Published in final edited form as:

Curr Protoc Toxicol. 2011 February ; CHAPTER: Unit2.18. doi:10.1002/0471140856.tx0218s47.

Role of integrative signaling through gap junctions in toxicology

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Measurement of Gap Junctional Intercellular Communication

Due to the central role of intercellular signaling through gap junctions play in coordinating signaling events controlling gene expression makes it an ideal biological endpoint to monitor the toxic effects of environmental agents, toxins and potential health hazards of pharmaceuticals. Although there are multiple intracellular signaling mechanisms controlling gap junctional pore sizes, the end-result of any of these regulatory mechanisms is simply that the channels will either be fully opened, partially closed, or fully closed. This fundamental nature of gap junction channels has allowed for the development of several bioassays that capitalizes on measuring the transfer of detectable molecules through multiple layers of cells allowing for simple measurements of gap junction function in intercellular communication.

Gap junctions are plaque-like protein structures that form contiguous channels between cells allowing for the passive diffusion of low molecular weight metabolites and second messengers between the molecular weight of 1 –1200 Da (Evans, W. H. *et al.* 2002). Each channel is made up of two connexons, each residing in separate contiguous cells (Evans, W. H. and Martin, P. E. 2002). The connexon is made up of six subunits that are termed connexins (Evans, W. H. and Martin, P. E. 2002). To date, the connexin gene family comprises 20 members in the mouse and 21 members in the human genome (Sohl, G. *et al.* 2004); (Sohl, G. *et al.* 2003). There are two commonly used nomenclature systems to classify this family of proteins. The first to be adopted was the connexin (Cx) system based on the predicted molecular weight (Sohl, G. and Willecke, K. 2004); (Sohl, G. and Willecke, K. 2003), such that Cx43 denotes connexins with a predicted molecular weight of 43 kDa. The other uses the Gja/Gjb nomenclature that is currently adopted by the NCBI data base (Sohl, G. and Willecke, K. 2004); (Sohl, G. and Willecke, K. 2003), but this system does contain some inconsistencies, thus we will be using the Cx terminology in this chapter.

The most frequently used techniques to measure GJIC involve the transfer of a low molecular weight fluorescent dye (< 1000Da) between contiguous cells, which are often modeled after the original report (El-Fouly, M. H. *et al.* 1987). These techniques are popular due to their simplicity, availability of equipment found in most biology programs, quick analyses and conducive to semi-high throughput. Thus, this unit will describe some basic protocols for measuring GJIC using fluorescent based dye transfer techniques and points to consider for adaptations to individual experimental needs.

Strategic Planning

The basic technique described in this chapter is versatile in that it is amendable to many cell types, but like all protocols the selection of cells and experimental designs will depend on the biological questions that are being addressed and will heavily influence the interpretations of the results. For example, in determining the tumorigenic potential of toxicants, one would not want to use highly differentiated cell types, i.e. primary cultures of cardiomyocytes, hepatocytes, etc..., but rather stem cells or progenitor cells that can give rise to neoplastic growth. If the emphasis is more on the initiating step of cancer, stem cells that lack the expression of gap junction genes should be selected. These experiments would be designed such that suspected initiators would be added prior the addition of the cell differentiating factor to determine if these suspected initiators would prevent the expression of gap junction genes and GJIC, a necessary step in differentiation of cells in a tissue. In contrast, determination of tumor promoting effects would require progenitor cells that already express the gap junction genes. The gap junction genes that are typically expressed in epithelial cell types are Cx43. Note that 90% of all cancers are epithelial derived carcinomas. Thus, a Cx43 epithelial cell model system would be a quite appropriate screening system for tumor promoters that block gap junction function or any other compound suspected in epithelial based growth and development diseases.

When designing experiments to determine the effect of toxicants on gap junction functions in metabolism or physiologic responses, then the selection of appropriate differentiated cell types is desired. For example, determining the effect of GJIC in toxicant-induced drug metabolism in the liver would require assessment of GJIC in primary cultures of hepatocytes. Another example would be determining the effect of toxicants on GJIC dependent cardiac function would require the primary culture of cardiac myocytes. Another critical consideration in the design of GJIC experiments is at what phase does one assess GJIC in cell culture. Typically GJIC will be done after cells have reached confluency and are no longer actively dividing. However, many cell types have abundant platelet derived growth factor (PDGF) receptors, thus cells grown in serum supplements growth medium, which contains high levels of PDGF, will need to be switched to serum free medium for several hours to allow the development of functional GJIC.

The Scrape-Load Dye Transfer Technique

This protocol has been adapted after the method of El-Fouly (El-Fouly, M. H., Trosko, J. E., and Chang, C. C. 1987). The specific protocol presented is for an epithelial cell line with oval cell characteristics derived from the livers of Fischer 344 rats (F344-WB). However, this protocol can be adapted to many cell lines with minimal to no modifications. This assay has been extensively used to determine changes in GJIC in cells that have been treated with many kinds of toxicants such as tumor promoters, endocrine disruptors, pesticides, etc.; and in normal cells transformed with transfected oncogenes, and for chemopreventive agents that either prevented dysregulation of GJIC by tumor promoters or restored GJIC in cancer cells or normal cells transformed with transfected oncogenes.

Materials

- Monolayer of cells (i.e. F344-WB from from Drs. J. W. Grisham and M. S. Tsao University of North Carolina-Chapel Hill-NC cultured in D-medium (Formula No. 78-5470EF, GIBCO Laboratories, Grand Island, NY) on 35 mm tissue culture plates (Corning Inc., Corning, NY), supplemented with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, NY), and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air.)

- phosphate buffered saline (PBS) buffer: 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4 , 1.47 mM KH_2PO_4
- or if cells tend to lift then use PBS with calcium and magnesium prepared by adding to PBS: 0.68 mM CaCl_2 , 0.49 mM MgCl_2 .
- 1.0 mg/mL Lucifer Yellow (LY)
- 1.0 mg/mL LY/1.0 mg/mL rhodamine–dextran (RD)

Note: Use this solution if there is a need to identify which cells are initially loaded with the dye. RD is a large dye that does not pass through gap junctions, while LY does pass through gap junctions.

