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Single-cell Microinjection for cell communication analysis

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Corresponding Author:	Luiz Alves, Ph.D. Institute Oswaldo Cruz Rio de Janeiro, Rio de Janeiro BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	alveslaa@gmail.com
Corresponding Author's Institution:	Institute Oswaldo Cruz
Corresponding Author's Secondary Institution:	
First Author:	Anael Viana Pinto Alberto, PhD
First Author Secondary Information:	
Other Authors:	Anael Viana Pinto Alberto, PhD
	André Gustava Bonavita, PhD
	Antonio Augusto Fidalgo Neto, PhD
	Filipe Berçot
Order of Authors Secondary Information:	
Abstract:	Gap junctions are intercellular channels that allow the communication of neighboring cells. This communication depends on the contribution of each neighboring cells with a hemichannel to form the gap junction. In mammalian cells, the hemichannel is formed by six connexins, monomers with four transmembrane domains and a C and N terminal within the cytoplasm. Gap junctions permit the exchange of ions, second messengers, and small metabolites. In addition, they have important roles in many forms of cellular communication within physiological processes such as synaptic transmission, heart contraction, cell growth and differentiation. We detail how to perform a single-cell microinjection of Lucifer Yellow to visualize cellular communication via gap-junctions in living cells. It is expected that in functional gap junctions, the dye will diffuse from the loaded cell to the connected cells. It is a very useful technique to study gap junctions since you can evaluate the diffusion of the fluorescence in real time. We discuss how to prepare the cells and the micropipette, how to use a micromanipulator and inject a low molecular weight fluorescent dye in an epithelial cell line.
Author Comments:	This version was improved in relation to the last one, we ordered professional microphones, camera and back light. We hope that this version could fill all requirements of JOVE. Thanks for the attention of the Editorial team My best Regards

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Gap junctions allow the passage of ions and second messengers to connected cells. Moreover, gap junctions also have some roles described in physiological processes as synaptic transmission, heart contraction and cell growth. In this manuscript, we describe the microinjection technique. This technique is performed to study communication of neighbor cells by functional gap junctions

Thank you for your consideration and I look forward to hearing from you as soon as possible.

Sincerely,

Luiz Anastacio Alves, MD, PhD
Laboratory of Cellular Communication, Oswaldo Cruz Institute,
Oswaldo Cruz Foundation - FIOCRUZ, Rio de Janeiro, Brasil.
Av. Brasil, 4365 - Manguinhos
21045-900 Rio de Janeiro, Brasil.
FAX: (55) (21) 25621816
E-Mail - alveslaa@gmail.com

Single-cell Microinjection for cell communication analysis

Anael Viana Pinto Alberto[1]*, Andre Gustavo Bonavita[1]*, Antonio Augusto Fidalgo Neto[1], Filipe Berçot[1] and Luiz Anastácio Alves[1&]

Alberto, AVP – aanael@gmail.com
Bonavita, GA – andrebonavita@gmail.com
Neto, AAF – fidalgo@kftox.com
Berçot, F – filipebercot@gmail.com
Alves, LA – alveslaa@gmail.com

[1]Oswaldo Cruz Foundation – Institute Oswaldo Cruz – Laboratory of Cellular Communication - Av. Brazil 4365 – 21045-900 - Rio de Janeiro, Brazil

* The authors contributed equally to this work

Corresponding author:
Luiz Anastacio Alves
Phone: 55-21-25621809

Key words: gap junctions, cellular communication, connexins, micro-injection, Lucifer yellow, hemichannel

Short Abstract

We describe here how to perform a single-cell microinjection of Lucifer Yellow to visualize cellular communication via gap-junctions in living cells, and provide some useful tips. We expect that this paper will help everyone to evaluate the degree of cellular coupling due to functional gap junctions. Everything described here could be, in principle, adapted to other fluorescent dyes with molecular weight below 1000 Daltons.

Long Abstract

Gap junctions are intercellular channels that allow the communication of neighboring cells. This communication depends on the contribution of a hemichannel by each neighboring cell to form the gap junction. In mammalian cells, the hemichannel is formed by six connexins, monomers with four transmembrane domains and a C and N terminal within the cytoplasm. Gap junctions permit the exchange of ions, second messengers, and small metabolites. In addition, they have important roles in many forms of cellular communication within physiological processes such as synaptic transmission, heart contraction, cell growth and differentiation. We detail how to perform a single-cell microinjection of Lucifer Yellow to visualize cellular communication via gap-junctions in living cells. It is expected that in functional gap junctions, the dye will diffuse from the loaded cell to the connected cells. It is a very useful technique to study gap junctions since you can evaluate the diffusion of the fluorescence in real time. We

discuss how to prepare the cells and the micropipette, how to use a micromanipulator and inject a low molecular weight fluorescent dye in an epithelial cell line.

