**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-YFPQL 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 YFPQL 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 Ca2+. 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.