Flow Cytometry and Scrape-loading/Dye Transfer as a Rapid Quantitative Measure of Intercellular Communication in Vitro

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ABSTRACT

We describe two flow cytometric assays performed on populations of cells which have been stained with various fluorescent tracer molecules by the scrape-loading technique. One assay uses a simple one-color analysis on a flow cytometer by quantitating the fluorescence intensity of scrape-loaded lucifer yellow CH (LY) in individual cells. The other assay utilizes a two-color analysis on a cell sorter whereby cells which are initially loaded (donors) are identified by their uptake of both rhodamine isothiocyanate-dextran and LY, whereas the recipients of dye transfer are identified as having LY only. Agents which have been shown to inhibit intercellular communication in other assays exhibit similar blocking activity in LY transfer and this is readily quantitated by flow cytometry. The two-color analysis has the added advantage of being able to identify both donors and recipients in a highly quantitative manner.

INTRODUCTION

Intercellular communication is an important process in the control of cellular growth and differentiation and tissue homeostasis (1-4). Aberrations in gap junction-mediated intercellular communication have been identified as a marker for abnormal growth and neoplastic transformation in vitro (5, 6). In addition, tumor promoters have been shown to induce alterations in gap junctional communication (4, 7-19) as well as cause disturbances in gap junction morphology both in vitro (20) and in vivo (21).

Because of the postulated importance of cell-cell communication in cellular growth control, many assays have been developed to measure the ability of cells to transfer low molecular weight substances across gap junctions (reviewed in Ref. 1). Several methods exist which can yield quantitative data in a rapid fashion (electrical coupling; intracellular dye injection), but these require a specialized microinjection apparatus which can be difficult to use. Another recently developed method utilizes fluorescence recovery after photobleaching (18). Although this technique allows for real time analysis of dye transfer of cells in situ, it utilizes instrumentation that is not yet widely available to most researchers. Other methods to measure intercellular communication include metabolic cooperation assays and [3H]uridine dye transfer assays, but these generally require several days or up to a week or more to perform the assay. Quantitation in these assays is often tedious (i.e., counting silver grains) or requires many tissue culture plates if the degree of inhibition of cell-cell communication is only slight (metabolic cooperation assays).

Each of these assays has its advantages, however. For instance, metabolic cooperation assays are useful especially in toxicology studies because the cytotoxicity of test agents can be determined simultaneously along with their effects on metabolic cooperation (9, 15, 16). The rapidity and ease with which one can observe cell-cell communication by using the scrape-loading/dye transfer method (7), and the general availability of flow cytometric analysis in most research centers, led us to investigate the possibility of combining scrape-loading/dye transfer with flow cytometry as an additional means of quantitating dye transfer on a population basis. We describe here two flow cytometric methods, one using a relatively inexpensive non-sorting flow cytometer and the other using a cell sorter which, when combined with the scrape-loading technique, can readily quantitate the degree of dye transfer between human diploid fibroblasts in vitro.

MATERIALS AND METHODS

Chemicals. LY3 and RITC-D (average molecular weight of 10,000, neutral charge) were purchased from Molecular Probes, Inc. (Eugene, OR). TPA and dieldrin were obtained from Sigma Chemical Co. (St. Louis, MO).

Cells and Cell Culture. The cells used in these experiments were a HDF strain obtained from fetal lung (AG 3542; gift of Dr. G. Raghu, University of Washington Seattle, WA). Cells were grown in modified Eagle's medium with Earle's balanced salts containing a 50% increase in vitamins and essential amino acids (except glutamine) and a 100% increase in nonessential amino acids. The medium was further supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum. Cells were incubated at 37°C in humidified air containing 5% CO2.

Scrape Loading. The scrape-loading/dye transfer technique has been described in detail (7). In brief, one day prior to an experiment, cells were plated into 35-mm tissue culture dishes (2 × 105 cells per dish). Test chemicals or vehicle controls were added to dishes 2 h prior to scrape loading. The cells were washed twice with PBS (Gibco, Grand Island, NY). The cultures were flooded with a PBS solution containing 2 mg/ml of LY only (for one-color analyses) or a solution containing LY (2 mg/ml) and RITC-D (2.5 mg/ml) for two-color analyses. Scrape loading was performed with a "comb" constructed from a 15-mm linear array of metal needles. The comb was rotated in 360° arcs such that concentric circles of cells were left on the dish. The dishes were then incubated for 5 min (except in cases in which rates of transfer were being investigated) to allow for equilibration of the dye across gap junctions. The dye mixture was then aspirated and dishes were washed with PBS containing EDTA (0.2 mg/ml) and trypsinized. Cells were then suspended in medium with 3% fetal bovine serum, stored briefly at 4°C to prevent LY efflux, and protected from light until flow cytometric analyses were performed.

