

Scrape Loading/Dye Transfer Assay

Pavel Babica, Iva Sovadinová, and Brad L. Upham

Abstract

The scrape loading/dye transfer (SL/DT) technique is a simple functional assay for the simultaneous assessment of gap junctional intercellular communication (GJIC) in a large population of cells. The equipment needs are minimal and are typically met in standard cell biology labs, and SL/DT is the simplest and quickest of all the assays that measure GJIC. This assay has also been adapted for *in vivo* studies. The SL/DT assay is also conducive to a high-throughput setup with automated fluorescence microscopy imaging and analysis to elucidate more samples in shorter time, and hence can serve a broad range of *in vitro* pharmacological and toxicological needs.

Key words Dye coupling, Dye transfer, Ex vivo assessment, Gap junctional intercellular communication assessment, High throughput, In vitro assay, Incision loading, Lucifer Yellow, Scalpel loading, Scrape loading, Tracers

1 Introduction

Dye coupling methods are by far the most frequently used assay for the assessment of GJIC, mainly because of their ease of use. Of all the techniques used to measure GJIC, the scrape loading/dye transfer (SL/DT) assay is the fastest and simplest. Most protocols are modification of the one first reported by El-Fouly et al. [1]. This technique has since been widely used to elucidate the GJIC status of many cell types in various biological circumstances in different scientific areas such as carcinogenesis, embryogenesis, growth control, or endocrine disruption (for review, *see* [2–6]). This visual method allows to assess GJIC in a large population of cells. It is therefore particularly useful when a large screen of multiple conditions is required or when different regions of a cell monolayer have to be compared within the same culture dish [2]. The SL/DT assay can be effectively used as a tool to determine the qualitative and quantitative presence or absence of GJIC as well as demonstrate the concentration-dependent inhibition of GJIC [3, 7–9].

The SL/DT assay relies on the introduction of small (MW <900), nonpermeable dyes (for review, *see* [2, 10]) into living cells that are traced in their intercellular movement through gap junctions. As the reference dye, dilithium salt of Lucifer Yellow hydrazine (LY, MW 457, negatively charged) is the most popular dye currently in use. This tracer has a high fluorescence efficiency, which ensures its detection in minute levels [10]. LY is introduced by scraping a monolayer of cells and becomes incorporated by cells along the scrape, presumably as a result of some mechanical perturbation of the membrane (Fig. 1). As normal permeability is reestablished, the LY becomes trapped within the cytoplasm and move from the dye-loaded cells into adjacent ones connected by functional gap junctional channels [2]. This dye transfer is monitored and quantified by fluorescent microscopy in multiple cells almost simultaneously. The amount of dye transferred from one cell to its neighbor that it is in contact with is dependent on the number of gap

