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# An iodide-yellow fluorescent protein-gap junction-intercellular communication assay

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**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 inhibitors[2,3,4]. Disrupted GJIC has been associated with nongenotoxic carcinogenesis[5,6]. As a potential therapeutic target, GJ involvement has been reported in particular subtypes of seizures[7,8], protection from cardiac and brain ischemia/reperfusion injury[9], migraine with aura[10], drug-induced liver injury[6,11], and wound healing[12]. 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 modulators[2,13,14,15].

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 migration[16]. Calcein-AM assays usually involve gap-fluorescence recovery after photobleaching[17,18]. 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 GJs[19]; however, it is technically demanding, time consuming, and expensive[20]. 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 ($YFP^{QL}$) and donor cells expressing an iodide transporter (SLC26A4)[21]. The two mutations carried by $YFP^{QL}$ allow quenching of fluorescence by iodide[22]. 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 $YFP^{QL}$ fluorescence. If GJs are closed or blocked by inhibitors, iodide cannot enter the acceptor cells to quench the fluorescence. The $YFP^{QL}$ 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 Cx43[21]. The LN215-$YFP^{QL}$ receptor and LN215-$I^-$ donor cells were generated by transduction with lentiviruses expressing $YFP^{QL}$ or SLC26A4[21,23].

**PROTOCOL:**

**1. Generation of lentiviruses expressing $YFP^{QL}$ 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% $CO_2$/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-YFP$^{QL}$ or pLenti6P-SLC26A4, 1225 ng of psPAX2 and 375 ng of pMD2.G to each. The two lentiviral plasmids have been previously described[21]. 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 $CO_2$ 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-YFP$^{QL}$ 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-YFP$^{QL}$, 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, YFP$^{QL}$, 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-YFP$^{QL}$ 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-YFP$^{QL}$ 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 $MgCl_2$, and 1 mM $CaCl_2$) and 500 mL of I-solution (10 mM HEPES, 140 mM NaI, 10 mM glucose, 5 mM KCl, and 1 mM $CaCl_2$).

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-YFP$^{QL}$ and LN215-I$^{-}$ cells

4.1. Culture LN215-YFP$^{QL}$ and LN215-I$^{-}$ cells in 100 mm plates separately in culture medium to reach the populations required for assay. LN215-YFP$^{QL}$ 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-YFP$^{QL}$ at 80,000 cells/mL and LN215-I$^{-}$ at 160,000 cells/mL.

4.5. Mix 7 mL of LN215-YFP$^{QL}$ 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% $CO_2$/95% air at 37 °C for 24 h. The LN215-YFP$^{QL}$ 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-YFP$^{QL}$ 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 assays[24]. 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% $CO_2$. 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 $YFP^{QL}$ quenching and GJIC activity as[23]

$$YFP^{QL}\ \text{quenching}\ (\%) = \left(1 - \frac{YFP\ Fluorecense}{YFP\ Fluorescence\ at\ 2\ s}\right) \times 100$$

$$\text{GJIC activity}\ (\%) = \left(1 - \frac{\%\ YFP\ quenching\ at\ 10\ s}{\%\ YFP\ quenching\ at\ 10\ s\ of\ control\ group}\right) \times 100$$

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-$YFP^{QL}$ 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-$YFP^{QL}$ 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-$YFP^{QL}$ 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 elsewhere[23].

**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 $YFP^{QL}$ 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, $YFP^{QL}$ 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 $YFP^{QL}$ quenching[21].

**Figure 2: Representative HTS results using the I-YFP-GJIC assay (A**) Suspensions of 1:2 mixtures of LN215-$YFP^{QL}$ 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 0.5[25]. See Zhang et al. for a description of the statistical analysis used to assessing the suitability of HTS assays[25]. 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 time[21]. LN215 and HOS, human osteosarcoma cells, cells need only 10 s to obtain a Z'-factor >0.5[21]. 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 variability[26].

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. $YFP^{QL}$ fluorescence is strongly affected by pH as well as by halides[22]. 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 GJIC[27]. On the other hand, serum starvation can eventually lead apoptosis[28], 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 anions[29], 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 $Ca^{2+}$, 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 $YFP^{QL}$ or SLC26A4 and selection with puromycin for at least 1 week[19]. 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 $YFP^{QL}$ 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 assay[23,30]. 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 established[23]. 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, $YFP^{QL}$, or cellular iodide permeability. As false GJ inhibition can result from inhibition of SLC26A4 or desensitization of $YFP^{QL}$ to iodide, every potential GJ inhibitor should be tested using cells that coexpress $YFP^{QL}$ 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 $YFP^{QL}$ 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 $YFP^{QL}$ or increases iodide permeability, then it will increase $YFP^{QL}$ 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 $YFP^{QL}$ protein.

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**DISCLOSURES:**

The authors have no conflicts of interest to disclose.