- surgical blade (#20 is recommended from Becton Dickinson Acute Care, Franklin Lake, NJ) or ultrafine micro-knife with a curved edge (Cat#10317-14, Fine Science Tools Inc. Foster City, CA) or acupuncture needle (size 0.25×0.33 mm [Jap gauge 5], Lhasa OMS, Inc., Weymouth, MA)
- 10% formalin solution ($\approx 4\%$ formaldehyde in PBS)
- epifluorescent phase contrast microscope or confocal microscope
- camera or CCD camera

General Procedure

1. Grow cells to confluency in 35 mm diameter or 48 well or 96 well cell culture plates. We have used culture plates from all major distributors with no observable differences in GJIC.
2. The cells of interest are removed from the incubator and the medium discarded by gently pouring off the solution into a waste container. (* See note below)
3. Then the cells are gently rinsed 3x with PBS using a pipette. The PBS is also discarded by gently pouring off the solution into a waste container. This and the subsequent steps are usually done at room temperature. We typically use a 25 ml pipette, which is sufficient for several 35 mm diameter culture plates or one 48 or 96 multi-well culture plate.
4. Add the LY dye solution that is sufficient to cover the cells.
5. Load the dye by gently rolling the surgical steel blade (#20 surgical blade and #4 handle; Bard-Parker, Franklin Lakes, NJ) over its rounded edge as indicated in Figure 2.18.1a., but do not slice through the monolayer as indicated in Figure 2.18.1b (** See note below).
6. Allow the LY dye to transfer, undisturbed, through several adjacent cell layers via gap junctions. For F344-WB cells, this is for 3 min. 3–5 min is usually sufficient time for dye transfer for most cell types.
7. Decant the dye and then gently rinse 3x to remove all extracellular dye. This is an important step considering that residual extracellular dye can result in background fluorescence.
8. Fix the cells by adding the 10% formalin solution that is sufficient to cover the cells. This can be done with a pipette. This step is optional and can be skipped for

*If cells were treated with a hazardous chemical, the culture medium must be properly discarded as hazardous waste according to the policy and procedures of the home institution.

any real time imaging, but the dye will continue to distribute through the cell layers and become overly diffuse to observe. The fixed cells can be air dried overnight and stored in the dark. Simply rehydrate the cells with the enough formalin solution to cover the monolayer of cells when needed for redetection of the dye spread through the cells. I have stored cells up to three years with no detectable decrease in dye intensity. Wrapping the plates in aluminum foil is the simplest way to store in the dark.

9. View the cells using an epifluorescent phase contrast microscope. You should see a spread of LY dye through several layers of cells in the control. If rhodamine–dextran was also added, this dye should remain in the cells that were originally loaded. Data capture of the fluorescent images can be done with a 35 mm camera or preferably with a CCD camera. Images at 200x usually give an excellent view of the dye spread that does not go beyond the microscopes field of view, thus offering the best option for quantification. If the dye goes beyond the field of view, then step down the magnification to 100x. Typical fluorescent images of the dye-spread in cells loaded with a scalpel blade, ultrafine micro-knife and acupuncture needle is shown in Figure 2.18.2)

Quantitation of GJIC

Quantifying the spread of dye can easily be done using very inexpensive low technology approaches, albeit more time consuming, or higher technology approaches using data imaging software systems, albeit more expensive. For a low cost approach, a traditional 35 mm camera (traditional or digital) can be attached to the camera port on the microscope and photographs of the fluorescent images can be taken. Then a ruler can be used to measure the distance the dye traveled from the line or point of loading in the photomicrographs. Typically for lines of dye loading by a scalpel or micro-knife, measuring the distance the dye travelled from dye front to dye front every 1 cm over a 5–10 cm length for 200X images (at 200x, 1 cm is equivalent to 50 μ m) gives an accurate representation of the data. Overlaying a transparency with a ruled edge over the dye loaded line marked at every 1 cm makes it easier to measure the distance of the dye front perpendicular to this dye loaded line. However, for very irregular dye fronts, more measurements might be needed, i.e. every 0.5 cm. The rate of dye migration can be computed by simply dividing the averaged distance the dye travelled by the time allowed for dye transfer in step-6. In measuring the dye spread in cells loaded with a point source, the averaged diameter of the resultant circle can be measured with a ruler through four evenly spaced measured diameters (8 arcs). Again, overlaying a transparency with ruled marks makes this easier. If the circle is very irregular more measurements might be needed. As before, the rate of dye migration can be computed by

****** This works quite well for culture plates that are 35-mm diameter or greater. However, this blade is too large for the smaller-diameter wells, so we use an ultrafine micro-knife or Integrative Signaling Through Gap Junctions 2.18.4 Supplement 47 Current Protocols in Toxicology Figure 2.18.1 The scrape-loading procedure. acupuncture needle to gently prick the cells. Typically, three to four areas are randomly selected, although the outer edges are omitted due to distortions at the edges of culture plates that do not allow good cell growth. This scrape load step is done at the lab bench without the use of a microscope; however, if there are any specific areas of special interest that were observed during microscopic examination, then use a marker to indicate where the scrape line is to be placed, or do the scrape or prick under the microscope.

This is a critical step if high quality images are to be obtained with fairly clean lines/points of loading and dye fronts. Although this technique is routinely referred to as scrape loading, the actual scraping/slicing of cells result in a large separation or empty hole between the cells that were loaded (Figure 2.18.1b) making quantification (to be described below) more variable and difficult, and resulting in more dead cells at the scrape. By gently rolling the blade (Figure 2.18.1A) or pricking the cells with a sharp point you get no separation of cells, probably because the membranes were not significantly compromised. A sharp blade or point is important in that it sticks to the plastic and prevents the blade or needle tip from sliding around and separating the cells similar to that seen in Figure 2.18.1b. Presumably, the cutting edge is simply traversing the very fluid plasma membrane without significantly compromising its physical-chemical properties and carrying dye along with it, of which a sufficient quantity is trapped inside of the cell when the instrument is retracted. Cells are not like balloons that will pop. Although this requires some practice, I have had inexperienced researchers from high school that easily mastered this technique, and is a routinely used by our undergraduate trainees.

dividing the averaged diameter by the time of dye transfer in step-6. This data is entered into a spread sheet program such as Excel for all further computations.

