A Method for Incorporating Macromolecules into Adherent Cells

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ABSTRACT We describe a simple method for loading exogenous macromolecules into the cytoplasm of mammalian cells adherent to tissue culture dishes. Culture medium was replaced with a thin layer of fluorescently labeled macromolecules, the cells were harvested from the substrate by scraping with a rubber policeman, transferred immediately to ice cold media, washed, and then replated for culture. We refer to the method as "scrape-loading." Viability of cells was 50–60% immediately after scrape-loading and was 90% for those cells remaining after 24 h of culture. About 40% of adherent, well-spread fibroblasts contained fluorescent molecules 18 h after scrape-loading of labeled dextrans, ovalbumin, or immunoglobulin-G. On average, 10^7 dextran molecules (70,000-mol wt) were incorporated into each fibroblast by scrape-loading in 10 mg/ml dextran. The extent of loading depended on the concentration and molecular weight of the dextrans used. A fluorescent analog of actin could also be loaded into fibroblasts where it labeled stress fibers. HeLa cells, a macrophage-like cell line, J774A.1, and human neutrophils were all successfully loaded with dextran by scraping. The method of scrape-loading should be applicable to a broad range of adherent cell types, and useful for loading of diverse kinds of macromolecules.

Methods for loading macromolecules into cytoplasm are essential to studies in several recent and evolving fields of eucaryotic cell and molecular biology. Transformation of cells with exogenous DNA, in vivo studies of the location and activity of cytoskeletal proteins and other macromolecules in living cells by fluorescent analog cytochemistry, and the analysis of cytoplasmic degradation of proteins all depend on the incorporation of large molecules into living cells.

Certain characteristics are desirable of any method for loading macromolecules into cells. The method should be simple, applicable to diverse cell types, and capable of loading a wide range of macromolecule species. A significant proportion of cells in a population should be loaded with the macromolecule and to a measurable extent. The macromolecules should be introduced, initially, into the cell's cytoplasm only. Cell function, morphology, and viability should not be extensively compromised by the loading method.

Several methods are now available for loading exogenous macromolecules into cell cytoplasm. Microneedles are used to inject diverse substances into individual cells (8; see 6 for a review). Brief exposure to a hypotonic medium loads protein in that medium into the cytoplasm of a cell population (2). Hyposomotic shock of pinosomes containing dextran or protein releases most of these substances into the cytoplasm of cells (14). Liposomes and red blood cells containing trapped substances can, after fusion with host cells, deliver such trapped substances into host cytoplasm (17, 7, 15). In addition, high voltage electric impulses enhance uptake of DNA into cells (13).

No single one of these methods now available fulfills all the criteria listed above, although each will continue to be useful for certain applications. We describe in this paper a new technique for loading macromolecules into the cytoplasm of adherent cells.

MATERIALS AND METHODS

Cell Culture and Preparation: Swiss 3T3 cells from American Type Culture Collection (Rockville, MD) were grown in Dulbecco's modified essential medium (base DME) (Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum (Gibco Laboratories), 0.3 mg/ml L-glutamine, 50 U/ml penicillin, and 0.05 mg/ml streptomycin (complete DME). 3T3 cells were passed through subcultures by trypsinization in 0.05% trypsin and 0.02% EDTA in Ca-Mg-free saline. Passage number of these cells ranged from 126 to 131.

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The macrophage-like cell line, J774A.1, from American Type Culture Collection, was grown in DME supplemented with 10% fetal calf serum (Gibco Laboratories) and L-glutamine, penicillin, and streptomycin as above. The J774A.1 cell line was passed through subcultures by scraping in culture medium with a rubber policeman (16).

HeLa cell cultures were the generous gift of Dr. J. F. Williams. Neutrophils from the blood of healthy human donors were isolated by the method of Boyum (3).

Fluorescein-labeled Dextrans: Unless otherwise stated, three dextrans labeled with fluorescein (FTC-dextran) (Sigma Chemical Co., St. Louis, MO) were used to load cells: 10,500 average molecular weight, molar dye/dextran ratio of 0.23; 39,000 average molecular weight, molar dye/dextran ratio of 1.3; and 65,000 (=70,000 mol wt referred to in Results) average molecular weight, molar dye/dextran ratio of 7.3.

Dextrans to be fractionated into a narrow range of molecular weights (by procedures similar to those of Caulfield and Farquhar [4]) were purchased from Pharmacia, Inc. (Piscataway, NJ) with average molecular weights of 40,000, 500,000, and 2,000,000. 1 g of each was labeled by a published method (5) with fluorescein isothiocyanate (FITC). Each molecular weight species (20 mg in 2 ml) was then fractionated separately by gel filtration chromatography on Sepharose CL-4B (1.5 × 95 cm) in 0.05M Tris-HCl, pH 8.0. The three central-most fractions of each broad fluorescence peak were pooled (7.5 ml total), concentrated 5-fold in an Amicon filter, and dialized against base DME for use in scrape-loading of cells. After use in scrape loading, 1 ml of each narrow range species from the first cellular wash (see below) were rechromatographed together on the Sepharose CL-4B column. Concentrations of dextran in the narrow range samples were determined by an anthrone reaction (18); concentrations of fluorescein covalently coupled to the dextrans were determined spectrophotometrically using a molar extinction coefficient for fluorescein at pH 8.0 of 6.8×10^4. The concentrations and molar dye to dextran ratios of the 40,000, 500,000, and 2,000,000 narrow range fractions used for scraping were, respectively: 2.5 mg/ml, 3.0; 2.0 mg/ml, 82.0; 1.0 mg/ml, 420.0

Fluorescein-labeled Proteins: Nonspecific goat immunoglobulin G labeled with FITC (FTC-IgG) was purchased from Sigma Chemical Co. It was dialyzed against base DME and used for scrape-loading at 19.6 mg/ml (molar dye/protein ratio of 3.8). Ovalbumin was labeled with FITC by previously published methods (22) and then dialyzed against base DME. Its concentration was 5 mg/ml and its dye/protein ratio was 0.9. Actin purified from rabbit skeletal muscle (19) was labeled with 5-iodacetamido-fluorescein (Molecular Probes, Cleveland, OH) as previously described (22). Its concentration for scrape-loading was 8.7 mg/ml; its molar dye/protein ratio was 0.6.