Introduction

Gap junctions are intercellular channels that allow the intercommunication among neighboring cells [1]. This communication connects two or more neighboring cells, where each one contributes with a connexon or hemichannel to form the intercellular channel. In mammalian cells, the connexon is formed by six connexins, monomers with four transmembrane domains and a C and N terminal within the cytoplasm [2]. Gap junctions not only permit the flow of ions, second messengers and small metabolites, but also contribute to many forms of cellular communication in many physiological processes, such as synaptic transmission, heart contraction, cell growth and differentiation[3-8]. In addition gap junctions have been associated with many diseases including cancer [9,10], muscular atrophy [11], some genetic diseases and demyelinating diseases [12].

This type of intercellular crosstalk can be evaluated by several methods[13-16]. In this paper, we show how to perform a single-cell microinjection of Lucifer Yellow to visualize cellular communication via gap-junctions in living cells. We discuss how to prepare the cells and the micropipette, the usage of the micromanipulator and the injection of Lucifer Yellow dye in a thymic epithelial cell line. Usually, this experimental procedure could be analyzed by the average of connected cells to the cell loaded with dye. In addition, this method could be used with other fluorescent dyes with molecular weight below the gap junctions` cut-off which is approximately 1000 daltons.

Protocol

1. Preparation of Cells

1.1. Maintain a culture of a thymic epithelial cell line (IT76M1) or cell to be tested in an incubator (37°C/5% CO_2).

1.2. Wash the cells with PBS 1x (repeat this item 3x).

1.3. Add Trypsin to the cells for 5 minutes.

1.4. Add medium (twice of the volume of trypsin added in item 1.3) with 10% FBS (fetal bovine serum) to the cells with trypsin and centrifuge (800 x g for 5 minutes).

1.5. Count the cells in a hemocytometer.

1.6. Adjust the density of cells according to the cell type as the cells have to be in close contact with each other to allow coupling. Note: In our case, we used $3x10^5$ cells per 35 mm Petri dish.

2. Micropipette preparation

2.1. Pull the micropipette as specified from a glass capillary micropipette (1.5 mm diameter) to a final 0.2 µm of diameter so as to attain a final resistance of approximately 30MΩ[17,18].

Note: Alternatively, injection pipettes can be purchased. The resistance depends on the cell size, for instance a higher resistance microelectrode would be necessary for pancreatic acinar cells, for example (100-150 MΩ) [19]. A common problem that could occur is the precipitation of Lucifer Yellow solution which can then obstruct the micropipette and may require prior filtration or centrifugation. Before injection, the micropipette should be analyzed under the microscope to detect if there is an obstruction or any type of disruption [13]. The micropipette can be tested by injecting LY with the micropipette tip inside a saline solution.

3. Testing the Micropipette

3.1. Prepare the Lucifer yellow solution (5%) in 150 mmol/L LiCI and load the micropipette using a syringe or by backfilling (put into it LY Solution).

3.2. Place the micropipette over the 35mm Petri dish with the IT76M1 cells on the microinjection workstation and submerge the tip of the glass micropipette into the cell medium. Focus on the micropipette and perform a dye flowing test by applying a pulse.

4. Single-cell Lucifer Yellow microinjection

4.1. Focus the microscope right above the cell layer using a high magnification (40x), then slowly lower the pipette to the cells using the micromanipulator.

4.2. Puncture the target cell when the tip is close enough to touch the cell membrane, and apply a small hyperpolarizing pulse to introduce the LY into the cell. The applied voltage will depend on the net charge of dye to be injected.

4.3. Capture cell images 3 minutes after dye injection or make a small movie with time lapse microscopy (30 fps).

Note: A similar approach could be seen in Hitomi et al (2015)[20]. To avoid communication by intercellular bridges (incomplete mitosis), a co-injection of rhodamin dextran (from 2 to 10 KDa), which does not pass through gap junctions but passes through intercellular bridges and certain types of nanotubules is recommended as shown in Figure 2.

Representative results

Thymic epithelial cell line IT-76MI were used to evaluate dye coupling by gap junctions as these cells were described to express functional gap junctions formed by connexin 43 [21]. Figure 1 shows the injection of Lucifer Yellow when applied in the one cell below

the tip of the pipette. After few minutes, connected cells become fluorescent (asterisks) indicating the diffusion of the fluorescent dye through the gap junctions. The number of cells and time to became fluorescent is directly associated with the degree of cellular communication among these cells.

Figure Legend:

Figure 1 – Lucifer Yellow injection in IT-76MI cells. A) The micropipette close to the cell membrane. B) The pipette is seen by fluorescence microscopy. C) A test pulse was generated to verify whether the electrode is actually injecting the dye. D) The pipette touched the cell membrane and it was charged with Lucifer Yellow dye. E) The cell charged allowed the dye to pass through gap junctions to at least five neighbor cells (indicated by the arrows), X20. F) A digital zoom was done to allow better visualization. Asterisks point out the cells that were charged in the contrast phase microscopy (Figure 1A).