Although inexpensive and a low cost method, we effectively used this approach for many years, however these measurements are tedious and significantly more time consuming than images acquired and digitized by the charge-coupled device (CCD) cameras, which are used in most research labs and are now becoming more common in teaching labs. Typical images acquired by a CCD camera can be seen in Figure 2.18.2 and Figure 2.18.3, which were specifically obtained using a Nikon epifluorescence microscope equipped with a Nikon Cool Snap EZ CCD camera (Nikon Inc., Nikon, Japan), and the images were digitally acquired using a Nikon NIS-Elements F2.2 imaging system. The area of the dye spread can be easily quantified using the numerous software packages on the market that can compute the areas of fluorescence. We have extensively used the 'Gel Expert' image analysis program (NucleoTech Corp, San Mateo, CA); however this specific software is no longer commercially available, and we are now using IMAGEJ, a free public domain imaging software package from the National Institute of Health (<http://rsbweb.nih.gov/ij/>).

The most common way of presenting dose and time response data is as a fraction of the control, which accounts for most variability that occurs from experiments independently conducted on different days, passage number and by different individuals, while the actual effects relative to the control are usually minimally variable. This is a very simple computation as shown in Eq. 1. For a more accurate measurement of a percent (%) inhibition of GJIC, we load the cells with both LY and RD and then compute % inhibition using Eq. 2.

$$\frac{Ae}{Av} = FOC \quad \text{Eq. 1}$$

$$\frac{(Ae - Ar)}{(Av - Ar)} \times 100 = \% \text{inhibition} \quad \text{Eq. 2}$$

Ae = area of the dye spread in cells exposed to an experimental variable, such as cells treated with a chemical at a specific dose or time

Av = area of the dye spread in the control, which is typically cells treated with the vehicle used to dissolve the chemical of interest;

note: if there is a vehicle effect then this data must also be presented

Ar = area of the dye spread in the RD acquired images

note: LY has a yellow fluorescent emission, and RD has a red fluorescent emission

Common Experimental Designs

- I. *Dose response*: Conduct these experiments in two phases. The first phase is highly iterative that uses only one plate per dose to expedite a very rough estimate of the dose response points of inflection. Typically these experiments are done at the higher soluble and noncytotoxic dose at 15 and 30 min incubation times (most compounds inhibit within 30 min, but if no inhibition is observed then longer times are used, up to 24 h). If a compound inhibits GJIC, then the doses are halved until no inhibition is observed. Then the second phase is initiated by selecting a more refined range of doses based on phase-1 results. At least two, preferably three

plates, are used for each selected dose, which is then independently repeated at least two more times. The results from the three independent dose-response experiments can then be averaged and statistically assessed and graphed. The actual steps are outlined in the following:

1. Rinse the cells 3x with PBS and then add fresh growth medium.
2. Add the compound or vehicle to be tested directly to the culture medium.

This is done by tipping the culture plate and then using a micropipette; deliver the desired volume directed more towards the wall of the dish followed by an immediate swirl of the medium for mixing. This technique prevents a localized group of cells from getting an instantaneous burst of chemical and solvent during the pipetting process.

3. Incubate for the designated time and dose.
4. Complete these experiments by following steps 2 through 9 as described under “*General Procedure*”.

Figure 2.18.3 are scrape load images that is a typical response of F344-WB cells treated with a tumor promoter, such as 12-O-tetradecanoyl phorbol 13-acetate (TPA).

- II. *Time response*: Also, conduct these experiments in two phases. The first phase is again highly iterative and uses only one plate per time to expedite a very rough estimate of the inflection points of the time response. These experiments are done at a dose where 90 to 100% inhibition of GJIC occurs as determined by the dose response experiments. Then the second phase is initiated by selecting a more refined range of times based on phase-1 results. Again, at least two, preferably three plates, are used for each selected time, which is then independently repeated at least two more times. The results from the three independent time-response experiments can then be averaged and statistically assessed and graphed. The actual steps are the same as outlined in “I. Dose response”.
- III. *Time recovery*: This is routinely done in screening for tumor promoters. The tumor promoting phase of cancer is a reversible process and any toxicant that contributes to this phase should also have a reversible affect on GJIC. Cells are first treated with the toxicant at a time and dose that induces 90–100% inhibition of GJIC. Then the medium containing the toxicant is poured off and the cells are rinsed 3X with PBS and then incubated with fresh, toxicant free medium, for various times (typically 5min to 24 h). These experiments can then be completed as outlined in “I. Dose response”, and again at least two, preferably three plates, are used for each selected time of recovery, which is then independently repeated at least two more times. The results from the three independent time-recovery response experiments can then be averaged and statistically assessed and graphed.
- IV. *GJIC regulatory mechanisms*

There are two primary mechanisms known by which endogenous and exogenous compounds dysregulate GJIC, one is dependent on the activation of mitogen-activated protein kinase kinase (MEK1/2) which can be activated by either protein kinase C-dependent pathways or by extracellular receptor kinase pathways. MEK1/2, like all kinases phosphorylates specific proteins, such as extracellular receptor kinases. The second is dependent on phosphatidyl choline specific phospholipase C (PC-PLC). PC-PLC is an enzyme that specifically hydrolyzes a phosphocholine from diacylglycerol phospholipid.