Flow Cytometric Analyses. Two different types of analyses were explored for quantitating differences in the degree of dye transfer that had occurred between cells.

The first method involved a single-color analysis on an arc-lamp illuminated flow cytometer (FACS Analyzer; Becton-Dickinson, Mountain View, CA). For these experiments cells were loaded with LY only. Excitation was at 400-505 nm (LP400 and SP505) and emission was detected at 515-545 nm (DF530/30). Fluorescence signals were displayed on log scale with simultaneous quantitation of electronic cell volume, also on log scale. Signals were digitized and stored on a DEC PDP-11/23 computer (Digital Equipment Corp., Maynard, MA) to give scattergrams of log volume versus log fluorescence intensity. Histograms were generated with histograms of log volume versus log fluorescence intensity.

3 The abbreviations used are: LY, lucifer yellow CH; HDF, human diploid fibroblasts; PBS, Dulbecco's phosphate-buffered saline; RITC-D, rhodamine isothiocyanate dextran; TPA, 12-O-tetradecanoylphorbol-13-acetate.
tograms of log fluorescence intensity (x-axis) versus cell number (y-axis) were formed from analysis of the scattergram by collapse of the volume/fluorescence data on the fluorescence axis after clockwise rotation of the axis by 45°. This rotation was necessary to remove volume bias from the fluorescence intensity signal (see Fig. 2).

The second method involved a two-color analysis of the fluorescence intensity of both LY and RITC-D on an Ortho Cytofluorograf (Model 50H/2151; Ortho Diagnostic Systems, Westwood, MA) equipped with an argon laser (Model 90-6; Coherent, Palo Alto, CA) tuned to 458 nm (LY excitation) and a dye laser (Model 599-OEM; Coherent) tuned to 570 nm (RITC-D excitation). Fluorescence emissions of LY and RITC-D were at 575–545 nm and above 590 nm, respectively. Analysis was gated on the basis of forward scatter to select cells of a nearly uniform size for subsequent fluorescence analysis, and thus a volume-based adjustment in fluorescence intensity was not performed.

RESULTS

One-Color Analysis. The principle of scrape-loading/dye transfer is shown in Fig. 1. LY dye solution was added to confluent monolayers of HDF and scrape loaded into the cells. As previously shown (7), LY readily transferred into adjacent nonscraped cells (Fig. 1A) except when the transfer was blocked by TPA (Fig. 1B).

When cultures which have been dye loaded are trypsinized and analyzed on the flow cytometer, both fluorescence intensity and volume can be simultaneously measured for each cell. The cells adjacent to the scrape have had their membranes transiently permeabilized to LY. When we compared the fluorescence of cells of different size, there was a direct relationship between cell volume and fluorescent intensity (data not shown), indicating that the cytoplasmic LY concentration had equilibrated with the LY/PBS solution, and the concentration of LY

![Graph](image-url)
was relatively constant within these cells. Since the fluorescence signals generated by these cells is the sum of their autofluorescence and LY fluorescence, and since both of these values are directly related to volume, we used an analysis which normalized for the volume-related effects. Unlike measurements which are only indirectly related to cell volume (such as light scatter), the electronic cell volume measurement is ideally suited to correctly perform such compensation. Taking the logarithm of both volume and fluorescence intensity, distributions with different volume versus fluorescence relationships (i.e., different slopes on linear plots) have distributions which are at an angle to the axes but are parallel with each other and displaced along the x-axis. This procedure is represented in Fig. 2. The rotation is made at a 45° angle because the gain of the log amplifiers for volume/fluorescence data onto the fluorescence axis after a counterclockwise rotation of the data by 45° to form a histogram of cell number (y-axis) versus fluorescence intensity (x-axis). This procedure is shown in Fig. 2. The rotation is made at a 45° angle because the gain of the log amplifiers for volume and fluorescence signals on the FACS analyzer are the same. This method of analysis allows one to remove volume bias from the fluorescence signals. Histograms of nonscraped and scrape-loaded HDF exposed to LY are shown in Fig. 2, B and D, respectively.