Figure 2 – Lucifer yellow injection in thymic epithelial cells (TEC). Phase contrast and fluorescence microscopy. A) and B) Human Thymic Epithelial cell. C) and D) Thymic Nurse Cell, E) and F) A Mouse Thymic Epithelial cell line, respectively. The insert in (D) shows the same cell (arrow) after an injection of rhodamine-dextran 10KDa (not permeable through gap junctions). The inserts in (E) and (F) show the absence of dye transfer in the TEC line pre-treated with octanol 1 mM (a gap junction blocker) for 10 min. In all panels asterisks mark the injected cells. (A)-(F) X 200. Reproduced from Alves et al., 1995[5].

Figure 3 - Gap junction communication increased by dexamethasone in a rat epithelial cell line. TEC were treated with 1µM dexamethasone, and coupling degree was evaluated by the Lucifer Yellow dye transfer assay. A) Microscopy fields (phase contrast and fluorescence, respectively, in the left and right panels) depicting the injected cell and those that were coupled when LY was injected (magnification 320X). In all panels asterisks mark the injected cells. B) Histograms showing the pattern of coupling of control and dexamethasone-treated cells. The analysis comprises of 100 microinjections per group. Reproduced from Alves et al., 2000[23].

Discussion

In order to verify the presence of functional intercellular gap junction, the use of tracers, which are membrane impermeable, although permeable by intercellular channels are required [16]. Fluorescein, the first fluorescent dye to observe cell-to-cell coupling [22], is permeable between non junctional membranes [3] and has therefore been substituted by Lucifer yellow dye [15]. Currently, to find the best choice among the many different types of florescent tracers depends on the scope and conditions of the experiment. The procedure of cell loading with fluorescent dyes permits the evaluation of morphology, function of single cells and the kinetic rate of transfer between cells. Furthermore, dye microinjection allows a better understanding of the physiological role

of gap junctions between cells[21,23], since the degree of cellular communication is related with the number of coupled cells.

Several key factors are crucial for successfully obtaining microinjection data. Normal cell homeostasis and integrity must be maintained, thus a short injection time (<1s) of dye and technical expertise with microinjection is needed. Key factors in getting a better resolution of the captured images is having a cooled CCD camera, the fluorescence filters in place and the use of a high NA objective[14]. Also, some tips could be useful as: 1 - cell culture dishes with grids are recommended to visualize a particular injected cell, also glass-bottom dishes should be used for high-resolution microscopy applications; 2 - it is recommended to make 10-20 pulled needles before starting microinjections; 3 - depending on the resistance of the electrode tip can be very helpful, loading the micropipette with the dye using capillarity by touching the pipette tip into the dye solution; 4 - it is not necessary to load the whole body of the pipette, a few microliters to fill the tip and 2 to 3 mm above the tip is sufficient; 5 - start the experiment with a less magnified lens and try to see the shadow of the pipette walls. Afterwards, move the pipette as close as possible and before touching the cell, shift to the higher magnification objective, such as 40x or greater; 6 - Some groups use a pneumatic microinjector. Pneumatic injection is not the best choice since it is not precise and could inject unknown volumes; with a pulse the protocol could be standardized to inject a known volume of dye. Additionally, it is recommended to inject into the membrane above the cytosol because of the depth; injection in the membrane above the nucleus could injure it and affect cell physiology. Finally, cells that are not too flat are preferable over flat ones.

In summary, this method is effective to study intercellular communication by gap junctions but needs expertise/experience and good material and equipment to obtain high quality data. We hope that this article and video help beginners to understand and perform this technique.

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Disclosures

The authors have no conflicts of interest.

