A Fluorescence Photobleaching Assay of Gap Junction–Mediated Communication Between Human Cells

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Gap junction–mediated communication between contiguous cells has been implicated in the regulation of cell proliferation and differentiation. This report describes a new technique to measure cell-cell communication, gap fluorescence redistribution after photobleaching, which is based on the diffusion-dependent return of 6-carboxyfluorescein–mediated fluorescence in a photobleached cell that is in contact with other fluorescently labeled cells. Fluorescence recovery rates are interpreted as dye transport across gap junctions. Results of experiments on normal human fibroblasts and human teratocarcinoma cells show that this technique can measure rapid dye transfer and detect inhibition of communication (between teratocarcinoma cells) by the tumor promoters 12-*O*-tetradecanoyl-phorbol-13-acetate and the pesticide dieldrin.

LOW-RESISTANCE PATHWAYS FOR INTERCELLULAR communication are formed after cell-cell contact (*1, 2*). These transport channels for low molecular weight cytoplasmic components [≤1.7K as measured with synthetic peptides (*1*)] have been termed gap junctions (*1–3*). It has been suggested that gap junction–mediated communication plays a significant role in the control of cell proliferation, tissue metabolism, and synchrony (*1, 2*). Investigators recently examined the role of gap junction transport in embryonic differentiation (*4*) and in the response to tumor promoters (*5*), toxic substances (*6*), and oncogene products (*7*). Several investigative methods have been used, including electrocoupling techniques (*1, 8*), intercellular transfer of fluorescein and other dyes (*1, 9, 10*), use of genetically deficient cells to measure "metabolic cooperation" (*1, 11*), and autoradiographic detection of the transfer of low molecular weight, radioactively labeled compounds (*1, 12*). In most instances a microelectrode or pipette is introduced into the cell to deliver the measurable partitioning agent. The possibility for cell trauma, the difficulty in extending this technology to all types of cells and cell aggregates, and the requirement for microinjection expertise have limited the usefulness of most transfer analytical techniques.

We describe here a new method that overcomes some of these problems and provides a means of flux quantitation. The method (i) takes advantage of the observation that fluorescein can be transferred from cell to cell by junctions (*9*) and (ii) extends the technique of fluorescence redistribution after photobleaching (FRAP) to whole cells. In this procedure, termed gap FRAP, cells in tissue culture are labeled with 6-carboxyfluorescein diacetate. All cells in the population are internally labeled by this stain, which is hydrolyzed to 6-carboxyfluorescein, a hydrophilic fluorescein derivative that is maintained in the cell (*10*). Any labeled cell may then be photobleached by a laser beam whose width is approximately equal to the cell diameter, or by a series of

