Glass beads load macromolecules into living cells

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Summary

We describe and characterize an exceptionally rapid and simple new technique for loading large numbers of cultured cells with large macromolecules. The culture medium of the cell monolayer is replaced by a small volume of the macromolecule to be loaded. Glass beads (75–500 μm diameter) are then sprinkled onto the cells, the cells are washed free of beads and exogenous macromolecules, and 'bead-loading' is completed. The conditions for bead-loading can readily be modified to accommodate cell type and loading objectives: for example, the amount of loading per cell increases if bead size is increased or if beads are agitated after sprinkling onto the monolayer, but at the expense of increased cell loss. As many as 97% of a population of bovine aortic endothelial (BAE) cells were loaded with a 10 000 M_r dextran; and 79% with a 150 000 M_r dextran using bead-loading. Various cell lines have been loaded using glass beads. Moreover, bead-loading has the advantage of producing loaded cells that remain adherent and well-spread, thus minimizing recovery time and permitting immediate microscopic examination.

Key words: cell-loading technique, microinjection, fibroblasts, endothelial cells, epithelial cells, glass beads.

Introduction

Techniques for loading impermeant molecules into the cytoplasm of living cells have become essential cell and molecular biological tools. Indeed, it is cell-loading techniques that make possible transformation of cells with foreign DNA (Celis, 1984) and fluorescent analogue cytochemistry (Taylor *et al.* 1985; Wang *et al.* 1982), to name just two of many possible examples.

Of the numerous cell-loading techniques now available, several depend on the cell's ability to heal wounds mechanically induced in its plasma membrane (Diacumakos, 1975; Fechheimer *et al.* 1986; Graessman *et al.* 1974; Klein *et al.* 1987; McNeil *et al.* 1984). Microinjection (Graessman *et al.* 1974) and scrape-loading (McNeil *et al.* 1984) are two such techniques, but both have important drawbacks. Microinjection, for example, is a tedious process, requires expertise and expensive apparatus, and is capable of loading only a relatively small number of cells. Scrape-loading, while avoiding these problems, produces loaded cells in suspension that must be replated, and allowed to recover and spread, before experimentation. This is a considerable disadvantage where labile probes subject to cytoplasmic redistribution, or degradative processes, are used.

We here describe and characterize a new technique, termed 'bead-loading'. It rapidly loads large numbers of cells with large macromolecules, and the loaded cells remain adherent and well-spread, a major advantage over scrape-loading. Moreover, several critical aspects of the technique can readily be modified so as to accommodate cell type and/or other loading objectives. Lastly, bead-loading appears to have an exceptionally broad applicability to tissue culture cell lines.

Materials and methods

Cell culture

Swiss/3T3 (CCL 92), obtained from American Type Culture Collection (Rockville, MD), were passage numbers 125 through 137. Bovine aortic endothelial cells (BAEC-11), a gift from Bill Atkinsin (Brigham and Women's Hospital), were passage numbers 25 through 36. Madin-Darby canine kidney cells (MDCK), a gift from Karl Matlin (Harvard Medical School), were passage numbers 15 and 16. J774.2 macrophage-type cells were a gift from Jay Unkeless (Rockefeller University). *Potorous tridactylis* kidney cells (PtK-2)-

(CCL 56) were obtained from American Type Culture Collection.

All cells, except J774.2, were cultured on 25 cm^2 flasks (Corning, Corning, NY) in Dulbecco's modified Eagle medium (DME) (Gibco, Grand Island, NY) supplemented with 100 units ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin (Irvine Scientific, Santa Ana, CA), and 10 % (v/v) calf serum (for Swiss/3T3), or foetal bovine serum (for all other cell lines except J774.2) (all sera from Gibco) in a 37 °C, 5 % CO_2 atmosphere. Cultures were passaged before confluence with trypsin–EDTA in Hank's Balanced Salts (Irvine). J774.2 cells were cultured in suspension in a spinner flask (Techne, Cambridge, UK) in DME supplemented with 10 % heat-inactivated foetal bovine serum. Cells were plated onto 22 mm square glass coverslips (VWR, Boston, MA) for bead-loading, or onto 60 mm dishes (Corning) for scrape-loading 24–48 h before experimentation. Unless noted otherwise, cultures were sub-confluent when used in loading experiments.

Loading methods

Fluorescein isothiocyanate-labelled dextran (FDx) (9000 M_r (FDx 9), lot no. 124F-5007, 0·35 mol FITC mol^{-1} dextran; and 156 900 M_r (FDx 150), lot no. 25F-0241, 3·48 mol FITC mol^{-1} dextran) (Sigma Chemical Co., St Louis, MO) was dissolved at a concentration of 10 mg ml^{-1} in calcium/magnesium-free phosphate buffered saline (CMF-PBS).

Scrape-loading. The scrape-loading method used here was a modification of the method previously characterized (McNeil *et al.* 1984). Sub-confluent cultures in dishes were rinsed with three 3-ml volumes of CMF-PBS (37 °C). FDx (150 μl) was added to the dish, the dish was swirled to distribute the FDx solution evenly, and then cells in the culture were scraped off of the dish with a rubber policeman. DME (37 °C, 5 % CO_2) was immediately added and the cell suspension was plated on 22-mm square coverslips. Cultures were placed in the incubator, washed with fresh culture medium 30 min later, and then returned to the incubator for various intervals ranging from 1 to 48 h.