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Figure 1

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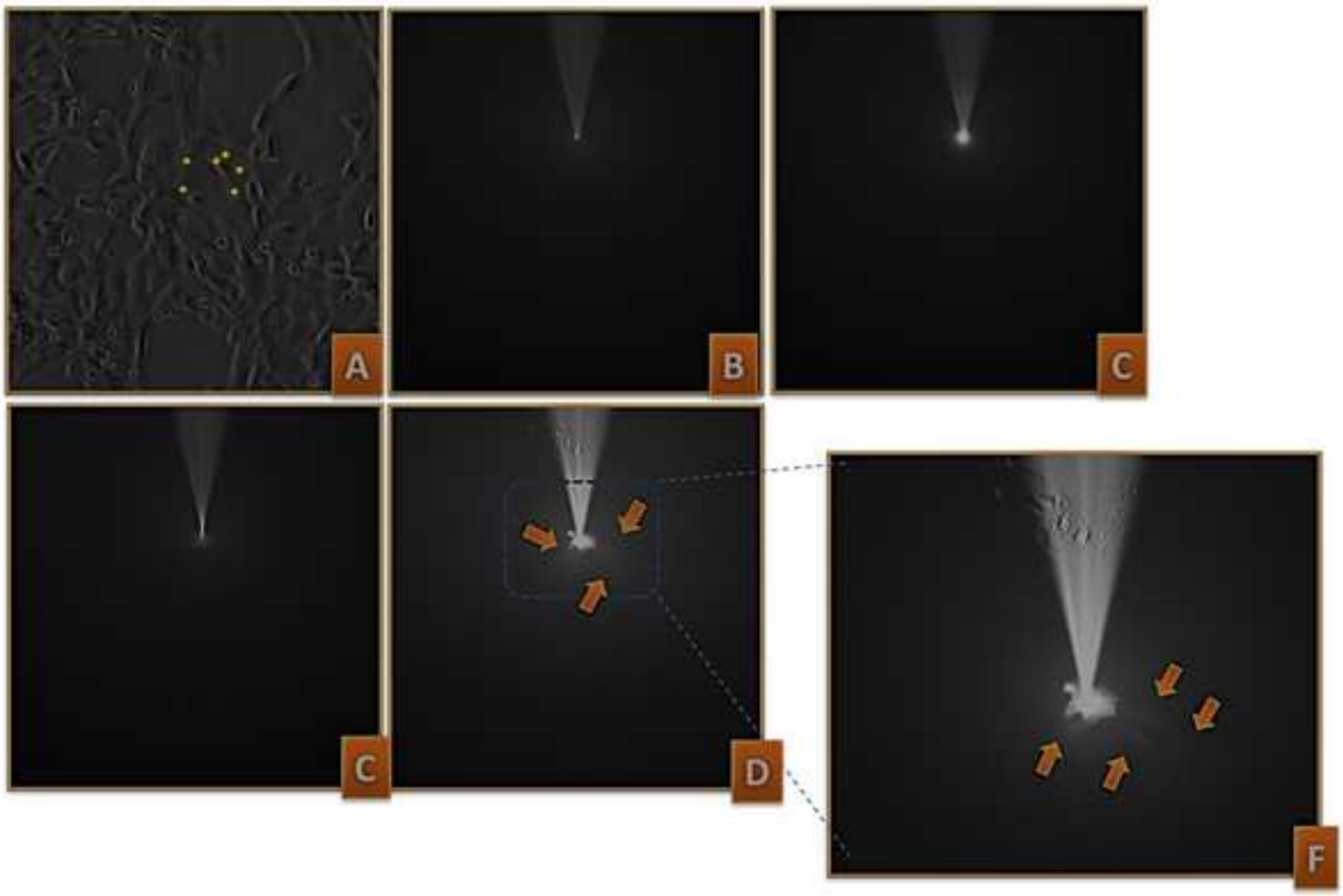

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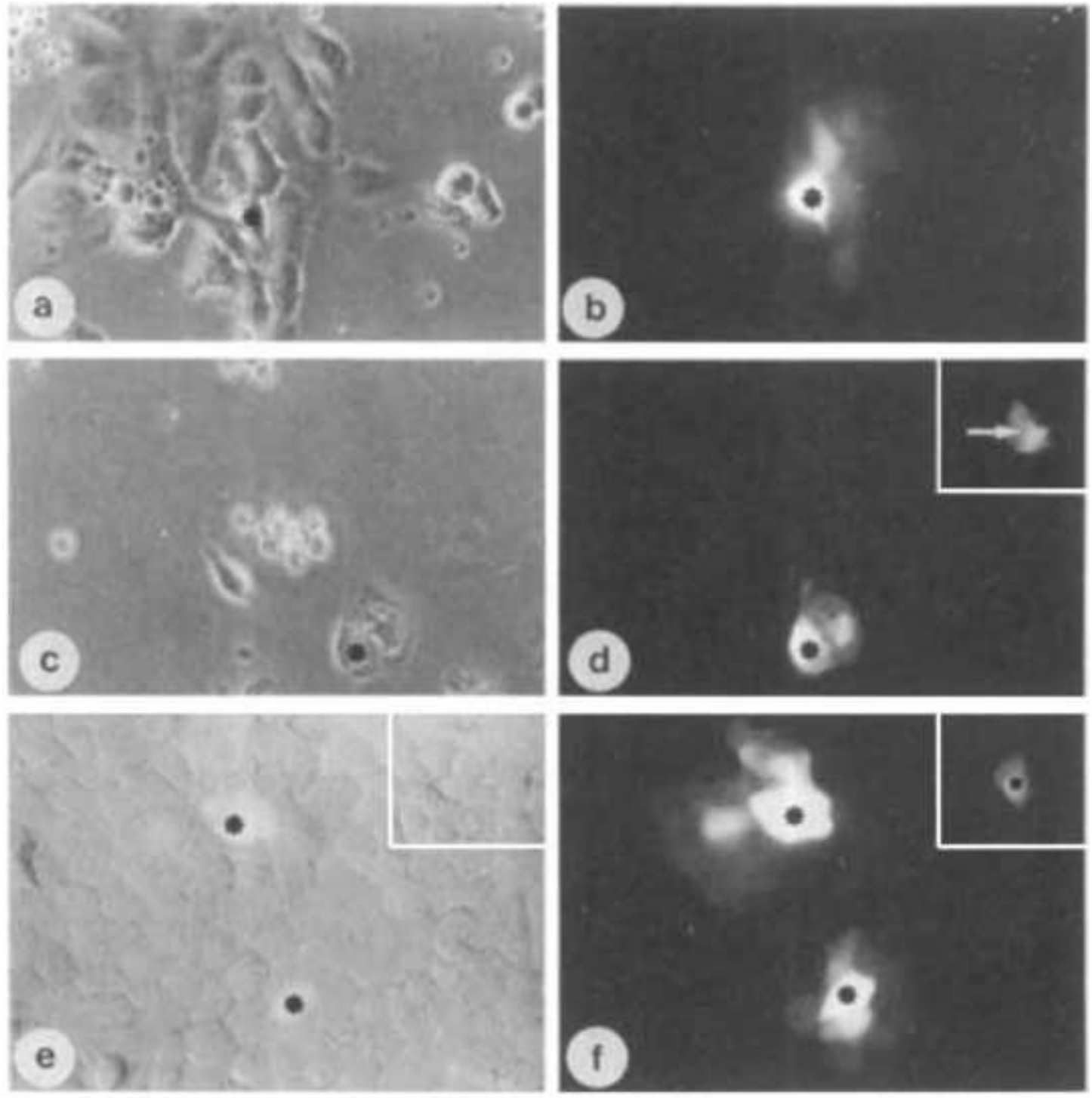

