. 7). As expected, there was also a large population of singly-stained protoplasts within the fused population. Examination of over 200 protoplasts in the mixed, unfused population with the fluorescent microscope showed no doubly-stained protoplasts, while a fusion frequency of approximately 1% was observed in the fused protoplast population (determined from both microscope and histogram analysis).

Cell Sorting

E. lathyris, *P. parodii*, and albino *P. inflata* protoplasts were analyzed and sorted on the Becton Dickinson FACS IV cell sorter based on fluorescence of endogenous or exogenously-applied fluorochromes. *E. lathyris* protoplast populations, unstained or stained with either or both fluorochromes, were analyzed for fluorescence using 520nm and 580nm band pass filters. All manipulations of the stained populations were done sequentially since only one PMT channel was available. The differentially-stained populations of protoplasts were clearly identified on the FACS IV (Fig. 13). Unstained protoplasts showed miminum fluorescence through either bandpass filter, while doubly-stained protoplasts fluoresced strongly through both filters. Singly-stained protoplasts showed strong fluorescence only through the associated bp filter (520 for FITC and 580 for RITC). There was some leakage of fluorescence signals between populations stained with FITC vs RITC, but the signals were of low enough intensity to be gated out electronically. The histograms demonstrate that

separation of differently stained protoplasts within a population will be feasible with two-parameter fluorescence on the FACS IV. A number of viable, intact protoplasts were collected (Fig. 14, A).

Large numbers of P. parodii and P. inflata leaf protoplasts (up to 10^6 per hr) were analyzed and sorted based on light scatter and chlorophyll fluorescence (or lack of it) (Fig. 15). Albino protoplasts had negligible fluorescence while P. parodii mesophyll protoplasts yielded a fluorescence histogram similiar to that of E. lathyris leaf protoplasts. Both Petunia populations had identical light scatter histograms indicating similiar protoplast size (confirmed by light microscopy). When the two populations were combined, four peaks were observed: chlorophyll-containing protoplasts, chlorophyll-deficient protoplasts, isolated chloroplasts, and debris. The two protoplast peaks were sorted and many (1000-2000) intact protoplasts were collected (Fig. 14, B-C). Sterilization of the FACS IV hydraulic system allowed for recovery of uncontaminated protoplasts.

Discussion

The ability to distinguish and physically separate protoplast hybrids from a melange of non-hybrid units is required for any protoplast fusion experiment. To date, many selection methods have been utilized, but none have had universal application due to either a very slow sorting rate (less than 200 protoplasts sorted per day) or a lack of appropriate genetic markers. A selection method based on flow cytometry and cell sorting of protoplasts tagged with non-genetic markers (fluorochromes) offers the possibility of quickly and efficiently retrieving members of a population of desired heterokaryons. In our laboratory we have made advances in

achieving this goal using the vital-stains FITC and RITC, a flow cytometer and a FACS IV cell sorter.

Protoplast staining efficiency using FITC and RITC was often 99 - 100% but was not consistent for all isolation attempts. This inconsistency of staining is contrasted by the 100% staining of *Nicotiana* protoplasts described by Galbraith and Mauch (1980). Most likely two factors in our experiments are responsible for this variation. The fluorescence intensity of isothiocyanate solutions is greatly diminished over time even when stored at 4 C in darkness; therefore, FITC and RITC solutions stored for one month will have a marked decrease in fluorescence intensity. The second factor is that ethanol, in which the stains are dissolved, is a source of free hydrogen ions that will protonate the terminal amines on plasma membranes, thereby decreasing the binding efficiencies of the isothiocyanate groups (FITC and RITC) to the membrane proteins (acidic media can have the same effect). Presumably Galbraith and Mauch (1980) avoided these problems by using only freshly prepared stains dissolved in acetone (not a proton donor) although they are not specific on this. Further work in our laboratory indicates that a much more intense and uniform staining of *E. lathyris* protoplasts is achieved when acetone is used as the stain solvent and at an elevated pH (pH 7.5 or greater). In agreement with Galbraith and Mauch (1980) we found the staining procedures to be non-toxic to the protoplasts. Although we used a species (*E. lathyris*) for which protplasts had not previously been isolated, we were able to induce callus formation and shoot production from FITC- and RITC-stained leaf protoplasts.

In order to minimize the overlap of the emission signals from FITC and RITC (Fig. 2) a large number of stain concentrations were tested in

conjunction with various voltage and amplifier levels on the flow cytometer and on the cell sorter. In many instances the FITC fluorescence masked that of RITC. The optimum concentrations of fluorochromes needed to maximize stain intensities while minimizing overlap was 2.2 to 3.6ul FITC per ml of enzyme- protoplast solution and 7.2ul RITC for both instruments. The stain concentratins gave consistantly good spectral separation of protoplast populations. Further improvement in separating fluorescence signals of FITC and RITC was achieved using log amplifiers coupled with electronic gating of lower intensity signals. The log amplifiers provide greater separation of the lower intensity signals where the overlap occurs. Another improvement was the addition of a two-color compensator to subtract FITC fluorescence from the RITC signal. Preliminary testing of the compensator has shown increased quality of the two fluorescence signals with much better resolution in the flow cytometric histograms.