Flow Cytometry: Those 3T3 cells to be analyzed immediately after scrape-loading by flow cytometry were first allowed to settle 20 min under gravity, and the supernatant solution taken for analysis. This was necessary to prevent clumps of cells from clogging the instrument's nozzle. Those 3T3 cells plated after scrape-loading for analysis 24 h later were released from 35-mm plastic culture dishes with trypsin (as for normal passage) and did not form clumps. Hence, the settling step was omitted before analysis by flow of plated cells.

Flow cytometric analyses were performed on a FACS 440 with a Consort 40 Data Management System (Becton Dickinson FACS Systems, Sunnyvale, CA). Excitation was provided by a 488-nm argon ion laser line (400 mW), and fluorescein fluorescence was detected with a narrow band fluorescein filter (#1793, Becton Dickinson). The amount of forward and right angle light-scattering, and linear and log fluorescein fluorescence were recorded for each cell in list mode (at least 20,000 cells per sample). A forward angle light-scattering threshold was set just below the peak of dead cells. For propidium iodide (PI)-exclusion analysis, a 50% beam splitter was used to measure linear fluorescein fluorescence with the normal filter, while linear PI fluorescence was measured using a 620 nm long pass filter. Cells were incubated with 100 μg/ml PI for 10 min before analysis.

Neutrophil Standards: Neutrophils isolated by leukophoresis for use as standards were the gift of Dr. J. Nusbacher (Pittsburgh Blood Bank). Red blood cells were removed by two cycles of osmotic lysis. The neutrophils (1.2×10^9 cells) were then resuspended in 5 ml of phosphate buffered saline (PBS) (Ca- and Mg-free) and squirted rapidly through a 25 gauge needle into 15 ml of a 70:30 mixture of ethanol and PBS. These fixed cells were then further diluted with 20 ml of ethanol/PBS, divided into four equal 10-ml portions, and FITC (from 1 mg/ml stock in 95% ethanol) added to a final concentration of 0.001, 0.01, or 0.1 μg per 10^6 cells. The fourth portion of neutrophils received no FITC. The cells were left overnight at 4°C to label with FITC, and then dialyzed for 72 h against 4 liters of cold ethanol/PBS. Labeled neutrophils were stored in the ethanol/PBS at −20°C until use.

The number of fluorescein molecules per labeled cell was estimated by spectrophotometry or fluorimetry of the extracts of the labeled neutrophils. 1 ml each of the labeled neutrophils, and unlabeled cells, were pelleted by centrifugation and then resuspended in 200 μl of cold PBS (Ca-Mg-free). Cell counts of each suspension were made in a standard microscope hemocytometer. To 180 μl of each neutrophil sample was added 200 μl of 10 mg/ml protease (Sigma Chemical Co.) in Tris-HCl, pH 8.0, and the mixture was incubated at 37°C for 1 h. To further reduce scatter, 60 μl of 10% SDS was added to the partially digested neutrophils. This mixture was thoroughly vortexed, and then diluted with a further 720 μl of Tris-HCl, pH 8.0. Using the unlabeled neutrophils as a blank, absorbance and fluorescence (excitation 495 nm; emission, 520 nm) was then measured from each sample. Fluorescein concentration was determined from absorbance using the molar extinction coefficient given above, or from fluorescence measurements using FTC-ovalbumin standards of spectrophotometrically predetermined fluorescein content.

Measurement of Radiolabeled Dextran Loaded into Fibroblasts by Scraping: Fibroblasts on four polystyrene dishes (60-cm dishes, each holding 2×10^5 cells) were scraped in 1.0 ml of 10.0 mg/ml 70,000-mol wt dextran [^{14}C]carboxyl, specific activity 1.2 μCi/mg (New England Nuclear, Boston, MA, Lot 1785-052) dissolved in base DME, or they were scraped in base DME only. These latter then received 1.0 ml of the dextran [^{14}C]carboxyl before their centrifugation in the first cold wash. Cells were washed as usual for scrape-loading, and then replated on dishes and placed in an incubator for 24 h. After three washes in 5 ml of complete DME, cells were trypsinized from the dishes, cell numbers were counted in a hemocytometer, and 0.45 ml of the cells (10^6) were mixed with an equal volume of 1.0% SDS, and with 10 ml of scintillation fluid. Radioactivity was measured with a Beckman LS7000 scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Background counts (30 dpm) were measured from samples of fibroblasts that received no dextran [^{14}C]carboxyl.

Scrape-loading of J774A.1 and HeLa Cells: The J774A.1 population was scraped directly from culture flasks (Nunc, Intermed, Roskilde, Denmark), but were otherwise processed as for fibroblasts (see Results). HeLa cells were loaded by scraping as for fibroblasts (see Results).

Scrape-loading of Neutrophils: Neutrophils freshly isolated from human blood were plated on 35-mm plastic petri dishes in phosphate buffered saline and 1% bovine albumin, and incubated for 30 min at 37°C. The cells were then scraped from their dishes in FTC-dextran in base DME, and processed as for fibroblasts.

Loading of Actin by Chilling followed by Scraping or Mild Agitation: 5-iodoacetamido-fluorescein actin (AF-actin) could not be easily loaded into fibroblasts by scraping in culture media, 37°C. Scraping of cells immediately after immersion in actin at 0°C was not suitable, because it killed nearly all the fibroblasts. We therefore chilled the fibroblasts for 10 min prior to initiating the loading by scraping or mild agitation. Fibroblasts were incubated for 10 min on ice on 35-mm plastic tissue culture dishes in base HEPES-buffered DME. This medium was then replaced with 100 μl of ice cold monomeric AF-actin in PBS (Ca-Mg-free), and the cells scraped from the dishes as above, or the actin solution was pipetted repeatedly for 1 min onto and off of the cells. Fibroblasts scraped from dishes were then layered on top of 2 ml of cold complete DME, and separated from free AF-actin by centrifugation. Those agitated by pipetting remained adherent to the slide, and were washed thoroughly with cold complete DME. Finally, the scraped fibroblasts were allowed to recover for 6 h in an incubator before microscopic observation; the agitated fibroblasts were allowed 3 h.