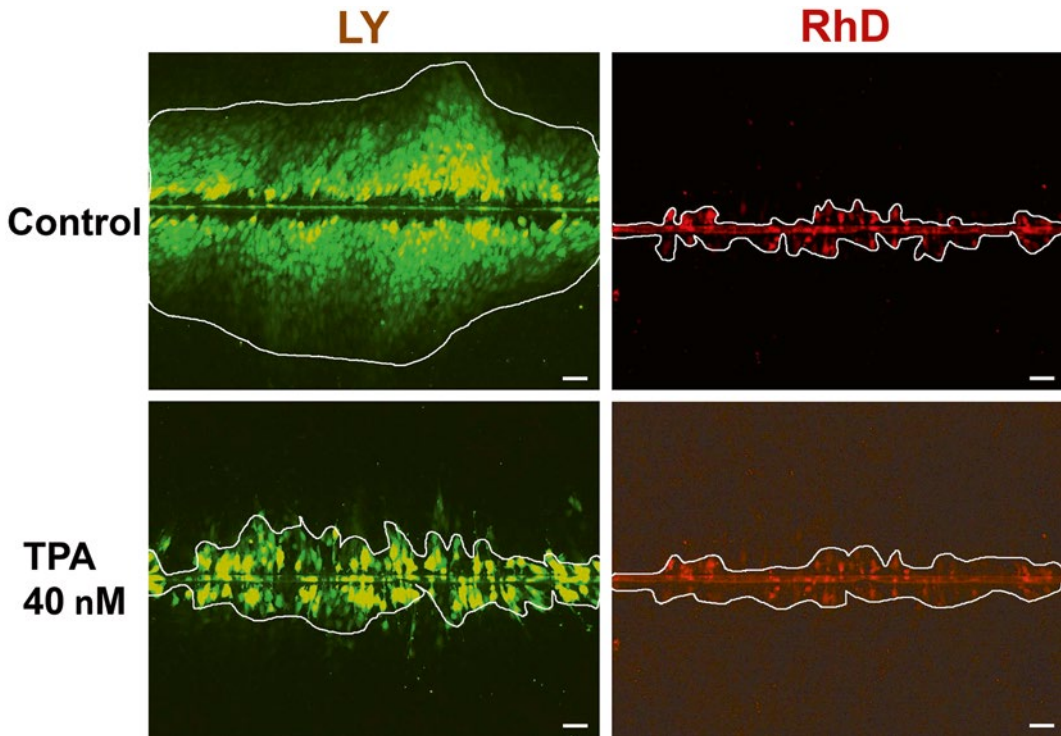


Fig. 1 Scrape load dye transfer analysis in mouse Sertoli cells. Images obtained by SL/DT assay, applying Lucifer Yellow CH dilithium salt (LY, MW 457), which transfers through functional gap junction channels, and rhodamine-dextran (RhD, MW 10,000), which is retained in the scraped cells. The GJIC function is evaluated by analyzing net transfer of LY (the area at which LY diffuses), excluding RhD-stained regions. GJIC after the 1-h exposure of mouse Sertoli TM4 cells to the model tumor promoter, TPA (12-O-tetradecanoylphorbol-13-acetate), at the concentration of 40 nM was reduced to FOC (the fraction of the control) = 0.13 when compared with the solvent control. Scale bar = 50 μ m

junctions that are coupled and the gating properties of individual channels. The distance or area at which the dye diffuses during a certain period away from the scrape line is a quantitative measurement of GJIC capacity. To determine which cells are initially loaded after the scrape, other fluorescent dyes (e.g., rhodamine-dextran, MW 10,000, or dialkylcarbocyanine) that are too large to traverse the gap junction channel are concurrently used with the diffusional dye to serve as an additional control (Fig. 1). These large fluorescent macromolecules that cannot diffuse across gap junctions are useful in ensuring that the intercellular transfer of the gap junction diffusible-reference dye is actually dependent on gap junctions and is not accounted for by alternative pathways, such as cytoplasmic membrane fusions, cytoplasmic bridge formation at the end of mitosis or due to membrane damage, which can occur after scraping [1, 11].

The SL/DT assay is an invasive technique but has been successfully demonstrated to assess compounds that disrupt GJIC. However, this assay is not conducive in studying GJIC in small cell populations, particularly between cell pairs, and also in cultures with low cell densities, or when the extent of junctional coupling is small, or when specific cells need to be observed [2]. In addition, the GJIC status of cell types of irregular shape is not easily quantified using this assay. For example, GJIC of neuronal cells or long spindly fibroblast cannot be easily quantified because the distance or area at which the dye diffuses cannot be easily “trackable” and quantified [11]. This approach is also not well suited to three-dimensional (3D) systems. The local activation of molecular fluorescent probe (LAMP) method has been recently improved (the so-called infrared-LAMP assay) and allows to examine cell-cell coupling in three dimensions [12, 13]. However, for the time being, two-dimensional cell culture systems still serve as a valuable tool in cell biology and toxicology research [5].

The major advantages of the SL/DT are as follows: (1) simplicity, (2) not a necessity of the special equipment or skills that are needed for other methods such as microinjection, (3) a rapid and simultaneous assessment of GJIC in a large number of cells, (4) conducive to a high-throughput setup with automated fluorescence microscopy imaging and analysis, and (5) its adaptation for in vivo studies followed by ex vivo assessment of GJIC in tissue slices from experimental animals [5]. An ex vivo GJIC assay, the incision loading/dye transfer method (IL/DT), is very similar to the in vitro protocol [14–16]. The IL/DT may be useful for rapidly screening tumorigenic compounds for setting doses for studies of carcinogenesis [14].

The basic technique described in this chapter has been adapted after the method of El-Fouly [1]. Rather than more invasive scrape with rubber policeman or wooden probe, the dye loading step in this protocol involves a clean cut with a sharp

blade, such as surgical scalpel. This modified technique can be thus called scalpel loading/dye transfer assay and is amendable to many cell types with minimal or no modifications. This assay has been extensively applied to determine changes in GJIC in a wide variety of mammalian (including human) cell types after treatment with many kind of toxicants such as tumor promoters, endocrine disruptors, pesticides, or developmental toxicants. Additionally, this assay has been successful in screening for compounds that can either prevent toxicant-induced disruption of GJIC or reverse the effects of these toxicants or endogenous oncogenes.