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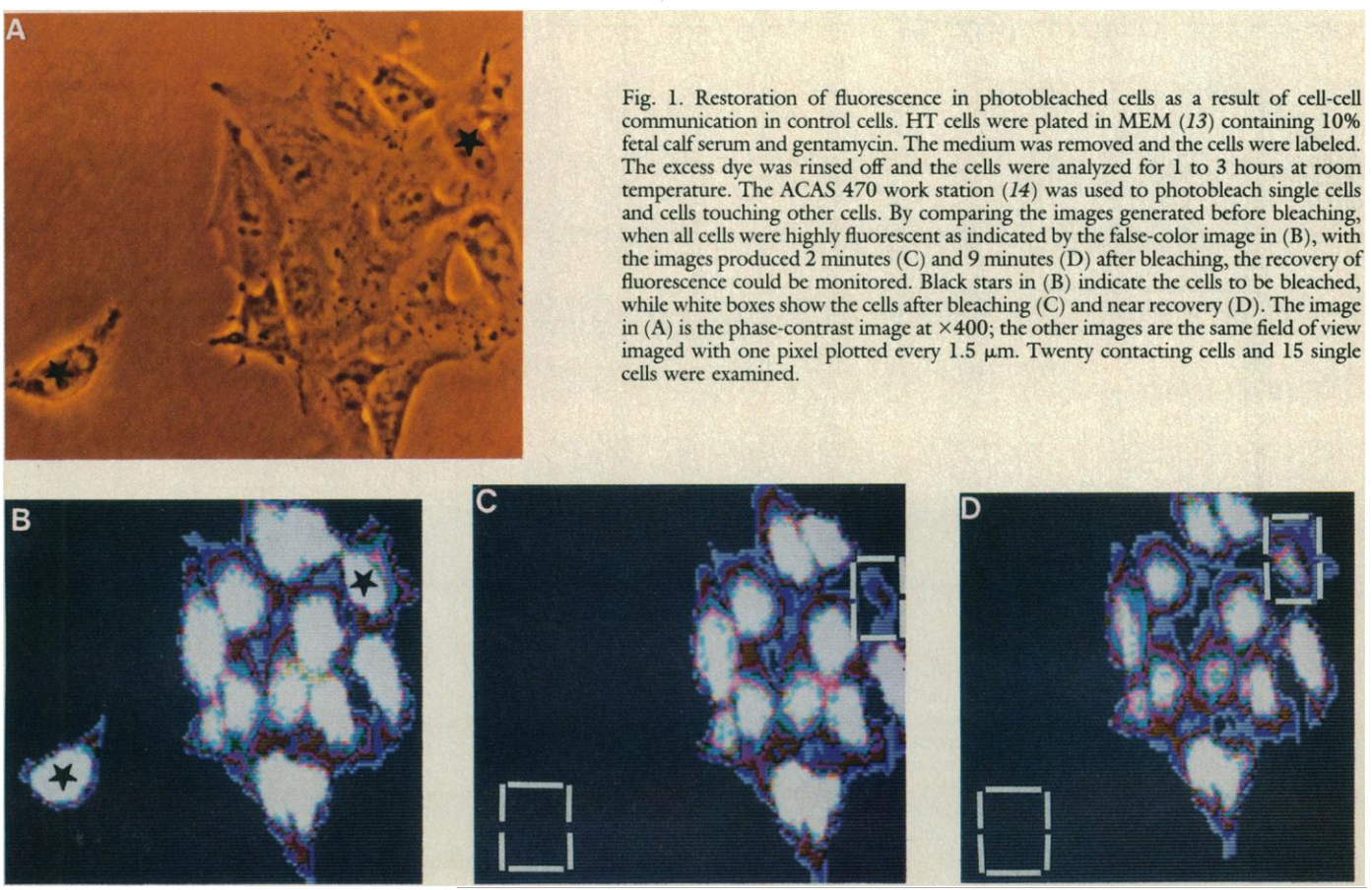

Fig. 1. Restoration of fluorescence in photobleached cells as a result of cell-cell communication in control cells. HT cells were plated in MEM (*13*) containing 10% fetal calf serum and gentamycin. The medium was removed and the cells were labeled. The excess dye was rinsed off and the cells were analyzed for 1 to 3 hours at room temperature. The ACAS 470 work station (*14*) was used to photobleach single cells and cells touching other cells. By comparing the images generated before bleaching, when all cells were highly fluorescent as indicated by the false-color image in (B), with the images produced 2 minutes (C) and 9 minutes (D) after bleaching, the recovery of fluorescence could be monitored. Black stars in (B) indicate the cells to be bleached, while white boxes show the cells after bleaching (C) and near recovery (D). The image in (A) is the phase-contrast image at ×400; the other images are the same field of view imaged with one pixel plotted every 1.5 μm. Twenty contacting cells and 15 single cells were examined.

laser pulses, each pulse with a diameter of about 1 μm. After photobleaching, the bleached dye molecules from one cell and the unbleached dye molecules from an adherent contacting cell may be redistributed through gap junctions. Monitoring the redistribution of these labeled reporter molecules as a function of time results in a single exponential recovery curve that yields a rate constant for dye transport.