Protoplast fusion frequency is not a critical factor for hybrid selection using flow cytometry and cell sorting since at a typical flow rate of 1,000 protoplasts per second, a one percent fusion frequency would provide 10 fused products per second. The frequency of fused E. lathyris protoplasts (based on the presence of both fluorochromes) was determined to be about 1% by light microscopy. This corresponds well with the 1.3% frequency of fused protoplasts as determined by integration of the unique region in the flow cytometric histogram for fused protoplasts (Fig. 11). Because of the great efficiency of the flow system, heterokaryon recovery should be possible even when fusion frequencies are as low as 0.01% (one fused product per 10 s of sorting).

Recovery and reanalysis of intact protoplasts sorted on the FACS IV suggest that the protoplasts are able to withstand the relatively harsh

conditions of the flow system (flow rate of 4 to 5 M/sec with interrogation by a 0.3 watt argon-ion laser for 1 to 5 usec). Viability of sorted protoplasts was indicated by cytoplasmic streaming in P. inflata protoplasts. Flow cytometric analysis of FACS-sorted protoplasts produced very similar histograms to those of non-sorted protoplasts (based on light scatter and chlorophyll fluorescence) which indicates, at a quantitative level, that reanalyzed sorted protoplasts were of similar size and cholorophyll content as non-sorted protoplasts.

Protoplast sorting with the FACS IV is partially hindered because the machine is designed for cells somewhat smaller, in general, than plant protoplasts. Small-bore nozzle tips constrict and can damage the protoplasts. The 50um size nozzle is particularly damaging because it approaches the diameter of protoplasts. The 70um tip is optimum for both sorting and minimizing protoplast damage, although a larger tip would likely increase protoplast viability. The larger tips (eg. 90um), however, are not suitable for sorting because of difficuties in reducing the drive frequency of the piezoelectric crystal to a low enough value for droplet formation within the range of the flow chamber viewing area. This can be partially overcome by raising the nozzle tip to provide a greater distance from the orifice to the point of observation for drop formation, but other technical difficulties may thus ensue. Another problem, plugging of the nozzle tip with protoplast clumps, can be minimized by rigorous filtering of the sample just prior to uptake into the flow system.

Flow cytometric analysis of fluorescent-labelled plant protoplasts provides clear resolution of differentially-stained populations. Heterokaryons can be identified, characterized, and distinquished from homokaryons and unfused protoplasts using non-toxic levels of fluorescent

dyes. Intact, viable protoplasts can be recovered after being sorted for fluorescence activity whether from chlorophyll or exogenous fluorochromes. Since this method of protoplast selection is not species, genotype, or explant specific, it should serve as a general method for identifying and selecting hybrid protoplasts.

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Figures

Fig. 1. Diagram of flow cytometer. B, beam-shaping lenses; FC, flow chamber; L, lens; PMT, photomultiplier tube. After Hawkes and Bartholomew (1977).

Fig. 2. Emission spectra for separate 5mg/ml solutions of fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) dissolved in absolute ethanol at 488nm excitation.

Fig. 3. Emission spectra for differentially-stained *E. lathyris* protoplasts at 488nm excitation. Stain concentrations per ml of enzyme-protoplast solution were 3.6ul for FITC-stained protoplasts, 7.2ul for RITC-stained protoplasts and 3.6ul FITC and 7.2ul RITC for doubly-stained protoplasts.

Fig. 4. *E. lathyris* mesophyll protoplasts stained with fluorescent dyes. A-B, Bright-field and epifluorescence photomicrographs of protoplasts stained with 3.6ul FITC/ml. Both FITC and chlorophyll fluorescence is present and can be clearly distinguished in color photographs. C-D, Bright-field and epifluorescence photomicrographs of protoplasts stained with 7.2ul RITC/ml. Only RITC fluorescence is visible. E-G, Bright-field and epifluorescence showing doubly-stained protoplasts (F, FITC fluorescence; G, RITC fluorescence). H-J, Population containing fused protoplasts. Arrows show a fused protoplast containing both stains. Protoplasts common to I and J not marked with an arrow show chlorophyll fluorescence

in I and RITC fluorescence in J . The differences are clearly distinquished in color prints. Bar=40 um.

Fig. 5-7. Flow cytometric histograms of differentially-stained populations of E. lathyris mesophyll protoplasts. The origin is in the back corner of the plot. 5, Protoplasts stained with RITC (10,000 analyzed). 6, FITC-stained protoplasts (7,000 protoplasts analyzed). 7, Protoplasts stained with both FITC and RITC (25,000 analyzed).

Fig. 8. A comparison of contour histograms of Figs. 5-7 (lower limits of Fig. 7 gated out to enhance contrast). FITC-stained protoplasts along 526:576 axis, RITC-stained along 576 axis, and doubly-stained in the central region.

Fig. 9-12. Mixed populations of FITC- and RITC-stained protoplasts. 9-10, Isometric and contour histograms of unfused protoplasts showing a minimum population around the origin (25,000 analyzed). 11-12, Isometric and contour histograms of fused protoplast with a sizable population of fused protoplasts (containing both stains) around the origin (5,000 analyzed). The fused population is similar to the doubly-stained protoplast histogram in Fig. 7.

Fig. 13. Sequential analysis of E. lathyris protoplasts, unstained or stained with either or both fluorochromes, on the FACS IV. Stain concentrations were 2.2ul FITC/ml enzyme-protoplast solution, 7.2ul RITC, or a combination of both. FITC-stained protoplasts

fluoresce brightly only through the 520 bp filter while RITC-stained protoplasts fluoresce only through the 580 bp filter. Doubly-stained protoplasts show fluorescence through both filters, and unstained protoplasts register no fluorescence. Histograms taken from Polaroid prints.

