A SIMPLE DYE-COUPLING ASSAY FOR EVALUATING GAP
JUNCTIONAL COMMUNICATION: THE IMPORTANCE OF
TRANSCRIPTION AND TRANSLATION ON THE
ESTABLISHMENT OF DYE-COUPLING

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ABSTRACT

A simple dye-coupling assay has been developed to
study gap junctional communication using a uterine cell
line. The new dye-coupling assay was found to be of
comparable sensitivity to an autoradiographic assay for
transfer of radiolabelled nucleotide. To investigate the
importance of protein synthesis in the establishment of
dye-coupling under the conditions of this assay, cul-
tures were treated with actinomycin-D and cycloheximide
to inhibit transcription and translation, respectively.
Inhibition of transcription had no significant effect on
dye-coupling. The protein synthesis inhibitor, cyclo-
heximide, also had no significant effect on dye-coupling
at concentrations up to 1 uM, even though protein syn-
thesis was inhibited to 19% of control.

Key Words: Gap junctions, intercellular junctions, pro-
tein inhibitors.

INTRODUCTION

Gap junctions mediate direct intercellular transfer
of low molecular weight substances in a wide variety of
cells (Loewenstein, 1981; Larsen, 1983). These communi-
cating junctions have been implicated in many important
functions, including the synchronized contraction of
uterine smooth muscle during labor (Garfield, 1988). We
have developed a rapid in vitro dye-coupling assay to
investigate intercellular communication between uterine
cells using an established cell line derived from a
human uterine leiomyosarcoma, SK-UT-1.
    In this dye-coupling assay, dye-donor cells were
labeled with both a permanent cell marker, latex beads,

and a transferable fluorescent dye derived from 5(and 6)-carboxy-2,7-dichlorofluorescein diacetate (DCCFDA). Donor cells were co-cultured with unlabeled dye-recipient cells, after which transfer of dye from the donors to the recipients was evaluated using an epifluorescence microscope. Parallel experiments with a previously published autoradiographic assay based on transfer of radiolabelled nucleotide demonstrated that the new dye-coupling assay was of comparable sensitivity.  Transcription and translation were inhibited with actinomycin-D and cycloheximide, respectively, to determine if the establishment of communicating junctions required protein synthesis.  This assay has recently been applied to an embryocarcinoma cell line (Loch-Caruso et al., 1988).

## MATERIALS AND METHODS

Cell Culture. SK-UT-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and used between passages 35-75. The SK-UT-1 cell line was originally isolated from a human mesodermal uterine tumor consistent with a grade III leiomyosarcoma, and is presumably of myometrial origin (Fogh et al., 1977). The cells were maintained in a growth medium containing Eagle's salts (Eagle, 1959), a two-fold enrichment of non-essential amino acids, a 1.5-fold enrichment of vitamins and essential amino acids (except L-glutamine), with the addition of 1 mM sodium pyruvate, 5.55 mM D-glucose, 14.3 mM sodium chloride, 11.9 mM sodium bicarbonate, and 5% fetal bovine serum, pH 7.4. Cultures were periodically checked for the presence of mycoplasma (Chen, 1977).

Chemicals. Actinomycin-D, ethidium bromide and cycloheximide were purchased from Sigma Chemical (St. Louis, MO).  The dye, 5(and 6)-carboxy-2,7-dichlorofluorescein diacetate (DCCFDA), was purchased from Molecular Probes (Eugene, OR). Radiolabeled uridine and leucine were purchased as sterile aqueous solutions (Amersham, Arlington Heights, IL). Fluorescein-conjugated latex beads (Fluoresbrite carboxylate microspheres, ca. 1 um diameter) were purchased from Polysciences (Warrington, PA). Stock solutions were prepared of actinomycin-D (5 mg/ml in absolute ethanol), DCCFDA (10 mM in dimethyl sulfoxide) and ethidium bromide (1 mg/ml in Dulbecco's phosphate-buffered saline solution (DPBS)), and stored refrigerated. Working solutions were diluted from the stock solutions immediately prior to use.