Additional Materials (also see Basic Protocol 1 and Support Protocol 1)

GJIC-dysregulating chemical, e.g.: 1-methylanthracene, 1-methylfluorene, benzoylperoxide, 18- β -glycyrrhetic acid, dicumylperoxide, fluoranthene, fluorene, pentachlorophenol, perfluorodecanoic acid, perfluorooctane sulfonic acid, phenanthrene, pyrene, 12-O-tetradecanoylphorbol-13-acetate (Sigma-Aldrich), 1,9-dimethylanthracene, 1-methylpyrene, perfluorooctanoic acid, 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) (Fluka), alachlor, lindane (Chem Service, <http://web1.chemservice.com/>), 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (DDT; Supelco)

1. Rinse the cells 3x with PBS and then add fresh growth medium.
2. Pre-incubate the cells with either the MEK1/2 inhibitor, U0126 (20 μ M, 30 min), or the PC-PLC inhibitor, D609 (50 μ M, 20 min).
3. After the designated pre-incubation time with either the MEK1/2 or PC-PLC inhibitor, add the GJIC dysregulating chemical. The time and dose of the GJIC-dysregulator is previously determined by the dose and time response experiments described above where 90 to 100% inhibition of GJIC occurs. Note that the MEK1/2 or PC-PLC inhibitor remains in the medium during this time period.
4. Complete these experiments as outlined in "I. Dose response".

Toxicants that dysregulate GJIC through a PC-PLC-dependent mechanism will result in D609 preventing the dysregulation of GJIC by the toxicant. Similarly, U0126 will prevent the dysregulation of GJIC by a toxicant that works through a MEK1/2-dependent mechanism. A MEK1 inhibitor that we have found to be equally as effective as U0126 is PD98059. Figure 2.18.4 is an example of what the fluorescent images would look like for these types of experiments. Please note that these are not the only possible mechanisms involved and similar experiments can be designed for other intracellular signaling proteins.

In Vivo Application

The scrape load-dye transfer technique as been adapted for *in vivo* analyses of tumor promoting and chemopreventive compounds (Sai, K. *et al.* 2000);(Upham, B. L. *et al.* 2009). The basic protocol is very similar to the *in vitro* protocol. All animal experimentation must first be approved by the home institutional animal care and use committee (IACUC). Below is a specific protocol for the incision loading/dye transfer assay for liver tissue: **NOTE:** All protocols that use live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study.

Materials

Liver of F344 rat treated with tumor-promoting (see Support Protocol 3) or chemopreventive compound (e.g., GJIC up-regulators: resveratrol, β -carotene, epigallocatechin gallate, retinoic acid, β -sitosterol; Sigma-Aldrich) (procedure can be adapted for other mammals) 1.0 mg/ml Lucifer Yellow (LY; see recipe) 10% (v/v) formalin ($\approx$ 4% v/v formaldehyde; see recipe) Plastic weigh plate Gauze Epifluorescent phase-contrast microscope Additional reagents and equipment for paraffin embedding (Zeller, 1989) and data analysis (Basic Protocol 1).

1. Remove a part of the left lobe of the liver and put on a plastic weigh plate covered with wet gauze.

2. Put a few drops of the LY-RD dye mixture onto the surface of the liver tissue.
Note: this is the same dye mixture used in the *in vitro* assays described above.
3. Make three to four incisions on the surface of each specimen with a sharp blade, and add an excess of dye-mixture into the incisions.
4. Incubate for the 3 min at room temperature.
5. After incubation, the tissue is washed with PBS three times and fixed in 10% phosphate buffered formalin overnight.
6. Slices are then quickly washed with water, and then processed using routine paraffin embedding techniques.
7. Sections (5 μ m) for GJIC-analysis are prepared by cutting the paraffin block perpendicular to the incision line.
8. As described before, the dye spread through the cells can be viewed using an epifluorescent phase contrast microscope and the data analyzed the same as for the *in vitro* technique. Figure 2.18.5 are examples of incision load-dye transfer images from the livers of Fischer 344 rats treated with either the vehicle (dimethylsulfoxide) or the tumor promoter, perfluorooctanoic acid. The detailed experiment for the treatment of these animals are in (Upham, B. L., Park, J. S., Babica, P., Sovadinova, I., Rummel, A. M., Trosko, J. E., Hirose, A., Hasegawa, A., Kanno, J., and Sai, K. 2009).

Reagents and Solutions

Note: Use Milli-Q-purified water or equivalent for the preparation of all stock solutions.

Sterile PBS

To 5 L of milli-Q water add: 80 g of NaCl, 2 g KCl, 11.5 g of Na₂HPO₄, 2 g of KH₂PO₄. Dissolve each ingredient before adding the next. After all compounds are in solution bring to a final volume of 10 L using milli-Q water. We usually dispense this solution into 500 mL autoclavable bottles with caps. The caps are loosely put on the bottles and autoclaved for 50 min/slow exhaust. After being autoclaved, the lids are tightened and the bottles indefinitely stored.

Sterile Mg-Ca-PBS

Prepare PBS according to the directions above except slowly add 1.5 g CaCl₂·6H₂O, dissolve and then add 1 g MgCl₂·6H₂O, dissolve and then continue as described above. Note: calcium chloride must be added before the magnesium chloride to avoid irreversible precipitation of the salts, and this should not be sterilized by autoclave but rather filter sterilization through a sterile 0.22 μ filter.

Formalin Solution

A 10 % phosphate buffered formalin solution ($\approx$ 4% formaldehyde) is prepared from a Baker Analyzed A.C.S. grade 100% stock solution in a chemical fume hood. Safety goggles and gloves should also be worn in the preparation of this solution. Simply dilute the stock solution 1:10 in PBS. Typically 100 ml (90 mL PBS + 10 mL stock) is sufficient for three months of experiments, which is the approximate shelf life of this solution.

LY Solution

Add 1.0 mg of Lucifer Yellow CH (LY, molecular weight of 457.25)-dilithium salt (Molecular Probes, Eugene, OR) per ml of PBS. If there is a need to which cells were

initially loaded with the dye, also add 1.0 mg rhodamine–dextran (RD, molecular weight of 10,000) (Molecular Probes, Eugene, OR) per ml of PBS. We typically make up 15 mL of the dye solutions and will reuse the dye for 10–15 experiments.

