**TITLE:**

An Iodide-Yellow Fluorescent Protein-Gap Junction‒Intercellular Communication Assay

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**KEYWORDS:**

Gap junction, gap junction intercellular communication, connexin, high-throughput screening, yellow fluorescent protein, iodide

**SUMMARY:**

Here, we present a protocol for a novel gap junction intercellular communication assay designed for the high-throughput screening of gap junction-modulating chemicals for drug discovery and toxicological assessment.

**ABSTRACT:**

Gap junctions (GJs) are cell membrane channels that allow diffusion of molecules smaller than 1 kDa between adjacent cells. As they have physiological and pathological roles, there is need of high-throughput screening (HTS) assays to identify GJ modulators in drug discovery and toxicology assays. A novel iodide-yellow fluorescent protein-gap junction-intercellular communication (I-YFP-GJIC) assay fulfills this need. It is a cell-based assay including acceptor and donor cells that are engineered to stably express a yellow fluorescent protein (YFP) variant, whose fluorescence is sensitively quenched by iodide, or SLC26A4, an iodide transporter, respectively. When iodide is added to a mixed culture of the two cell types, they enter the donor cells via the SLC26A4 transporter and diffuse to the adjacent acceptor cells via GJs where they quench the YFP fluorescence. YFP fluorescence is measured well by well in a kinetic mode. The YFP quenching rate reflects GJ activity. The assay is reliable and rapid enough to be used for HTS. The protocol for the I-YFP-GJIC assay using the LN215 cells, human glioma cells, is described.

**INTRODUCTION:**

Gap junctions (GJs) act as intercellular channels to allow the diffusion of small molecules of <1 kDa such as nutrients, metabolites, and signaling molecules between adjacent cells. The junctional elements include a hemichannel or connexon in each cell, and each connexon constitutes six connexins (Cxs)1. GJs and Cxs have been used in toxicology assays of carcinogens such as polycyclic aromatic hydrocarbons (PAH), which are GJ inhibitors2,3,4. Disrupted GJIC has been associated with nongenotoxic carcinogenesis5,6. As a potential therapeutic target, GJ involvement has been reported in particular subtypes of seizures7,8, protection from cardiac and brain ischemia/reperfusion injury9, migraine with aura10, drug-induced liver injury6,11, and wound healing12. High-throughput screening (HTS) assays are required to identify GJ-modulating chemicals or antibodies for drug discovery, for toxicology assays, and to identify novel cellular regulators of GJ activity. HTS assays can also be used to investigate structure-activity relationships of GJ modulators2,13,14,15.

Some GJIC assays include dye transfer or dual patch clamp techniques. Lucifer yellow CH (LY) and calcein acetoxymethyl ester (calcein-AM) have been used in dye-transfer assays. Cells are not permeable to LY, which is introduced by microinjection, scrape loading, or electroporation. Once inside the cell, LY spreads into neighboring cells *via* GJs and GJ activity is assayed by the extent of the LY migration16. Calcein-AM assays usually involve gap-fluorescence recovery after photobleaching17,18. Calcein-AM is a cell-permeant dye that is converted intracellularly into impermeable calcein by an intrinsic esterase. The assay requires a confocal microscope to observe the transfer of calcein-AM into a cell from those surrounding it following laser photobleaching. If functional GJs are present, calcein-AM in adjacent cells enters the photobleached cells and the fluorescence is recovered. GJ activity is assayed by the extent of fluorescence recovery of the photobleached cells. Dye-transfer assays are laborious and time consuming or have low sensitivities. Dual patch clamping is an electrophysiological method that measures junctional conductance. It is relatively sensitive, with a direct dependence of conductance on the number of open GJs19; however, it is technically demanding, time consuming, and expensive20. The I-YFP-GJIC assay was developed for use in HTS.