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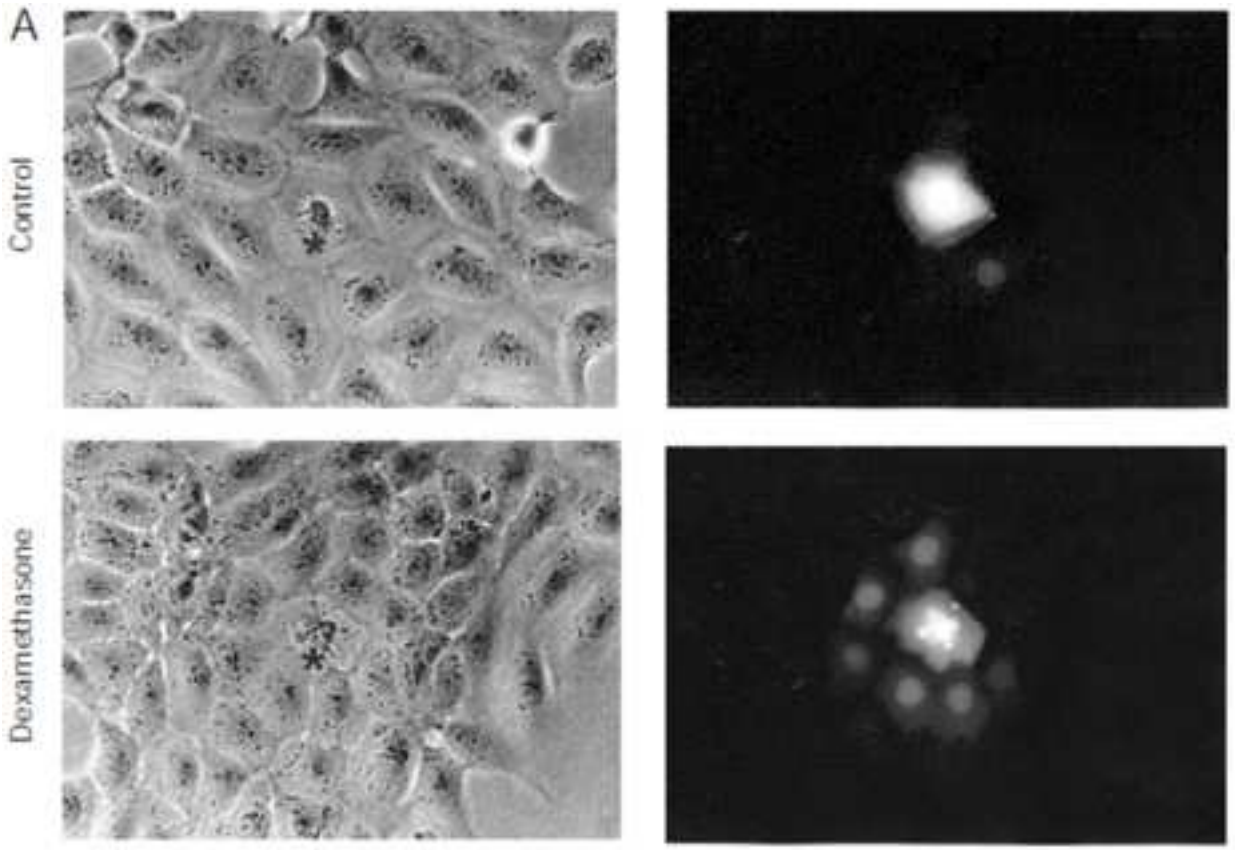