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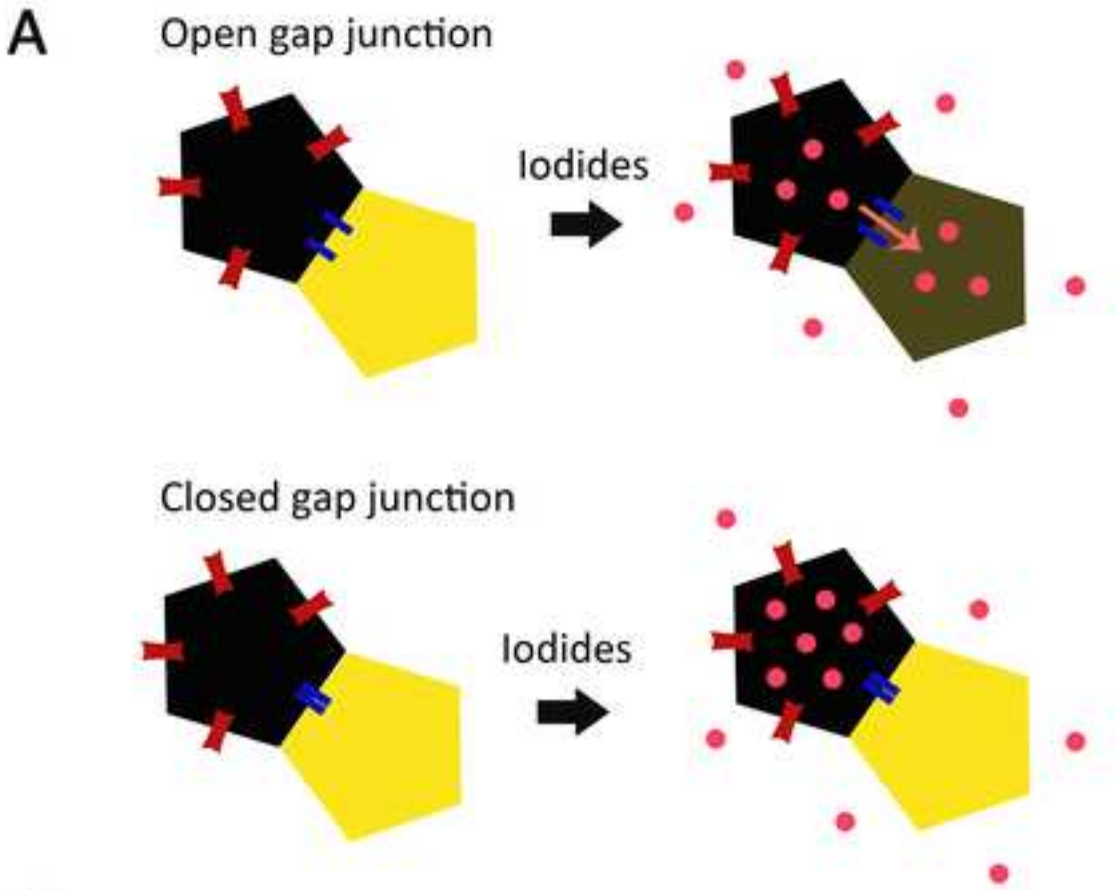