Plating Efficiency Assays: Fibroblasts plated 3 d previously on 60-mm culture dishes were either scraped from the dishes in 10 mg/ml FTC-dextran in base DME, or trypsinized from the dishes as usual for subculturing. One-half of the trypsinized cells and all scraped cells were then washed twice in cold, complete DME as usual for scrape-loading. These washed cells and remaining unwashed, trypsinized cells were then diluted so that, on average, 100, 50, and 10 viable cells of each sample were plated onto three replicate 35-mm dishes. Media in the dishes was changed every 3 d. On the 12th day the plates were fixed and stained with Diffquick (American Scientific Products Div., McGraw Park, IL), and the number of colonies of cells in each dish counted.

Light Microscopy: A Zeiss Photomicroscope II was used with a 40 × phase water immersion lens for phase optics; a 40 × planapo water immersion lens for Nomarski optics; or a 63 × (NA = 1.25) planapo water immersion lens for fluorescence microscopy. Images were recorded on Ilford HP-5 35-mm film exposed at 800–3200 ASA and developed in Diafine, or with a Zeiss 3-stage image intensifying camera and a video recorder.

RESULTS

Scrape-loading of Fibroblasts

Approximately 10^5 fibroblasts present at a subconfluent density on 60-mm tissue culture grade polystyrene culture

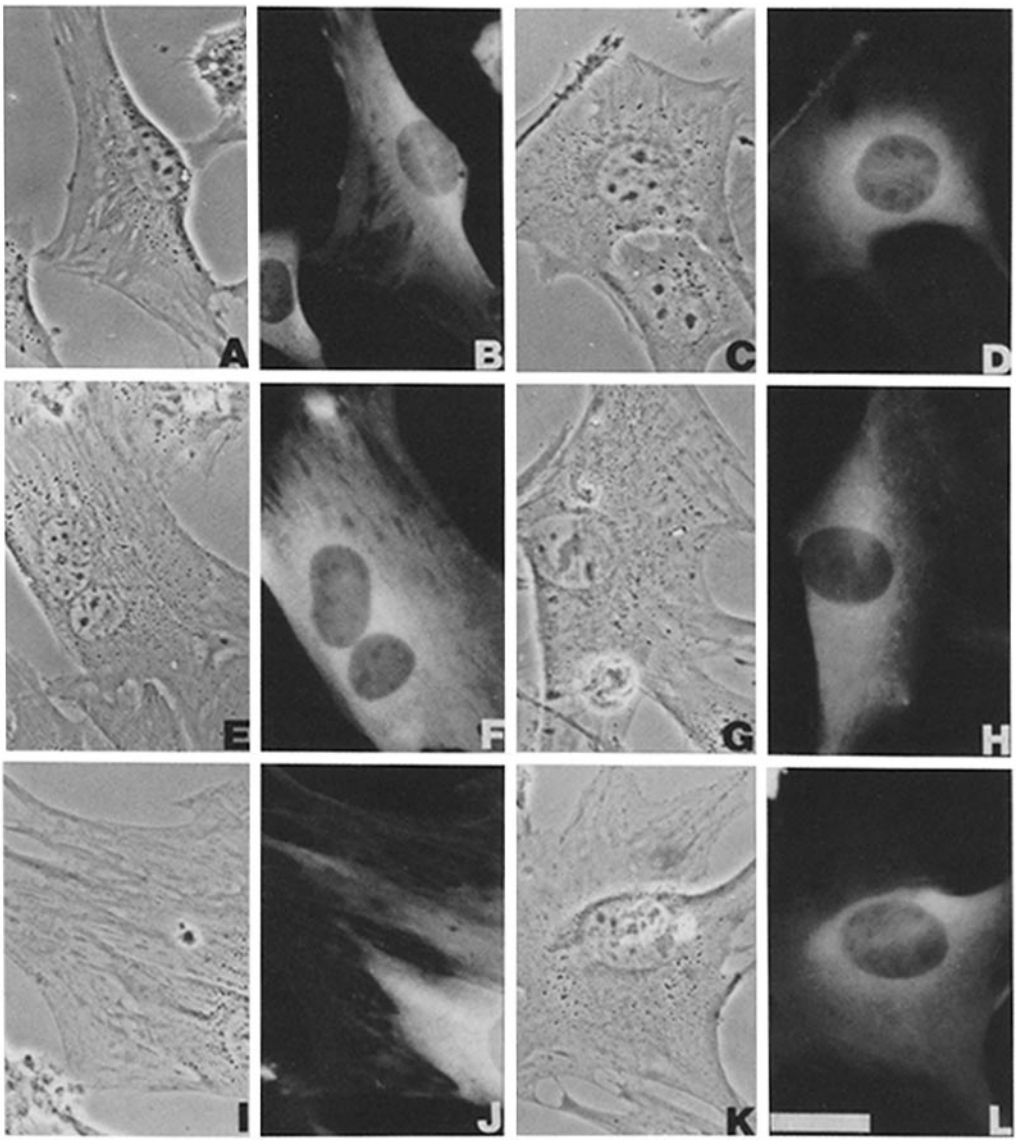


FIGURE 1 Phase contrast (*A, C, E, G, I, K*) and fluorescence (*B, D, F, H, J, L*) images of living fibroblasts 18 h after scrape-loading with FTC-dextran, 70,000-mol wt, 10 mg/ml. Fluorescence images were recorded on Ilford HP5 film. Bar, 10 μm.

dishes were used in the loading procedure. Culture medium aspirated from the cells was replaced with 0.5 to 1.0 ml of the fluorescent molecules dissolved in base (without serum) DME at 37°C. The cells were then immediately scraped from the plastic surface with a rubber policeman, and pipetted directly into 10 ml of ice cold complete DME. Cells were exposed to the fluorescent molecules at 37°C for a maximum of 10 s. The loaded cells were washed in the first and a second 10 ml of ice cold DME by centrifugation (200 g for 5 min), and were then analyzed by flow cytometry or plated on microscope slides or plastic culture dishes for culture and later analysis.