Bead-loading. All glass beads (Sigma) were acid-washed unless otherwise indicated. The 450–500 μm beads (referred to as 450 μm beads) were acid-washed in 5 M-HCl followed by several rinses in distilled water. The 75–150 μm beads (referred to as 75 μm beads) had been acid-washed as purchased from the manufacturer. Beads were alkali-washed by soaking them overnight in 4 M-NaOH and then washing with distilled water until the pH of the water washes was stable at ≈7·0. Approximately 0·23 g (≈1000 beads) of the 450 μm or 0·12 g of the 75 μm beads were used in those experiments where the number of beads was held constant. These weights were chosen because they were just sufficient to cover the coverslip with a single layer of beads.

Cultures growing on coverslips were rinsed three times with CMF-PBS (37 °C) by dipping them in three successive baths of this medium. The culture was wicked free of excess CMF-PBS after the third rinse. We have found it convenient to then place the coverslip on a silicone/rubber sheet (North American Reiss Corp., Bellemead, NJ) supported by a glass slide, although the coverslip can also be held with forceps throughout the procedure. FDx (37 °C) was pipetted onto the culture, drawn off, and repipetted onto the coverslip in order to assure thorough mixing at the liquid interface with the cells. Beads were then carefully and evenly sprinkled onto the coverslip from a 6 mm × 50 mm culture tube (Kimble, Toledo, OH) held 1–3 cm above the horizontally oriented coverslip surface. Unless otherwise stated, the beads were caused to roll around on top of the culture until evenly distributed over its surface by gently rocking the coverslip three to six times, and afterwards were rinsed off the culture by dipping the coverslip in a PBS bath. Cultures were returned to the culture medium in their original dishes and allowed to recover at 37 °C and 5 % CO_2 for various intervals.

Photomicrography

Fluorescein fluorescence and phase-contrast micrographs of living cells were taken on Plus-X (Kodak, NY) film (rated 125 ASA) using a Zeiss Photomicroscope III equipped with Zeiss Neofluor 16 and 40× phase-contrast objectives. Micrographs of horseradish peroxidase (HRP) reaction product within cells were taken on Plus-X film using a Zeiss dissecting microscope.

Horseradish peroxidase labelling of cells

HRP (grade I, Boehringer Mannheim, Indianapolis, IN) was dissolved at 20 mg ml^{-1} in CMF-PBS and used for bead-loading as described above. At intervals after bead-loading, cultures were fixed in 2 % formaldehyde, 2·5 % glutaraldehyde in 0·1 M-Sorenson's phosphate buffer and HRP reaction product was then developed as described (Adams, 1977).

Measurement of protein and fluorescein dextran content of cell populations

One hour after various loading procedures, cultures on coverslips were rinsed six times in PBS. Excess PBS was wicked off of the coverslip between rinses. Cell protein and loaded fluorescein dextran were then extracted by incubating coverslips in 0·5 ml of 0·05 % Triton X-100 and 0·02 % NaN_3 in water. After harvesting extracted cells from the coverslips with a rubber policeman, 0·2 ml of the resultant lysate was used for protein determination in a Lowry assay (Lowry *et al.* 1951) and the remaining 0·3 ml was diluted with 1·7 ml of 0·05 % Triton X-100, 0·02 % NaN_3, 1 mg ml^{-1} BSA, 10 mM-Tris·HCl (pH 8·0). The fluorescence intensity of the latter solution was measured with a SPF-500C spectrofluorometer (SLM Instruments, Urbana, IL). Relative fluorescence values thus measured were converted to molar dextran concentrations using known ratios of molar dye to dextran and assuming an extinction coefficient for fluorescein at pH 8·0 of $6{\cdot}8\times10^4$ units mol^{-1}. A 'loading index' was then calculated as picomoles dextran loaded per mg cell protein.

Control cultures received FDx, but not beads (or received FDx, but were not scraped). Controls were used as a blank for fluorescence.

Flow cytometry

Cultures on coverslips were rinsed six times in CMF-PBS as described above and trypsinized with 250 μl trypsin–EDTA. Calf serum was added to give a final concentration of 10 %. Suspensions were centrifuged at 4 °C and 1000 revs min^{-1} for 3 min. The pellet was resuspended in 300 μl PBS supplemented with 10 % calf serum and then analysed in list mode on a Coulter EPICS V (Hilaheah, FL) flow cytometer

(Dana Farber Cancer Institute). Relative fluorescence values measured by flow cytometry from cells were calibrated in terms of absolute numbers of fluorescein molecules per cell using as standards microbeads labelled with known numbers of fluorescein equivalents (Quantitative Fluorescein Microbead Standards Kit, Flow Cytometry Standards Corporation, NC). Numbers of dextrans loaded per cell could then be calculated from the known molar ratio of fluorescein to dextran.

Results

The bead-loading technique

The bead-loading procedure is a simple and very rapid (<2 min) technique for loading cell populations. Cells were grown on coverslips or Petri dishes to any desired density, and their culture medium was replaced with a small volume of a solution of the macromolecule to be loaded. Then glass beads were sprinkled onto the cells. Many were thereby loaded with macromolecules and, after washing, could be returned to normal culturing conditions or used immediately in experiments.

Despite its simplicity, there are several important and readily controlled variables of bead-loading that strongly influence the amount of loading and the yield of viable cells. These critical variables and other characteristics of bead-loaded cells are therefore examined in depth in the following sections.

