Video Article

Single-cell Microinjection for Cell Communication Analysis

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/50836/

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[3,4,5,6,7,8]. 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[13,14,15,16]. 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 1,000 daltons.

Protocol

1. Preparation of Cells

1. Maintain a culture of a thymic epithelial cell line (IT76M1) or cell to be tested in an incubator (37°C/5% CO_2).
2. Wash the cells with PBS 1x (repeat this item 3x).
3. Add Trypsin to the cells for 5 min.
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 min).
5. Count the cells in a hemocytometer.
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 3 x 10^5 cells per 35 mm Petri dish.

2. Micropipette Preparation

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 30 MΩ[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

1. Prepare the Lucifer Yellow solution (5%) in 150 mmol/L LiCl and load the micropipette using a syringe or by backfilling (put into it LY Solution).
2. Place the micropipette over the 35 mm 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

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.
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. Alternatively, some other dyes could be used with this technique as shown in Table 1.
   Note: In principle any hydrophilic dye with MW less than 1KDa could be used. However, the rate of transfer could vary according to the weight and hydrophilicity. Additionally, unspecific transfer of the dye used must be evaluated.
3. Capture cell images 3 min 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 2** shows the injection of LY in thymic epithelial cells and the inserts (insert d) show the co-injection of LY and Rhodamine Dextran (10KDa). As expected the LY passes to a neighboring cell, although the Rhodamine Dextran does not because of its high molecular weight. In addition, the presence of a GJ blocker (insert f), octanol, blocked the passage of the dye to the surrounding cells. Alternatively, one can evaluate the degree of coupling of a specific cell or the effect of a drug on the function of GJ. Figure 3A shows the injection of LY in TEC cells with or without dexamethasone. Then 100 cells were injected, in the presence of dexamethasone and the percentage of 1 or 2 cells communicating was similar in relation to the control without the drug. However the number of 3 or 4 cells communicating was higher when compared to control. Five or 6 cells and 7 or 8 cells with GJ communication in the presence of dexamethasone was not observed in the control, thus indicating that dexamethasone increased the degree of coupling in TEC cells.

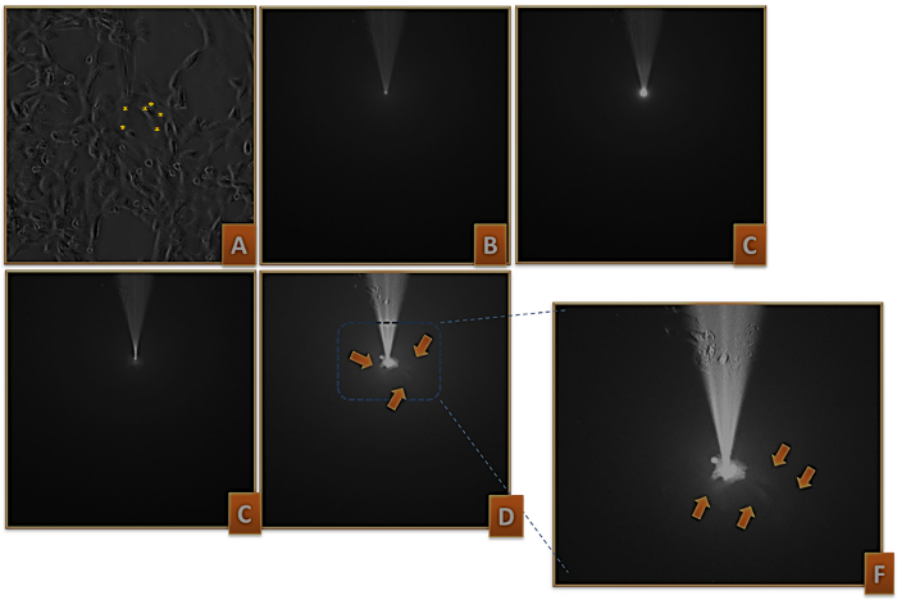

Figure 1: Lucifer Yellow injection in IT-76MI cells. (**A**) The micropipette close to the cell membrane. (**B**) The pipette is seen by fluorescence microscopy. (**C**) A test pulse was generated to verify whether the electrode is actually injecting the dye. (**D**) The pipette touched the cell membrane 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). Please click here to view a larger version of this figure.