Dye-Coupling Assay. Dye-donor cells were grown to near confluence, rinsed with DPBS containing 1 mg/ml glucose (PBSG), and treated for 4 h at $37^\circ$C with fluo- rescent latex beads in PBSG (1-2 x $10^8$ beads/sq. cm growth surface area). Under these conditions, each donor cell internalized approximately 5-20 beads, which were then retained throughout the experiment. Because the beads were too large to pass through gap junctions, they provided a readily identifiable and permanent marker to distinguish the donor cells from the recipient cells. The cultures were then rinsed free of unincorporated beads with PBSG, and treated for 10 min with 15 uM DCCFDA in PBSG at $37^\circ$C. DCCFDA is a membrane-permeant, non-fluorescent dye (529.3 MW) that is metabolically activated to a fluorescent and lipophobic state after hydrolysis of the acetate ester linkages (Rotman and Papermaster, 1966). The lipophobic fluorescent dye is retained by many cells for several hours, yet is small enough to pass through gap junctions (Loewenstein, 1981). The two chlorine substitutions make this dye much less sensitive to fluorescent photobleaching than the parent material, 5(and 6)-carboxyfluorescein (unpub- lished observation). The cultures were rinsed twice with PBSG to remove unincorporated and surface-bound dye, and then rinsed an additional two times with calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS). Cells were harvested by trypsin digestion, pelleted by low-speed centrifugation, rinsed with medium and resus- pended in fresh medium. A sample of the cell suspension was counted to determine both the total number of cells and to estimate viability through exclusion of trypan blue (Patterson, 1979).

Dye-recipient cells were plated 1-2 days prior to the assay in 60 mm tissue culture dishes (Corning) at a density of $10^4$ cells/sq. cm. of growth surface. Dye- donors, at a density of 2,500 cells/sq. cm. of growth surface, were added to the unlabeled, dye-recipient cultures along with fresh medium, with or without (controls) a test substance. Depending on the experimen- tal requirements, cultures were allowed 30 min to 5 h for donor cell attachment, spreading, and the opportuni- ty to form permeable junctions. After co-culture the medium was aspirated and replaced with ice-cold PBSG with or without (controls) the test substance. The plates were then put on ice to retard establishment of additional dye-coupling.

Plates were scored for dye-coupling by examination of at least 100 contacting cell pairs at 400-fold magni- fication using a Nikon Diaphot-TMD microscope equipped with an epifluorescence unit. The donor-recipient cell pairs were first examined under bright-field conditions to verify their close association, and then under blue

excitation (450-490 nm) for evidence of dye-coupling.
Under light of this spectral band, the beads appeared as
intensely bright dots which were clearly distinguishable
from the DCCFDA-derived label.  To evaluate changes in
the frequency of dye-coupling, the percentage of con-
tacting donor-recipient cells that showed evidence of
dye-coupling was compared to control cultures.

Autoradiographic Visualization of Radiolabelled Nu-
cleotide Transfer. Radionuclide-donor cells were labeled
for 4 h in PBSG containing both $^3$H-uridine (10 uCi/ml)
and fluorescent latex beads (1-2 x $10^8$ beads/sq. cm.
growth surface). Similar to the dye-coupling assay, the
cells were then seeded into cultures of unlabeled recip-
ient cells. After 1-5 h of co-culture the medium was
aspirated, and each plate was rinsed with DPBS and
placed on ice. The cultures were fixed with ice-cold 7%
trichloroacetic acid for 30 min, rinsed twice with ice-
cold 7% trichloroacetic acid, once with distilled water
and air dried. The plates were coated with Kodak NTB2
emulsion, air dried and exposed for 7-10 days in the
dark. The plates were developed using Kodak D19, rinsed
with distilled water, fixed with Kodak acid fixer,
rinsed with tap water, post-stained with hematoxylin and
eosin, and examined under a 50% (v/v) mixture of glycer-
ol and DPBS.