**Figure 1** illustrates the components and steps of the I-YFP GJIC assay, which utilizes acceptor cells expressing an iodide-sensitive YFP variant bearing H148Q and I152L (YFPQL) and donor cells expressing an iodide transporter (SLC26A4)21. The two mutations carried by YFPQL allow quenching of fluorescence by iodide22. Iodides are added to co-cultured acceptor and donor cells; they do not enter the acceptor cells, but are taken up by the SLC26A4 transporters present on the donor cells. Iodides in the donor cells diffuse through functioning GJs into adjacent acceptor cells where they quench the YFPQL fluorescence. If GJs are closed or blocked by inhibitors, iodide cannot enter the acceptor cells to quench the fluorescence. The YFPQL quenching rate reflects GJ activity. The I-YFP GJIC assay procedure is neither complicated nor time consuming. It is compatible with HTS and can be used to test the effects of a large number of compounds on GJ activity in a relatively short period. It requires only acceptor and donor cells, and two balanced salt solutions. The protocol described below is based on LN215 cells whose major Cx is Cx4321. The LN215-YFPQL receptor and LN215-I− donor cells were generated by transduction with lentiviruses expressing YFPQL or SLC26A421,23.

**PROTOCOL****:**

**1. Generation of lentiviruses expressing YFPQL and SLC26A4**

1.1. Grow HEK293T human embryonic kidney cells to 80% confluency on 100 mm culture plates. Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin is the culture medium used throughout the protocol to maintain HEK293T and other cells mentioned below.

1.2. Coat 6-well culture plates by adding 2 mL of 0.005% of sterile poly-L-lysine (PLL) solution to each well for 10 min. Aspirate the PLL solution and rinse the surface twice with 2 mL of sterile water.

1.3. Wash the HEK293T cells with 10 mL of phosphate buffered saline (PBS) and treat the cell monolayers in each 100 mm dish with 2 mL of 0.25% trypsin-EDTA solution at 37 °C for 3 min. Add 5 mL of culture medium and resuspend the cells.

1.4. Count the cells in a hemocytometer and adjust the density of the cell suspension to 250,000 cells/mL in culture medium and add 500,000 cells in 2 mL of culture medium to each lysine-coated well of 6-well plates. Incubate the cells in a humidified 5% CO2/95% air atmosphere at 37 °C for 24 h and then replace the culture medium with DMEM without penicillin or streptomycin.

1.5. In a 1.5 mL tube, dilute 20 µL of transfection reagent with 500 µL of DMEM without serum or antibiotics. Mix gently by pipetting and let stand at room temperature for 5 min.

1.6. Meanwhile, pipette 250 µL of DMEM into each of two 1.5 mL tubes and then add 1500 ng of pLVX-EIP-YFPQL or pLenti6P-SLC26A4, 1225 ng of psPAX2 and 375 ng of pMD2.G to each. The two lentiviral plasmids have been previously described21. Add 250 µL of diluted transfection reagent to each plasmid tube, mix gently and incubate for 20 min at room temperature.

1.7. After 20 min, add 500 µL of transfection reagent and plasmid complexes in the 1.5 mL tubes dropwise to each culture plate well in step 1.4 and mix by rocking the plate back and forth. Incubate cells at 37 °C in a CO2 incubator for 12 h.

1.8. Replace the medium with 2.5 mL of fresh medium and incubate for an additional 48 h. Then place the culture plate on ice for 5 min to keep the conditioned medium containing lentivirus chilled to maintain infectivity.

1.9. Harvest the media containing lentiviruses and transfer to 15 mL conical tubes. Centrifuge at 3,000 x *g* at 4 °C for 3 min and then remove floating HEK293T cells from the supernatant by filtration at 0.4 µm.

1.10. Store the media containing lentiviruses at 4 °C for use within 2 days. For later use, store 200 µL aliquots at −80 °C.

**2. Generation of LN215-YFPQL and LN215-I− cells by lentiviral transduction**

2.1. Grow LN215 cells in 100 mm culture plates to 80% confluency in DMEM supplemented with 10% fetal bovine serum (FBS) 100 U/mL penicillin, and 100 μg/mL streptomycin as described above.

NOTE: If the I-YFP-GJIC assay is conducted using a different cell line, use the appropriate culture medium. LN215-YFPQL, and LN215-I- cells can be provided by the University-Industry foundation, Yonsei University. Please contact the corresponding author.

2.2. One day before transduction, wash the cells twice with 10 mL of PBS, treat with 2 mL of 0.25% trypsin-EDTA at 37 °C for 3 min. Resuspend the cells in 5 mL of culture medium with a 10 mL serologic pipette and adjust the density to 50,000 cells/mL. Add 20,000 cells in 400 μL of media to each well of a 24-well culture plate for treatment as no virus control, YFPQL, and SLC26A4 cells.