2 Materials

1. Cells of interest and appropriate media.
2. Cell culture plates, e.g., 35 mm dishes, or multiwell plates, e.g., 6/12/24/48/96 wells.
3. Phosphate buffered saline (PBS) buffer with calcium and magnesium (CaMg-PBS; *see Note 1*): 137 mM NaCl (8 g/L), 2.68 mM KCl (0.2 g/L), 8.10 mM Na₂HPO₄ (1.15 g/L), 1.47 mM KH₂PO₄ (0.2 g/L), 0.68 mM CaCl₂ (0.075 g/L), 0.49 mM MgCl₂ (0.047 g/L). The pH is adjusted to 7.2. The CaMg-PBS is filter sterilized and can be stored at room temperature.
4. LY-dye solution: 1 mg/mL Lucifer Yellow CH dilithium salt (LY, MW 457) (*see Note 2*) and 1 mg/mL rhodamine-dextran (RhD, MW 10,000, optional, *see Note 3*) in CaMg-PBS. The solution is filter sterilized and can be stored for weeks in the dark at 4 °C (*see Note 4*).
5. Surgical scalpel blade or micro-knife with a curved, flat, or needle blade as appropriate (Fig. 2) to fit into the cell culture plasticware used (*see Note 5*).
6. 10% Formalin solution (i.e., approximately 4% formaldehyde) in CaMg-PBS (*see Note 6*).
7. 25 mL pipettes with a pipette aid or manual bulb, automatic pipette 100–1000 µL or Pasteur pipette with a bulb, waste container, parafilm, aluminum foil.
8. Inverted epifluorescent microscope or confocal microscope with appropriate filters (LY: excitation at 428 nm, emission at 536 nm; RhD: excitation at 555 nm, emission at 580 nm).
9. Camera, CCD or CMOS camera coupled to the microscope, computer, image acquisition and analysis software.

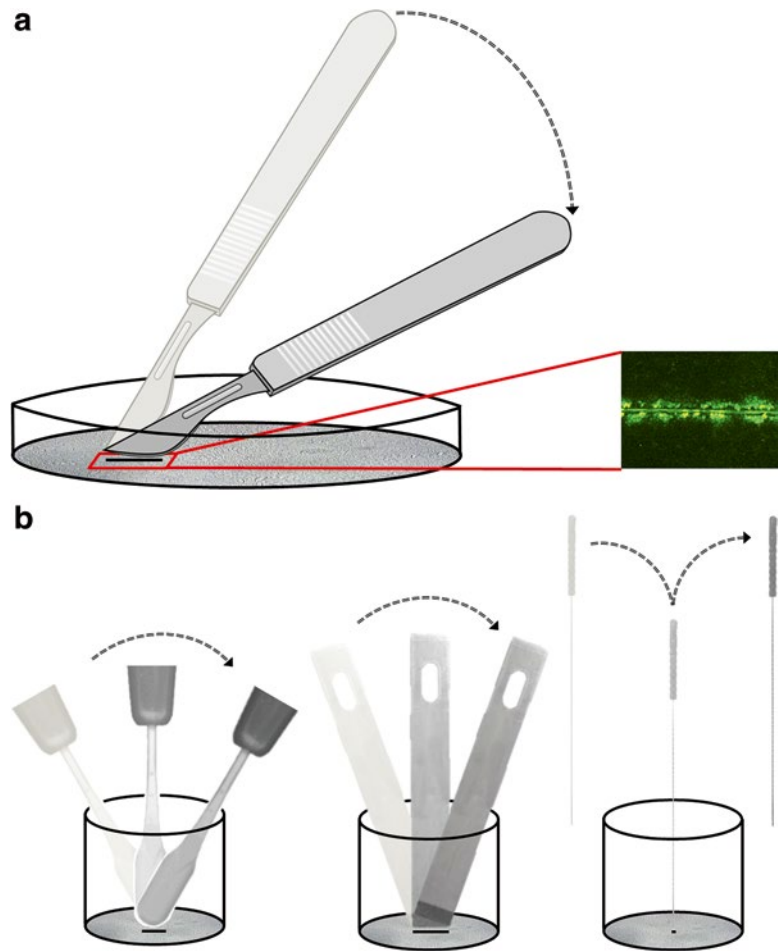


Fig. 2 *The scrape loading procedure. (a)* Scrape loading technique done by surgical scalpel with a curved blade in 35 mm dishes. *(b)* Different types of blades suitable for SL/DT assay in microplate wells, including a curved blade (*left*), a flat blade (*middle*), and an acupuncture needle (*right*)

3 Methods

3.1 General Procedure

1. Grow cells to confluency in suitable cultivation medium under appropriate conditions for the desired cell type (*see Note 7*).
2. Visually check the health of the cells for expected morphologies and sterile conditions prior to the start of the experiments.
3. Remove cells from the incubator and discard medium by gently pouring off the medium or by siphoning with a pipette into a waste container (*see Notes 8 and 9*).