Microscopy of the Scrape-loaded Cells

By 9–18 h after scrape-loading, the typical fluorescent fibroblast was fully adherent and had assumed characteristic morphologies (Fig. 1, *A, C, E, G, I, K*). Up to 40% fibroblasts loaded in 10 mg/ml FTC-dextran (70,000-mol wt) were clearly fluorescent at this time, and to an extent readily recordable on 35-mm film, with a SIT camera or with an image intensifier (Fig. 1, *B, D, F, H, J, L*). Dextran of molecular weight 30,000 and above was excluded from most nuclei and other cellular organelles for at least 20 h, unlike dextran of 10,500-mol wt which clearly entered the fibroblast nucleus. Most cells containing FTC-dextran were uniformly fluorescent 18 h after scrape-loading (Fig. 1, *B, D, F, H, J, L*), although limited autophagocytosis was sometimes indicated by fluorescent cytoplasmic vesicles.

Cells scrape-loaded with FTC-ovalbumin or FTC-IgG (Fig. 2) were also readily visible by fluorescence microscopy.

Viability of the Scrape-loaded Fibroblast

The viability of scrape-loaded cells was estimated by flow cytometry and by a plating efficiency assay. Two measures of cell viability were recorded in the flow cytometer from the scrape-loaded fibroblasts. These were forward angle light scatter and fluorescence of cells incubated with the nucleic acid dye, propidium iodide, which stains nuclei of dead cells only. Table I shows that between 50 to 60% of fibroblasts were living immediately after scrape-loading; and that 24 h later, after plating on tissue culture dishes and subsequent trypsinization, 90% or more were viable. In a typical experiment, if we started with 10^5 cells before loading, we obtained 2×10^4 live and fluorescent cells 24 h after loading.

As a test of the longer term viability of those cells surviving

scrape-loading, we used a plating efficiency assay. The plating efficiency of viable scrape-loaded cells (assessed by trypan blue exclusion) was compared with that of viable cells released from dishes by trypsinization (Table II). The plating efficiency of scrape-loaded cells measured after 12 d was not significantly different from that of cells released by trypsinization and then washed twice in cold medium, although both were ~10% lower than trypsinized cells plated without the cold washes.

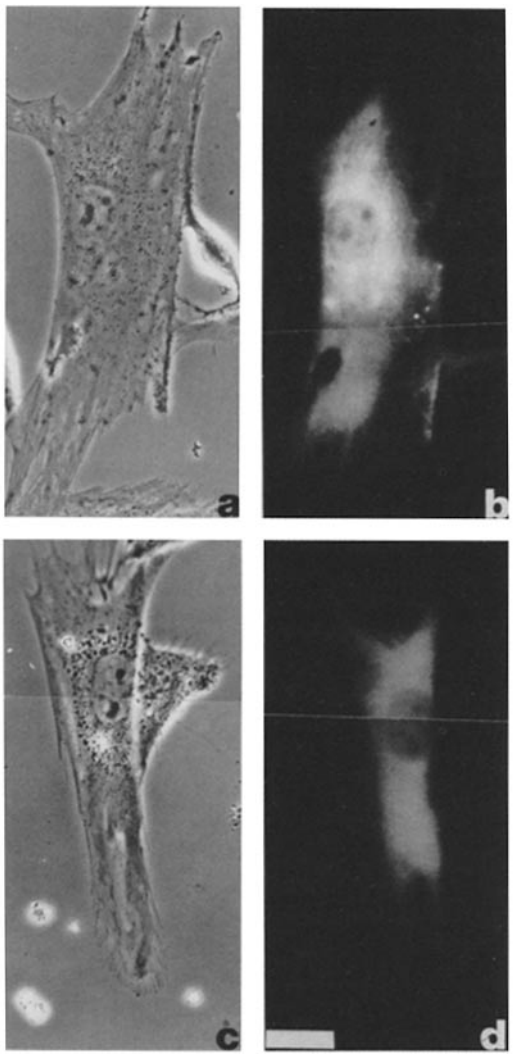


FIGURE 2 Phase (*A* and *C*) and fluorescence (*B* and *D*) images of fibroblasts 18 h after scrape-loading with FTC-IgG (nonspecific). Fluorescence was recorded with a Zeiss three-stage image intensifying television camera. Bar, 10 μm.

Flow Cytometric Analysis of the Population of Scrape-loaded Cells

Fluorescein fluorescence and forward scatter were measured by flow cytometry from fibroblasts scrape-loaded in 10 mg/ml FTC-dextran, 70,000-mol wt (Fig. 3). The extent of loading was heterogeneous, both immediately after scraping and 24 h later: fluorescence intensities ranged more than 100-fold across the population of loaded cells. Elevated fluorescence of the scrape-loaded cells was not due to FTC-dextran incompletely washed out of the cell medium, or to pinocytosis of FTC-dextran during the cold washes. As a control, FTC-dextran was added to the first wash of the control cells to a concentration equivalent to that in the first wash of the scrape-loaded cells. Enhanced fluorescence was not detected in this control population (Fig. 3).

TABLE II
Plating Efficiency of Scrape-loaded Fibroblasts

Experiment*	Number cells plated per dish	Plating‡ efficiency	Average plating efficiency at all densities
Trypsinized, no cold washes	10	60	52
	50	50	
	100	46	
Trypsinized, with two cold washes	10	40	42
	50	37	
	100	48	
Scrape-loaded, with two cold washes	10	50	40
	50	32	
	100	39	

* Details of plating efficiency assay and experimental treatments are given in Materials and Methods.
‡ Plating efficiency = number of plaques formed/number of cells plated × 100. Each value represents the mean of three replicates from each of two experiments.