Experiments were performed with human teratocarcinoma (HT) cells plated in a modified minimum essential medium (MEM) (*13*) containing 10 percent fetal calf serum and gentamycin in 35-mm culture dishes. After 24 to 48 hours of growth the cells were washed with phosphate-buffered saline (PBS) containing calcium (0.9 m*M*) and magnesium (0.5 m*M*) and stained with 6-carboxyfluorescein diacetate (7 μg/ml for 10 minutes at room temperature).

The dye and labeling conditions do not affect cell viability, and the same cells can be restained for several days. All measurements are performed at room temperature for 1 to 3 hours. A tissue culture plate of labeled cells is placed on a high speed computer-controlled two-dimensional stage of the ACAS 470 work station (*14*). The stage moves the cells in a defined manner above the objective (×40) of an inverted epifluorescence microscope. The microscope objective focuses the argon ion laser beam (excitation wavelength, 488 nm) to a 1-μm spot that excites fluorescence in individual cells at 1.5-μm steps in a two-dimensional raster pattern. The single point emission from each excited step is recorded as an intensity by a photomultiplier tube. The digital signals representative of fluorescence intensity are stored in the computer with the source x-y location. The emitted intensities are color-coded and presented on a computer video screen as false color images of the fluorescence distribution in the analyzed cell.

Figure 1 shows HT cells stained with 6-carboxyfluorescein diacetate. Figure 1A is a phase view of the cells, while a computer-generated false color image of fluorescence distribution is shown in Fig. 1, B to D. The color contour map of each cell shows concentric color rings that merge to a white center signifying the nuclear volume (white represents the highest fluorescence intensity). The circular color bands suggest a homogeneous distribution of dye throughout the cytoplasmic compartment. Before photobleaching, an image of the initial dye distribution was produced (Fig. 1B). After this event, the intensity of the laser beam was significantly increased and a series of bleaches (~1 μm in diameter) was initiated to photochemically destroy the dye in a single contacting cell and in an isolated, unconnected cell (Fig. 1, B to D). An imaging scan was then performed. Destruction of dye fluorescence emission was observed in both bleached cells (Fig. 1C). A time-dependent series of image scans was then generated. Recovery of fluorescence occurred only in the contacting cell and not in the isolated cell (Fig. 1D). When cells were rebleached similar recoveries were seen, suggesting that there was no significant photochemical damage affecting cell-cell communication and that repetitive transport measurements of the same cell could be performed.