2.3. After 24 h of incubation at 37 °C, transduce two wells by replacing the culture medium with 400 μL of a 1:1 mixture of pLVX-EIP-YFPQL or pLenti6P-SLC26A4 lentivirus and fresh culture medium supplemented with polybrene at a final concentration of 4 μg/mL. For no virus controls, replace with culture medium.

2.4. Incubate the cells at 37 °C for 15 h, aspirate the medium containing lentiviruses, add fresh culture medium, and incubate the cells for an additional 72 h.

CAUTION: To prevent contamination of lentivirus between wells, use new tips or pipets for each well when you aspirate culture medium containing lentivirus or dispense fresh growth medium.

2.5. Wash the cells in each well twice with 0.5 mL of PBS, treat with 300 μL of trypsin-EDTA for 3 min. Resuspend the cells in 2 mL of culture medium and plate in six-well culture plates with 2 μg/mL puromycin.

2.6. Culture the cells in media containing 2 μg/mL puromycin until all cells in the control well are dead (round-shaped or floating when observed in microscope), which usually takes a week. Refresh the culture media containing puromycin every other day during the selection period. If LN215-YFPQL or LN215-I- cultures become confluent before selection has completed, transfer the cells to 100 mm plate and continue selection as in step 2.5.

**3. Preparation of solutions required for the assay**

3.1. Prepare 500 mL of C-solution (10 mM HEPES, 140 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM MgCl2, and 1 mM CaCl2) and 500 mL of I-solution (10 mM HEPES, 140 mM NaI, 10 mM glucose, 5 mM KCl, and 1 mM CaCl2).

3.2. Adjust the pH of both solutions to 7.4 with 1 N NaOH, sterilize the solutions by filtrations at 0.4 µm for storage. Store at 4 °C for up to 1 month. Check the pH before using.

**4. Plating the LN215-YFPQL and LN215-I− cells**

4.1. Culture LN215-YFPQL and LN215-I− cells in 100 mm plates separately in culture medium to reach the populations required for assay. LN215-YFPQL and LN215-I− cells in 40% and 80% confluency in 100 mm plates, respectively, are sufficient for a 96-well plate assay.

4.2 One day before conducting the I-YFP GJIC assay, wash each 100 mm culture plate with 10 mL of PBS. Treat each plate with 2 mL of 0.25% trypsin-EDTA solution and incubate at 37 °C for 5 min. Resuspend the cells in each plate in 4 mL of culture medium and transfer to 15 mL conical tubes.

4.3. Pellet the cells by centrifugation at 1,000 x *g* for 3 min. Discard the supernatant and resuspend each cell pellet with 5 mL of culture medium. Break up any cell clumps into single cells by pipetting up and down about 20 times with a 10 mL serological pipette.

4.4. Count the cells in a hemocytometer, and dilute the cells in the culture medium to make cell suspensions of LN215-YFPQL at 80,000 cells/mL and LN215-I− at 160,000 cells/mL.

4.5. Mix 7 mL of LN215-YFPQL and 7 mL of LN215-I− cell suspensions in a reservoir. Add 100 µL of the mixture to each well of a 96-well cell culture plate using a multichannel pipette.

NOTE: To add 100 µL of the mixture in each well of 96-well cell plate, about 10 mL of mixed cell suspension is needed. It is recommended to make more cell suspension than needed.

4.6. Incubate the cells in humidified 5% CO2/95% air at 37 °C for 24 h. The LN215-YFPQL and LN215-I− cell culture should be 100% confluent when the assay is conducted.

**5. Conducting the I-YFP assay**

NOTE: Use a fluorescence microscope with 20x magnification, and a GFP filter to check the 96-well plates to be sure there are no clumps of LN215-YFPQL or LN215-SLC26A4 cells and that the cell cultures are fully confluent and well distributed before conducting the assay.

5.1. At least 30 min before doing the assay, turn on a microplate and set to 37 °C.

5.2. Wash the tubing of an automated injector with 3 mL of 70% ethanol, 3 mL of distilled water, and then 3 mL of I-solution at a flow rate of 135 µL/s.

5.3. Warm the C- and I-solutions to 37 °C in the water bath.

NOTE: As 100 µL of each solution is needed for each well of the 96-well plate, about 10 mL of each solution is needed for each assay. An additional 10 mL of the I-solution is needed for priming each plate (total of 20 mL) and an additional 25 mL of the C-solution is needed for washing each plate (total of 35 mL).