Incorporation of Acid-Insoluble Radiolabel. To
monitor RNA synthesis, cultures grown in 16 mm multi-
well dishes were incubated for 3.5 h with 5 uCi/ml $^3$H-
uridine. To monitor protein synthesis, the actinomycin-D
treated cultures were incubated for 3.5 h in 60 mm
dishes with 8 uCi/ml $^3$H-leucine, and the cycloheximide
treated cultures were incubated for 3.5 h in 35 mm
dishes with 25 uCi/ml $^3$H-leucine. The radionuclide-
labeled cells were rinsed with ice-cold PBS, placed on
ice and fixed with trichloroacetic acid as described for
autoradiography. An alternate fixation protocol utilized
a freshly prepared mixture of methanol and acetic acid
(3:1) for 15 min (Stein and Yanishevsky, 1979). The
cytoskeleton and other macromolecules were dissolved by
incubation for 16-18 h in 0.1 N NaOH at 37°C. The pH was
adjusted to neutrality by addition of an equal volume of
0.1 N HCl, the contents were mixed thoroughly, and the
mixture was allowed to stand at room temperature for at
least 2 h. Triplicate samples were counted in a Beckman
LS8000 scintillation counter to determine acid-insoluble
radioactivity.

DNA Quantification. DNA was quantified with ethidi-
um bromide using a modification of the method of Le Pecq
and Paoletti (1966, 1967). Briefly, triplicate samples

from each plate were mixed with ethidium bromide dis-
solved in a Tris/EDTA solution to provide final concen-
trations of 10 ug/ml ethidium bromide, 160 mM Tris and
16 mM EDTA.  Fluorescence at 612 nm was measured on a
fluorescence spectrophotometer using 306 nm excitation
wavelength and compared to a control curve generated
with calf thymus DNA dissolved in O.1 N NaOH. The stand-
ard curve for the controls always had a correlation
coefficient greater than 0.99.

        Statistical Methods. Growth curve data were ana-
lyzed using a two-factor analysis of variance (ANOVA),
followed by Tukey's method for multiple comparisons
(Devore, 1982). Other data were analyzed using the
Kruskal-Wallis or chi-square test for homogeneity, as
appropriate, followed by post-hoc group comparisons
using the methods described in Devore (1982).


                        RESULTS


        Dye Coupling. The dye chosen for this assay,
DCCFDA, was of low toxicity to the SK-UT-1 cells as
indicated by DNA measurements. Concentrations of DCCFDA
below 25 uM did not alter DNA content at 2 h, 2 days and
5 days post-treatment (Fig. 1). A significant reduction
in growth rate occurred only at the highest concentra-
tion tested, 100 uM (p<0.001). The labeling treatment
selected for use with SK-UT-1 cells in this assay, 15 uM
DCCFDA for 10 min, allowed adequate detection of dye-
coupling with no apparent toxicity.
        Dye-coupling was readily detected by the presence
of fluorescent dye in the bead-free recipient cells that
were in contact with bead- and fluorescent dye-labeled
donor cells (Fig. 2). Pre-plating the recipient cells a
day or two in advance allowed unambiguous interpretation
of dye-coupling, since the recipients were maximally
flattened and had often replicated to form small colo-
nies. In such cases, the dye was often distributed
throughout the entire group of inter-connected cells
from a single donor cell. The percent of dye-coupled
cell pairs increased in proportion to the duration of
co-culture until about 3-3.5 h (Fig. 3). Since no fur-
ther increase in dye-coupling was observed with addi-
tional incubation up to 4.5 h, 3.5 h was selected as an
optimal duration of culture for the experiments with the
test substances, actinomycin-D and cycloheximide.
        In order to compare the dye-coupling assay used in
these investigations to a standard published procedure
for junctional communication, parallel autoradiographic
studies were performed using transfer of radiolabelled
nucleotide (Pitts and Simms, 1977). This procedure has

been reported to be a highly sensitive method to study
intercellular communication (Pitts and Kam, 1985).
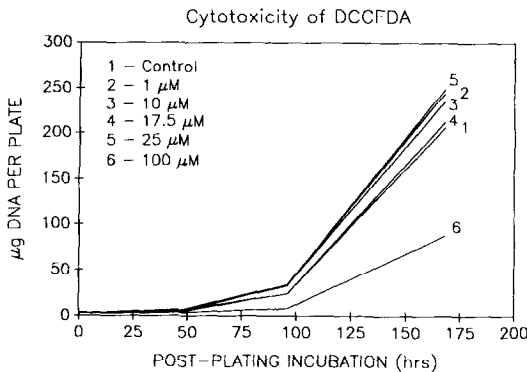