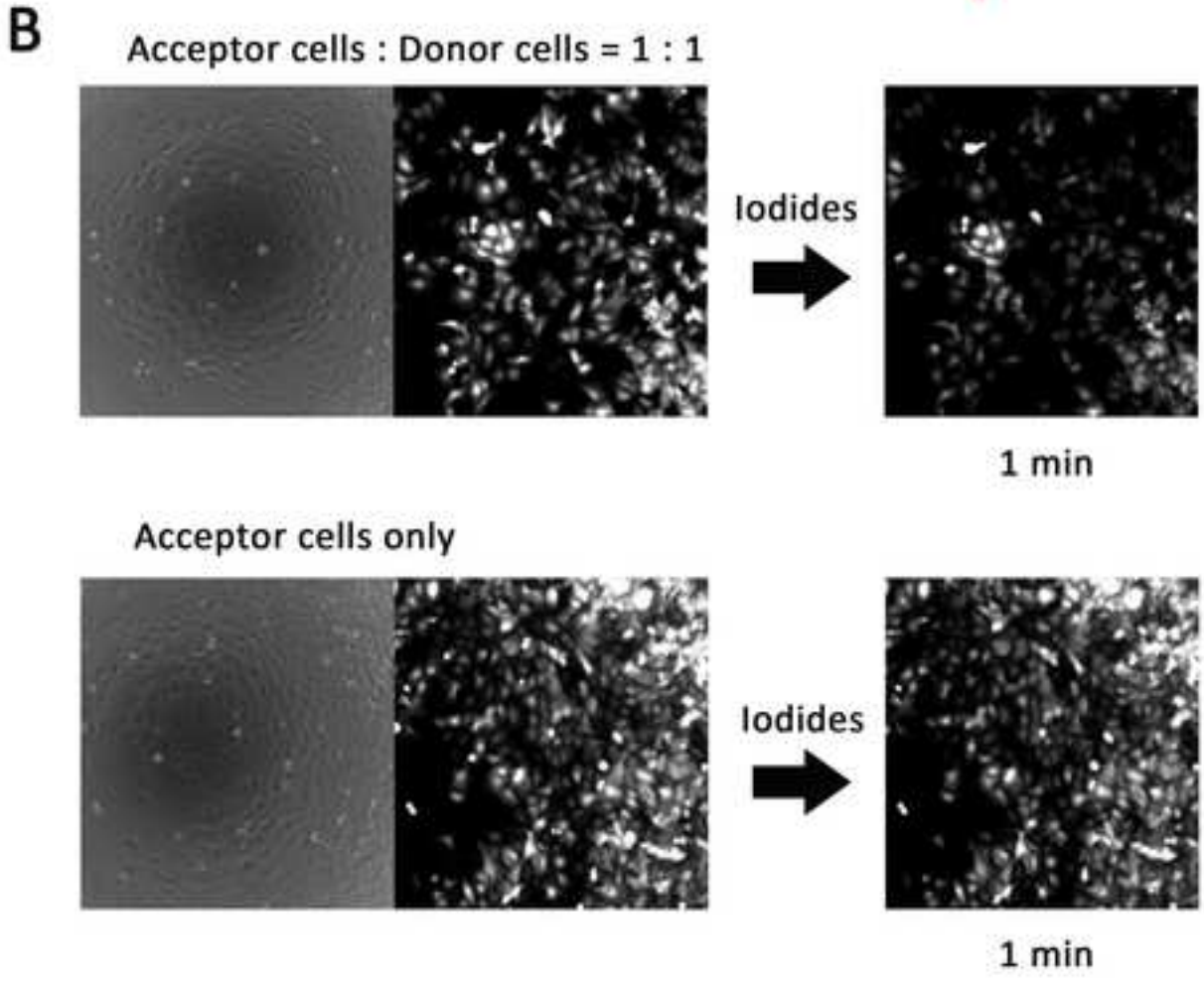

Figure

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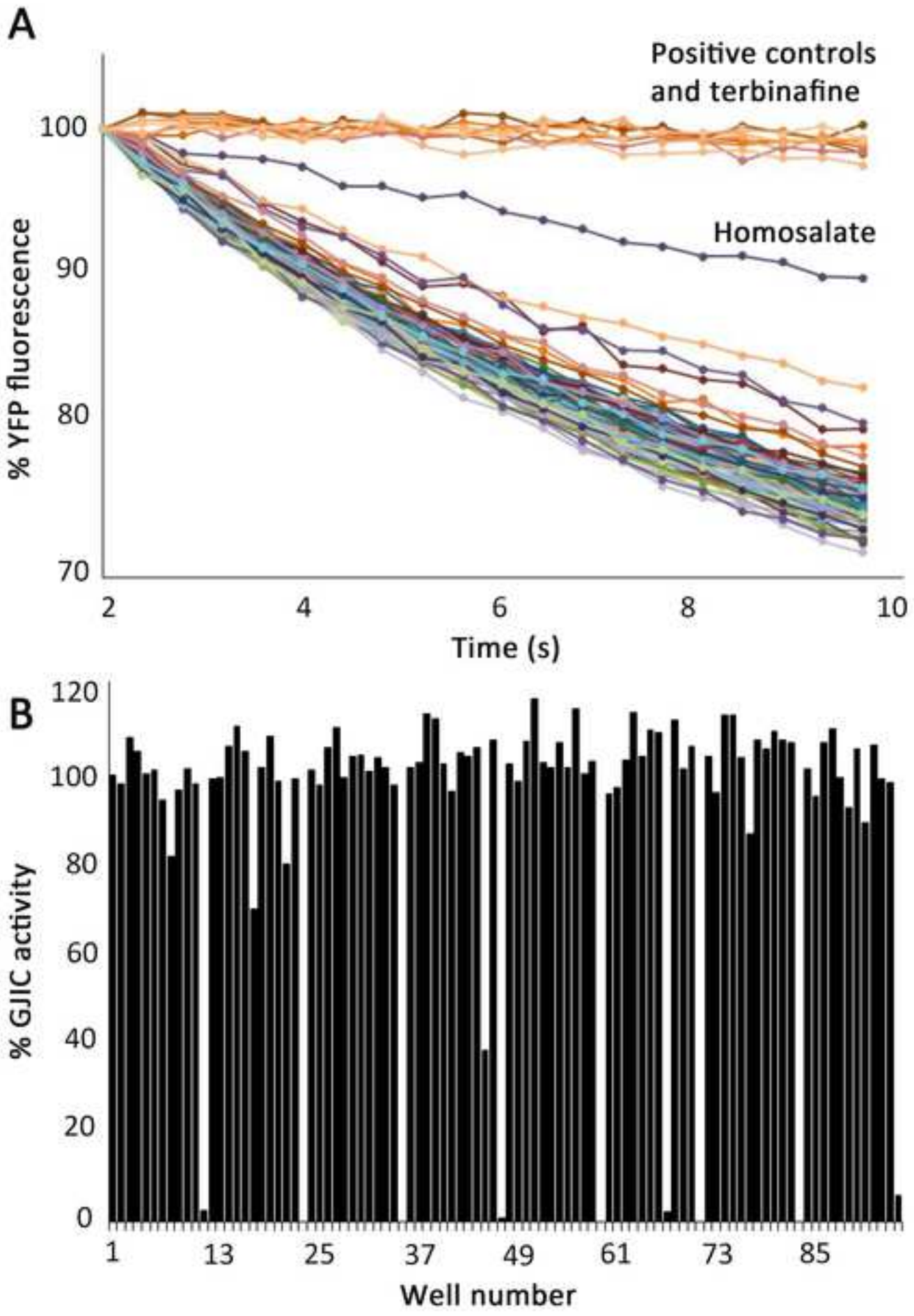