Fig. 3 shows the cell density dependence of LY transfer. Since the transfer of LY from initially loaded cells to adjacent cells is diffusion mediated, the numbers of LY positive cells increases as the cell density increases. This is due to a combination of more cells being loaded and greater numbers of cells receiving dye by transfer as the probability of cell-cell contact increases.

The time course for LY transfer in confluent cultures of HDF is shown in Fig. 4. As one can see from Fig. 4, transfer is rapid and nearly complete after 3 min.

The effect of TPA and dieldrin on dye transfer in confluent cultures of HDF is shown in Fig. 5. A dose-dependent block in dye transfer is caused by the tumor promoter TPA (Fig. 5A). A similar dose-dependent effect on dye transfer was seen in confluent cultures of HDF cells preexposed to dieldrin (Fig. 5B). Both of these observations are consistent with previously obtained results in metabolic cooperation assays (see "Discussion").

Two-Color Analysis. Since the numbers of LY donor and LY recipient cells are difficult to determine with the one-color analysis (some donor cells may have intermediate fluorescence due to equilibration with several recipients), it would be preferable to distinguish between LY donor and LY recipient cells. As described previously (7), one way to mark the donor cells is to label them with a second fluorophore which, by virtue of its high molecular weight, cannot cross gap junctions.

Cell sorter analysis of LY and RITC-D loaded cells placed in suspension after scrape loading is shown in Fig. 6. Fluorescence of LY and RITC-D is quantitated independently by excitation from two spatially separated laser beams. There are three regions of interest when this type of analysis is carried out. The initially loaded cells appear as a subpopulation of cells that are both bright red and intermediate to bright green. The recipients of dye transfer are a subpopulation with intermediate to bright green fluorescence and "negative" red fluorescence (actually...
Fig. 6. Isometric displays of HDF exposed to LY (green fluorescence) and RITC-D (red fluorescence) and analyzed by dual laser excitation on a cell sorter. A, nonscraped cells exposed to LY and RITC-D; B, cells scraped in the presence of LY only; C, cells scraped in the presence of RITC-D only; D, cells scraped in the presence of both LY and RITC-D. d, RITC-D positive donors; r, LY positive recipients; n, nonlabeled cells.

dimly fluorescent due to nonspecific binding to RITC-D to cells). The third subpopulation of nonloaded and nonrecipient cells have low green fluorescence and dim red fluorescence. Thus the enumeration of LY recipients and LY donors is easily made with this type of analysis.

As with the one-color analysis above, this transfer was dependent upon cell density (Figs. 7 and 8). There is an increasing number of cells in the LY recipient subpopulation as the density of the scraped cultures is increased. Fig. 8 also shows that when TPA is added to these cultures before scrape loading there is a dose-dependent reduction in the numbers of LY recipient cells.

As in the single-color analysis with the flow cytometer, the distribution of cells into these various subpopulations could be affected by the addition of dieldrin to confluent cultures of HDF (Fig. 9). A dose-dependent decrease in the numbers of LY recipients is caused by exposure to dieldrin, indicating a block in LY dye transfer.

DISCUSSION

The results obtained by combining flow cytometry with the scrape-loading/dye transfer technique demonstrate the utility of this approach in providing a quantitative measure of dye transfer. The combination of these two procedures provides an efficient method for assessing the physiology of gap junctional communication in vitro under a variety of physiological, pharmacological, and toxicological conditions.

The data presented in Figs. 1 and 2 were suggestive of dye transfer in high density cultures that had been scraped in the presence of LY. This interpretation is reinforced by experiments which showed a cell density dependence (Fig. 3) and a dramatic block in dye transfer induced by the tumor promoter TPA (Fig. 4A). Many studies have shown that TPA can inhibit cell-cell communication in vitro (8, 9, 12, 13, 19). It is clear from the results obtained with both single-color and two-color analyses that this blocking effect can be rapidly and efficiently quantitated with scrape-loading/dye transfer and flow cytometry.

This effect can be shown for compounds other than TPA, as evidenced by the results obtained when HDF were exposed to dieldrin. Dieldrin has been shown to be a tumor promoter for liver in vivo (22), a neurotoxin (23), and also an inhibitor of metabolic cooperation in Chinese hamster V-79 cells (10) as well as in human teratocarcinoma cells (11). Again, there was a clear dose-dependent reduction in the number of cells with LY fluorescence in the single-color analysis (Fig. 4B) and a marked reduction in the number of LY recipients in the two-color analysis (Fig. 8), indicative of a block in dye transfer.