Morphology of bead-loaded cells

Cells were examined by phase-contrast and fluorescence microscopy at various intervals after bead-loading with FDx 9 (10 mg ml^{-1}). A substantial proportion of the population of bead-loaded BAE cells were highly fluorescent after loading, and could readily be photographed on 35 mm (125 ASA) film. It was obvious, moreover, that the intensity of fluorescence was highly variable from cell to cell in the population. Fluorescent BAE cells (e.g. cells successfully loaded) exhibited well-spread, normal phase-contrast morphologies at 1, 18 h and 48 after bead-loading (Fig. 1A–C). That is, the fluorescent cells were indistinguishable from neighbouring, non-fluorescent cells, as well as from cells in cultures not subject to bead-loading. Bead-loading also readily introduced the much larger FDx 150 into BAE cytoplasm (Fig. 1D). Remnants of cells lost during bead-loading were sometimes present on the substrata of bead-loaded cultures and these increased in frequency as loading conditions were made more stringent (see below).

Bead-loading was successfully applied to a variety of cell types: Swiss/3T3, J774.2, PtK-2 and MDCK, grown at either sub-confluent or confluent densities (Fig. 1E–L). It was sometimes possible to introduce fluoresceinated dextrans into cells of confluent monolayers without noticeably interrupting monolayer continuity (Fig. 1E,F,I–L). This generally required that beads were used without being agitated after sprinkling onto the monolayer (see below). However, we have noticed that the requirements for successful bead-loading vary considerably from one cell line to another. For example, only a small percentage of cells in MDCK monolayers (Fig. 1K,L) were loaded with the 75 μm beads, despite the fact that these beads were agitated vigorously by tapping the monolayer coverslip and associated beads on a solid surface (see below). Under these same conditions, loading of cells in a BAE monolayer would approach 100 %.

Proteins also could be introduced into cytoplasm by bead-loading. Fig. 2, for example, shows a low-magnification image of a BAE culture 15 min after bead-loading with HRP (M_r 40 000) using 450-μm beads. The majority of the cells in this subconfluent population were loaded with the HRP.

Effects of bead size and bead agitation on loading

The size of beads used, and the level of agitation imparted to beads resting on the monolayer, both strongly influenced the extent of loading of cells. Large, 450 μm glass beads improved loading several fold (depending on cell type) over smaller, 75 μm beads (Tables 1, 2). Increased agitation of the 75 μm beads, caused by tapping on the benchtop of coverslips held in a vertical orientation, increased loading by 8·4-fold over undisturbed coverslips, and by 2·7-fold over coverslips rocked 5–10 times while held in a horizontal position (Table 1). Using the 450 μm beads, loading could be increased simply by increasing the number of times coverslips supporting beads were rocked from side to side (Table 1).

Cell yield and viability after bead-loading

Bead-loading sometimes resulted in loss of cells from the population. The magnitude of such losses depended strongly on cell type, bead size and agitation level imparted to the beads (Tables 1, 2). Yield of BAE cells was generally 80–90 % if 75 μm or 450 μm beads were used without agitation (Table 1 and data not shown), but fell much lower if the 450 μm beads were agitated by repeated rocking of the culture coverslip onto which they had been sprinkled (Tables 1, 2).

Viability of cells remaining on the coverslip after bead-loading was always greater than 95 %, whether assessed by Trypan Blue exclusion or flow cytometric analysis of forward-angle light scattering of trypsinized cells. Moreover, fluorescent, mitotic cells and post-mitotic daughter cells were frequently observed after bead-loading with fluoresceinated dextrans, indicating that loaded cells could divide (not shown). There was little change over a 24 h period in the fluorescence