Fig. 14. Protoplasts sorted and collected with the FACS IV. A, *E. lathyris* mesophyll protoplast (bar=10 um). B-C, *P. parodii* mesophyll and *P .inflata* albino protoplasts, respectively. Cytoplasmic streaming indicated protoplast viability. (bar=30 um).

Fig. 15. Light scatter and fluorescence histograms for *P. inflata* (albino) and *P. parodii* (leaf mesophyll) protoplasts. The first light scatter peak is debris plus free chloroplasts while the second is the protoplast population. The albino protoplasts have a very low fluorescence intensity peak due primarily to debris while the mesophyll protoplasts have a low intensity peak due to individual chloroplasts fluorescence and a high intensity fluorescence peak from intact protoplasts (chlorophyll fluorescence). Histograms taken from Polaroid prints. LS, light scatter; CF, chlorophyll fluorescence.

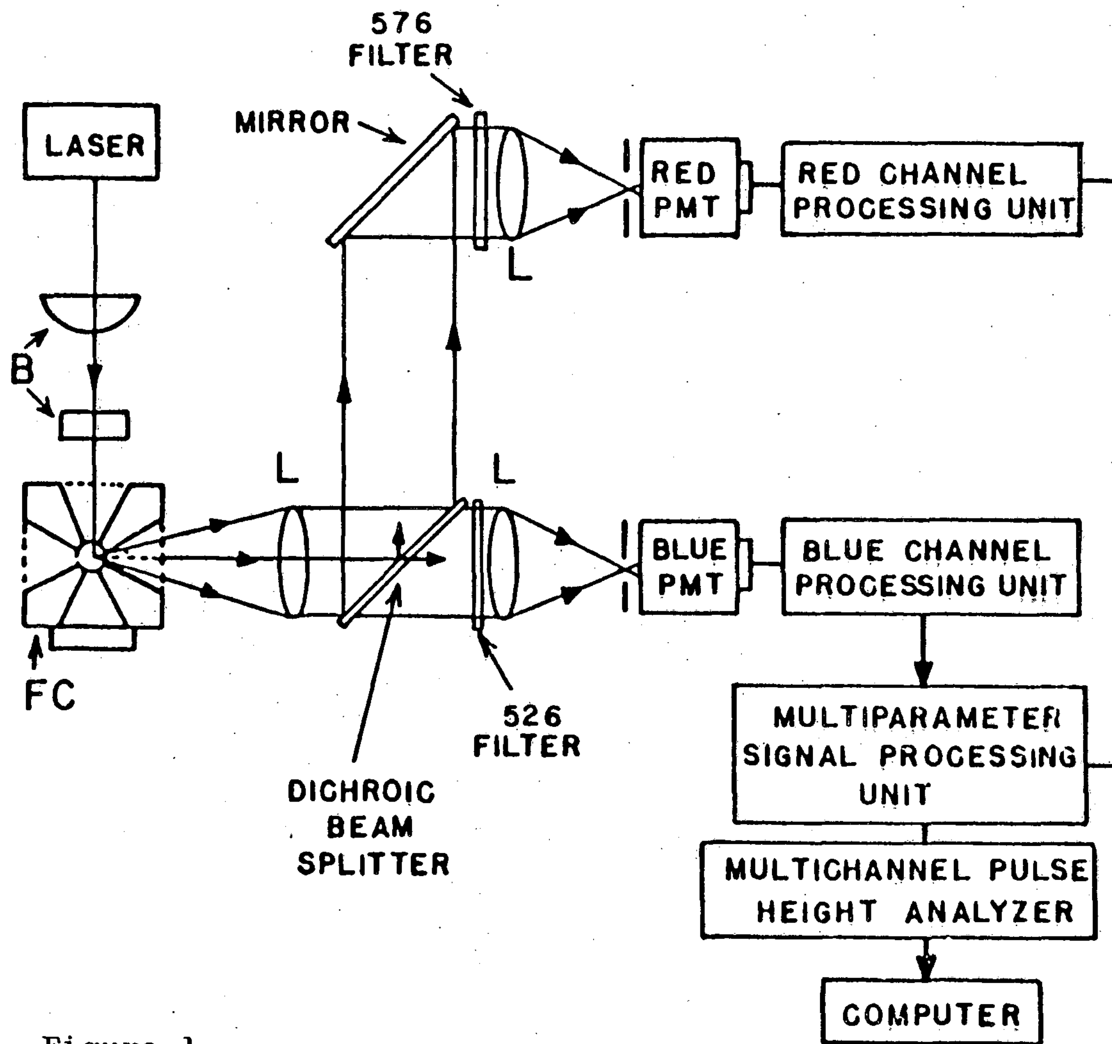


Figure 1

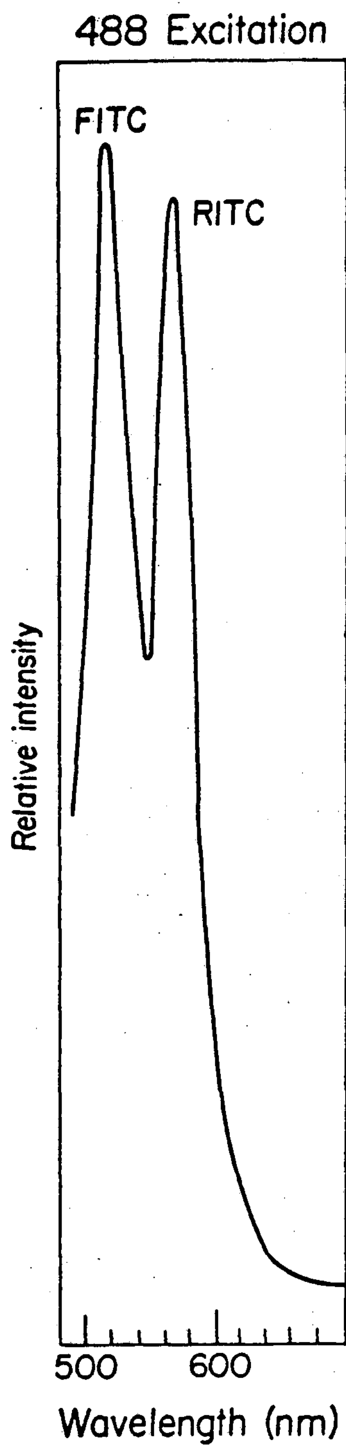
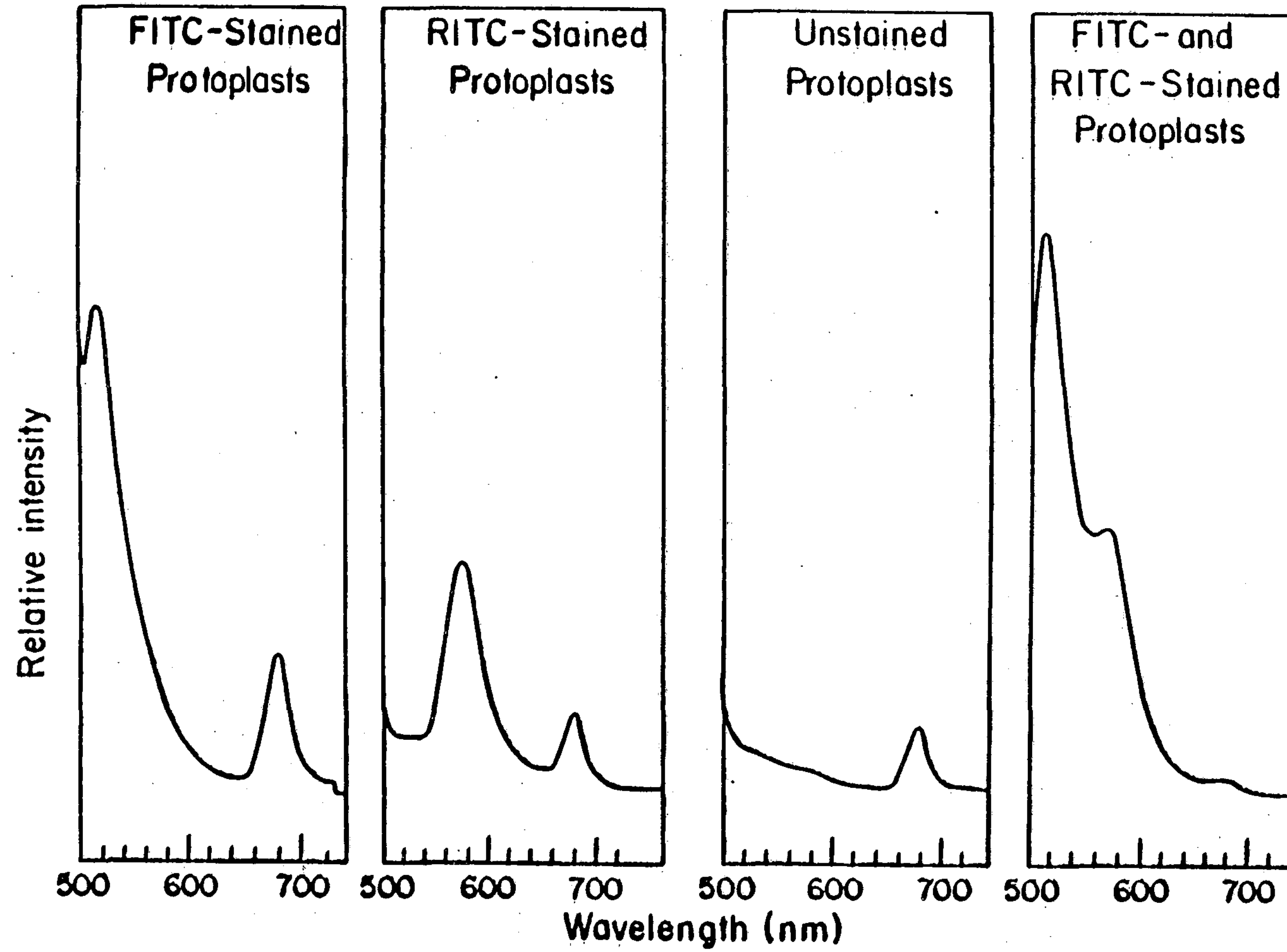