Table

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| Well No. | Position | Chemical | Well No. | Position | Chemical |
|---|---|---|---|---|---|
| 1 | A01 | DMSO | 49 | E01 | DMSO |
| 2 | A02 | difloxacin hydrochloride | 50 | E02 | propofol |
| 3 | A03 | betamethasone valerate | 51 | E03 | oleandomycin phosphate |
| 4 | A04 | erythromycin | 52 | E04 | mianserin hydrochloride |
| 5 | A05 | cyproheptadine hydrochloride | 53 | E05 | valsartan |
| 6 | A06 | liothyronine | 54 | E06 | salsalate |
| 7 | A07 | theophylline | 55 | E07 | hydrocortisone |
| 8 | A08 | tolnaftate | 56 | E08 | rifaximin |
| 9 | A09 | trimethobenzamide hydrochloride | 57 | E09 | adrenolone hydrochloride |
| 10 | A10 | cefamandole nafate | 58 | E10 | imiquimod |
| 11 | A11 | dimethyl fumarate | 59 | E11 | nonoxynol-9 |
| 12 | A12 | CBX | 60 | E12 | CBX |
| 13 | B01 | DMSO | 61 | F01 | DMSO |
| 14 | B02 | piracetam | 62 | F02 | ranolazine |
| 15 | B03 | gluconolactone | 63 | F03 | danthron |
| 16 | B04 | azlocillin sodium | 64 | F04 | acedapsone |
| 17 | B05 | choline chloride | 65 | F05 | atomoxetine hydrochloride |
| 18 | B06 | atorvastatin calcium | 66 | F06 | desoxycorticosterone acetate |
| 19 | B07 | oxyphencyclimine hydrochloride | 67 | F07 | tramadol hydrochloride |
| 20 | B08 | propafenone hydrochloride | 68 | F08 | terbinafine hydrochloride |
| 21 | B09 | fluconazole | 69 | F09 | topiramate |
| 22 | B10 | lovastatin | 70 | F10 | gemifloxacin mesylate |
| 23 | B11 | bleomycin (bleomycin b2 shown) | 71 | F11 | pravastatin sodium |
| 24 | B12 | CBX | 72 | F12 | CBX |
| 25 | C01 | DMSO | 73 | G01 | DMSO |
| 26 | C02 | acesulfame potassium | 74 | G02 | levalbuterol hydrochloride |
| 27 | C03 | teniposide | 75 | G03 | metformin hydrochloride |
| 28 | C04 | tannic acid | 76 | G04 | pregabalin |
| 29 | C05 | carprofen | 77 | G05 | topotecan hydrochloride |
| 30 | C06 | hydroxychloroquine sulfate | 78 | G06 | phenoxybenzamine hydrochloride |
| 31 | C07 | pentoxifylline | 79 | G07 | arecoline hydrobromide |
| 32 | C08 | mepivacaine hydrochloride | 80 | G08 | mepartricin |
| 33 | C09 | nilutamide | 81 | G09 | pantoprazole |
| 34 | C10 | aminolevulinic acid hydrochloride | 82 | G10 | loperamide hydrochloride |
| 35 | C11 | aniracetam | 83 | G11 | podofilox |
| 36 | C12 | CBX | 84 | G12 | CBX |
| 37 | D01 | DMSO | 85 | H01 | DMSO |

| | | | | | |
|---|---|---|---|---|---|
| 38 | D02 | metaxalone | 86 | H02 | levodopa |
| 39 | D03 | chloroguanide hydrochloride | 87 | H03 | ethisterone |
| 40 | D04 | clarithromycin | 88 | H04 | enrofloxacin |
| 41 | D05 | modaline sulfate | 89 | H05 | sparteine sulfate |
| 42 | D06 | protirelin | 90 | H06 | testosterone propionate |
| 43 | D07 | theobromine | 91 | H07 | pyridostigmine bromide |
| 44 | D08 | rosiglitazone maleate | 92 | H08 | enilconazole sulfate |
| 45 | D09 | losartan | 93 | H09 | betamethasone sodium phosphate |
| 46 | D10 | homosalate | 94 | H10 | azaserine |
| 47 | D11 | salicylanilide | 95 | H11 | acrisorcin |
| 48 | D12 | CBX | 96 | H12 | CBX |

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| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| 96-well plate | SPL | 30096 | |
| Calcium chloride ($CaCl_2$) | Sigma | C5670 | I-solution |
| D-(+)-Glucose | Sigma | G7021 | C-solution, I-solution |
| Dimethyl sulfoxide (DMSO) | sigma | 276855 | |
| HEPES | Sigma | RES6003H-B7 | C-solution, I-solution |
| Lipofectamine 2000 | Invitrogen | 11668-027 | transfection reagent |
| Magnesium chloride hexahydrate ($MgCl_2$ $6H_2O$) | Sigma | M2393 | C-solution |
| Microplate reader | BMG LabTech | POLARstar Omega 415-1618 | |
| pMD2.G | Addgene | #12259 | |
| Polybrene | sigma | H9268 | |
| Poly-L-lysine solution | sigma | P4707 | |
| Potassium chloride (KCl) | Sigma | P5405 | C-solution, I-solution |
| psPAX2 | Addgene | #12260 | |
| Puromycin Dihydrochloride | sigma | P8833 | |
| Sodium chloride (NaCl) | Sigma | S5886 | C-solution, I-solution |
| Sodium hyroxide (NaOH) | Sigma | S2770 | |
| Sodium Iodide (NaI) | Sigma | 383112 | I-solution |

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Author(s): Jou Hye Yeo, Jinu Lee

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**Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*We appreciate the opportunity to improve the manuscript. We revised the manuscript following the editor's and the reviewers' recommendations. We have incorporated all changes and submitted a clean version of the manuscript. After revising the manuscript, we sent it to a language editing service for checking.*

2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

*We have rephrased the summary to clearly state the goal of the protocol, its application, and potential uses.*

3. Please rephrase the Abstract to more clearly state the goal of the protocol.

*We revised the abstract to state the goal of the protocol more clearly.*

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

5. 2.1: Please specify the acceptor and donor cells as well as the culture medium that are used in the protocol. We need specific details for filming.