TABLE I
Viability of Scrape-loaded Fibroblasts

Experiment	Scraped* FTC-dextran	Parameter‡ monitored	Percent live	Percent live§ and fluorescent	Percent dead§ and fluorescent	Percent of Live‖ that are Fluorescent
Immediately after Scrape-loading	yes	PI	61.5	11.5	0.5	19.7
	no	PI	58.5	1.8	0.4	3.0
	yes	SC	51.4	35.0	13.0	68.0
	no	SC	60.6	2.6	1.3	4.3
24 h after Scrape-loading	yes	PI	89.8	40.6	0.8	41.8
	no	PI	97.2	0.3	0.9	0.3
	yes	SC	90.0	39.9	0.8	47.0
	no	SC	85.0	0.6	0.1	0.7

* Fibroblasts were scraped in 70,000-mol-wt FTC-dextran at 10 mg/ml (= yes) or in base DME without FTC-dextran (= no).
‡ *PI*, propidium iodide fluorescence; *SC*, forward angle light scattering.
§ Percent of all cells analyzed (includes dead cells).
‖ Obtained by dividing percent live and fluorescent by percent live. These are the cells selected for microscopic analysis.

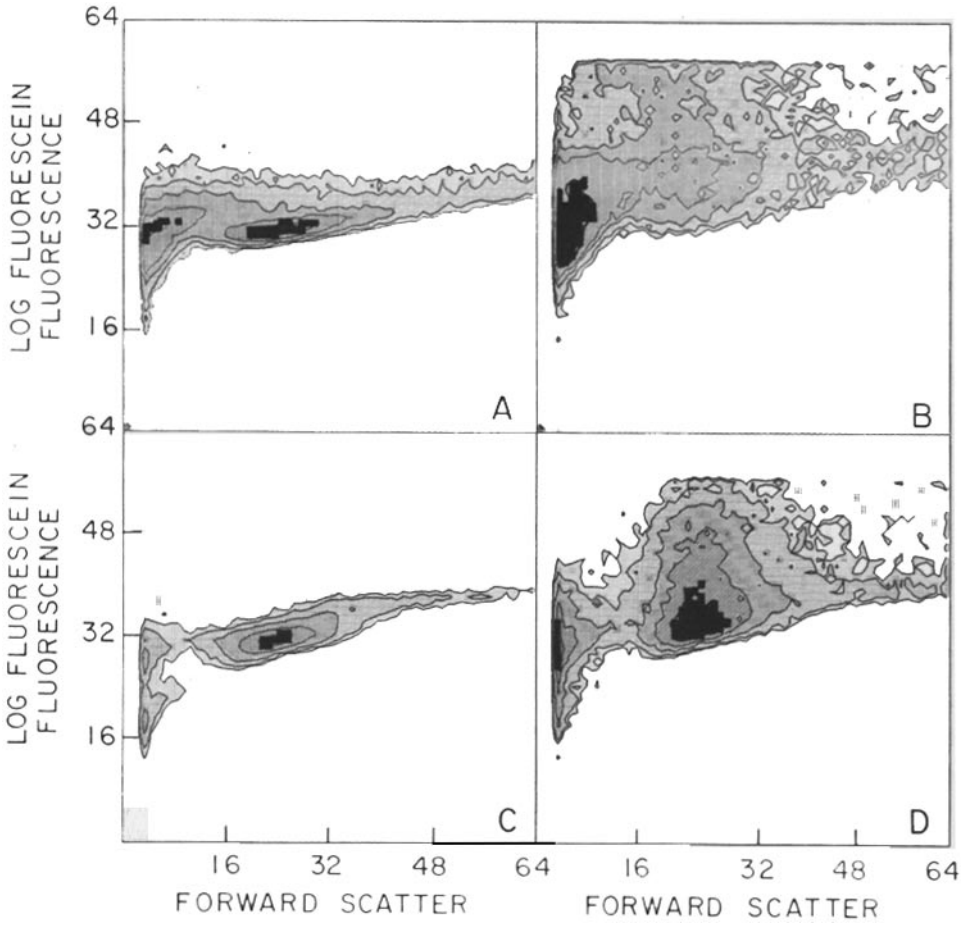


FIGURE 3 Correlated dual parameter flow cytometric histograms relating forward angle scatter to the log of fluorescein fluorescence of fibroblasts scraped in base DME (*A* and *C*) or in 10 mg/ml FTC-dextran, 70,000-mol wt (*B* and *D*). Contour lines are drawn connecting those histogram bins containing 95, 90, 75, 50, and 20% of the total number of events. Fibroblasts in *A* and *B* were analyzed immediately after scrape-loading, those in *C* and *D* 24 h later after plating on plastic culture dishes and release from dishes by trypsinization. To the first wash of cells scraped in base DME only (*A* and *C*) was added FTC-dextran to a concentration equivalent to that present in the first wash of those fibroblasts scraped in FTC-dextran (*B* and *D*). The low scattering cluster to the left in each histogram is dead cells and debris. Each histogram contains 25,000 events.

Measurement of the Number of Dextran Molecules Loaded by Scraping

The number of fluorescein or dextran molecules loaded by scraping into the average fibroblast was calculated by flow cytometry from the calibration curve presented in Fig. 4*A*. Approximately 2×10^8 FITC molecules or 2.8×10^7 dextran molecules were loaded on average into fibroblasts scraped in 70,000-mol wt FTC-dextran at 10 mg/ml. The relative frequency at which cells were loaded to a measured level with FTC-dextran is given in Fig. 4*B*.

A radiotracer (^{14}C-labeled dextran) was used as an independent means of measuring the amount of 70,000-mol wt dextran, at 10 mg/ml, loaded by scraping (Table III). The resulting number of dextran molecules loaded per cell is calculated as 4.7×10^7.

Effects of Dextran Concentration and Molecular Weight on the Extent of Scrape-loading

The average amount of dextran loaded into fibroblasts was a nonlinear increasing function of the concentration of FTC-dextran present in the medium during scraping (Fig. 5). This nonlinearity was probably due to increasing viscosity with dextran concentration.