Figure 2



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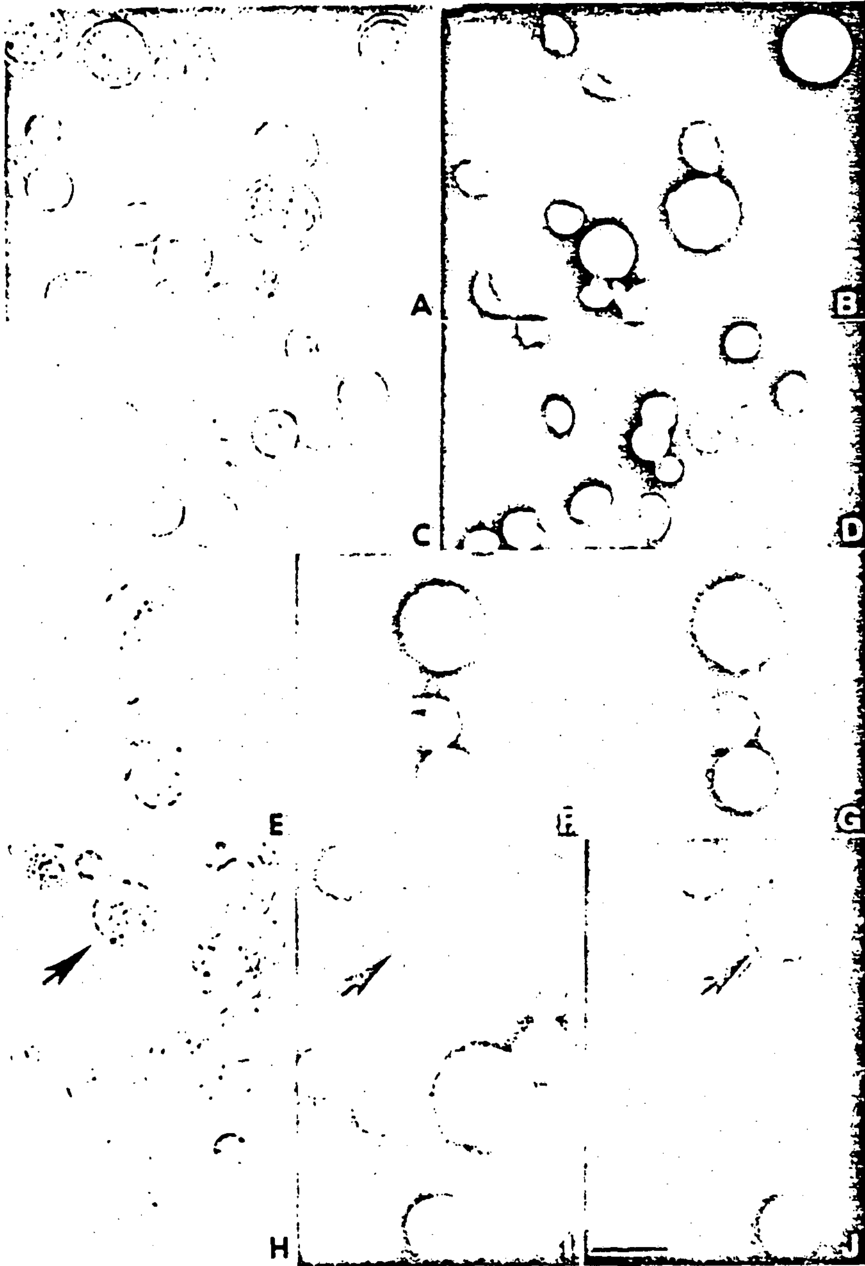
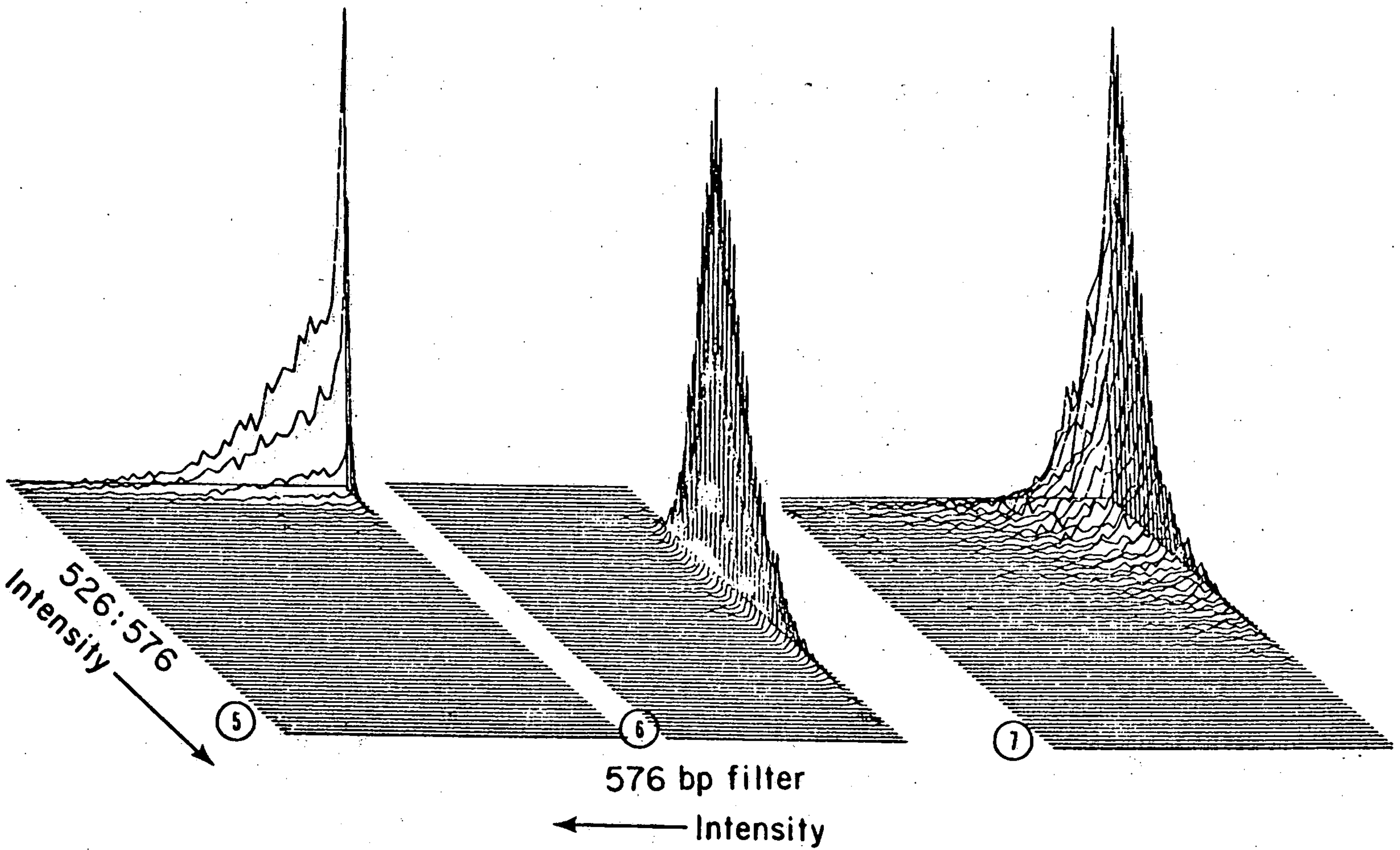