*The acceptor and donor cells are now identified as LN215-YFP$^{QL}$ and LN215-I$^{--}$ throughout the manuscript. Culture medium is identified in protocol step 1.1.*

6. 2.2: Please describe how to detach the cells with trypsin including the concentration of trypsin used and the reaction conditions.

*The details of the trypsin digestion method are given protocol section 1.3.*

7. 2.3: What solvent is used to dilute cells if needed?

*Cell diluent used to suspend the acceptor and donor cells was the culture medium and that is now noted throughout.*

8. 2.4: Please specify the volume. How to resolve cell clumps into single cells?

*The volumes of reagents and cell suspensions have been included in each protocol step as needed. The method of breaking up cell clumps when plating the acceptor and donor cells is given in section 4.3. "Break up any cell clumps into single cells by pipetting up and down about 20 times with a 10 mL serological pipette."*

9. 3.1: Please describe how to prime the microplate reader with ethanol, DI water and I-solution.

*The detailed priming procedure is included in the steps of protocol section 5.*

10. 3.4: Please specify the vehicle or drugs of interest used in this step.

*The vehicle can be different from solvents used to dissolve the tested chemicals. Most chemicals in compound libraries are dissolved in DMSO. We added this description in the revised version in protocol section 5.*

11. Figure 1: Please add panel labels A and B.

*We have added images of the cells in a representative I-YFP-GJIC assay as Figure 1B.*

*Figure 1A and B of the original manuscript have been replaced with a single figure with upper and lower panels.*

12. Figure 2: Please explain what different lines represent in the figure legend. How are the quenching rates determined? Please specify in the corresponding step in the protocol.

*We have added a description of what each line of the graph in Figure 2A represents in the figure legend. Quenching rate (%) and GJIC activity (%) were determined using the equations included in protocol section 6. The calculation step has been included in the legend of Figure 2B.*

Reviewers' comments:
NOTE: Please note that while we do not require in depth results for publication in JoVE, the results must accurately demonstrate the efficacy of the proposed method. Additionally, the results must substantiate all claims presented within the manuscript. Please ensure that all claims you make are fully backed by your results or references to previous works.

Reviewer #1:

Manuscript Summary:
The authors describe a method they previously used to measure gap junction intracellular communication and propose this method to be advantageous for high throughput screening of gap junction modulating molecules. The method is sound and may be of interest to investigators of gap junction communication. The authors argue superiority of their method to traditional methods of GJIC measurement including FRAP and path clamping.

Major Concerns:
1. While the method appears sound, because this is a methods paper I would expect more detailed methods such that an independent investigator would be able to apply this method without other resources. The authors fail to note where they obtained the Lentiviral particles expressing I-YFP and SLC26A4. Because this is a methods paper its critical to include information on the development of these donor and acceptor cells and specifically how another investigator may obtain them.

*The description of the development of the donor and acceptor cells in protocol sections 1 and 2 has been expanded to include details of how to produce the lentiviral*

*particles expressing $YFP^{QL}$ and SLC26A4. The cell selection and transfer methods are also included.*

2. The authors mention (line 87) that "Iodides that are used in this assay are not involved in physiologic events in the cells" without a citation. I am familiar with some physiologic effects of iodides (gastric cells for instance secrete iodides into the stomach lumen under specific conditions for instance) And cretinism IS caused by a congenital deficiency of Iodine. I would like some clarification about potential physiologic effects of Iodides, why they are not involved in physiologic events in these cells, and examples of cells in which they might exhibit a physiologic role such as gastric or thyroid cell lines perhaps?

*Thank you for this informative and helpful suggestion. Originally we wanted to mention that iodide is not a signaling ion like $Ca^{2+}$. We agree that is not necessary and have omitted the description in the revised manuscript.*

Minor Concerns:
1. The excitation and emission settings of the plate reader should be noted.

*The excitation and emission settings are now included in protocol section 5.8.*

2. figure 2 Please list compounds that appeared to be potential hits for blocking GJIC and compounds that were not

*Thank you for this suggestion. Figure 2 and Table 1 include the compounds tested and potential hits for GJ blockers. The positive control (carbenoxolone) is also given in Figure 2A in the revised manuscript.*

3. please have a native English reader read the manuscript and adjust verb tense and make plural/ singular word adjustments where needed

*Thank you for this suggestion. The manuscript has been carefully reviewed by an experienced editor whose first language is English and who specializes in editing*

*papers written by scientists whose native language is not English.*

Reviewer #2:

Manuscript Summary:
Yeo and Lee developed a I-sensitive YFP assay to study gap junction (GJ) function between donor cells and recipient cells. According to the authors this assay could be ranked as high throughput assays to test many chemicals for GJ blockers. I thought that this is interesting approach, but need to have several critical controls to consolidate the assay. Here are some issues/concerns to improve the manuscript.

Major Concerns:
-the assay needs to be validated with important positive and negative control experiments. For example, known GJ blockers, carbenoxolone, flufenamic acid, Octanol, heptanol, mefloquine, etc. are among those commonly used uncoupling compounds. Other control experiments should also be included to avoid any compounds change the I-permeability in the recipient cells. This is very critical, may be better to be a control for every compound tested to avoid false positives!