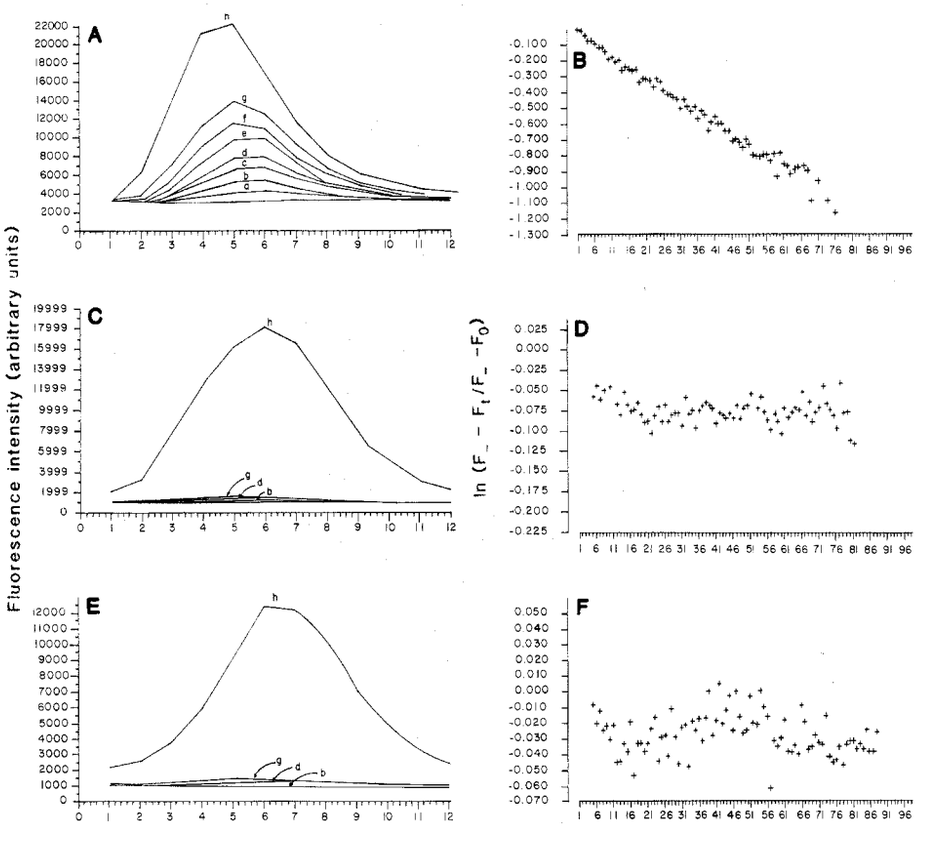

Fig. 2. Fluorescence recovery in touching HT cells (A and B), in an isolated single cell (C and D), and in a touching cell exposed to the tumor promoter dieldrin (E and F). Single cells were scanned (*15*) with a beam approximately 30 μm in diameter. Photobleaching and recovery calculations were performed as described by Peters (*16*). In (A), scan h represents the fluorescence intensity of the intracellular compartment before bleaching. Scan a represents the fluorescence intensity in the cell 50 seconds after bleaching, with b to g representing recoveries at 100, 200, 400, 600, 800, and 900 seconds, respectively, after bleaching. In (C) and (E), scan h represents the level of fluorescence intensity before bleaching, while scans b, d, and g are for 100, 400, and 900 seconds after bleaching. In (B), (D), and (F) the data are plotted as described in (*16*) and the text. The fluctuations in (D) and (F) represent a low signal, as shown by the ordinate values when compared to (B).

To corroborate these results the experiment was performed on a FRAP instrument (*15*). Bleaching in this instance was performed by defocusing a laser beam (width approximately equal to the cell diameter). The beam was scanned across a labeled cell by a galvanometrically controlled mirror (*15*), resulting in the fluorescence intensity profile presented in Fig. 2A, scan h. Bleaching the cell resulted in the complete loss of fluorescence (Fig. 2A, scan a). Subsequent scans demonstrated fluorescence recovery (Fig. 2A, scans b to g). Recovery curves were analyzed (*16*) for passive transport of fluorescent dyes through pores. Considering that the rate-limiting step for dye transport is gap junction permeation (*1*), kinetics should follow the equation

$$\frac{C_e - C_t}{C_e - C_0} = e^{-kt} \qquad (1)$$

where C_e, C_0, and C_t are dye concentrations in the cell at equilibrium, zero time, and time t, respectively. The rate constant k is related to the permeability coefficient P by $P = (V/A)k$, where V and A are the volume and area, respectively, of the cell. As with all photobleaching experiments, measured fluorescence intensities are representative of the dye concentration at time t. Accordingly, Eq. 1 may be represented as