Figure 4



Figures 5,6, & 7

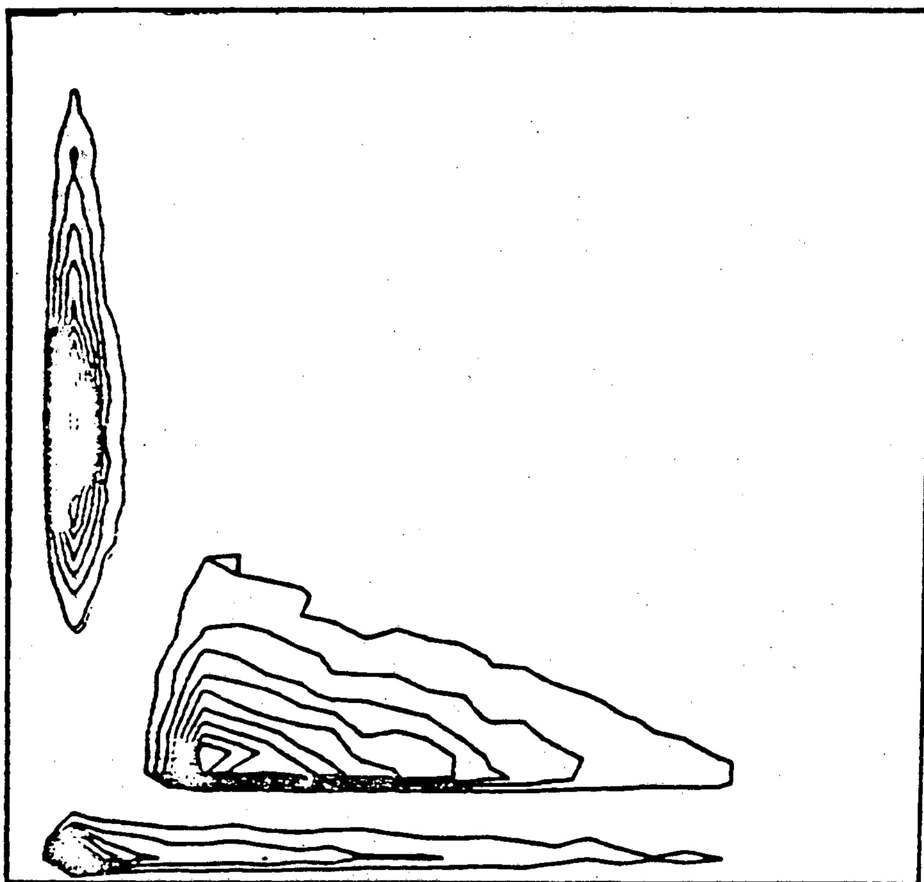
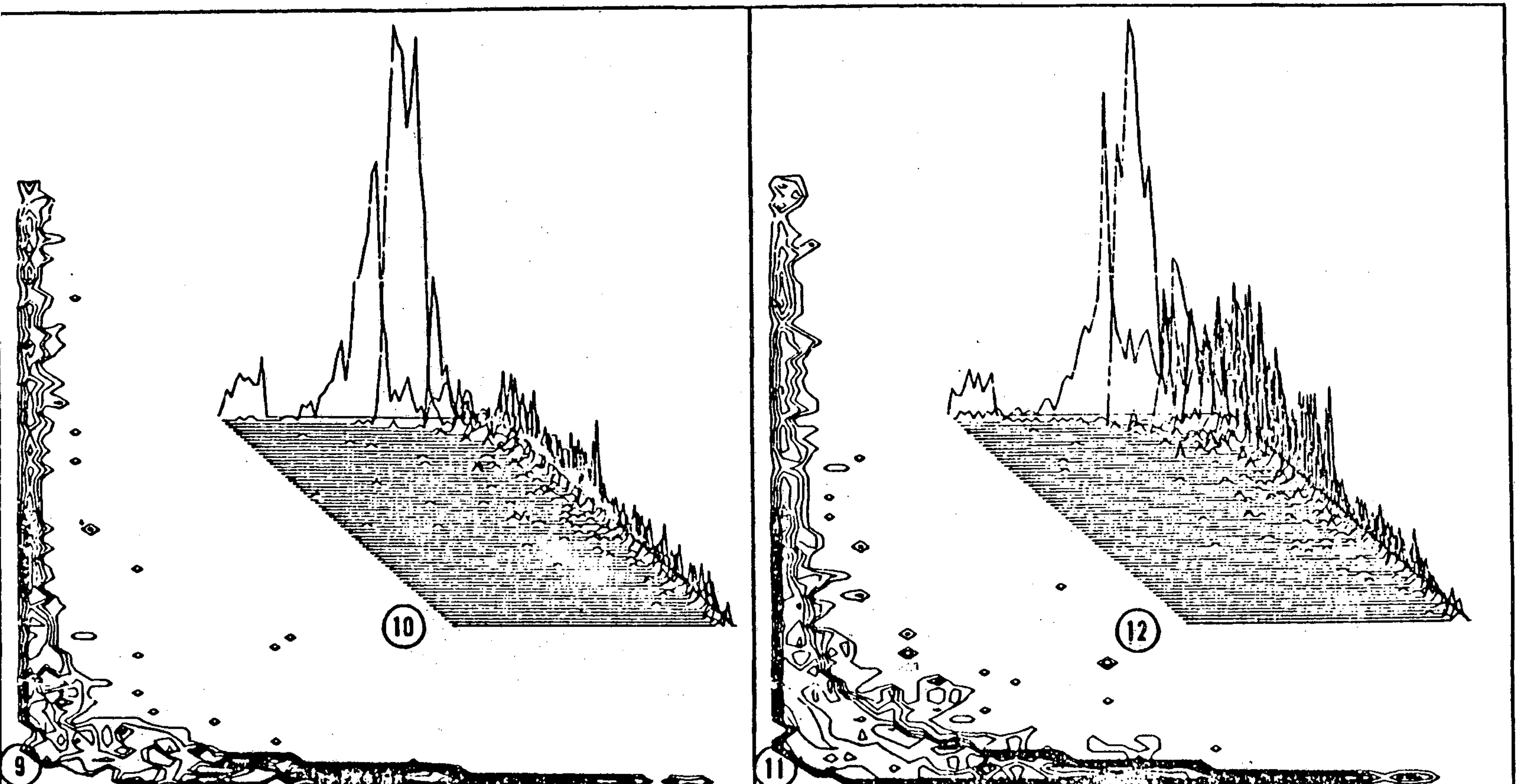