**Fig. 1:**    Cytotoxicity of DCCFDA.   Aliquots of freshly
harvested SK-UT-1 cells were suspended in PBSG contain-
ing DCCFDA at a concentration of 0 (Control), 1, 10,
17.5, 25 or 100 uM. The cells were incubated for 5 min
at ambient temperature, pelleted and rinsed with PBSG.
Each pellet was resuspended in medium, and aliquots of
the cell suspension were added to 35 mm wells of 6-well
plates. At 2, 48, 96 and 168 h post-plating, three
plates from each group were processed for quantification
of DNA using ethidium bromide. A large reduction in DNA
was evident only at 100 uM (p<0.001).

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The results in Figure 4 show that this dye-coupling
assay provided results of comparable sensitivity.

     **Transcriptional Inhibition.** To evaluate the impor-
tance of transcription on the development of dye-cou-
pling, cultures were treated with actinomycin-D, a
potent transcriptional inhibitor (Takusagawa et al.,
1982). Actinomycin-D had no significant effect on dye-
coupling, even though RNA synthesis  was reduced in a
concentration-dependent manner with treatments from 0.1
to 10 uM, as measured by the incorporation of acid-
insoluble ^{3}H-uridine (Table 1). At the two highest
concentrations tested, 5 and 10 uM actinomycin-D, incor-
poration of acid-insoluble ^{3}H-uridine was reduced to 1%
of controls, yet dye-coupling was still 92% and 80% of
controls, respectively. Actinomycin-D did not alter cell
attachment as measured by DNA content, nor did it inhib-
it total protein synthesis at concentrations up to 10
uM, as measured by incorporation of acid-insoluble [3]H-
leucine (data not shown). The lack of an effect on total

protein synthesis may be due to stabilization of exist-
ing mRNA, allowing normal rates of translation in spite
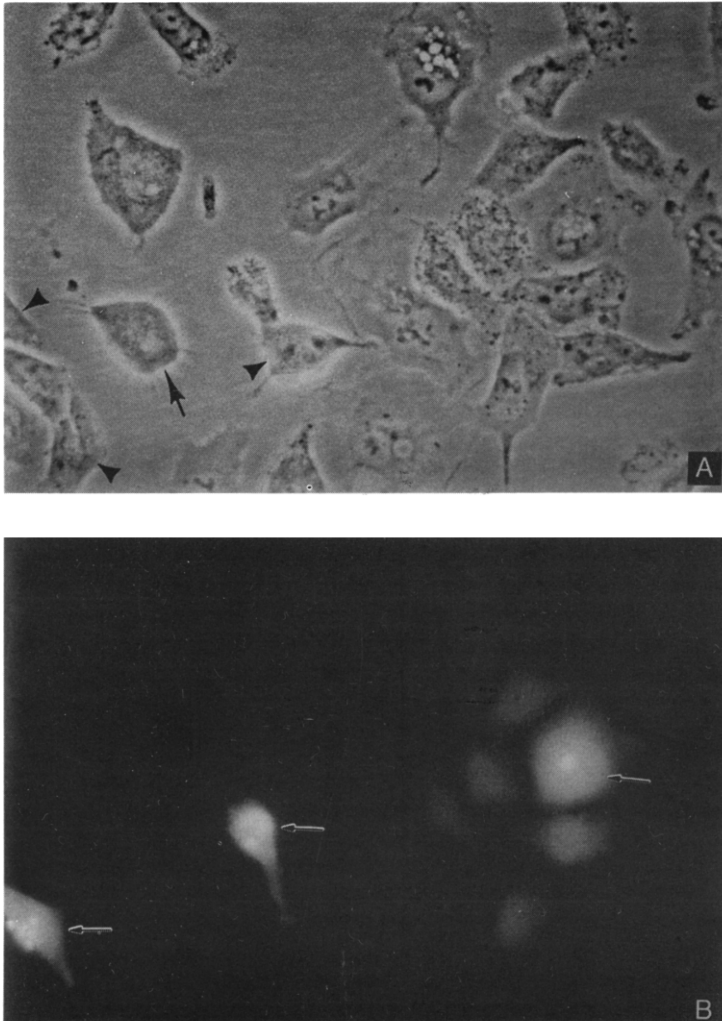