Notes on Stock Solutions of Chemicals

Water soluble compounds are dissolved in PBS. Compounds not soluble in water are usually dissolved in 100% acetonitrile. Concentrations of stock solutions usually range from 5 to 30 mM, depending upon the solubility of the compound in acetonitrile and the final dose needed. The volumes of the stock solutions are added directly to the culture medium in each plate/well. Vehicle controls are added to the cells at a volume of acetonitrile equivalent to the volume of chemical stock solution used in the treatment. Acetonitrile is used because it is non-cytotoxic and non-inhibitory up to 2% (v/v) (Upham, B. L. *et al.* 1994). For very hydrophobic compounds, such as polychlorinated biphenyls, we use dimethylsulfoxide to dissolve the compound. We find that DMSO is sometimes inhibitory above 1% (v/v). We often use stock concentrations of either 20 mM or 2 mM because we typically use 35 mm diameter cell culture plates containing 2 mL of medium, thus making the $C_1V_1 = C_2V_2$ calculation simple, such that a desired final concentration, for example 20 μ M, will require the addition of 2 μ L of a 20 mM stock and 20 μ L of a 2 mM stock each into the 2 mL medium in the plate, respectively. This approach can be used for other final volumes. Particularly for research labs with undergraduate trainees, selecting a stock concentration that makes the $C_1V_1 = C_2V_2$ calculation that can be mentally done for a desired final volume much easier for the students.

D609 and U0126 Stock Solutions

For a 2 mL stock solution of 20 mM D609 (tricyclodecan-9-xanthogenate) add 10.7 mg of D609 to 2 mL of PBS. For a 2 mL stock solution of 20 mM U0126, add 15.2 mg of U0126 to 2 mL of acetonitrile.

Commentary

Background

In vitro bioassays have become invaluable in greatly expanding our basic knowledge of cell functions and have become an essential component in toxicological assessments of environmental and food born contaminants as well as in safety evaluations of new pharmaceuticals. There is no single *in vitro* bioassay system that can comprehensively meet these demands, which has resulted in the adoption of many different *in vitro* bioassay systems. Many of these *in vitro* assay systems were developed based on key signaling pathways known to be involved in specific pathologies. Although there a finite number of signal transduction pathways involved in the control of gene expression, these finite numbers are extensive with new pathways constantly being discovered making the task of choosing which signaling pathway specific *in vitro* assay system quite complex. However, many, if not most, signal transduction pathways are further modulated by cooperative intercellular signaling systems through gap junctions. Although these intercellular signaling systems are also numerous and multiple pathways control the closure and opening of the gap junction function, the final outcome is simply an intercellular channel system that is either opened, partially closed or completely closed, which are endpoints that can be easily measured using various *in vitro* bioassay systems.

The homeostasis of a tissue depends on intercellular signaling between cells. Transient closure of cells is a natural response to mitogenic stimuli, but autoregulatory pathways will reestablish intercellular communication. However, the chronic closure of gap junction channels is a strong indicator of pathological conditions. Thus, bioassays that assess GJIC

are an excellent starting point to evaluate the toxic potential of compounds. As noted above, the cell systems used will greatly influence the interpretation of results. The F344-WB, Cx43 model system we typically use is appropriate for screening tumor promoters.

The dysregulation of GJIC results in uncontrolled cellular growth in a tissue, which typically originates with the stem and progenitor cells, that can lead to the development of tumors (Trosko, J. E. *et al.* 2002);(Yamasaki, H. *et al.* 1999). There are several lines of evidence that support the hypothesis that inhibited GJIC activity is related to carcinogenesis (Trosko, J. E. *et al.* 2004);(Trosko, J. E. *et al.* 1993);(Trosko, J. E. 2003);(Yamasaki, H. *et al.* 1993);(Yamasaki, H. 1996);(Yamasaki, H., Krutovskikh, V., Mesnil, M., Tanaka, T., Zaidan, D. M., and Omori, Y. 1999). Most cancer cells have defective gap junctional intercellular communication (GJIC) (Yamasaki, H. *et al.* 1996), and tumor promoters and oncogenes inhibit GJIC, while tumor suppressor genes and chemopreventative compounds enhance GJIC (Trosko, J. E. and Ruch, R. J. 2002);(King, R. E. *et al.* 2005). A QSAR model that scanned through a large population of molecules and a wide variety endpoints demonstrated that inhibition of GJIC is strongly linked to carcinogenic process in rodents, uncontrolled cell proliferation and differentiation, embryonic lethality or teratogenesis (Rosenkranz, H. S. *et al.* 2000);(Rosenkranz, M. *et al.* 1997). Gap junction genes transfected into cancer cells restore their normal growth regulation and morphology (Trosko, J. E. and Ruch, R. J. 2002). Connexin 32 knock out mice (Cx32, is a gap junction protein of the liver) administered with a single dose of the tumor initiator, diethylnitrosamine, two weeks after the birth of the mice had 3.3 to 12.8 times increase of preneoplastic foci as compared to the wild-type mice, while mice not treated with diethylnitrosamine resulted in no significant increase in preneoplastic lesions indicating that the deletion of Cx32 promoted the carcinogenic effects of diethylnitrosamine (Evert, M. *et al.* 2002), and these knockout mice also exhibit increased levels of radiation and chemical-induced liver and lung tumor formation (King, T. J. *et al.* 2005);(King, T. J. *et al.* 2004b);(King, T. J. *et al.* 2004a);(King, T. J., Gurley, K. E., Prunty, J., Shin, J. L., Kemp, C. J., and Lampe, P. D. 2005);(King, T. J. and Lampe, P. D. 2004b). Tumor induction utilizing X-ray radiation resulted in a statistically significant increase in overall tumor burden in Cx32-deficient mice compared with wild-type mice due to tumorigenesis in several tissues such as the lung, liver, adrenal, lymph and small intestine (King, T. J. and Lampe, P. D. 2004a). These results collectively indicates the role of gap junctions in cancer.