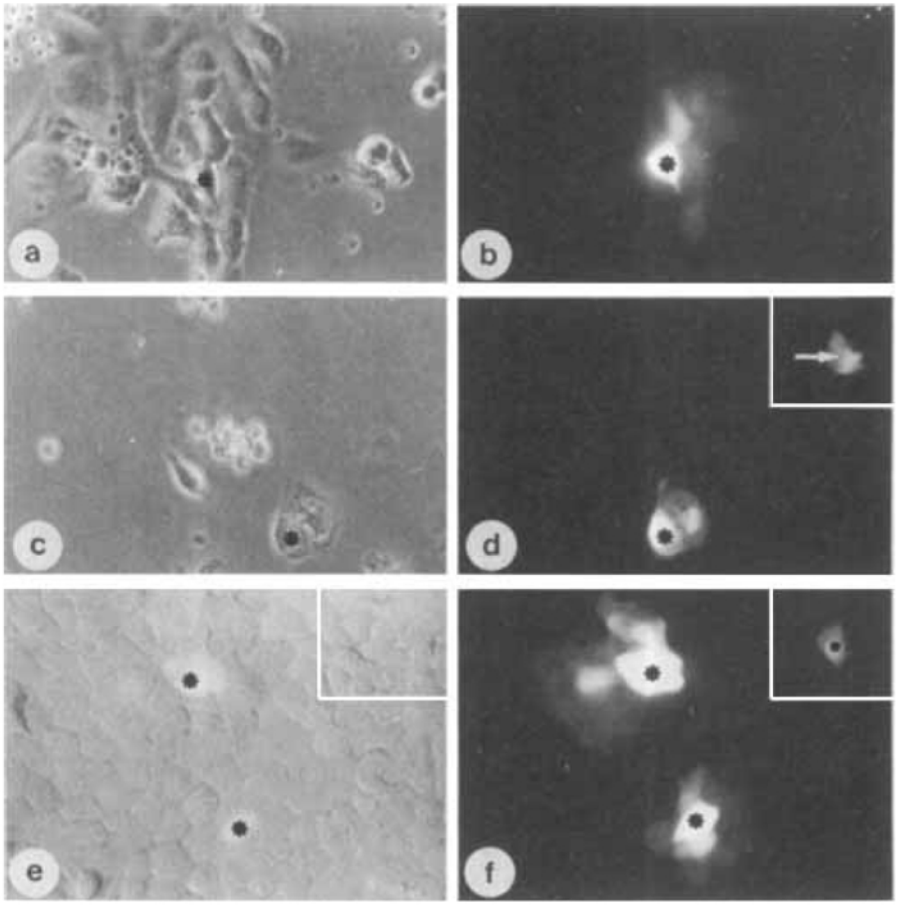

Figure 2: Lucifer Yellow injection in thymic epithelial cells (TEC). Phase contrast and fluorescence microscopy. (**A** and **B**) Human Thymic Epithelial cell. (**C** and **D**) Thymic Nurse Cell, (**E** and **F**) A Mouse Thymic Epithelial cell line, respectively. The insert in (**D**) shows the same 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]. Please click here to view a larger version of this figure.