**Fig. 2:** Dye-coupling in SK-UT-1 cells, (A) phase-
contrast and (B) dark field. Donor cells (B, small
arrows) were labeled with both fluorescein-conjugated
latex beads and DCCFDA, then co-cultured with unlabeled
cells. At 3.5 h post-plating, the culture was examined
for evidence of dye-coupling. Extensive dye-coupling is
shown between donor and recipient cells (B, right side),
as well as the lack of dye-coupling to several contact-
ing unlabeled cells (A, arrowheads, compared to B, dark
field). Note also the absence of dye in a non-contacting
unlabeled cell (A, large arrow). X360.

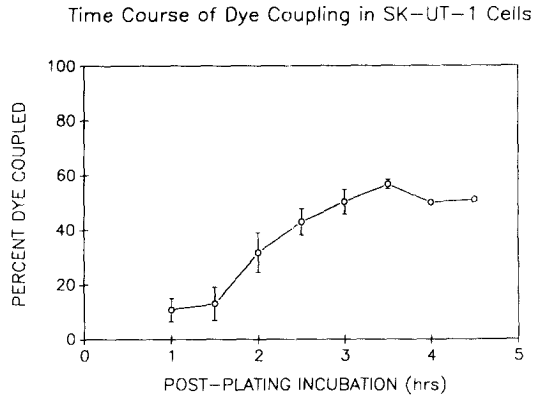Time Course of Radiolabel Coupling in SK–UT–1 Cells
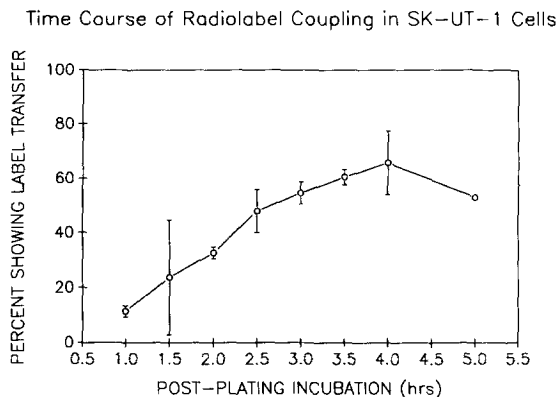


**Fig. 3:**   Time Course of Dye-Coupling in SK-UT-1 Cells.
The frequency of dye-coupling between SK-UT-1 cells
increased in a time-dependent manner until about 3.5 h
post-plating. Donor cells were labeled with latex beads
and DCCFDA, then co-cultured with unlabeled cells. At
various times after plating the donor cells, cultures
were examined for evidence of dye-coupling. The data are
compiled from five experiments, and represent the means
± SE from two to six cultures at each point except for
the 4.5 h point, which represents a single culture.

of blocked transcription (Raghow, 1987). These experi-
ments demonstrated that dye-coupling was established in
this assay independent of active transcription.

        Translational Inhibition. The importance of trans-
lation was evaluated in cultures treated with the pro-
tein synthesis inhibitor, cycloheximide (Obrig et al.,
1971).   As expected, cycloheximide suppressed protein
synthesis as measured by the incorporation of $^3$H-leucine
into acid insoluble material (Table II).   At the lowest
concentration, 0.1 uM cycloheximide, incorporation of
acid-insoluble $^3$H-leucine was reduced to 27% of controls
(p<0.05), with maximal reduction to 12% of controls at 1
mM (P<0.001). The reduction of protein synthesis pro-
duced by cycloheximide treatment was rapid, since inhi-
bition of $^3$H-leucine incorporation was comparable wheth-
er $^3$H-leucine was added at the time of donor cell plat-
ing or following 0.5, 1 or 2 h post-plating (data not
shown).
        As shown in Table II, cycloheximide did not signif-
icantly alter dye-coupling at concentrations up to 1 uM,
although considerable (73-81%) inhibition of $^3$H-leucine
incorporation was observed at these concentrations.