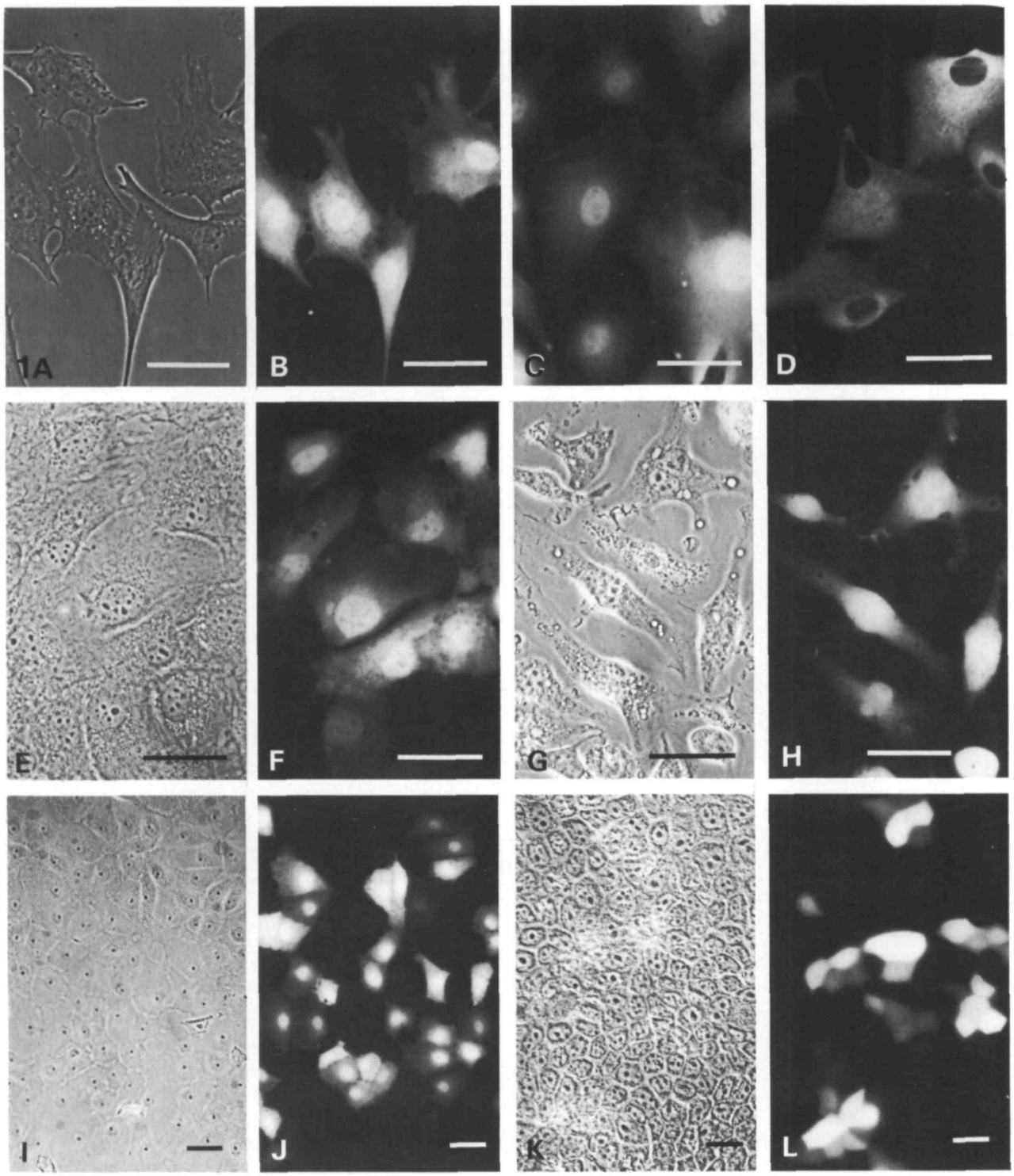

Fig. 1. Living cells of various types photographed after bead-loading. A,B. BAE cells 1 h after bead-loading in FDx 9 (10 mg ml^{-1}) using alkali-washed, 75 μm beads. C. BAE cells 48 h after loading as in A and B. D. BAE cells 4 h after bead-loading in FDx 150 (20 mg ml^{-1}) using 75 μm beads. E,F. Swiss/3T3 cells, confluent, 4 h after bead-loading in FDx 9 using 450 μm beads. G,H. J774.2 cells 1 h after bead-loading in FDx 9 using 450 μm beads. I,J. PtK-2 cells 1 h after bead-loading in FDx 9 using 450 μm, alkali-washed beads. K,L. MDCK cells 1 h after bead-loading in FDx 9 using 75 μm beads. See text for further details regarding loading conditions. Bar, 40 μm.

profiles of bead-loaded populations (Fig. 3B,C), indicating that cells loaded by this technique were not preferentially lost from the population.

Scrape-loading is ineffective in PtK-2 cells, causing loss of 87 % of the population. Possibly this is because the PtK-2 cell, which is highly flattened or spread on

Table 1. *Effect of bead size and agitation on loading and cell yield*

Bead size (μm)	Agitation	Yield cells (% of control)¶ Protein	Yield cells (% of control)¶ Counts	Loading index (pmol FDx 9 per mg cell protein)
75	None*	84 (4)		145 (4)
	Rock†	72 (4)		450 (4)
	Rock and tap‡	57 (4)		1219 (4)
450	None*	80 (4)	79 (2)	514 (4)
	Rock 2×§	56 (4)	47 (2)	1000 (4)
	Rock 4×§	31 (4)	34 (2)	1305 (4)
	Rock 6×§	13 (4)	12 (2)	1417 (4)

Numbers in parentheses represent numbers of replicate samples.

* To keep agitation to a minimum, the BAE culture was maintained in a horizontal orientation after addition of beads and then transferred to a beaker of PBS, lowered into the PBS, and then tilted vertically to rinse away beads.

† Beads were sprinkled onto the coverslip and it was then gently rocked (three to six times) until the beads were evenly distributed over its surface.

‡ After the beads were evenly distributed (as above), the coverslip, held vertically and supported by a silicone/rubber sheet on a glass slide, was firmly tapped once on the benchtop, displacing the majority of the beads to the lower end of the coverslip.

§ Beads were sprinkled onto the coverslip, which was then rocked to and fro the indicated number of times.

¶ Protein was determined by Lowry assay. Cells excluding Trypan Blue were counted on a haemocytometer.

Table 2. *Bead-loading and scrape-loading of cell lines*

Cell type	Method	Yield cells (% of control)* Protein	Yield cells (% of control)* Counts	Loading index (pmol FDx 9 per mg cell protein)
Swiss/3T3	450 μm beads	28 (4)	33 (6)	296 (4)
	75 μm beads	74 (4)	52 (6)	110 (4)
	Scrape-loading	44 (4)	31 (6)	246 (4)
BAE	450 μm beads	25 (8)	34 (2)	420 (5)
	75 μm beads	68 (14)	50 (3)	248 (12)
	Scrape-loading	19 (15)	32 (4)	177 (10)
PtK-2	450 μm beads	58 (9)	43 (8)	266 (5)
	75 μm beads	86 (6)	62 (8)	49 (2)
	Scrape-loading	13 (7)	12 (6)	103 (4)

Numbers in parentheses represent numbers of replicate samples. Beads were sprinkled onto the coverslip and it was then gently rocked (three to six times) until the beads were evenly distributed over its surface.