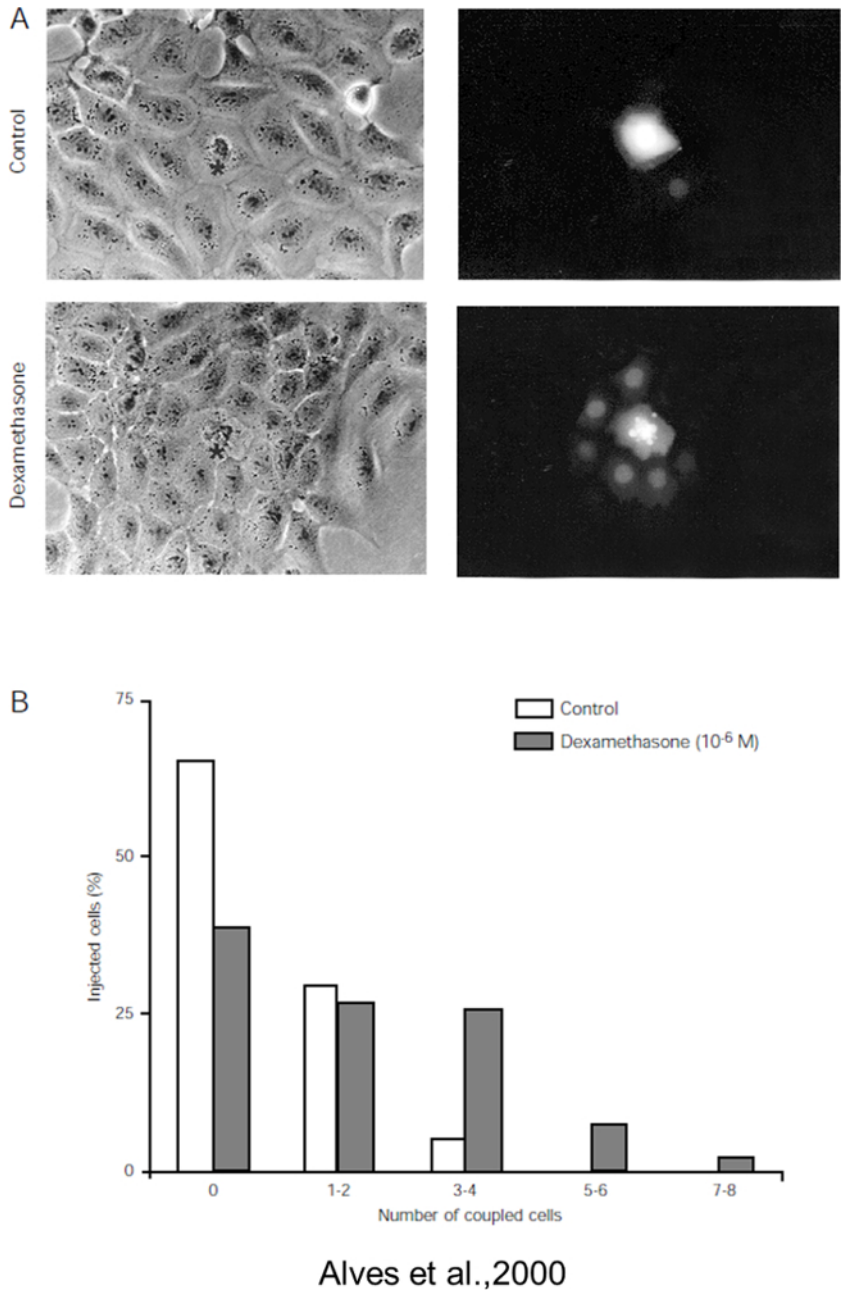

Figure 3: Gap junction communication increased by dexamethasone in a rat epithelial cell line. TEC were treated with 1 µM dexamethasone, and coupling degree was evaluated by the Lucifer Yellow dye transfer assay. (**A**) Microscopy fields (phase contrast and fluorescence, respectively, in the left and 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]. Please click here to view a larger version of this figure.

DYE	MW	Excitation/Emission
hydroxycoumarin carboxylic acid	206	386/488
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

Table 1: Dyes currently used for microinjection experiments. Here in, some used dyes with molecular weight and the excitation/emission wavelength.

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 (<1 s) 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.

Disclosures

The authors have no conflicts of interest.

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