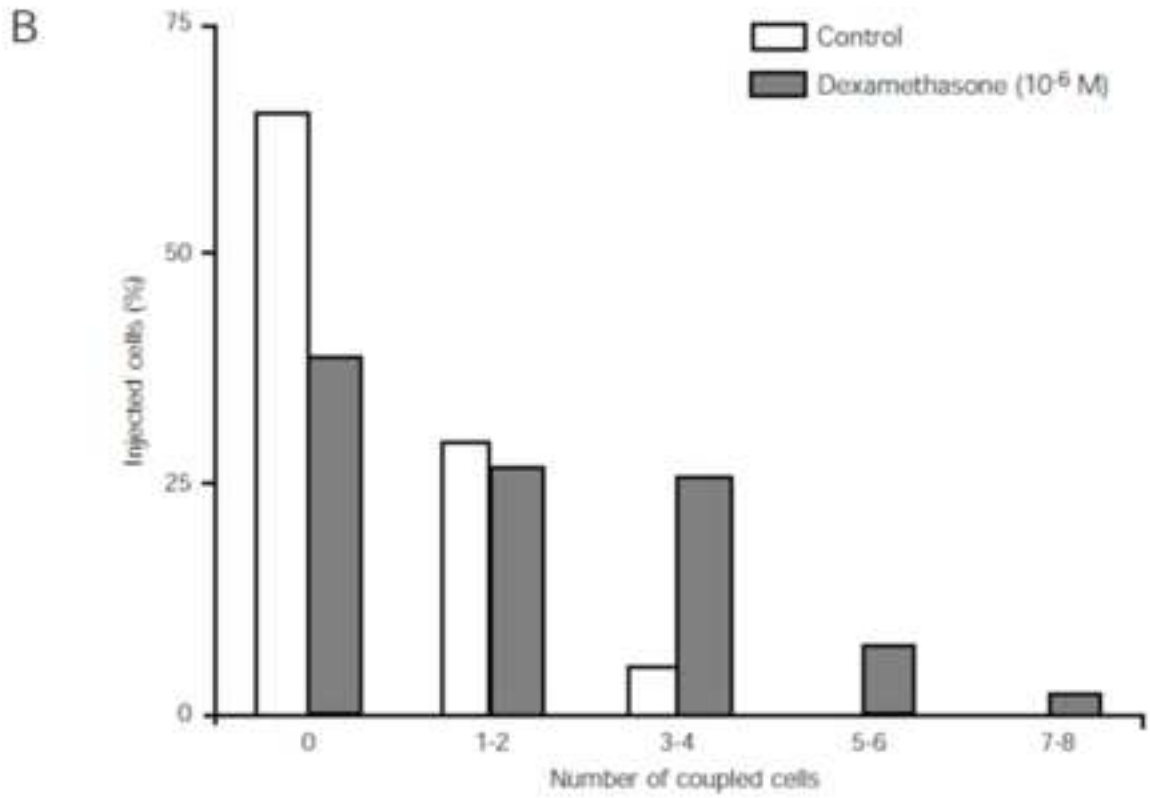

Alves et al.,2000

Table

DYE	MW	Excitation/Emission
Hydroxycoumarin carboxylic acid	206	386/448
Calcein blue	321	360/449
4',6-diamidino-2-phenylindole dihydrochloride	279	358/461
Carboxyfluorescein	376	492/517
ethidium iodide(bromide)	314	518/605
Lucifer yellow CH	443	428/536
Alexa fluor 488	570.5	495/519
Calcein	622	494/517
Propidium iodide	414	535/617

*Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/Material	Company	Catalog Number
Lucifer yellow	Sigma	L0259
Lithium Chloride	Sigma	L4408
PBS tablets	Sigma	P4417
RPMI	Sigma	R4130
Bovine fetal serum	Cultilab	
Trypsin	Sigma	T4799
Microscope	Nikon	TE-2000
vibration-insulated table	Newport	VH3036W-OPT
Micromanipulator	Narishige	MMO-203
Current Generator	Digitimer	DS2

Comments

For microinjection experiments, one needs an inverted fluorescence microscope and filters for fluorescent microscopy
A vibration-insulated table is needed to protect the experiments from vibration and avoid cell damage
This equipment allows precision adjustments of the micropipette, which is needed for cell micro injection.

To produce the dye flow through the micropipette, a current below one nano ampere was given using a current generator with an electrode inside the micropipette or an amplifier which has a capacitance compensation circuit (old electrometer) or current injection functions of new patch clamp amplifiers, and the ground wire submersed in the plate dish. Alternatively, the dye can be injected by a pneumatic microinjector, following the factory recommendations.