Altered GJIC has also been implicated teratogenesis (Trosko, J. E. *et al.* 1982), reproductive dysfunction (Gilula, N. B. *et al.* 1976);(Larsen, W. J. *et al.* 1986);(Ye, Y. X. *et al.* 1990), alteration of muscle contractions in heart and uterus (Cole and Garfield, 1986; DeMello, 1982) and neuropathy (Bergoffen, J. *et al.* 1993). Obviously, the interruption of GJIC disrupts the homeostasis of the organism leading to various chronic diseases, depending on the cell- and tissue-type where communication is altered.

Techniques have been developed to measure intercellular communication that includes assays based on metabolic cooperation, electrical coupling and fluorescent dye transfer between cells. The first standardized bioassay for GJIC was the metabolic cooperation assay, first developed by Yotti *et al.* (Yotti, L. P. *et al.* 1979). This bioassay was based on metabolic cooperation between Wild-type Chinese hamster V79 cells (6-thioguanine-sensitive) and 6-thioguanine-resistant cells that were co-cultured at high densities. The 6-thioguanine (6-TG) resistant cells do not metabolize 6-TG to a toxic metabolite, thus conferring resistance; however co-culture with the 6-TG sensitive cells results in the transfer of the toxic metabolite through gap junctions resulting in cell death. Inhibition of GJIC results in the rescue of 6-TG resistant cells by preventing the transfer of the toxic 6-TG metabolite from the 6-TG sensitive cells. The advantage of this assay is that it is minimally invasive, while a major disadvantage is that it requires one week for completion, which

makes extensive dose, time dependent assessments of toxicants and mechanistic studies, etc. impractical.

Electrical coupling of cells through gap junctions play vital roles in the physiological functions of cells that depend on coordinating electrical potentials between cells. These measurements are done using dual patch voltage clamp protocols to measure electrical conductance across gap junctions (Spray, D. C. *et al.*; (Spray, D. C. *et al.* 1981); (Spray, D. C. *et al.* 1984), and are commonly used by electrophysiologists. However, these sophisticated techniques require significant technical training. Although these techniques play a vital role in electrophysiological research, they are not routinely done in cell biology and toxicology labs, and are not always applicable to screening for epigenetic events related to growth and development diseases that stem from less differentiated cell types.

The most common and versatile means by which GJIC is measured are fluorescent dye transfer techniques. The most frequently used technique is the scrape load-dye transfer technique, which was described in this chapter unit. This technique is a simple procedure that does not require sophisticated and extensive training by cell biologists. As mentioned before, this technique has been easily mastered by our undergraduate and high school level research trainees. Although simple, this technique can be incorporated into a wide range of experiments that address questions on gap junction function and its role in cell development, toxicology and human diseases. The simplicity and short time (≈ 5 –10 min) needed to assay GJIC also makes this procedure amenable to semi-high-throughput analyses. However, this procedure is more invasive than the metabolic cooperation assay.

Incorporation of a fluorescent dye into a cell by a sharp or pointed edge is not the only means to introduce the dye. The fluorescent recovery after photobleaching (FRAP) assay (Wade, M. H. *et al.* 1986) has also been frequently used in many labs. This assay preloads all cells with a fluorescent dye by incubating the cells with a methylester of a fluorescent dye, such as 6-carboxyfluorescein, and as the dye incorporates into the plasmamembrane, methyl esterases on the cytosolic side will hydrolyze the ester resulting in a water soluble version of the dye trapped inside of the cells. Microscopes equipped with laser technologies that can focus the beam onto single cells can be used to photobleach these cells, followed by post-scans to assess the rate of reuptake of the fluorescent dye from neighboring cells. This technique has been described in detail by (Trosko, J. E. *et al.* 2000); (Wade, M. H., Trosko, J. E., and Schindler, M. 1986). The great advantage of this technique is that specific cell types, i.e. dividing vs. nondividing cell, can be selected for cell specific studies. Also, the GJIC assay can be coupled with many other cell cytometric fluorescent based methods studying cell specific functions, such as changes in calcium and pH. Although considered by many as a more quantitative method than the SL-DT assay, I have compared the two techniques and find that they are comparable. A major disadvantage of this assay is the very significant expense in the purchase of these laser equipped microscopes, which are typically confocal. Another disadvantage is that it is much slower. Typically I can conduct approximately 20 assays using 35 mm diameter culture plates in an 8 h day using FRAP, but I can conduct 200 assays using SL-with 35 mm diameter culture plates in this same time period. The FRAP technique cannot be speeded up using 48 and 96 multi-well culture plates because the time committed to selecting cells, bleaching and post scans would be independent of the number of plates, but this is not true for the SL-DT technique where multiple cutting edge tools can be designed to load multiple wells in the 48 and 96 well culture plates, thus offering a potential to do more than 200 assays per day. Like the SL-DT assay, this procedure is also invasive in that the lasers will generate reactive oxygen species (ROS). To minimize ROS production, one has to balance laser strength and efficient photobleaching of the cells.

More recently another technique has been developed; the local activation of molecular fluorescent probe (LAMP) method (Dakin, K. *et al.* 2005), which is based upon a new generation of caged coumarin-like fluorophores. Similar to the FRAP assay, all cells are preloaded with dyes containing a methyl ester, however, these dyes become fluorescent upon subsequent local illumination with a small dose of ultraviolet light, which probably produces significantly lower ROS than FRAP. This technique has been modified to examine GJIC in 3 dimensions (Yang, S. *et al.* 2009);(Dakin, K. *et al.* 2006), which will potentially become a valued technique as *in vitro* cell biology and toxicology will become increasingly dependent on three dimensional *in vitro* culture systems.

Another commonly used technique to load fluorescent dyes is microinjection. One major advantage of this technique is the ability to select a cell, which can also be done using FRAP, and then monitor which cells the dye transfers to, which cannot be done using FRAP. Thus, if the experimental questions involve determination of heterologous GJIC (communication between different cell types) *vs.* homologous GJIC (communication between same cell types), then microinjection is the desired technique. Disadvantages for this technique are that it requires the expense of a microinjection apparatus, a higher level of skill and experience to use this apparatus, highly invasive (often 50% of the cells are killed in the process), and requires longer times (similar to FRAP) to measure GJIC. As we have been experimenting with needles, we have found that acupuncture needles are very effective in loading cells with LY dye and these could potentially be used along with a microscope to identify cells of interest and load the dye.