* Performed as described in Table 1.

the substratum, is severely traumatized when it is scraped off from a substratum to which it apparently adheres very tightly. In any case, bead-loading with fluoresceinated dextrans readily produced fluorescent, viable PtK-2 cells at a much higher yield (4- to 6-fold) than scrape-loading (Table 2).

Influence of bead surface properties on cell yield and loading

As purchased, the glass beads we used for loading had been washed in acid (HCl). These acid-washed beads clearly adhered strongly to the cells: the 75 μm beads were observed to coat confluent monolayers through the usual washes with which we conclude bead-loading, and such beads detached from the cells only after 5–10 min in serum-containing culture medium.

To alter bead surface properties so as to render them less adherent to cells, we washed the beads in alkali (4 M-NaOH). The 75 μm beads treated in this way did not noticeably adhere to cells. Despite their lack of adherence to cells, both the 75 μm and the 450 μm alkali-washed beads were capable of loading cells (data not shown) and they moderately increased cell yield (1·3- to 1·5-fold). This latter effect was most apparent when the beads were agitated by rocking of the coverslip.

Volume requirements of the carrier solution used for bead-loading

If the volume of carrier solution required for loading can be minimized, so too can the amount of macromolecule required for loading. We have therefore determined how the extent of bead-loading is affected by the

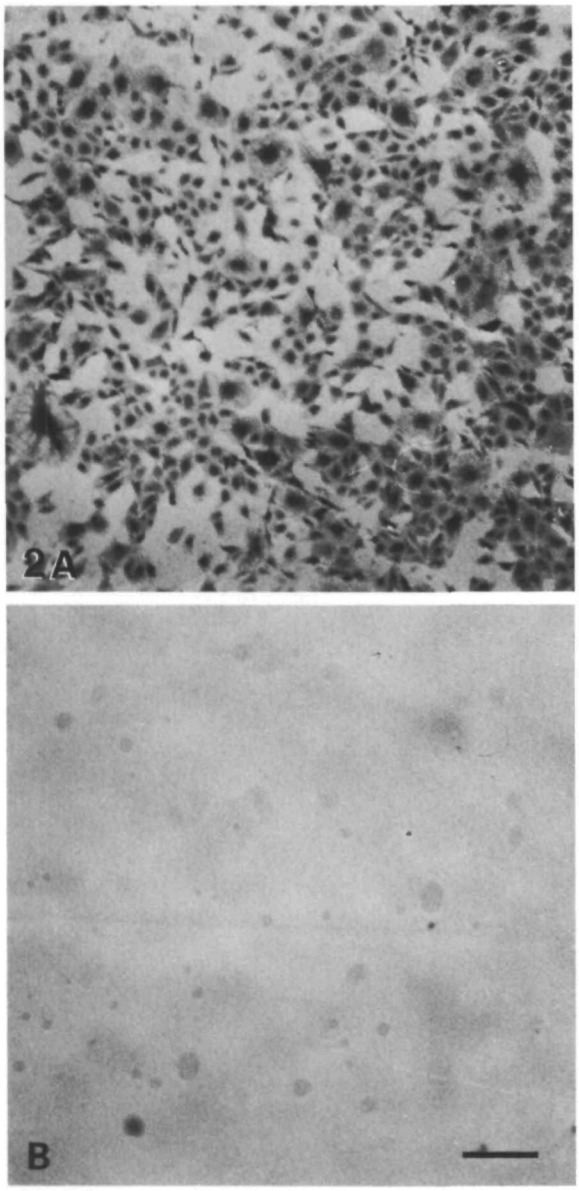

Fig. 2. A. BAE cells fixed 15 min after bead-loading with HRP using 450 μm, alkali-washed beads. Most of the cells on this nearly confluent coverslip (photographed on a dissecting microscope) are darkened by the HRP reaction product, indicating that most were loaded with HRP. B. Control cultures, which received HRP but no glass beads, were not visibly darkened with reaction product. Identical photographic procedures were used throughout for A and B. Bar, 100 μm.

volume of carrier solution used, e.g. by that volume present on the coverslip during addition of beads. We found, using 450 μm beads, that 200 μl of a solution provided a greater (3·8-fold) loading index than 20 μl. But the smaller volume still yielded many cells that were highly fluorescent when viewed in the microscope. Moreover, using the 75 μm beads, the loading index actually increased by almost twofold as the volume of carrier solution was reduced from 200 to <20 μl. Therefore, substantial bead-loading can be achieved with no more than the volume necessary to prevent drying of the cells during the brief loading procedure. It is difficult to say accurately how small this volume might be, but we were able to use <20 μl for the 22 mm coverslip.