We also examined how the molecular weight or size of the dextran molecule affected the measured extent of loading (Fig. 6). Fibroblasts were scraped in three narrow (see *inset* Fig. 6) range molecular weight fractions of dextran (40,000, 500,000, and 2,000,000) diluted before dialysis (see Materials and Methods) to give equivalent concentrations of fluorescein in the scraping medium. Fluorescence was then measured from the fibroblast populations by flow cytometry, and the mean number of fluorescein molecules loaded calculated from the standard curve. A sharp decline in the mean number of fluorescein (Fig. 6 *top*) or dextran molecules (Fig. 6 *bottom*) loaded per cell was measured between the 40,000- and 500,000-mol wt species. However, 10^5 molecules of 500,000-mol wt dextran entered the average fibroblast.

Scrape-loading of Other Mammalian Cells

FTC-dextran, 70,000-mol wt was successfully loaded by scraping into HeLa cells (Fig. 7), a macrophage-like cell line, J774A.1 (Fig. 8), and into human neutrophils (not shown). While the viability of these cells was not rigorously assessed after loading, all resumed normal morphologies and were adherent and motile after scraping. Moreover, the J774A.1 is routinely subcultured by scraping (16).

Loading of Actin into Chilled Fibroblasts

AF-actin, which could not easily be loaded by scraping of fibroblasts at 37°C, was loaded by a combination of cell chilling and scraping or slight mechanical agitation (see Materials and Methods). AF-actin was observed 3 h after loading by these methods as distinct fibrous structures in some cells (Fig. 9*A*), while its cytoplasmic distribution was more uniform in others (9*B*). These results are similar to those from fibroblasts microinjected with fluorescent actin analogues (10, 12). We have not yet quantified the efficiency of loading by chilling

with agitation.

DISCUSSION

Evaluation of the Method

We showed that exogenous macromolecules can be introduced into adherent cells by scrape-loading. The technique is technically simple and is inexpensive. Some 40% of a fibroblast population was loaded to an extent distinguishable from control levels, and the extent of this loading ranges by more than two orders of magnitude across the population. Dextran

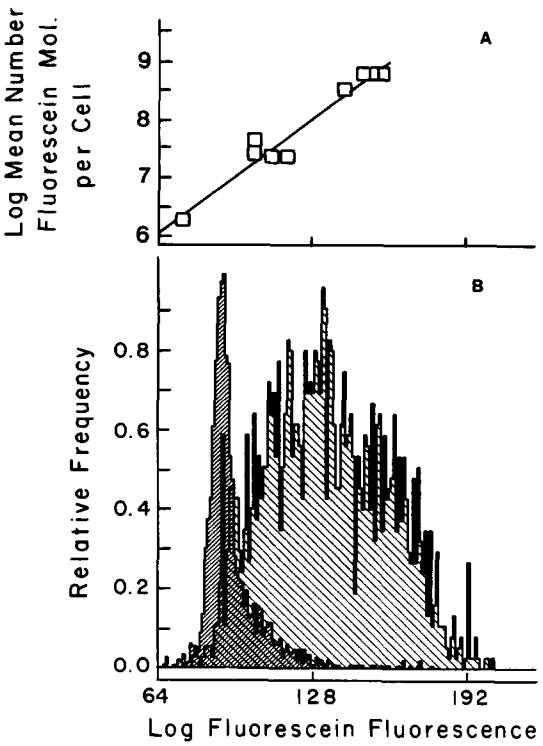


FIGURE 4 (*A*) Standard curve relating log fluorescein fluorescence measured by flow cytometry on FTC-labeled neutrophils to the numbers of fluorescein molecules per labeled neutrophil (see Materials and Methods for details). (*B*) Relative frequency at which fibroblasts from a population scraped in base DME (////) or 10 mg/ml FTC-dextran, 70,000-mol wt (▨) were loaded with the indicated quantity of log fluorescein fluorescence. The number of fluorescein molecules per scrape-loaded cell for a given region of the histogram can be obtained by projecting it upward to the standard curve and then subtracting the value similarly obtained for the unloaded cells.

TABLE III

Radiotracer Measurements of the Extent of Scrape-loading of Dextran 70,000-mol wt into Fibroblasts

Sample	DPM per 10^5 cells*	Calculated number dextran molecules loaded per cell
Control cells scraped in DME only‡	60	—
Experimental cells scraped in [^{14}C]dextran	389§	4.7×10^7

* Mean of three replicates.

‡ Control fibroblasts were scraped in base DME only, then dextran [^{14}C]-carboxyl was added to their first cold wash to a concentration equivalent to that in the first wash of the experimentals. Experimentals were scraped in 1.0 ml of 10 mg/ml dextran [^{14}C]carboxyl. See Materials and Methods for details.

§ These DPM represent loaded dextran [^{14}C]carboxyl molecules only and were obtained by subtracting the DPM of controls from the DPM of experimentals. The specific activity of the dextran [^{14}C] was 1.2 μCi/mg.

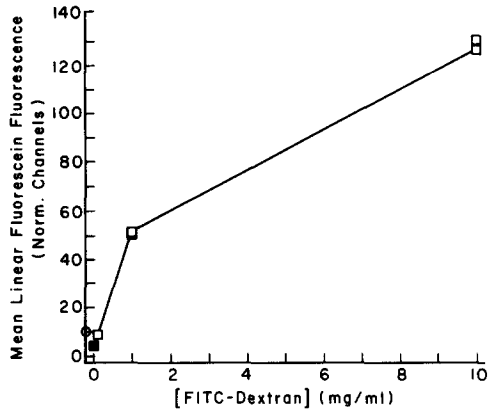


FIGURE 5 Flow cytometric measurements of the mean linear fluorescein fluorescence of fibroblasts as a function of the FTC-dextran (70,000-mol wt) concentration present during scraping (□). Two controls scraped in base DME are indicated: one received FTC-dextran in the first wash after scraping at a concentration equal to that in the 10 mg/ml experiment (○); the other received no FTC-dextran in the first wash (■). Mean fluorescence values were calculated from histograms obtained by flow cytometry of 10,000 cells.