*We replaced the old data set with a new one that includes negative (DMSO) and positive (carbenoxolone, CBX) controls in Figure 2A of the revised version. When we conduct high throughput screening with the assay in 96-well plates, eight wells of DMSO and eight wells of CBX are always included. We have also added a discussion of possible reasons for false positive results in the revised penultimate paragraph of the Discussion.*

-the authors should provide some information on the type of GJs in the model cells. Which GJ is expressed in the cell lines used? Is it only one connexin expressed or multiple connexins? There are 21 different connexins in the human and 20 in mouse. GJs of different connexins show different permeabilities to cations or anions, I do not think that all GJs are permeable to iodide. Authors should discuss more along this line.

*We recently reported that the major connexin in LN215 cells is Cx43 and have included a citation of that study in the last paragraph of the Introduction. As the permeability ratio of atomic cations and anions in Cx43 channels was reported as 1.0 (Harris and Locke, 2009, reference 31 in the revised manuscript), the GJIC activity of LN215 cells could be assayed with this technique. However, this assay may not be*

*appropriate for cells with connexin channels that have low iodide permeability. This has been included in the third paragraph of the revised Discussion.*

Minor Concerns:
Page5, mentioned that this assay could obtain time course and dose-response relationship, could you provide some experimental evidence to show this instead of just saying.
I noticed that there is a preceding publication on the same assay, not if there is any improvement over the previous publication by the same authors. The authors should at least provide the novel aspect of this ms.

*Time course and dose-response relationship data have been previously described in references 23 and 29, which are cited in the penultimate paragraph of the revised Discussion. Since JoVE aims to present and illustrate detailed experimental procedures, we have included the citations, detailed description of procedures, and unpublished screening results with the names of the tested chemical in the revised version.*

Reviewer #3:

Manuscript Summary:
In this paper, the authors delineate a protocol for assessing gap junctional (GJ) communication using the iodide-yellow fluorescent protein (I-YFP). The cell-based assay consists of donor cells expressing an iodide transporter and acceptor cells containing a mutant YFP with high sensitivity for iodide. The assay requires plating the donor and acceptor cells and adding iodide which then is transported into the donor cell, permeates to the adjacent cell via gap junctions and quenches YFP fluorescence in adjacent cells.

This assay was presumably "developed to be used as an HTS assay system to identify compounds that modulate" gap junction channels, but there are significant problems.

Major Concerns:
The assay does not demonstrate that compounds have a direct action on GJ coupling.

Quenching of YFP- fluorescence, as the authors themselves note can be due to a number of effects, including an action on the iodide transporter itself. This necessitates testing the positive hits with a second assay. This is not ideal for a HTS assay

Quenching of YFP fluorescence could also be due to an effect of cellular processes that indirectly

affect GJ coupling (or iodide transporter activity). For example, exogenous compounds could affect phosphorylation of connexin proteins, which can strongly regulate GJ channel activity, through an action on kinases or phosphatases. The assay does not control for non-channel mediated effects. The complement of kinases etc could be different in different cells, leading to variability.

*We agree that the I-YFP-GJIC assay does not directly measure GJIC and that because the GJIC activity measured by the assay can be affected by various indirect effects. A second assay might be needed. However, since time, cost, and quality of assay are the important aspects of HTS (Curr Opin Pharmacol 9:580–8), this assay is of considerable value. We have described the possible off-target effects and additional methods to rule out false positives attributable to each off-target mechanism. The use of a second assay with different off-target mechanisms is discussed in the revised manuscript as well as in the original report of the assay (BMC Biotechnol 15:90). We also agree that this assay cannot discriminate between the effects by direct interaction with GJs and those by affecting connexin expression, GJ assembly, GJ trafficking, GJ stability, GJ channel gating, and so on. This is common to all dye transfer-based GJ assays. Only electrophysiological assays which have the highest time resolution among GJ assays can provide some clues about mode of action.*

How does variability of coupling in a dish impact the permeation of iodide and YFP quenching?

*We agree that it would be helpful to answer this question. As this assay depends on reading the fluorescence of the cells in each well, the assay of GJIC activity is the sum of the GJIC activities of all the cells in each well, which results in low variability. This is described this in the first paragraph of the revised Discussion.*

Some connexins can form hemichannels, which can provide a pathway for the entry of iodide in acceptor cells. How will the authors control for this?

*If a chemical enhances the iodide uptake of acceptor cells by opening or increasing hemichannels or by any other mechanisms, the GJIC activity will be overestimated. That is one of the off-target effects. After hits for GJ activators are identified via HTS using the I-YFP-GJIC assay, YFP quenching rates of cultures of acceptor cells only and cocultures of acceptor and donor cells should be compared to rule out hemichannel activators or other false positives. This off-target issue has been included in the*

*Discussion of the revised version.*

No images of donor cells and iodide quenching was provided. It would be helpful to assess the protocol's robustness.

*We have included the phase contrast and fluorescence images of a representative I-YFP-GJIC assay in Figure 1B of the revised version.*

Reviewer #4:

Manuscript Summary:
Dr. Lee has developed an exciting new assay for assessing GJIC, previously published as indicated in reference 12. The assay is based on establishing two cell subtypes of an established cell line that involves a donor cell subtype transfected with the Iodide transporter gene that allows cells to transport I into the cytoplasm, and second cell subtype transfected with YFP. The principle of the assay involves co-culturing both cell subtypes to 100% confluence, then adding iodide to the cell incubation medium in which the uptake of iodide in the acceptor cell will transfer through gap junctions or other intercellular channels to the acceptor cells resulting in the quenching the YFP fluorescence. Inhibition of intercellular communication will reduce this quenching effect by preventing the diffusion of I from the donor cell to the acceptor cell. This assay is very amendable to high throughput screening (HTS) and is very relevant to publication in JoVE. I highly recommend that JoVE publish this paper, but after major revisions. Dr Lee's original paper was very well written and organized, but the current paper is not. This JoVE paper needs to be more independent from their previously published paper, which will require more data. The following are recommended revisions.