Quantitative fluorescence properties of individual cells within the population of bead-loaded cells

The fluorescence of each of 20000 cells subject to various bead-loading conditions was measured and recorded by flow cytofluorometry. The amount of FDx 9 and FDx 150 introduced into cytoplasm by bead-loading clearly varied considerably from cell to cell in the population: in the case of the smaller dextran, measured single-cell fluorescence spans a range of almost three orders of magnitude (Fig. 3A,B). Flow cytofluorometry shows that use of the larger, 450 μm beads considerably shifted the population distribution of fluorescence intensities upwards compared to that obtained using 75 μm beads (Fig. 3C,D). Table 3 shows that a very high percentage of cells were successfully loaded using glass beads. For example, 86% of cells subject to bead-loading with FDx 9 using 75 μm beads were more fluorescent than controls (which received FDx 9 solution but not beads).

Flow cytofluorometry was also used to compare bead- and scrape-loading (Fig. 3E). Scrape-loading introduced more fluorescence on average than did bead-loading using 75 μm beads and standard levels of agitation. Clearly, however, there are present in such bead-loaded populations individual cells loaded to levels comparable to the highest achieved by scrape-loading. Use of the larger, 450 μm beads resulted in a distribution of fluorescence intensities as high as, or for some cells, slightly higher than those obtained by scrape-loading. Moreover, the 450 μm beads reproducibly produced a sub-population of cells that were loaded to a higher level than any produced by scrape-loading (Fig. 3D,E). Very similar results to those in Fig. 3 were obtained by flow analysis for Swiss/3T3 and also PtK-2 cells.

Approximately $4{\cdot}3\times10^7$ molecules of FDx 9 were introduced on average into each BAE cell using 450 μm beads and a 10 mg ml^{-1} solution of this dextran (Fig. 4). The extent to which a dextran molecule was loaded using beads was a function of its molecular weight. Nevertheless, $>10^5$ molecules of FDx 150 were introduced on average per BAE cell using 450 μm beads.

Discussion

We have described and characterized a new technique, termed 'bead-loading', for introducing macromolecules into cell cytoplasm. Some favourable characteristics of

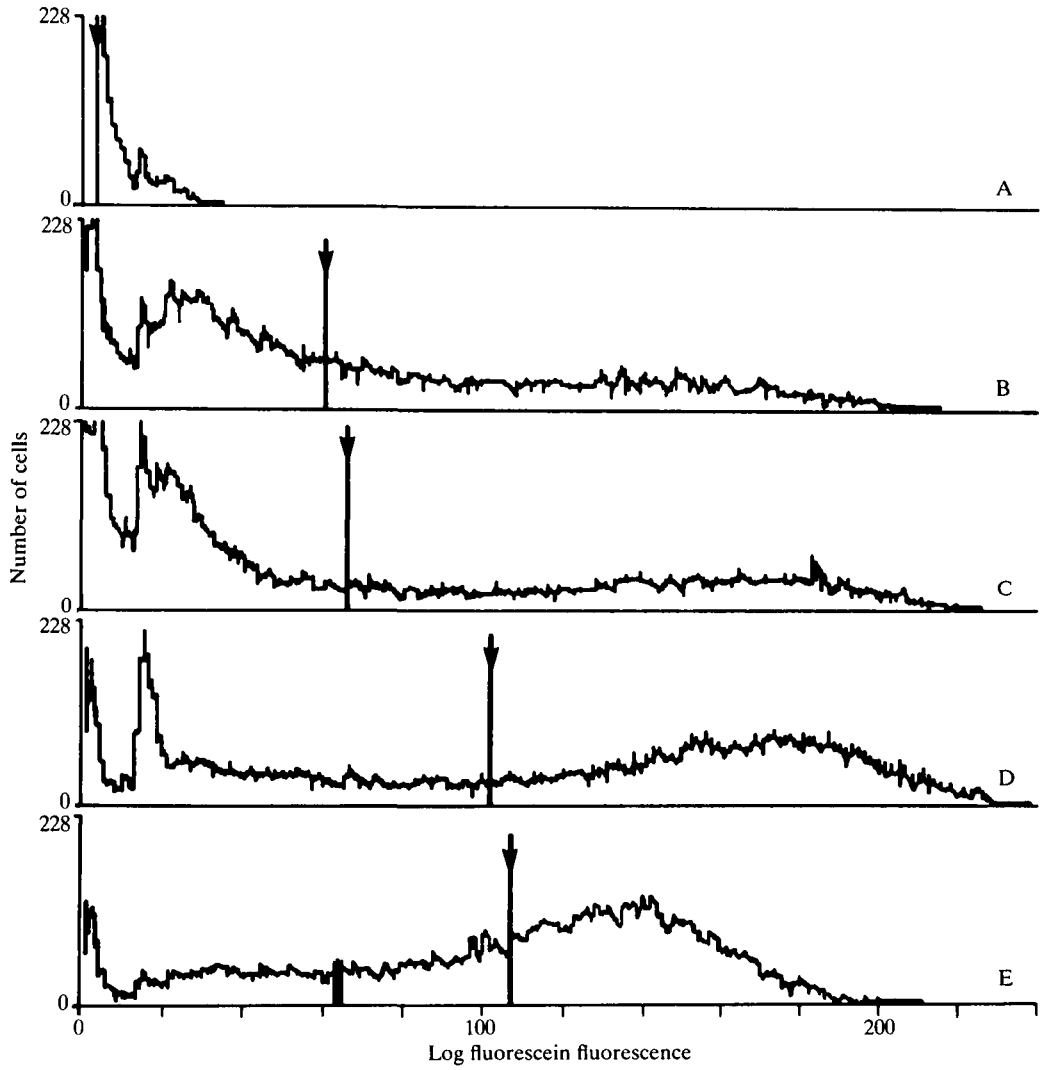

Fig. 3. Flow cytometric measurements of the frequency distribution of fluorescence intensities within BAE cell populations after loading. A. Untreated controls. B,C. Cells 1 h and 24 h, respectively, after bead-loading with FDx 9 using 75 μm beads. D. Cells 24 h after bead-loading with FDx 9 using 450 μm beads. E. Cells scrape-loaded with FDx 9. Arrows indicate mean fluorescence intensity of each population of 20 000 cells; the solid bar in E indicates the relative fluorescence of the microbead standards labelled with $1{\cdot}8\times10^{6}$ fluorescein equivalents. All experiments were performed on co-cultures of BAE cells and using a single 10 mg ml^{-1} solution of FDx 9 for loading.