4. Rinse cells gently three times with CaMg-PBS using a pipette (*see Note 10*) and discard the CaMg-PBS by gently pipetting off into a waste container.
5. Add sufficient LY-dye solution (warmed up to 37 °C) to cover the cell monolayer (*see Note 11*).
6. Load the dye into the cells by gently placing the tip of a surgical steel blade with a curved edge in contact with the cell monolayer and then rolling the blade in one direction over its curved edge as indicated in Fig. 2a (*see Note 12*). If using multiwell plates, use a micro-knife with a curved or flat blade, or an acupuncture needle (*see Note 5*) to gently prick the cells (Fig. 2b).
7. Typically, three cuts are done for each dish or multiwell plate well. The areas for cell loading are randomly selected in the central part of the plate/well (*see Note 13*).
8. Incubate the dish or multiwell plate, undisturbed and under minimum illumination for 3–6 min at room temperature to allow the LY dye to travel through several adjacent cell layers (3 and more) via functional gap junctions (*see Note 14*).
9. Cover the plate, to limit the exposure of the LY-dye solution to light during the incubation to avoid fluorescence photobleaching of the dye.
10. Aspirate the LY-dye solution from plate/wells (*see Note 15*) and then rinse cells three times with CaMg-PBS to remove all extracellular dye (*see Notes 10 and 16*).
11. Fix the cells by adding sufficient 10% formalin solution to cover the cells (*see Notes 11 and 17*).
12. The cells can be viewed immediately using an inverted epifluorescence microscope or confocal microscope with appropriate filters.
13. Acquire three representative LY/RhD images per plate/well (*see Note 18*).
14. A bright field or phase contrast image should be acquired for each field of view (*see Note 19*).
15. Fixed cells can be air-dried overnight, stored in the dark for extended periods (months with no detectable decrease in dye intensity), and rehydrated with formalin solution for viewing.
16. Store the plates sealed in parafilm and covered in aluminum foil.

3.2 Quantification of GJIC Using Morphometric Software

1. The degree of GJIC can be measured using a variety of methods (*see Note 20*) [17].
2. The fluorescent distance or the area of the dye spread can be quantified using a morphometric software package. We use ImageJ, a free public domain imaging software package from

the National Institute of Health (<http://imagej.nih.gov/ij/>), with a subroutine for determining fluorescence area of a fluorescent image.

3. To obtain a corrected fluorescence area value for LY and RhD, the fluorescent areas of digitized images (e.g., Fig. 1) are subtracted from background fluorescence obtained at an area of the monolayer well away from any scrape lines within the same test plate (*see Note 21*).
4. To calculate the net transfer of LY, the areas of LY and RhD fluorescence are subtracted from each other for each field of view (i.e., $\text{Area}^{\text{LY}} - \text{Area}^{\text{RhD}}$) (*see Note 22*).
5. The net LY areas of individual images can then be normalized to the averaged net area from negative or solvent control dishes to obtain the fraction of the control (GJIC-FOC)

$$\text{GJIC FOC}_{\text{Treatment}} = \frac{(\text{Area}_{\text{Treatment}}^{\text{LY}} - \text{Area}_{\text{Treatment}}^{\text{RhD}})}{(\text{Area}_{\text{Control(Averaged)}}^{\text{LY}} - \text{Area}_{\text{Control(Averaged)}}^{\text{RhD}})}$$

6. GJIC-FOC values of individual images can be then grouped by the individual dishes or treatment conditions for further data evaluation and statistical analyses to allow relative comparisons between the control and the treatments, for concentration and time response analyses, or for comparisons between independent experiments.