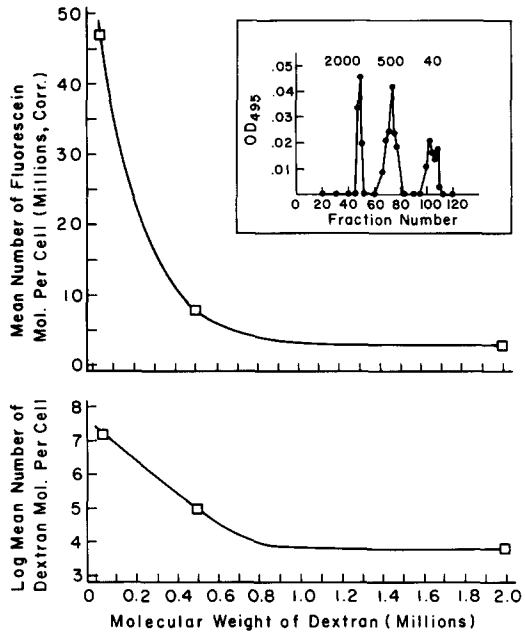
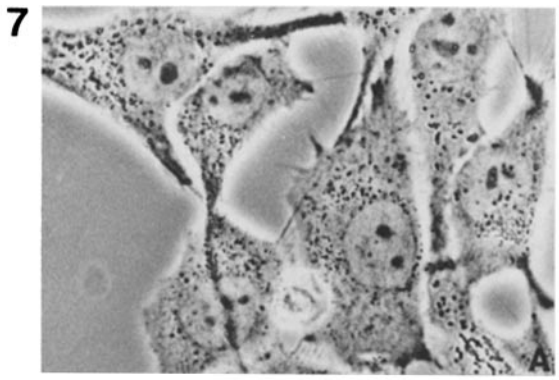
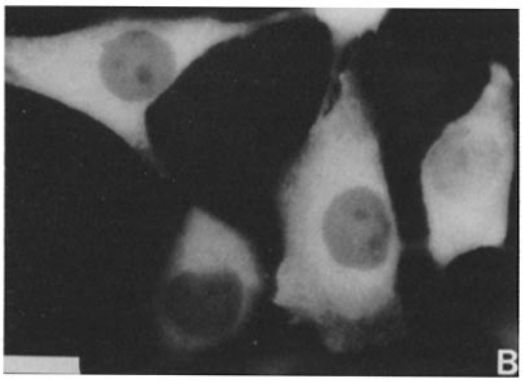


FIGURE 6 (*top*) Flow cytometric measurements of the mean number of fluorescein molecules loaded per cell scraped in three narrow range molecular weight FTC-dextrans. Numbers of fluorescein or dextran molecules per cell were determined from the standard curve of Fig. 4. Loading is compared for fibroblasts scraped in equivalent molar concentrations of fluorescein (bound to dextrans), and not on a weight or molar basis of dextran. Since small differences in fluorescein concentration were present during scraping, the plotted values were corrected to a molar fluorescein concentration of 1.8×10^{-5}, the lowest concentration used. (*inset*): Fractionation, after their use in scrape-loading, of the three narrow range molecular weight FTC-dextrans on Sepharose CL4B (1.1-ml fractions). Details of chromatography are in Materials and Methods. (*bottom*) Mean number of dextran molecules loaded per cell as a function of the molecular weight of dextran present during scraping.





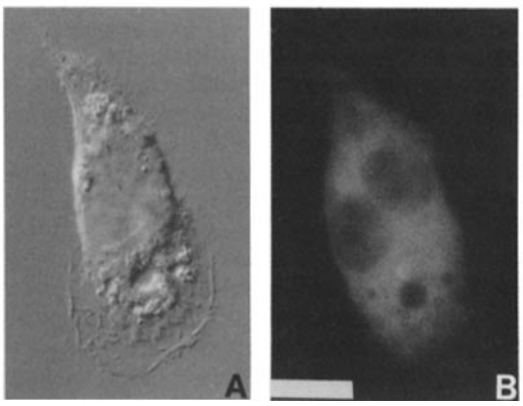


FIGURE 7 and 8 Phase (*A*) and fluorescence (*B*) images of HeLa cells loaded by scraping in 10 mg/ml FTC-dextran, 70,000-mol wt. Fluorescence was recorded on 35-mm film. Bar, 10 μm. Fig. 8: Nomarski (*A*) and fluorescence (*B*) images of a J774A.1 cell loaded by scraping in 10 mg/ml FTC-dextran, 70,000-mol wt. Fluorescence was recorded on 35-mm film. Bar, 10 μm.

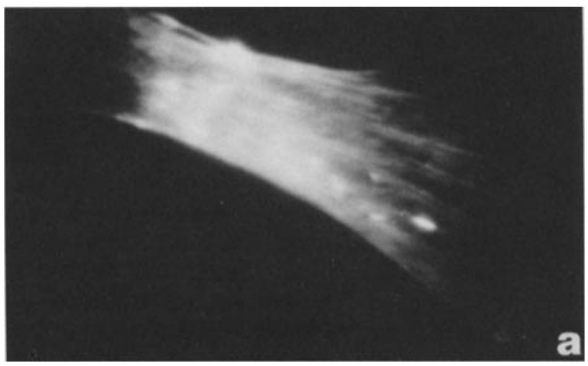
molecules as large as 2,000,000-mol wt were loaded by scraping, although to a considerably lesser extent than 40,000 mol wt dextran. The loaded dextran molecules were located, initially, in the cells' cytoplasm as indicated by the uniform fluorescence of such cells. The viability of cells surviving the loading procedure was reasonably high, whether assessed by dye exclusion in the flow cytometer or, in the longer term, by a plating efficiency assay.

Application of the technique requires attention to several points: (*a*) almost 40% of the fibroblasts scraped from substrata were dead. Flow cytometry showed that these dead cells were not a significant part of the population classified as loaded cells. But this initial loss should be considered when estimating the yield of loaded cells to be expected from a known starting population; (*b*) a volume of the macromolecule in solution must be provided that is sufficient to cover the cells during scraping. We have not determined how small this volume can be, but we have successfully used as little as 100 μl of a FTC-dextran solution (10 mg/ml) to load fibroblasts on 35-mm plastic dishes: (*c*) the slowly motile fibroblasts required as many as 12 h to resume normal well-spread morphologies after scraping and subsequent cold washes. Thus, studies of cell structure or function dependent on a loaded molecule and normal morphology cannot necessarily begin immediately after the scrape-loading procedure. This was not a problem for cells that recovered more quickly from scrape-loading, such as the motile J77A.1 cells and neutrophils.