Figure 8



Figures 9,10,11 & 12

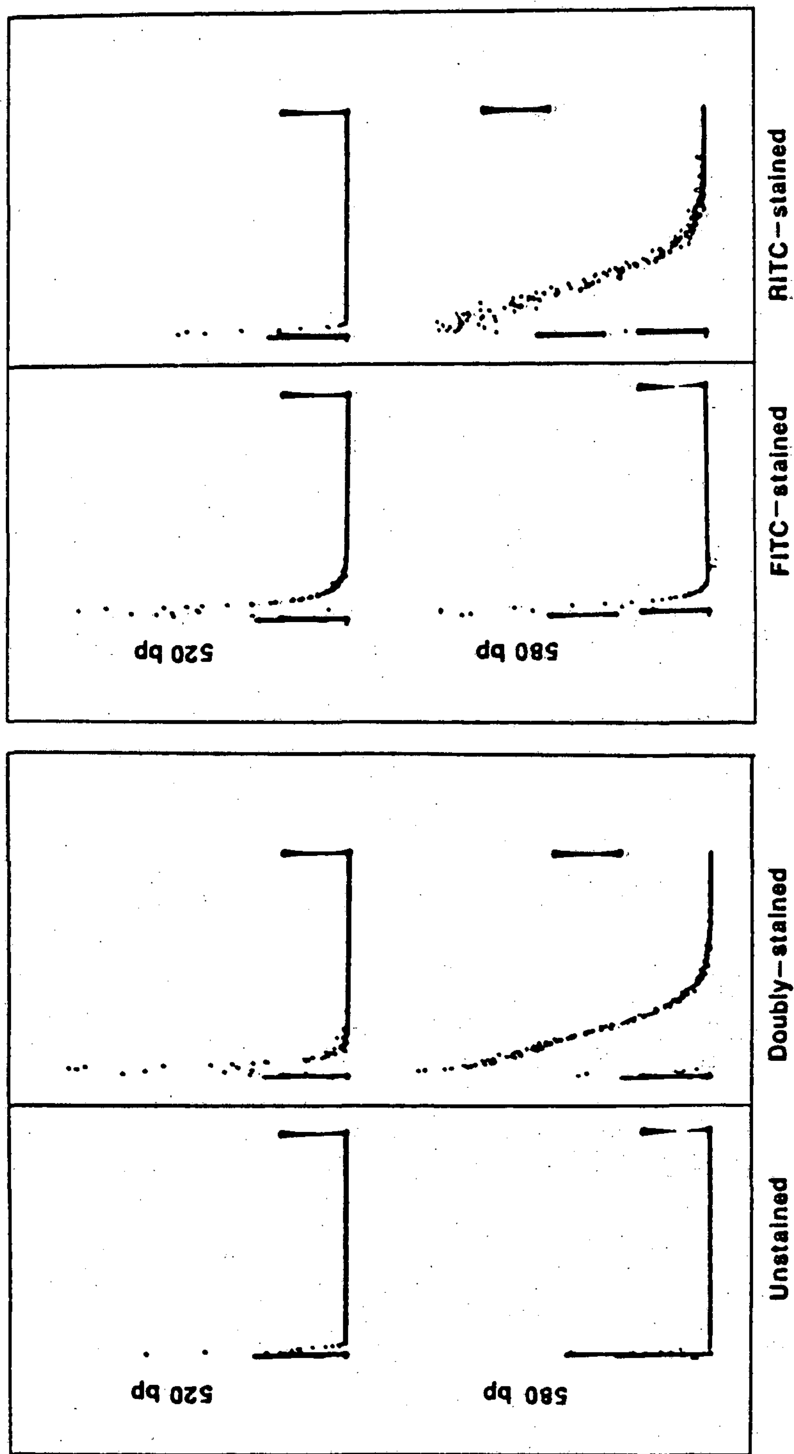


Figure 13

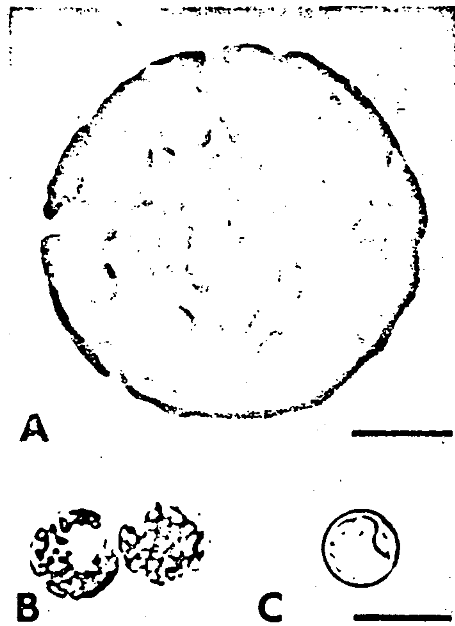