The least invasive bioassays of GJIC are the preloading and parachute techniques. These techniques require preloading cells with methyl ester- fluorescent dyes. In the preloading technique, the loaded cells are suspended together with unloaded counterparts and are then plated and allowed to form a confluent monolayer. The spread of the dye can then be observed with an epifluorescence microscope (Goldberg, G. S. *et al.* 1995). In the parachute assay, one group of cells are loaded in suspension and then added to a monolayer of unloaded cells, and again the spread of the dye is observed with an epifluorescence microscope (Ziambaras, K. *et al.* 1998). However, these techniques are technically more challenging for higher throughput analyses, are more tricky from a cell culture perspective in that the cells must be immediately plated at near confluency and requires a time lag for the reattachment of cells in the preload method and attachment and connection with nonloaded cells in the parachute assay. However, these techniques look promising and probably will be used more in the future to validate the commonly used assays that are more invasive, such as the SL-DT technique.

Critical Parameters

As noted above, one critical parameter is selecting the appropriate cell type for the hypotheses to be addressed. For example, one would not select a cancer cell line to screen for tumor promoters, considering that cancer cell lines either have no GJIC or greatly reduced GJIC. Instead, a normal, noncancerous cell line exhibiting GJIC must be selected. Also, the quality of the cells that are selected is important, which requires good laboratory practices in tissue culture. The LY solution must be warmed to 37°C before use on the cells. Before we start our experiments, the cells are checked for health by looking for typically expected cell morphology, and then at least two plates are selected for a SL-DT assay, one is an untreated and the other is a vehicle treated plate to be sure that they exhibit functional GJIC. Often we will also treat one plate with a well established inhibitor of GJIC to be sure the cells are appropriately responding to a known GJIC-dysregulator. If any of these measured parameters fail, then trouble shooting is needed before one proceeds with the GJIC experiments. Also, experiments must be conducted at noncytotoxic doses of the chemical to be tested. Therefore, cytotoxicity experiments should be conducted prior to the

GJIC experiments. The simplest is to treat cells with increasing doses of the chemical and observe the effects of the chemical on cell morphology. We also use the neutral red uptake, trypan blue exclusion and mitochondrial reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to test for cytotoxic doses.

The selection of culture plate size is not critical for assessing GJIC, however, if other parameters are to be measured such as identifying specific proteins, DNA or RNA then larger plates will be needed. We find the 35 mm diameter plates produce sufficient amount of cells when we need to use Western, Southern and Northern blots. If we are to extract proteins, DNA or RNA from the same plate that an SL-DT assay was done, we will skip the formalin fixation step. For general screening of compounds, dose and time response experiments the smaller sized culture plates are sufficient. However, to assess the greatest variability between cells within a well/treatment, assessing GJIC along several long cuts by a scalpel in the larger 35 mm diameter plate would be desirable.

Troubleshooting

The most frequent problem affiliated with the SL-DT assay conducted by investigators new to the technique is background fluorescence. The most common contribution to background fluorescence is an insufficient rinse of the extracellular dye after it has been removed from the cells. Thus, improved rinsing techniques will prevent this problem. Another common problem is that cells are treated with a cytotoxic dose of the chemical sometimes resulting in increased background fluorescence and more commonly an uptake of dye in cells that were not loaded with a sharp edge indicating compromised cell membranes. The obvious solution to this problem is to decrease the dose of the toxicant to non-cytotoxic levels. Another cytotoxic response of cells to a chemical is the detachment of the cells from the plate during the rinse step, and again one needs to decrease the dose of the chemical. Some cell types do not adhere strongly to the plastic and we found that rinsing in Ca/Mg-PBS prevents this problem. However, Ca/Mg-PBS is not needed for the F344-WB cells. Another cause for background fluorescence results from cells that have been confluent for more than a day, in which they will begin laying down significant extracellular matrices that will bind to the LY. However, we have not found this to be a problem when quantifying the data because this background staining appear as clear thread like structures that are very distinct from the dye fronts needed for measurement of GJIC.

Another common problem with those beginning GJIC bioassays with the SL-DT method is a spreading of the cells at the point of dye loading. This is most commonly caused by blunt edges and shaky hands. Practice and sharp edges or points is the best solution to this problem. I believe that the major feature of a sharp edge or point is not that it is best for the cells but rather it bites into the plastic, thereby preventing the edge from slipping and creating large gap between the loaded cells. Dim images are another common problem that is almost always due to the LY solution, especially if it is recycled. Fresh LY solutions will usually alleviate this problem. Particularly with high school and undergraduate research trainees, blurry images can occur. Training on the proper use of phase contrast epifluorescence microscopes, alignment of the phase contrast rings, clean objectives, etc... and practice in focusing images is important for minimizing this problem.

The biggest problem that can occur is that the untreated cells are not communicating. If the cells being used are known to exhibit GJIC, then a new batch of cells will need to be grown. Problems with the CO₂ level, temperature and humidity in the growth chambers occur then these can ultimately influence the outcome on GJIC. Again, good laboratory practices (GLP) in basic tissue culture practices include daily log sheets tracking these parameters. Also, mycoplasma can unknowingly contaminate cell lines and should be a factor to be considered in lack of GJIC, and periodically checking for mycoplasma contamination is also part of

GLP in tissue culture. Another GLP for tissue culture is that any new batch of medium and PBS must first be tested of cell lines to be used before distributing to the lab. A bad batch of medium or PBS could certainly affect GJIC.