Time Course of Dye Coupling in SK—UT—1 Cells



**Fig. 4:** Time Course of Radiolabel Coupling in SK-UT-1 Cells. The frequency of radiolabel transfer between SK-UT-1 cells increased in a time-dependent manner until about 4 h. Donor cells were labeled with latex beads and $^3$H-uridine as described in Methods, then co-cultured with unlabeled cells. At various times after plating the donor cells, cultures were fixed and processed for autoradiography. The data are compiled from three experiments, and represent the means $\pm$ SE from two to three cultures at each point except for the 5 h point, which represents a single culture.

However, dye-coupling was inhibited in a concentration-dependent manner at higher concentrations of 10-1000 uM cycloheximide despite the fact that further inhibition of total protein synthesis was minimal at these higher concentrations, being reduced from 81% at 1 uM to 89% at 1 mM. Transcriptional effects of cycloheximide were also evaluated by measuring incorporation of $^3$H-uridine into acid-insoluble material. In these experiments it was observed that treatment with cycloheximide reduced $^3$H-uridine incorporation in a concentration-dependent manner over the range tested, 1 to 100 uM (p<0.05; Table II). No cycloheximide effects on cell attachment were noted by measures of DNA content (data not shown).

**TABLE I.** THE EFFECT OF ACTINOMYCIN-D
ON RNA SYNTHESIS AND DYE-COUPLING[a]

| Actinomycin-D (uM) | Acid-Insoluble ^{3}H-Uridine (dpm/ug DNA) | | Dye-Coupling (% Control) |
|---|---|---|---|
| 0 | 37,030±2915[b] | (100%) | 100[c] |
| 0.05 | n.d.[d] | | 109 |
| 0.1 | 6,943±229 | (19%)[e] | 103 |
| 0.5 | 3,088±107 | (8%)[e] | 98 |
| 1 | 1,799±92 | (5%)[e] | 83 |
| 5 | 362±40 | (1%)[e] | 92 |
| 10 | 209±15 | (1%)[e] | 80 |

[a] Incorporation of ^{3}H-uridine into acid-insoluble material and dye-coupling were performed in parallel cultures as described in Materials and Methods, 3.5 h after co-culture of donors and recipients.

[b] Each value is the mean ± SE (% control) of triplicate samples taken from each of four cultures.

[c] Each value represents 100 donor-recipient cell pairs from single cultures except for controls which were done in duplicate. The unadjusted mean ± SE of dye-coupling in the controls was 37 ± 9%. Data from one experiment are shown, although similar experiments verified the results.

[d] n.d., not determined.

[e] Significantly less than control and all other exposures ($p < 0.001$).

DISCUSSION

    This dye-coupling assay provides a relatively rapid, simple, and inexpensive method to investigate gap junction-mediated intercellular communication. Comparisons with similarly-modelled autoradiographic experiments demonstrated that this assay is of comparable sensitivity, avoids the use of potentially hazardous radiochemicals, and is significantly more rapid. Likewise, there are several advantages to the use of this assay as compared to dye-coupling assays that use microinjection of fluorescent dyes. Since DCCFDA is fluorescent only after metabolic cleavage of the ester side-chains, it provides a measure of viability of the dye-donor cells (Rotman and Papermaster, 1966). Similarly,

**Table II.** THE EFFECT OF CYCLOHEXIMIDE ON
PROTEIN SYNTHESIS, RNA SYNTHESIS AND DYE-COUPLING[a]

| Cyclo-heximide (uM) | Acid-Insoluble ^{3}H-Leucine (dpm/ug DNA) | Acid-Insoluble ^{3}H-Uridine (dpm/ug DNA) | Dye-Coupling (% Control) |
|---|---|---|---|
| 0 | $11,992 \pm 2,159$[b] (100%) | $32,492 \pm 1,912$[b] (100%) | 100[c] |
| 0.1 | $3,254 \pm 387$[d] (27%) | n.d.[e] | 113 |
| 0.5 | $2,813 \pm 964$[d] (24%) | n.d. | 89 |
| 1 | $2,282 \pm 263$[f] (19%) | $28,085 \pm 1,820$[d] (86%) | 94 |
| 10 | $1,762 \pm 165$[g] (15%) | $23,158 \pm 1,414$[g] (71%) | 60[g] |
| 100 | $2,092 \pm 511$[g] (17%) | $18,266 \pm 56$[g] (56%) | 50[g] |
| 1000 | $1,433 \pm 29$[g] (12%) | n.d. | 31[g] |