The amount of dye transfer measured by the flow cytometer would be best expressed quantitatively as the percentage of LY positive cells less the percentage of initially loaded cells. Since
Fig. 7. Isometric displays showing effect of density and TPA on LY dye transfer in HDF as determined by dual laser analysis performed on a cell sorter. A, B, and C, cells at low, medium, and high densities (0.25, 0.7, and 2.1 x 10⁴ cells/cm², respectively), scrape loaded with LY and RITC-D. Cells were plated and scraped as described in Fig. 3. D, effect of a 2-h preexposure to 10 ng/ml TPA on the transfer of LY in scrape-loaded high density HDF.

Fig. 8. Effect of density and TPA on LY dye transfer in HDF. The ratio of LY positive cells (dye transfer recipients) to RITC-D positive cells (dye transfer donors) is shown as a function of both cell density and preexposure to TPA at 0, 0.1, 1.0, or 10.0 ng/ml for 2 h.

this last quantity is difficult to determine with a one-color analysis (i.e., the cells with intermediate fluorescence intensity could be either recipients of dye transfer or donors that have transferred most of the dye originally loaded into them), a more practical index for one-color studies is the percentage of LY positive cells. The percentage of LY positive cells can be determined by first defining LY negative cells as those that fall within a region determined for nonscraped cells. Any cells with fluorescence greater than this region can be considered to be either recipients or donors of LY, and comparisons are then made of the percentage of LY positive cells under different experimental conditions. The problem with this analysis is that one must be careful to scrape the control and test plates equally so as not to bias the result with different numbers of initially loaded cells. This effect can be seen by comparing the histograms in Figs. 3 and 4, where there is an obvious difference in the numbers of cells with high LY fluorescence.

Because of the attendant problems with quantitating the numbers of LY donor cells when using a one-color analysis, we...
have also demonstrated a two-color analysis on a cell sorter equipped with a dual excitation source. The principles of LY dye transfer do not differ in this analysis from the flow cytometric data presented above. However, when the cells are simultaneously exposed to a high molecular weight marker that can be loaded into cells but not transferred across gap junctions, they can be identified as initially loaded cells. The numbers of LY donors (cells with bright red fluorescence) can thus be readily quantitated. The numbers of LY recipients can be quantitated equally as well, for they have predominantly green fluorescence due to LY transfer. Thus, the analysis becomes highly quantitative and can be expressed as the numbers of LY recipients (cells with LY only) per LY donor (cells with LY and RITC-D). Although the use of the cell sorter with two-color analysis requires more sophisticated instrumentation and tuning, the results are more quantitative than those obtainable with a one-color analysis because of the ability to readily identify and quantitate these dye donor and recipient cells. An additional benefit of using the sorter is the ability to sort cells from the population which remain negative after scrape loading. This should allow for the selection of variants in the population with reduced metabolic cooperation for further study.

We are currently investigating the use of other large molecular weight fluorophores other than RITC-D so that a two-color analysis can be performed on equipment with a single excitation source. A combination with potential prospects might be a low molecular weight phycobiliprotein together with LY. The phycobiliproteins can be excited at wavelengths close to those used to excite LY and yet have emission characteristics which allow good separation of their signals. Evaluations of phycobiliprotein subunits and fragments are in progress.

Use of flow cytometry in quantitating dye transfer by the techniques described here has potential applications in not only toxicology and chemical carcinogenesis research (i.e., the screening of putative tumor promoters and the investigation of chemically induced perturbations in gap junctions) but also in investigating the effect of disturbances in gap junctional communication on embryonic development and differentiation (24).

Furthermore, this approach may have utility in the field of cancer diagnosis. In a series of experiments designed to determine the communication competence of a host of continuous cell lines, it was found that there was a good correlation between the ability of these cells to undergo cell-cell communication with feeder layers of normal cells and their ability to be growth arrested by the normal cells (6, 25). Since abnormalities in cell-cell communication might be an early marker of transformation (5, 6, 21, 25), an exciting possibility would be to use the techniques described here to assess the cell-cell communication potential of premalignant tissues as a prognostic indicator.

REFERENCES


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