bead-loading are the following. Large numbers of cells can be loaded by a simple procedure that requires only a few minutes to complete. Bead-loading is versatile as regards cell type: fluoresceinated dextrans were successfully introduced into Swiss/3T3, BAE, J774.2, PtK-2 and MDCK cell cytoplasm. Molecules as large as 150 000 M_r are readily loaded by the technique. Under certain conditions most of the cells in populations of some cell lines are permeabilized by bead-loading: using 450 μm beads, 97 % of the cells in BAEC cultures were loaded above control levels with a 9000 M_r dextran; 79 % with a 150 000 M_r dextran. The conditions for bead-loading can readily be modified to accommodate cell type and/or loading objectives (see below). Finally, cells loaded by the procedure remain adherent and well-spread. It is therefore possible, for example, to make microscopic examinations and/or measurements of cellular fluorescence immediately after the bead-loading procedure.

There are currently four general strategies for loading impermeant molecules into living cells. Cells can be loaded by causing them to fuse with a suitable membrane-delimited vehicle (usually a red blood cell ghost or liposome) that has itself been loaded with macromolecules (Doxsey *et al.* 1985; Godfrey *et al.* 1983; Pagano & Weinstein, 1978; Schlegel & Rechsteiner, 1975). Cells can be reversibly permeabilized to macromolecules by electric shocks, a technique used predominantly for transfection (Neumann *et al.* 1982). Cells allowed to pinocytose macromolecules in hypertonic medium can be loaded by osmotic lysis of such pinosomes (Okada & Rechsteiner, 1982); or, more

Table 3. *Percentage of loaded cells in BAE populations after bead-loading and scrape-loading*

M_r (×10 $^{-3}$)	Technique	% Loaded*
9	75 μm beads	86
9	450 μm beads	97
9	Scrape-loading	99
150	75 μm beads	32
150	450 μm beads	79
150	Scrape-loading	81

Beads were sprinkled onto the coverslip and it was then gently rocked (three to six times) until the beads were evenly distributed over its surface.

* Determined by flow cytofluorometric analysis of 20 000 cells from populations taken 24 h after standard (see Materials and methods) bead-loading or scrape-loading. A cell was classified as loaded if its fluorescence intensity exceeded that of 95 % of the cells from control cultures, which received FDx solution but not beads (or received FDx but were not scraped).

simply, cells can be loaded by osmotically shocking them in the presence of macromolecules (Borle & Snowdowne, 1982). Finally, cells can be reversibly permeabilized to macromolecules by mechanical disruptions of plasma membrane, with or without subsequent pressurized injection of macromolecules (Celis, 1984; Fechheimer *et al.* 1986; Klein *et al.* 1987; McNeil *et al.* 1984).

Each of the four above strategies has advantages and limitations. Fusion-mediated loading introduces exogenous plasma membrane into that of the target cell; carrier vesicles can undergo phagocytosis rather than fusion; and, if virus-mediated, fusion-mediated loading is restricted to appropriately infected or transfected cells. Osmotic lysis of pinosomes requires that a cell line is actively pinocytotic, and appears to require a very high concentration of the molecule to be loaded. Electric and osmotic shocks of cells have not been characterized quantitatively and are therefore difficult to evaluate as generally useful loading techniques. The bead-loading method relies on mechanical disruption of plasma membrane to achieve loading, and therefore shares certain disadvantages in common with other techniques utilizing this strategy. Bead-loading, for example, results in loss of cells. The extent of this loss depends on cell type and loading conditions, but can be kept within the range 10 to 20 %. Microinjection of Swiss/3T3 cells has been reported to cause an ≈40 % loss of injected cells (Pastri *et al.* 1986). Surprisingly, such loss, as opposed to viability of those cells remaining at various times after microinjection, has seldom been documented. Bead-loading generally causes less cell loss than does scrape-loading (McNeil *et al.* 1984), especially in certain cell lines, such as the PtK-2. Certainly, it is essential in applying bead-loading, and any other bulk-loading or micro-injection method to