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Rio de Janeiro, June 19, 2016

Manuscript. No.: **JoVE50836R5**
Journal of Visualized Experiments

Dear Dr. Sephorah Zaman**:**
Please find enclosed the revised manuscript entitled "**Single-cell Microinjection for cell communication analysis**" First of all, we thank the reviewers for their generosity in the corrections and for the comments that have contributed to improve our article. As you will see, we have followed most of the reviewers` suggestions. The critiques were addressed point by point below. The major changes are labeled in red font in the manuscript.

We hope that the manuscript is now publishable in JOVE. We look forward to hearing from as soon as possible.

Sincerely,
Anael Viana P. Alberto
Cellular Communication Laboratory
Oswaldo Cruz Institute-Oswaldo Cruz Foundation-FIOCRUZ
Rio de Janeiro/Brazil

LIST OF CHANGES IN THE REVISED VERSION AND COMMENTS

Editorial comments:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made minor copy edits to your manuscript and formatting changes to comply with the JoVE format. Please maintain these changes. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word.

Response: The request was followed by the authors.

2) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Response: We made a new revision.

3) Please disregard the comment below if all of your figures are original.
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: We added a figure published by our group and we will follow the request.

4) Please address the following points with respect to your video component:
a) There are still some title cards that are centered and some with the text in the bottom right of frame. If these are all meant to be chapter title cards, they should be numbered and centered. If just the centered ones are meant to be chapter title cards, just number those.

Response: It was changed as requested.

b) 8:52 - A numbered chapter title card reading "Conclusion" should be inserted here.

Response: The request was followed by the authors.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this manuscript, Alberto et al. described the microinjection technique in details with visualized examples. Microinjection is a powerful technique for delivery exogenous materials including proteins, peptides, DNA, drugs into cells, making it is possible to transduce cells that normally are difficult to transfect or infect, such as primary neurons. As a single cell technique, it is also challenging by its manipulation and injection. This manuscript is very helpful to visualize the updated microinjection.

Major Concerns:
N/A

Minor Concerns:
In Discussion, about the critical factors for successful microinjection, the author can add comments about injection site, such as cytosol or nucleus. This would greatly affect the injection results.

Response: We agree with the reviewer and this comment was addressed in the discussion.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The authors show how to microinject cells with fluorescent markers to evaluate transfer to other cells through gap junctions. This approach has been used by many scientists for many many years.

Major Concerns:
Images are not the best quality. Increase contrast and do not use red asterisks on black background. The square showing the phase pix in the video adds little or nothing and is not referred to. There are no scale bars. Information on how often the images were captured in the timelapse should be included. Discussion of how gap junction function can be carefully characterized by injecting dyes of different MWs and colors should be described. Also, one should always include an injection marker that is not gap junction permeable like high MW rhod or fluorescein conjugated antibodies as a positive control for injection and show expts where cells are treated with drugs to close gap junctions as a negative control.

Response: We agree with the reviewer all requests were added in the paper. And a figure with different dyes and the presence of a gap junction blocker was added in the paper.

Minor Concerns:
Video should be shorter.

Response: The original video had 15 minutes and the editorial office requested it to be shorter, so we cut off some minutes already.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The manuscript describes a step by step procedure to assess functional gap junction activity utilizing microinjection and fluorescent imaging. This approach has been used previously so the degree of novelty is limited, however, it is useful for the community to have access to a step by step protocol and visualization.

Major Concerns:
*Line 50, "giving some tricks" Does this mean "useful tips" to the readers?

Response: We thank the reviewer for the comment and we changed the text.

*Lines 92-93
"this method could be used with other fluorescent dyes with molecular weight below the gap junctions` cut off which is approximately 1000 daltons." Have authors tested if any dye with molecular weight above 1000 daltons? This would be a very important control, especially in the context in which moelcules larger than 1kDa have been reported to be transported in a gap junction-dependent manner (i.e. miRs).

Response: We agree with the comment and we added a figure in the manuscript of an experiment with the addition of rodhamine dextran 10KDa that is too big to pass through gap junction. Moreover, we also added in the same figure an injection of LY in the presence of a gap blocker (octanol).

*Line 98-112
In the Section 1: Preparation of Cells, steps from 1.1 to 1.5 are dispensable. The cell density information 3x106 per 35 mm dish may be critical. Author should make it clear that the density of the cells need to be adjusted according to the cell type and the most importantly the cell density needs to be adjusted so that cells contact each other.

Response: We thank for the suggestion and we shortened this item and added the information requested.

*Line 117, "Pull the micropipette as specified"
Provide more detailed information about "as specified", or cite reference(s) from which readers can obtain information.

Response: The reference was cited in the manuscript.

*Line 131, "Prepare the Lucifer yellow solution (5%) in 150 mmol/L LiCl"
Why did authors choose 150 mM LiCl? Is there any possibility that injected Li ion alters membrane potentials, which can alter gap junction functionality? Or, isn't it possible that injected Li ion may inhibit glycogen synthetase kinase beta (GSK beta) and change the cellular physiology?