5.4. Aspirate the growth medium or invert the plate to empty it; tap out residual medium.

NOTE: Residual fetal bovine serum in the growth media causes background fluorescence and a decline in assay quality.

5.5. Add 200 μL of C-solution to each well from a reservoir using a multichannel pipette. Aspirate the C-solution or invert the plate to empty and tap out residual solution.

5.6. Add 50 µL μL C-solution, 1 µL of 2.5 mM chemical stock (see **Table 1**) or dimethylsulfoxide (DMSO) as a vehicle, and then 50 µL μL C-solution to each well with a multichannel pipette.

NOTE: Most reagents in a chemical library are dissolved in DMSO and up to 1% (v/v) is allowed in most cell-based assays24. As DMSO has a higher density than water, reagents dissolved in DMSO tend to go down to the bottom when added to the culture plate wells, which disturbs the concentrations of the assay solutions. This can be circumvented by adding 50 µL of C-solution, reagents in DMSO, and 50 µL C of -solution in order. The last 50 µL of C-solution is for mixing.

5.7. Incubate the cells at 37 °C in air, not in 5% CO2. The incubation time can be modified, but 10 min is usually sufficient for ion-channel modulators to act.

5.8. During incubation, set the microplate reader program to inject 100 μL of I-solution to each well at 1 s and to measure the fluorescence for 10 s at 0.4 s intervals. Set the reader to read fluorescence from the bottom. The recommended injection speed is 135 µL/s. Set the excitation wavelength to 485 nm and read the emission at 520 nm.

NOTE: Detailed settings for the microplate reader programs are as follows.

5.8.1. Click the **Manage protocols** button.

5.8.2. Select the **Fluorescence Intensity** in the measurement method section, and **Well** **Mode** in the reading mode section. Next, click the **OK** button and then **New** button. New tab will appear.

5.8.3. In the **Basic Parameters** menu, set the excitation wavelength to 485 nm and the emission at 520 nm. Select the **Bottom Optic** to read fluorescence from the bottom. Set measurement start time to be “0 s”, number of intervals to be “25”, number of flashes per well, and interval to be “20”, and interval time to be “0.4 s”.

5.8.4. In the **Layout** menu, draw region of the plate to be read.

5.8.5. In the **Concentrations/Volumes/Shacking** menu, set the microplate reader to inject 100 µL of I-solution to each well with 135 µL/s injection speed.

NOTE: Avoid faster injection speeds because they can result in detachment of cells.

5.8.6. In the **Injection time** menu, set the injection start time to be 1 s. Then, click the **Start** **Measurement** button.

5.9. After incubation, place the 96-well plates in the microplate reader and start the measurement by clicking **Start Measurement** button again.

**6. Calculation of GJIC activity**

6.1. Calculate the percentages of YFPQL quenching and GJIC activity as23

YFPQL quenching (%) =

GJIC activity (%) =

NOTE: In principle, GJIC activity should be calculated from the difference of the percentages of YFP fluorescence in wells with acceptor cells and donor cells and the corresponding acceptor-cell only wells. However, as LN215-YFPQL cells show negligible YFP quenching by iodide after 10 s, we do not take the background YFP quenching into account when conducting HTS using LN215-YFPQL and LN215-I- cells.

**REPRESENTATIVE RESULTS:**

Twenty-nine 96-well culture plates were used to screen 2,320 chemicals to identify novel GJIC modulators by I-YFP GJIC assay using the LN215-YFPQL and LN215-I− cells. The results obtained with a representative plate are shown in **Figure 2**. The percentage of YFP fluorescence in each well is shown as a line graph in **Figure 2A** and the percentage of GJIC activity in each well is shown in the bar graph in **Figure 2B**. The negative and positive controls and the 80 chemicals that were screened are shown in **Table 1**. Each well was treated with 25 μM of a compound for 10 min. Terbinafine completely inhibited GJIC and homosalate inhibited approximately 50% of GJIC. Terbinafine was confirmed as a GJ inhibitor. Its dose-response, reversibility, and other experimental results have been published elsewhere23.