*We thank the reviewer for this positive and careful evaluation. We agree that major revisions were required to improve the manuscript.*

Major Concerns:

1. Please show a fluorescence image of these cells with normal GJIC and inhibited GJIC. This should be Fig. 1B, and current Fig 1 should be Fig. 1a. Also, in Fig. 1, make all the receptor cells yellow.

*We revised Figure 1 as recommended. The acceptor cell with quenching of the YFP*

*fluorescence by iodides is now included the upper panel of Figure 1A in dark yellow.*

2. In Fig. 2; please list the chemicals used along with a key. The version of the figure I saw was low resolution. This needs to be addressed. Also replot the 10 sec data point as bar graphs for each chemical as this will be great way to visualize differences in GJIC activity among the different chemical treatments.

*A revised assay data set is now included in Figure 2 that was obtained in one of the 29 96-well plates that we screened for GJ modulators. Negative (DMSO) and positive (carbenoxolone) controls were treated in the first and the last rows. The chemicals tested in the 80 remaining wells are shown in Table 1 and in the new line graphs in Figure 2A. The % GJIC activity observed in the plate is shown in the bar graph in Figure 2B.*

3. Be specific as to how the background quenching of fluorescence was measured. Also, show variability among the different wells for the control, a chemical that showed low variability and a chemical that showed high variability in a figure of %YFP quenching at 10 s vs well number. This will be useful for those considering this assay for HTS and how variability is either dependent on chemical type of independent of chemical type.

*Background quenching of the fluorescence can be measured in wells with only acceptor cells or with acceptor cells cocultured with donor cells without the iodide transporter, i.e., original cell line, in this case, LN215. As the background level was not significantly high in the HTS assays that we conducted, it was ignored. However, when confirming hits for GJ activators, the background quenching should be measured and considered to rule out false positives. We have added a note regarding this issue in protocol section 6.1.*

*As mentioned above, data from eight wells with DMSO and eight with 25 µM carbenoxolone are included in the revised manuscript. Their variation is shown in Figure 2A, B. The high Z' factor (BMC Biotechnol 15:90) reflects low variability. We agree that the data of chemicals with high and low variabilities are useful, but we do not have variability data except for DMSO and carbenoxolone.*

4. Many readers will not have experience with HTS, thus unfamiliar with the Z-factor, a stat many might have learned and forgotten. Thus, an introduction of the Z-factor and how it is used to

assess quality control of HTS, and then a short discussion on the Z-factor's limitations and its comparison to standardized mean difference (SSMD). Why was SSMD not used to measure the differences between positive and negative controls?

*We agree that a more extensive discussion of Z' and Z factors would be helpful for readers. However, JoVE aims at publishing experimental methods. Consequently, the discussion should emphasize the methods themselves. We believe that an extended description of the statistical factors is outside the scope of JoVE.*

5. Describe the key parameters used for the microplate reader

*The key parameters used for the microplate reader have been added in protocol section 5.8 of the revised version.*

6. Show data for optimization of cell ratios for this cell model system.

*The data for determination of the optimal cell ratio is not a representative outcome following the use of the protocol. It was an intermediate step in the development of the protocol. We believe that it is appropriate to refer to the original article rather than repeating the data in this manuscript.*

7. Pick a chemical used in Fig. 2 and show a time and dose response curve, and time recovery, as these are basic data typically reported for GJIC experiments assessing chemicals.

*We have confirmed terbinafine as a GJ inhibitor and have previously published the experimental data (Toxicol Appl Pharmacol 307:102–107). The dose-response and reversibility data and other results regarding GJIC inhibition by terbinafine were included in the previously published article (Toxicol Appl Pharmacol 307:102–107). We included unpublished result of an HTS of one 96-well plate including terbinafine data in Figure 2 of the revised manuscript.*

8. Why were glioma cells chosen for HTS. This is a cancerous cell type. Most cancer cells have

some level of decreased GJIC. A more normal cell line would be optimal in developing a HTS system. Intracellular regulation of GJIC in cancer cells can be quite different from non-cancerous cells, thus toxicological interpretations of results could be significantly different between non-cancerous vs. cancerous along with the value to risk assessors. These points must be discussed and put into a more realistic framework on the use of these assays for risk assessment.

*We appreciate the reviewer's helpful comments. We agree that it is ideal to use primary cells such as primary astrocytes rather than glioma cells like LN215. However, establishing stable cell cultures is necessary. Primary cells can be passaged for a limited number of times and are not ideal for this assay. If technical advances eventually make it possible to grow primary cells such as astrocytes, hepatocytes, and keratinocytes for longer periods and in sufficient numbers, then they might be used in this assay. As mentioned by the reviewer, GJs are important pharmacological and toxicological targets. We agree that GJIC assays using primary cell cultures would be more useful for both pharmacological and toxicological assessment than assays in cancer cells. The ability to use primary cell cultures and their potential advantages is discussed in the revised version.*

9. Another reason for depleting FBS, is that growth factors in the FBS often inhibit GJIC, thus needs to be removed. However, FBS depletion for too long can lead to apoptosis. Let readers know if apoptosis was an issue at these depletion times.