Comparisons with Other Techniques

The scrape-loading technique compares favorably with other methods. Microinjection is a tedious process, allowing, even in skilled hands, loading of only a limited number of cells, and it is difficult to control the amount of macromolecule injected. By varying the concentration of macromolecules present during scraping (Fig. 5), and by sorting scrape-loaded cells in the flow cytometer, one could select for a population loaded to a known extent (Fig. 4).

The hypo-osmotic shock method described by Okada and Rechsteiner (14) is characterized in some depth. Pinosomes containing the macromolecule to be loaded are subject to osmotic lysis, and so release their macromolecules into cyto-



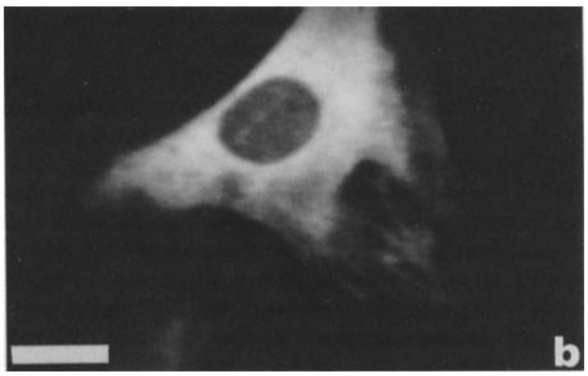


FIGURE 9 Fluorescence images of fibroblasts 3 h after loading with AF-actin by chilling followed by agitation. Cells are shown with (*A*) and without (*B*) intense fluorescence localized on stress fibers. Fluorescent paracrstals of AF-actin such as that in cell (*A*) are sometimes observed in microinjected cells, and are not therefore an artifact peculiar to the loading method used here. Fluorescence was recorded with the Zeiss three-stage camera. Bar, 10 μm.

plasm. As the authors point out, however, it would not be possible to efficiently load molecules that bind tightly to the plasma membrane and thus pinosomal membranes. Furthermore, not all of those pinosomes containing molecules to be loaded are lysed by the osmotic shock: 20% of pinocytosed horseradish peroxidase appeared in a lysosomal pellet after osmotic lysis.

Two problems are associated with cell loading by fusion with red blood cells (17, 7) or liposomes (15). First, the membrane of the loaded cell is contaminated with red blood cell or liposome membrane. Therefore, artifacts might be encountered in studies of membrane functioning of cells loaded by fusion. Second, although many fluorescent molecules loaded by the technique do diffuse throughout the cytoplasm of the loaded cell, a significant proportion sometimes appear to remain concentrated at the former site of membrane fusion, in association with the red blood cell membrane (7).

A Possible Mechanism of Scrape-Loading

Loading by scraping depends on a mechanical perturbation of the plasma membrane. This perturbation consists, we suggest, in the transient opening of holes in the plasma membrane at those sites of tightest cell adherence to plastic substratum, the close and focal contacts. Exogenous macromolecules would therefore enter cytoplasm during scraping by diffusion through such holes. The size of these holes may be large, perhaps as large as the attachment plaques. However, an additional barrier to diffusion may be the actin-based cortical gel which is discussed below. Our studies with narrow range fractions of dextran indicate that, operationally, the pores opened by scraping are probably not much bigger than 48 nm, twice the radius of gyration of the 5×10^5 mol wt dextran. Because fibroblasts undergoing tail retraction in tissue culture appear to leave behind small bits of their plasma membrane and cytoplasm during locomotion (1, 9), such transient holes in plasma membrane may be a normal part of the life of the motile tissue culture cell.

We have shown that chilling of fibroblasts followed by scraping or slight mechanical agitation loads actin into the cytoplasm. We suggest that prechilling causes a decrease in the gel structure of the cortex in addition to limiting the rate of actin assembly and reducing the strength of cell adhesion to substratum. Therefore, plasma membrane and gelled cell cortex may both present barriers to the diffusion of macromolecules into scraped cells.

Possible Applications of the Scrape-loading Technique

Possible applications of the scrape-loading technique are numerous. Functional, fluorescently-labeled molecules of cellular origin can be reincorporated by scrape-loading into living cells to serve as real-time reporters of native molecular activity in a wide variety of cellular processes (21, 20). This technique of fluorescent analog cytochemistry will benefit from the ability to load a large number of cells with a range of analog concentrations. Antibodies to a variety of antigens can be loaded. Since the technique loads a large range of numbers of fluorescent molecules, and since scrape-loaded cells can be sorted by flow cytometry according to degree of loading, the cellular response to a particular dose of antibody or other inhibitor could be determined (see 10 for a review of injecting antibodies). RNA and DNA could possibly be introduced into cytoplasm by scrape-loading. Although of high molecular weight, the result of loading even a very small number of these molecules should be detectable. We are presently studying transfection by scrape-loading of viral DNA into fibroblasts.

Scrape-loading should be applicable to many types of normally adherent cells or any cell that can be induced to adhere strongly to a glass or plastic substratum. The important variable in achieving suitable loading while maintaining acceptable cell viability will probably be the strength of cell attachments to the substratum. Cells adhering too strongly could be scraped during spreading before full development of cell-substratum contacts, or after cell chilling to reduce cell-substratum adhesion. Those not adhering strongly enough could be plated on, for example, a poly-L-lysine-coated substratum. For those macromolecules that must be loaded in the cold, such as actin, we have described how chilling and slight mechanical agitation can result in fibroblast loading. Furthermore, slight mechanical perturbation to plasma membrane might be produced by any number of other physical means for cells not strongly adherent to a glass or plastic substratum (Fechheimer, Denny and D. L. Taylor, unpublished results).

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