For new cells that have not been previously tested for GJIC, detection of GJIC can be difficult if the growth conditions are not yet optimized. One frequent problem is that many cells have abundant PDGF receptors, thus conducting GJIC experiments with cells grown in medium supplemented with fetal bovine serum (FBS) poses a problem due to the high levels of PDGF in FBS inhibiting GJIC even after they have reached confluency and contact inhibition. Usually transferring the cells to FBS free medium for 2–4 hours will overcome this problem. Due to the unnatural environment that occurs in two-dimensional cell culture, some cell types may not establish GJIC in the traditional medium and plastic. Experimenting with various substrates, i.e. collagen coatings, and microenvironments, will be necessary to find the proper conditions for establishing functional GJIC. Actually, functioning GJIC should be a criterion in establishing any new cell line that exhibits GJIC *in vivo*. Although passage number (the number of times cells have been trypsinized and recultured) has little to no affect on GJIC in F344-WB cells, passage number does play a more significant role in functioning GJIC in other cell types and must be assessed and implemented in the design of GJIC experiments.

Ultimately, new tissue culture techniques will need to be developed in the future for establishing more natural, multicellular three dimensional (3D) culture systems along with new GJIC assay systems in 3D. Establishing 3D culture systems and assays of GJIC in 3D is one of our current focuses in our lab. However, for the time being, two dimension cell culture systems will still serve as a valuable tool in cell biology and toxicology research.

Anticipated Results

This is a straight forward technique that results in a spread of dye through several layers of cells. One should expect a bright image of a minimum spread of LY dye of three to four cell layers and sometimes up to ten cell layers. Assessment of GJIC with only three cell layers will lead to higher variability as a function of the dependent variables; therefore it is preferable to use cells that communicate through at least five cell layers to minimize variability. Typically the variability for untreated cells and cells exhibiting 100% inhibition is less than 5% (note: we typically get higher variability with the acupuncture needle of 7–10%), but much higher variability can be seen at the inflection points of dose and time response curves. Sometimes accurate determination of dose or time needed for 10, 50 and 90 % inhibition might require more than three replicates. Also, variability within one cell culture plate or one well of a multiwell plate should be 5% or less. For F344-WB cells, three minutes is sufficient time for a reasonable dye spread, but other cell types may require five to ten minutes for sufficient dye spread.

Time Considerations

Most SL-DT assays require a 3 min dye transfer time, resulting in a total time of the assay of 5–6 min. Usually for new trainees, doing three 35 mm diameter plates is very manageable. The time required to make the cuts are usually less than 10 seconds per three plates. For the more experienced, 10 plates are quite manageable. For multiwell plates, it is advisable to construct a multiple blade or acupuncture pin holder to do multiple wells at one time and again, approximately 5–10 minutes would be required for an SL-DT of one multiwell culture plate. If different time periods for the different wells of one multiwell plate are planned, then using a Pasteur pipette hooked to a vacuum system, such as a vacuum pump or aspirator, will be the best way to remove solutions quickly from the wells. The vacuum system design must have a collection flask, and again the collection of any hazardous waste

must be properly disposed according to the procedures and policies of the home institution. Another approach in the use of the multiwell plate is to plan the experiments such that they all end at the time SL-DT needs to be assayed, such that every well is decanted and rinsed at the same time.

Although these assays are simple and relatively quick, efficient time management of complex experiments is still important. For example, time dependent experiments requiring longer times will require initiating the longer experiments in the morning and the shorter timed experiments later in the day such that they all can be terminated around the same time for the SL-DT assays. If you estimate that you can do all the SL-DT assays in one hour; then be sure the termination of the timed experiments will end in 10 minute intervals for each group of plates to be assayed over the one hour designated for the GJIC assays.

Acknowledgments

Financial support from NIEHS grants #R01 ES013268-01A2 and its contents are solely the responsibility of the author and do not necessarily represent the official views of the NIEHS.

Abbreviations

GJIC	gap junctional intercellular communication
SL-DT	scrape load-dye transfer
IL-DT	incision load-dye transfer
FRAP	fluorescent recovery after photobleaching
PC-PLC	phosphatidyl choline specific phospholipase C
MAPK	mitogen activated protein kinase
ERK	extracellular receptor kinase
MEK1/2	MAPK/ERK kinase1/2
ROS	reactive oxygen species

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Figure 2.18.1.
The scrape-loading procedure.

Figure 2.18.2.

SL-DT fluorescent images at 200X of F344-WB cells loaded with the LY dye using a scalpel blade, micro-knife edge and acupuncture needle.

Figure 2.18.3.

SL-DT images at 200X of a dose response of F344-WB cells to the model tumor promoter, TPA. The first five panels are fluorescent images and the last panel is a phase contrast image. The phase contrast image indicates that 6 μg/L TPA did not alter the health of the cells.

Figure 2.18.4.

Mechanistic determinations. SL-DT fluorescent images were at 200X of F344-WB cells treated with 1-methylantracene (1-MeA, 70 μ M, 15 min), or pretreated with the phosphatidyl choline specific inhibitor, D609 (50 μ M, 20 min) followed by the addition of 1-MeA (70 μ M, 15 min), or epidermal growth factor (EGF, 5 ng/mL, 30 min), or pretreated with the Mek1/2 inhibitor, U0126 (20 μ M, 30 min) followed by the addition of EGF (5 ng/mL, 30 min). The vehicle was acetonitrile (0.35% v/v).

Figure 2.18.5.

Ex vivo scrape load dye transfer of Lucifer Yellow in liver slices from F-344 rats fed with either, the vehicle or perfluorooctanoic acid (PFOA, a tumor promoting peroxisome proliferator). A single intraperitoneal (i.p.) administration of either the vehicle, DMSO, or 100 mg/kg PFOA was administered to the rat for 24 h. The rats were euthanized and the liver removed for the IL-DT. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institutes of Health Sciences of Japan to assure that the rats were treated humanely and with regard for alleviation of suffering. Further information on these experiments were reported in (Upham, B. L., Park, J. S., Babica, P., Sovadinova, I., Rummel, A. M., Trosko, J. E., Hirose, A., Hasegawa, A., Kanno, J., and Sai, K. 2009).