[a] Incorporation of ^{3}H-leucine into acid-insoluble material and dye-coupling were performed in parallel cultures and incorporation of ^{3}H-uridine was performed in a separate experiment; measurements were made 3.5 h after co-culture of donor and recipient cells.

[b] Each value is the mean $\pm$ SE (% Control) of triplicate samples from each of two or three cultures.

[c] Each value represents 100 donor-recipient cell pairs from single cultures except for controls which were done in duplicate. The unadjusted mean $\pm$ SE of dye-coupling in the controls was $62 \pm 1\%$. Data from one experiment are shown; similar experiments verified the results.

[d] Significantly less than control ($p < 0.05$).

[e] n.d., not determined.

[f] Significantly less than control ($p < 0.01$).

[g] Significantly less than control ($p < 0.001$).

because a relatively early event after cell death is the loss of membrane integrity with consequent loss of low-molecular weight materials (Patterson, 1979), overt toxicity can be readily observed by the presence of bead-labeled cells that lack fluorescent dye. Other advantages include the ability to perform the assay without requiring special skills or equipment, beyond basic tissue culture techniques and an inverted fluorescence microscope.

Since the donor cells must attach to the substratum, spread out, recognize adjacent recipient cells, and form communicating junctions before successful dye-coupling can be observed, the ultimate expression of dye-coupling is dependent on the events that occur in the early establishment of junctional communication (Burger and Misevic, 1985). Our experiments with inhibitors of transcription (actinomycin-D) and translation (cycloheximide) investigated the importance of protein synthesis in these early events.

Actinomycin-D inhibited transcription by more than 98% but did not significantly alter dye-coupling, providing clear evidence that active transcription is not required for cells to establish dye-coupling under the conditions of this assay. Dye-coupling was not inhibited by cycloheximide treatments of 1 uM or lower, despite considerable suppression of protein synthesis at these concentrations. While higher concentrations of cycloheximide did reduce dye-coupling, this effect occurred with relatively slight further reductions in protein synthesis. These data suggest that the reduced dye-coupling observed at higher concentrations of cycloheximide may be due to some effect of cycloheximide other than inhibition of protein synthesis. Although cycloheximide partially inhibited transcription in addition to its effects on translation, the absence of a significant effect on dye-coupling by actinomycin-D suggests that transcriptional inhibition did not contribute to cycloheximide's effect on dye-coupling.

The data from these experiments suggest that the development of junctional communication in this assay may depend on pre-existing protein. This mechanism is supported by reports that some cells in vitro can form gap junctions within minutes of contact (Johnson et al., 1974; Epstein et al., 1977; Dahl et al., 1980; Campbell and Albertini, 1981; Kachar and Reese, 1985), in some cases unaffected by treatments with protein synthesis inhibitors (Epstein et al., 1977, Cox et al., 1976). In experiments with mouse embryos and frog larvae, gap junction development was inhibited by treatment with transcription and translation inhibitors, but only if the treatments began early enough in development (Decker, 1976; McLachlin et al., 1983; Levy et al., 1986;

McLachlin and Kidder, 1986).  Finally, the observation
that cytoplasts are able to communicate effectively many
hours after cell enucleation provides support that
transcription and/or translation of proteins is not
required for establishment of junctional communication
in all cases (Cox et al., 1976; Bols and Ringertz,
1979).

In summary, we have developed a rapid, simple and
sensitive dye-coupling assay which should prove useful
as a method to detect changes in junction-mediated
intercellular communication.  The data from the present
experiments suggest that neither active transcription
nor translation is absolutely necessary for the estab-
lishment of dye-coupling in this system.  This assay has
been successfully adapted to other cell types in our
laboratory (Loch-Caruso et al., 1989; Loch-Caruso,
1990).

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