4 Notes

1. Calcium chloride must be added before the magnesium chloride to avoid irreversible precipitation of the salts. This buffered solution should not be sterilized by autoclave because of salt precipitation but should be rather filter sterilized through a sterile 0.22 μm filter. Calcium and magnesium cations are added to the PBS buffer to maintain cell adhesion and prevent a monolayer of cells from lifting, i.e., detaching from the bottom of culture dishes or plates. However, some cell types may be sensitive to this level of Ca^{2+} . This problem may be alleviated by preparing the LY-dye solution in CaMg-free PBS.
2. Some junction channels exclude anionic molecules like LY, for example, connexin 45 [2, 18, 19]. For these channels, smaller cationic dyes such as biotin conjugate (e.g., Neurobiotin [16, 20] or Biotin [21]) are recommended. To be detected, biotin conjugates should be visualized with either streptavidin coupled to a fluorochrome (cyanine dyes, fluorescein, or rhodamine) or to horseradish peroxidase. If the connexin channels of a particular cell type are unknown, then SL/DT using LY as the transfer dye is not enough to elucidate GJIC status in these cells.

3. Use LY-dye solution with RhD if there is a need to identify which cells are initially loaded with the dye. RhD is a large dye that does not pass through gap junctions, while LY does pass through gap junctions.
4. The LY-dye solution must be warmed to 37 °C before use on the cells.
5. Surgical scalpel with a curved blade is suitable for 35 mm dishes (Fig. 2a). Micro-knives and blade holders with curved blades, flat blades, or ultrafine needle blades (e.g., acupuncture needles) with dimensions fitting multiwell plate wells are suitable for SL/DT in setups allowing for higher throughput (Fig. 2b).
6. The formalin solution should be prepared in a chemical fume hood and safety goggles and gloves should be worn. Shelf-life of this solution is approximately 3 months.
7. The growth phase at which a SL/DT experiment is done is critical. Typically, cells which have reached confluency and are no longer actively dividing (“contact inhibition”) are the most suitable for the SL/DT assay. For new cells that have not been previously assessed for GJIC, the growth conditions, i.e., cell seeding density as well as culture time, must be optimized. In addition, the passage number must be noted during GJIC experiments because the passage number can play a significant role in functioning GJIC in a given cell type. Many cells have abundant PDGF (platelet-derived growth factor) receptors. PDGF inhibits GJIC in several cell lines [22], thus conducting GJIC experiments with cells grown in medium supplemented with fetal bovine serum (FBS) poses problem due to the high levels PDGF in FBS. Transferring the cells to FBS free for 2–4 h can overcome this problem. Due to the unnatural two-dimensional environments of the traditional in vitro assays, some cell types may not establish GJIC in the traditional medium or plastic. Some cell types need specific culture conditions to express functional GJIC such as extracellular matrix-coated plates (e.g., mammary CID-9 cell line [23]) or low or high calcium medium (e.g., mouse epidermal cell line [24]). Some cell types such as mouse testicular cell lines TM3 and TM4 can detach from the bottom of tissue culture plates during the washing steps. Growing cells on gelatin-coated plates can overcome this problem.
8. Culture medium containing hazardous waste must be properly disposed.
9. This and the subsequent steps are usually done on the lab bench at room temperature.
10. We typically use a 25 mL pipette, which is sufficient volume to rinse several 35 mm cell culture dishes or a 6- to 96-well multiwell plate.

11. We typically use about 1 mL of solution per 35 mm cell culture dish or per well of 6- or 12-well plate, 0.5 mL per well of 24-well plate, 0.25 mL per well of 48-well plate, 0.1 mL per well of 96-well plate.
12. The key principle of proper loading technique is to put the tip or apex of the blade to the bottom of the dish or multiwell plate well and then roll the blade over its cutting edge against the cell monolayer or to gently prick the monolayer with the tip of a thin acupuncture needle. This action is minimally invasive and provides a very clean line or spot of loading. Do not slice or scratch the monolayer, but only apply gentle pressure to minimize physical effects of this step. A sharp blade or point is important to prevent a large separation or empty hole between the cells that were loaded resulting in high variability.
13. This scrape loading step is done at the lab bench without use of a microscope. If you want to assess GJIC in any specific area of special interests that you found during microscopic examination, then use a marker to indicate where the scrape line needs to be placed, or use the microscope. The three cuts per dish or well should be aligned in parallel, and in the case of multiwell plates also geometrically parallel with the base of the well.
14. The optimal incubation time varies between different cell types, depending on the level of communication and attachment properties of the cells [8]. Incubation up to 10 min might be required for some cell cultures. For a set experiment duration, different rates of dye diffusion through homotypic channels is correlated to the number of gap junction channels [25]. If processing several dishes or multiwell plates in parallel, work in a timely manner to make sure that the incubation time after the dye loading step will be the same for all dishes or wells.
15. LY-dye solution can be collected and reused. We reuse the dye solution for approximately 10 experiments, when stored in dark and refrigerated. We filter the solution through 0.22 μm syringe filter, if needed.
16. The washing step is very important step because of the reduction of background fluorescence. Even in the absence of a scrape line, some LY can be incorporated into the cells as evidenced by nonspecific background fluorescence. It also binds to cell components after aldehyde fixation [10].
17. The fixation of cells is optional and can be skipped. The cells in CaMg-PBS or medium can also be observed without fixation, but the dye will continue to travel through the cell layers and become overly diffuse to observe.