**FIGURE LEGENDS AND TABLE TITLE**

**Figure 1: The components and steps of the I-YFP GJIC assay (A**)The yellow and dark yellow pentagons represent acceptor cells expressing YFPQL before and after quenching. The black pentagons are donor cells expressing SLC26A4. The blue bars are GJs and the red bars are SLC26A4 transporters. The pink circles are iodides. When the GJs are open (upper panel), iodides pass through SLC26A4 and enter the donor cell. Iodides migrate to adjacent acceptor cells *via* GJs. Iodides quench the YFP fluorescence of the acceptor cells. If the GJs are closed (lower panel), iodides entering the donor cells cannot move to the neighboring acceptor cells, and are retained only in the donor cells. The YFP fluorescence of the acceptor cells is not significantly reduced after the addition of iodides (**B**) Phase contrast and fluorescent images obtained when doing an I-YFP-GJIC assay. When acceptor and donor cells were plated at a ratio of 1:1, YFPQL quenching was observed 1 min after iodides were added (upper panel). When only acceptor cells were plated, iodide treatment for 1 min did not lead to significant YFPQL quenching21.

**Figure 2: Representative HTS results using the I-YFP-GJIC assay (A**)Suspensions of 1:2 mixtures of LN215-YFPQL and LN215-I− cells were plated and incubated for 24 h, as described in protocol section 4. Cells were then washed and treated with vehicle or chemicals as in protocol section 5. All chemicals were assayed as 25 μM samples from 2.5 mM stock solutions in DMSO for 10 min. Each well contained 1 μL DMSO and 100 μL of C-solution. The first and last rows of the plate were assigned to negative (vehicle) and positive (carbenoxolone) controls. The remaining 80 wells were used to screen chemicals listed in **Table 1**. After treatment, the I-YFP-GJIC assay was conducted well-by-well as in protocol section 5. The percentage of YFP fluorescence in each well at each time was normalized to the value at 2 s and plotted against time. The lines in the figure represent the changes in YFP fluorescence in each well. The YFP fluorescence in most well at 10 s ranged from 70% to 80%. Terbinafine and homosalate were potential hits for GJ inhibitors (**B**)The bar graph shows the percent GJIC activity of each of the 96 wells. The percent GJIC activity was calculated as shown in protocol step 6.1 and plotted in the bar graph against the well numbers.

**Table 1. List of chemicals screened in this study.**

**DISCUSSION:**

The I-YFP-GJIC assay can be used for HTS because it is robust, rapid, and inexpensive. An HTS assay is considered robust if the Z’-factor is above 0.525. See Zhang et al. for a description of the statistical analysis used to assessing the suitability of HTS assays25. When LN215 cells were used, the Z’-factor was >0.5 without any assay optimization. If other cell types are used in the assay and its Z’-factor is <0.5, the robustness can be improved by extending the assay time21. LN215 and HOS, human osteosarcoma cells, cells need only 10 s to obtain a Z’-factor >0.521. The assay requires only acceptor and donor cells, the I-solution, and the C-solution. No additional reagents, such as LY and calcein-AM are needed. Another advantage of this GJIC assay is that it measures the total GJIC activity of the cells in a single well, which results in low between-well variability. In microinjection, gap-fluorescence recovery after photobleaching (FRAP), and dual patch clamp assays, a cell in the assayed area may significantly affect the measurement. Although scrape loading assays evaluate GJIC over a relatively wide area, the scraping-loading procedure can introduce significant variability26.

The critical steps for successful conduct of the I-YFP-GJIC assay are as follows. The pH of the C- and I-solutions must be adjusted to 7.4 before each use. YFPQL fluorescence is strongly affected by pH as well as by halides22. A pH difference between the two solutions can lead to aberrant change in YFP fluorescence after addition of the I-solution. The donor and acceptor cells must be completely dissociated before plating. Clumps of donor or acceptor cells disturb the assay. The confluence of the mixed culture should be 100% to maximize formation of GJs between cells. The ratio of acceptor to donor cells also affects the assay results as described by Lee et al*.*21. As only acceptor cells have YFP fluorescence, the ratio and arrangement of acceptor and donor cells can be easily checked with a fluorescence microscope before conducting the assay. It is important to wash the co-culture with C-solution to remove residual medium before treatment with vehicle or chemicals. Residual serum or phenol red from the culture medium may be a source of background fluorescence or an unwanted fluorescence quencher, respectively. They may thus interrupt the I-YFP-GJIC assay. Growth factors present in the FBS can also inhibit GJIC27. On the other hand, serum starvation can eventually lead apoptosis28, which can also be accelerated by chemical treatment. In our experience, the acceptor and donor cells were healthy after treatment by most screened chemicals at 25 μM in C-solution without serum for 10 min. Some chemicals were not toxic even after treatment for 4 h. As chemical toxicity varies, the condition of the cells should be carefully checked when treatment for longer periods is required. If the cells are not healthy, an obvious reduction of YFP fluorescence that occurs within 1 s after introduction of the I-solution may result from cell detachment. That can be confirmed by microscopic observation of the involved well.