$$\frac{F_- - F_t}{F_- - F_0} = e^{-kt} \qquad (2)$$

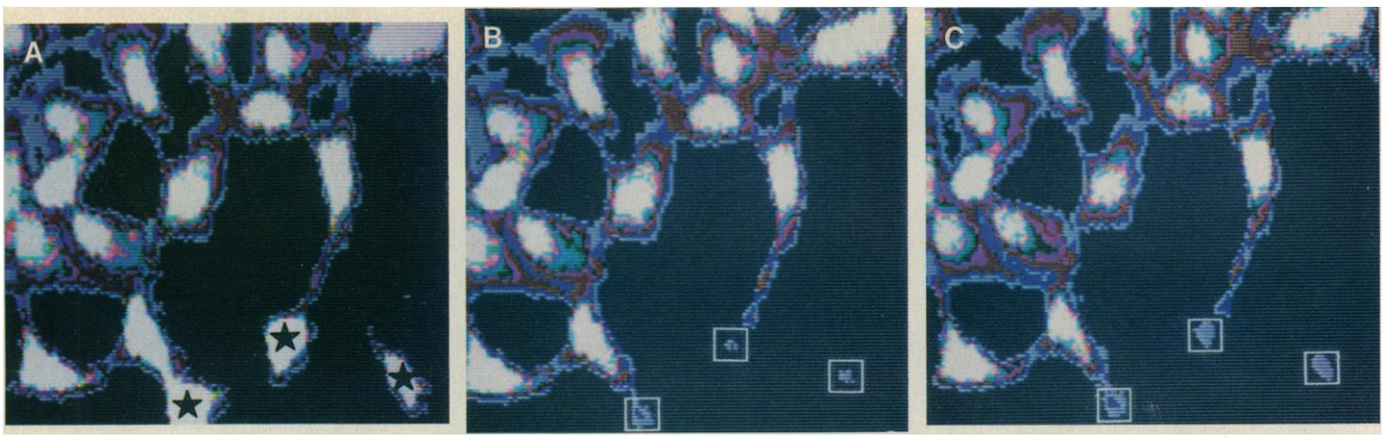

Fig. 3. Recovery of fluorescence in cells in the presence of dieldrin (7 μg/ml). Several cells were photobleached within one field (A) and monitored for recovery 2 minutes (B) and 18 minutes (C) after bleaching. Thirteen contacting and seven nontouching cells were examined. This concentration of dieldrin is nontoxic (*19*). Stars and boxes denote cells before bleaching and after bleaching and recovery, respectively.

where F_-, F_0, and F_t are fluorescence signals before photobleaching, after, and at time t. A representative analysis of the recovery curves in Fig. 2A is presented in Fig. 2B. The half-time of recovery is calculated to be ~7.5 ± 1.5 minutes for four measurements. This is approximately what was observed with the ACAS 470–based imaging method and appears to be consistent with most reports suggesting that the recovery time for BALB/c 3T3 cells (*9*), blastomeres (*4, 17*), and HEL-37 cells (*18*) is on the order of minutes. For pancreatic islet cells, however, a higher rate of transfer was reported (*19*). Figure 2, C and D, shows the results of a control experiment that demonstrated the lack of fluorescence recovery in a photobleached isolated single cell.

Further support for the effectiveness of this method is provided by measurements of gap junction communication in the presence of a tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the pesticide dieldrin (*20*). Several investigators have found that these compounds and other tumor promoters inhibit junctional communication between cultured mammalian cells (*5, 6, 21*). Dieldrin blocked the recovery of fluorescence of photobleached cells in culture (Fig. 2, curves a and e, and Fig. 3). When the culture was washed and incubated at 37°C for 4 hours in medium without dieldrin, the cells recovered after photobleaching (Fig. 4) at rates similar to the control rates, suggesting that dieldrin blockage is reversible. TPA also blocked fluorescence recovery in exposed cell cultures. Inhibition of transport by TPA may be related to its tumorigenicity, since 4-phorbol-12,13-didecanoate, a phorbol ester, is nontumorigenic (*22*) and did not block transport. This result was also reported by Fitzgerald *et al.* (*18*), who used dye microinjection techniques.

Fluorescence photobleaching technology has demonstrated its usefulness in analyzing the transport properties of molecules in membranes (*23*), cellular organelles (*24*), and the cytoplasm (*25*). Gap FRAP extends this method to investigations of gap junction–mediated cell-cell communication. Previous studies have had to rely on microinjection methods with subsequent loss of temporal resolution (*9, 17, 18*). Furthermore, appropriate controls or reexperimentation on the same series of microinjected cells was not possible. Gap FRAP permits multiple measurements of the same cell without traumatic cell manipulations. Perhaps the full potential of gap FRAP rests in its ability to make possible measurements of all anchored cell types and cell configurations. The possibility of continuously monitoring communication patterns in the same developing blastomere, a measurement that would appear to be enormously difficult with existing methods, may now be considered.