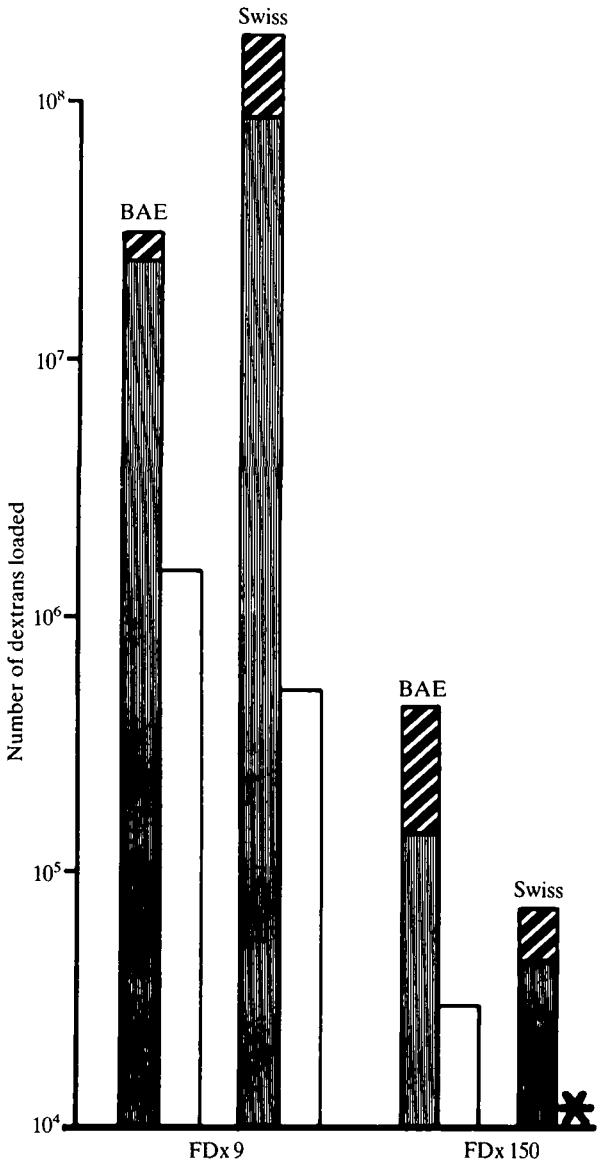

Fig. 4. Average number of molecules of dextran loaded per cell (measured by flow cytofluorometry) for the 9000 M_r dextran (FDx 9) and for the 150 000 M_r dextran FDx 150). Unfilled bars represent cells loaded using the 75 μm glass beads; vertical striped bars represent cells loaded by scraping; diagonal striped bars represent cells loaded using the 450 μm glass beads. The number of molecules of FDx 150 loaded per cell using the 75 μm beads was less than 10^4 (asterisk). It should be noted that, for reasons of signal optimization, both dextrans were used at 10 mg ml^{-1}, rather than at equimolar concentrations. Therefore, the number of FDx 150 molecules loaded is probably underestimated by as much as 16·7-fold relative to FDx 9. This cannot, however, account for the greater than 1000-fold difference measured between numbers of FDx 9 and FDx 150 molecules loaded into the Swiss cells, or for the almost 100-fold difference measured for BAE cells. BAE stands for bovine aortic endothelial cells; Swiss for Swiss/3T3 cells.

the study of cell function, to demonstrate that that function has not been compromised in the loaded cell.

The bead-loading technique will be immediately useful for single-cell (microscopic) analyses requiring loading of, for example, fluorescent probes into cytoplasm. It may be useful for transfection: the related technique of scrape-loading has recently been used successfully in this application (Fechheimer *et al.* 1987). For population averaging studies, bead-loading presents the potential problem that the number of molecules introduced per cell varies widely from cell to cell in the population. This was clearly indicated by flow cytofluorometry as a three log-scale spread in the relative fluorescence intensities of bead-loaded cells. Flow cytofluorometry also shows, however, that larger (450 μm) beads can be used to compact considerably and also to skew this spread towards higher intensities (e.g. greater loading per cell). Alternatively, fluorescence-activated cell sorting could be used to select for those cells loaded to the most desirable levels. Heterogeneity of loading would sometimes be of advantage: one might require, for example, to measure on a cell-by-cell basis a particular cellular response to loading of various doses of impermeable molecules.

The mechanism of bead-loading is probably identical to that suggested for scrape-loading: exogenous molecules are able to enter cytoplasm by diffusion through a transiently disrupted plasma membrane. The mechanical forces driving membrane disruption are clearly increased by larger, heavier beads and by imparting velocity to beads in contact with cells. The effects of bead size and agitation level are of practical significance because their variation allows for adjustments in the extent of loading achieved, or for the requirements of a particular cell line. However, such adjustments entail a compromise: increased loading is traded for decreased cell yield.

The surface properties of the beads do not seem to be critical for successful loading. We found, for example, that use of alkali-washed, rather than acid-washed, 450 μm beads resulted in higher cell yields but had little effect on loading. Thus, although the adherence to cells characteristic of acid-washed beads could facilitate the membrane disruption leading to cell loading, it is not a necessary component of this process. Practically speaking, one could use alkali-induced alterations in bead surface properties to increase cell yield, particularly for cells that are weakly adherent to the substratum, and/or to limit the loss of those valuable macromolecules that might otherwise adhere to the oppositely charged, acid-washed bead surface.

Various mechanical methods have now been used successfully for loading cells. This seems to indicate that cells possess an impressive mechanism for healing (sealing) wounds to their plasma membrane. Yet we understand very little about how cells accomplish such wound-healing, beyond what can be deduced from the physical/chemical properties of the plasma membrane itself. Nor can we say whether wound-healing is a frequently called upon cellular capability *in vivo*, or merely of practical benefit to certain cell biologists. These are interesting questions for future research. Meanwhile, the bead-loading method should find widespread applications in cell and molecular biology, where study of intact, living cells is a rapidly growing experimental strategy.

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