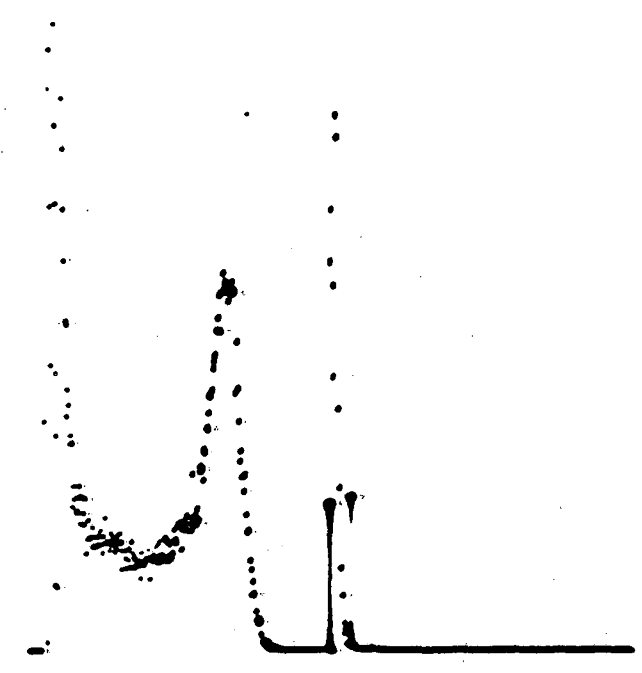


Figure 14



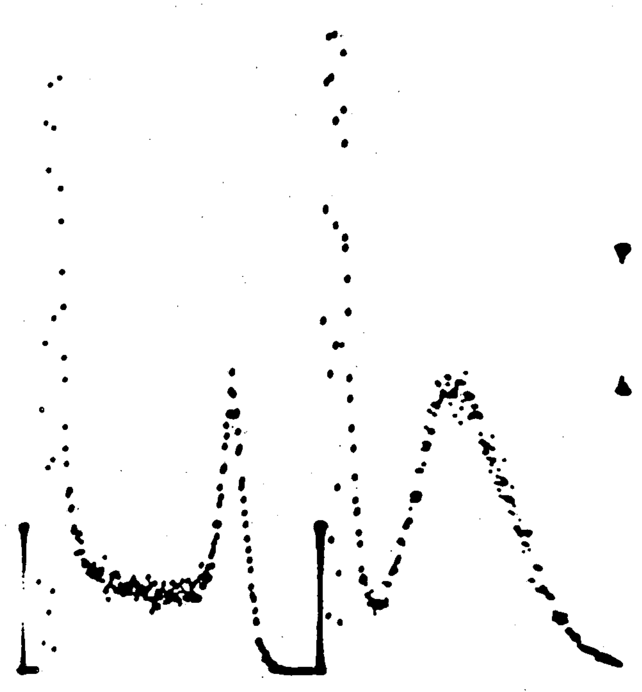


Figure 15

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