Since the original hypothesis that altered intercellular communication might play a

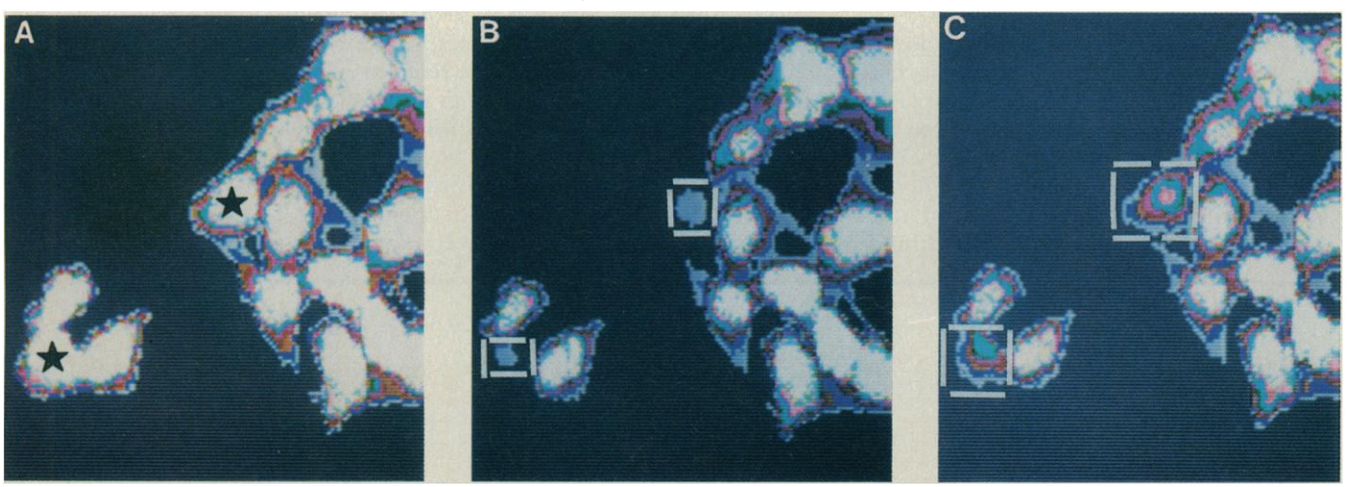

Fig. 4. Recovery of fluorescence in cells treated with dieldrin and then washed. The images shown were generated before bleaching (A), 2 minutes after bleaching (B), and 9 minutes after (C). Four contacting and three single cells were examined. Stars indicate analyzed cells.

role in carcinogenesis (*1*)—specifically in the tumor promotion phase of carcinogenesis (*1, 5, 6*)—practical attempts have been made to develop short-term, in vitro assays as substitutes for whole animal screens for the detection of this class of toxic chemicals (*5, 6, 9*). Our results with the confirmed tumor promoters TPA and dieldrin demonstrate that such an assay with human cells may be possible using gap FRAP. Gap FRAP technology should provide a powerful new tool in elucidating the nature of cell-cell communication in developmental and tumor biology, toxicology, and neurobiology.

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26. Supported by grants from Dow Chemical U.S.A., M.S.U. Center for Environmental Toxicology, Biotechnology Center of M.S.U. (J.E.T.); NIH grant SBIR IR 43 CA/39945-01 (Meridian Instruments); and NIH grant GM 30158 (M.S.). We thank R. Olinger, D. Towson, and L. Ward of Meridian Instruments for valuable discussions and technical assistance and S. Suter for technical assistance.

25 September 1985; accepted 14 February 1986