Response: The reviewer question is relevant! The Lucifer yellow is a lithium salt, and in general depending of the concentration has not a good solubilization in KCl saline solution. Moreover no electrophysiological changes was reported in LY dye coupling experiments (J. Cell. Mol. Med. Vol 16, No 1, 2012 pp. 22-31). This technique was used for years in a wide spread way. (Curr. Protoc. Toxicol. (2009) 41:2.17.1-2.17.10 - Assessment of Gap Junctional intercellular communication; Bioelectrochemistry 69 (2006) 187–192 - Automatic positioning of a microinjector in mouse ES cells and rice protoplasts). Also, depending on how the injection was made it can alter the gap junction functionality, because of that the experiment is repeated many times and the observation of the morphology of the cell during the experiment are important.

*Line 148-151, "Avoid communication by intercellular bridges (incomplete mitosis), a co-injection of rhodamin dextran (from 2 to 10 KDa), which does not pass through gap unctions but passes through intercellular bridges and certain types of nanotubules is recommended."

If authors have images that indicate the utility of rhodamin dextran (2-10KDa), these images should be included into the figures.

Response: It was added in the results.

*Line 161-162, "After few minutes, connected cells become fluorescent (asterisks) indicating the diffusion of the fluorescent dye through the gap junctions."
As discussed by the authors in the previous paragraph (line 148-151), this diffusion of dye to the neighboring cells might be through intercellular bridges. In order to rule out this possibility, the data from co-injection of rhodamin dextran and lucifer yellow are required.

Response: We added a figure 2 with the co-injection of rodhamine dextran 10KDa and LY.

*Line 177-178, "since the degree of cellular communication is related with the number of coupled cells"

It would be nice if author can show the data of quantification of number of coupled cells after injection described in this paper in the presence and absence of gap junction inhibitor(s).

Response: We added the figure 3 with an experiment showing the quantification of coupled cells.

Minor Concerns:
A recent paper described a very similar approach in a highly quantitative fashion to analyze importance of gap junction in cancer stem cells (Hitomi, M., et al. Cell Rep 11, 1031-1042. 2015) and should be cited.

Response: The paper is very interesting and was cited in the manuscript.

Additional Comments to Authors:
N/A

Reviewer #4:
Manuscript Summary:
This is an interesting and well documented study.

Major Concerns:
N/A
Minor Concerns:
N/A
Additional Comments to Authors:
N/A

Reviewer #5:
Manuscript Summary:
This is a straight-to-the point description of the dye microinjection technique. Well done.

Major Concerns:
None

Minor Concerns:
The dye coupling technique may not always test for gap junction functionality if not adapted to the connexin type. For example, Lucifer Yellow will not diffuse through Cx30 channels while they are fully functional.

Additional Comments to Authors:
I suggest to the authors to add a table of the commonly used fluorescent dye and their characteristics (excitation/emission wave lengths, MW and charge).

Response: A table was added in the manuscript.

Reviewer #6:

Manuscript Summary:
The manuscript presented a single-cell microinjection method applying hyper polarizing pulses to visualize cellular communication via gap-junctions in living cells. The video spent too much time introducing the microinjection setup. This should be cut down and the time be used for introducing more details of the biological protocol. Otherwise, the value of the video/manuscript will be rather limited to readers.
Comments on Protocol:
1.line110, the authors may give the reason for why the operator needs to count cell numbers or give the acceptable cell density for the injection experiment.

Response: We changed the protocol manuscript. We give a protocol specific for IT76 cells, but according to the type or size it have to be adjusted.

2.Line 117-118, the authors should introduce measurement or calibration method for the resistance of the micropipette with a diameter of 0.2 um instead of simply stating "to have a final resistance of approximately 30MΩ. " If the value is derived from other publications, please cite references.

Response: The reviewer is right! We added 2 more references to guide readers

3. Line 136-137, the authors may give the magnitude value of the pulse to test the micropipette.

Response: The pulse has to be between 1-2 nA. We added this information to the manuscript (J. Cell. Mol. Med. Vol 16, No 1, 2012 pp. 22-31)

4. Line 147-151. As the authors stated that the "communication by intercellular bridges" are also attributed to the cell screening results, the author should carefully evaluate the effect on the experimental results or add the injection experiments on "rhodamin dextran (from 2 to 10 KDa)" to evaluate its effect.

Response: We added the figures with rhodamine dextran 10KDa and another with a gap blocker.

5. The injection success rate and transfer distances derived in the experiments are important to discuss for proving the effectiveness of the proposed method.

Response: **The dye coupling rate is an indirect measure of how much the cell is expressing functional connexins. This technique was used in hundreds of paper studying gap junctions since the 80´s.**

6. Line195. The authors should discuss comparisons of using pneumatic injectors and the proposed method.

Response: It was added in the discussion.

Major Concerns:
N/A

Minor Concerns:
N/A