*The potential influence of serum deprivation on apoptosis and the consequent effects on the assay has been added in the second paragraph of the revised Discussion.*

10. Please use the following, more original reference for the FRAP technique in line 65 next to ref 9 (Wade et al. 1986)

*We appreciate the reviewer's kind request and have changed the reference as recommended.*

11. Reference #1 is incomplete. I assume the following was the intended reference: (Goodenough 1996).

*Thank you for pointing this out. We corrected the error.*

12. The paragraph, lines 45-51 is not logically laid out. Why these examples. Expand on the role of gap junctions in human diseases with appropriate references. In essence, build a better argument on why gap junctions ought to be used in toxicological assessments of chemicals. Include the use of GJIC assays in structure activity relationships such as shown in (Upham et al. 1998; Upham et al. 2003; Upham et al. 2009; Weis et al. 1998). Use reviews on gap junction in diseases to expand on this section: (Trosko et al. 1998; Vinken et al. 2009; Yamasaki et al. 1993; Yamasaki 1996; Yamasaki et al. 1999).

*Thank you for pointing this out and for the suggestions for improving the paragraph. We have expanded the discussion of the rationale for using gap junction activity to screen chemical toxicity. The recommended references were helpful for expanding the discussion of structure–activity relationships of GJ modulators and the relevance to HTS-compatible GJIC assays. We have also included additional diseases that are possible targets of GJ modulators, with appropriate references.*

13. Rewrite the discussion. In particular, the last paragraph is awkward. Transitions from one line of thought to another was lacking here and throughout the m.s. Discuss important parameters, issues needed for quality assurance. Discuss sensitivities and limitations of this technique.

*The manuscript has undergone major revisions, including the Introduction and Discussion. Parameter and methodological issues required for quality assurance have been added in the second paragraph of the Discussion. Descriptions of assay sensitivity and the causes of false positive results have been added in the fifth and the final paragraphs of the Discussion.*

Minor Concerns:

14. Line 49-51: HTS is not required to screen potential toxicants, and toxins. Many compounds have been screened with current techniques. Although not essential, HTS will allow for more extensive screening of compounds in shorter time frames.

*We appreciate the reviewer's valuable comment and have noted the increased*

*throughput and shorter time frame of this assay compared with current techniques for screening toxicants in the Introduction.*

15. Line 57: Opsahl-Rivedal paper is an acceptable reference, but please cite the original dye transfer assay (El-Fouly et al. 1987).

*We have changed the citation in the revised version.*

16. Line 65: The statement that the dye transfer assays are all laborious and time consuming is not true. Please reword. The microinjection and FRAP are laborious and slow, the scrape load assays are quite simple and fast.

*We reworded the statement in lines 63–64 of the revised version.*

17. Lines 86-87: Too soon to use I-solution and C-solution. At this point, one asks what are these solutions. Reword and use chloride vs iodide solutions, the latter of which will be added to begin the assay. Also change this in the abstract on line 39. In essence you use only two solutions.

*We have changed "C-solution and I-solution" to "two solutions" in the abstract and added a brief description of C-solution and I-solution when they appear first in the revised final paragraph of the Introduction.*

18. Line 102: What was the normality of the NaOH solution?

*A 1N NaOH solution was to adjust the pH of solutions. That has been added in protocol section 3.3 of the revised manuscript.*

19. Line 102: What was the pore size of the filters?

*We used a filter with 0.4 µm pore size. That has been added in protocol section 3.3*

*of the revised manuscript.*

20. Line 103: be more specific. Maybe use XX number of months for maximum shelf life.

*We have added "up to one month" in protocol section 3.2 of the revised version.*

21. Line 108: What is the appropriate medium. One can think this might be the time to add the C-solution. Be more specific for your cell type, and add a note that each cell type requires a particular medium.

*Thank you for pointing this out and for the recommendation. The protocol was revised to include greater detail of the procedures for the I-YFP-GJIC assay using LN215 cells. The culture medium is described in protocol section 1.1. The use of specific media for cells other than LN215 has also been added.*

22. Line 114-116: As indicated above, show your optimization experiment and reference your figure for the 1:2 ratio. Also, indicate the final volume of medium used in each well. I am also assuming you used 96 well culture plates, so please let the readers know this. Were the cells distributed to the wells robotically or with an octapipette.

*Thank you for this suggestion. We have provided the information about the final volume of the medium in each well of the 96-well culture plates (100 µL). The use of multichannel pipettes and the distribution methods have been revised in protocol section 4 of the revised manuscript. We gave an opinion about the optimization data in critique no. 6. We hope that you agree.*

23. Line 118-119: How were the clumps resolved? With pipettes?

*We broke up cell clumps into single cells by pipetting up and down about 20 times with 10 mL serological pipettes as described in protocol section 4.3 of the revised manuscript.*

24. Line 128: A pet peeve of mine. "perform, performed and performing" have been misused for so long, we accept these term instead of "do, does, doing", but we should not. E.g, "My son is performing in a band". Please state "At least 30 min before doing the assays….."

*Thank you for this suggestion. We changed "perform" to "conduct" or "do" throughout the manuscript.*

25. Line 131: how much C-solution per plate?

*The amounts of C-solution (and I-solution) have been specified more clearly. We described the required volumes of the solutions in detail in the protocol 5.3 of the revised manuscript.*

26. Check manuscript for passive language and use a more active tense. E.g, line 133, "Incubate the cells for 24 h

*Thank you for this suggestion. The steps in the assay protocol have been revised to include a more active voice in preference to the passive voice.*

27. Line 148: Please give more details on the parameters used in the microplate reader.

*The description of microplate reader programming has been revised as suggested in protocol section 5.8.*