18. The cells from experimental conditions where the highest and lowest level of GJIC are expected (e.g., negative and positive controls) should be used first to adjust or check the microscope and image acquisition settings. The plates should be positioned so the line or spot of dye-loaded cells will be in the center of the microscope field of vision, and the line also parallel with the horizontal line of image field. Camera exposure time and other image acquisition settings (e.g., excitation source intensity or fluorescence attenuator, camera binning, image brightness, contrast and gamma correction) should be adjusted in a way that LY- or RhD-stained cells can be clearly discriminated from the background, i.e., from the cells whose fluorescence intensity is comparable to all the other cells in regions distant from the cut. However, the background cells should not turn out completely black in the image (too dim images), since such condition might lead to underestimation of GJIC. The used combination of the objective magnification (typically 5–20× objective), digital camera (e.g., C-Mount adapter magnification, size of the imaging sensor), and other image acquisition settings should allow to fit within one image (one field of view) not only all LY-stained cells in the direction perpendicular to the cut, but also part of the background, so to assure most accurate quantification GJIC and to prevent underestimation of communication in the cells from the experimental conditions with the most intense GJIC.
19. Bright field or phase contrast images from the same field of view as LY/RhD fluorescence images can offer additional visual information on the cells, such as the effects of experimental treatments on cell morphology, growth, confluency, and attachment, and also provide additional information to discriminate between the reduction of LY-stained area due to inhibition of GJIC, as compared to reduced dye transfer as a function of subconfluency or cell detachment issues.
20. An alternative method of quantitating GJIC is by counting the rows of fluorescent cells from the scrape line. This method is useful and more appropriate when comparing populations of different cell type, size, and growth state [26].
21. The most frequent problem is the high intensity of background fluorescence, so the cells stained by LY due to the dye transfer cannot be discriminated from the cells in the background. The most common contributions to background fluorescence are as follows: (1) an insufficient rinse of the extracellular dye, (2) treating cells with cytotoxic concentrations of the chemical (an uptake of dye by cells that

were not scraped or near to scrape line indicating disrupted cell membranes or detachment of the cells from the plate during the rinse step), and (3) “overconfluent cells,” i.e., cells have been confluent for more than one day and have started to produce significant extracellular matrix (LY binds to extracellular matrix). Dim images are almost always problem related to the photobleaching of LY solution, especially if it is being recycled and reused. In the short term, this issue can be compensated for by increasing exposure time for fluorescence image acquisition. Fresh LY solutions will usually alleviate this problem.

22. A more simplified version of the SL/DT assay is to load cells with only LY and not the RhD. The results from these experiments typically give FOC values very similar to those that include RhD. However, when measuring the dye fronts, the loaded cells cannot be identified, and thus cannot be subtracted from the calculations resulting in values that are always above zero. This issue can be circumvented by introducing a positive control into the experimental design, i.e., treatment with a known inhibitor with GJIC (such as 12-O-tetradecanoyl-13-phosphol acetate), which will induce complete inhibition of GJIC. The net LY dye transfer can be then calculated by subtracting the average area of LY-stained cells in the positive control from LY-stained areas of all images. Adjusted areas of the experimental treatments can be then compared to the averaged adjusted area of the negative or solvent control:

$$\text{GJIC FOC}_{\text{Treatment}} = \frac{\left(\text{Area}_{\text{Treatment}}^{\text{LY}} - \text{Area}_{\text{Positive control (Averaged)}}^{\text{LY}} \right)}{\left(\text{Area}_{\text{Negative control (Averaged)}}^{\text{LY}} - \text{Area}_{\text{Positive control (Averaged)}}^{\text{LY}} \right)}$$

This approach is suitable only for in vitro models with well characterized GJIC, where complete inhibition of GJIC can be reproducibly induced and reliably recognized. However, this greatly simplifies the assay, particularly at the microscopy step where only one dye needs to be assessed.

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