The cells used in the I-YFP-GJIC assay should meet several requirements. They should express GJs endogenously or be induced to form GJs. As channels composed of Cx43 are almost equally permeable to atomic cations and anions29, iodides can migrate through the channels present in I-YFP GJIC in LN215 cells. If the Cx channels in the cells of interest have low iodide permeability but freely allow diffusion of Ca2+, the I-YFP-GJIC assay may not be possible. The absence of iodide uptake activity is a requirement. The cells that express functional anion channels and uptake iodide rapidly are not appropriate for this assay. Cell growth is another requirement. The acceptor and donor cells are generated by transduction of lentiviruses expressing YFPQL or SLC26A4 and selection with puromycin for at least 1 week19. Cells that grow slowly or have limited growth such as primary hepatocytes are not suitable for this assay. Adhesion of cells to the surface of the plastic culture ware is also important. Cells that do not adhere firmly, such as HEK293 cells, are easily detached by introduction of the I-solution. This can be overcome by coating 96-well plates with PLL before plating. However, coating plates is a tedious step, and avoiding those types of cells is recommended. In principle, primary cells like astrocytes, hepatocytes, and keratinocytes are toxicologically, physiologically, and pharmacologically more relevant than cancer cells like glioma, hepatoma, and basal cell carcinoma cells. However, their limited growth rates make them inappropriate for the assay. Future advances in cell culture that allow growth of primary cells that meet the above requirements for longer periods, would broaden the toxicological, physiological, and pharmacological assessments possible with this assay.

The I-YFP GJIC assay can be used not only for HTS but also for determining whether a cell type endogenously expresses functional GJs. If the cells do not have GJs, the YFPQL quenching rate observed in cocultured acceptor and donor cells would not be different from that observed when only the acceptor cells were plated. If there was a significant difference in the quenching rates, then the original cells express functional GJs. As the difference tends to increase with time as shown in **Figure 2A**, this assay has a greater sensitivity to detect weak GJIC activity than scrape-loading or gap-FRAP assays. Time course data, dose-response relationships, and assessing the reversibility of GJ modulators can be obtained with the I-YFP-GJIC assay23,30. By inducing the expression of a Cx of interest into the cells devoid of functional GJs and then generating acceptors and donors, I-YFP specific Cx-GJIC assays can be established23. This assay thus makes it possible to determine IC50 values of a chemical on a specific type of Cx.

Like most HTS assays, the I-YFP-GJIC assay can produce false positive results resulting from changes in SLC26A4, YFPQL, or cellular iodide permeability. As false GJ inhibition can result from inhibition of SLC26A4 or desensitization of YFPQL to iodide, every potential GJ inhibitor should be tested using cells that coexpress YFPQL and SLC26A4. If a potential GJ inhibitor is an SLC26A4 blocker or a YFP desensitizer, YFP quenching is attenuated. On the contrary, a true GJ inhibitor does not affect YFP quenching. Electrophysiological methods or purified YFPQL can discriminate between SLC26A4 blockers and YFP desensitizers. GJ activators that sensitize YFP to iodide or increase iodide permeability via anion channels, Cx hemichannels, or by nonspecific toxic effects will also give false positive results. It should be determined whether potential GJ activators enhance YFP quenching when only acceptor cells are plated. If a chemical sensitizes YFPQL or increases iodide permeability, then it will increase YFPQL quenching in acceptor cell cultures without donors. The two types of false GJ activators can be distinguished in other GJ assays, such as scrape loading or FRAP or using purified YFPQL protein.

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**DISCLOSURES:**

The authors have no conflicts of interest to disclose.

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