Video Article

A Functional Assay for Gap Junctional Examination; Electroporation of Adherent Cells on Indium-Tin Oxide

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Abstract

In this technique, cells are cultured on a glass slide that is partly coated with indium-tin oxide (ITO), a transparent, electrically conductive material. A variety of molecules, such as peptides or oligonucleotides can be introduced into essentially 100% of the cells in a non-traumatic manner. Here, we describe how it can be used to study intercellular, gap junctional communication. Lucifer yellow penetrates into the cells when an electric pulse, applied to the conductive surface on which they are growing, causes pores to form through the cell membrane. This is electroporation. Cells growing on the nonconductive glass surface immediately adjacent to the electroporated region do not take up Lucifer yellow by electroporation but do acquire the fluorescent dye as it is passed to them via gap junctions that link them to the electroporated cells. The results of the transfer of dye from cell to cell can be observed microscopically under fluorescence illumination. This technique allows for precise quantitation of gap junctional communication. In addition, it can be used for the introduction of peptides or other non-permeant molecules, and the transfer of small electroporated peptides via gap junctions to inhibit the signal in the adjacent, non-electroporated cells is a powerful demonstration of signal inhibition.

Video Link

The video component of this article can be found at http://www.jove.com/video/51710/

Introduction

The application of electrical current to a cell causes the formation of pores on the cell membrane, by a process termed electroporation. The pores allow the passage of a variety of nonpermeant molecules through the membrane. The electrical field can be controlled precisely, so that the pores formed are very small and reclose rapidly, with minimal disturbance to the cellular physiology. Interestingly, adherent cells can be grown on a glass slide coated with conductive and transparent indium-tin oxide (ITO) and electroporated *in situ*, that is on the surface where they grow, without being detached to be electroporated in suspension. Cells can grow very well on this surface, and as they are attached and extended, detailed microscopic observation is possible. Using this technique, small nonpermeant molecules can be introduced instantly and into essentially 100% of the cells, which makes this technique especially suitable for studies on the activation of components of a pathway following ligand stimulation of a receptor (reviewed in[1]).

Electroporation has been used mostly for the introduction of DNA (also called electrotransfection). However, electroporation *in situ* can be valuable for the introduction of a large variety of molecules, such as peptides[2-4], oligonucleotides, such as antisense RNA, double-stranded DNA decoy oligonucleotides to inhibit transcription factor binding, or siRNA[3,5,6], radioactive nucleotides[7-10], proteins[1112] or pro-drugs[13]. Following electroporation, cells can be lysed for biochemical analyses or fixed and stained with antibodies.

The conductive ITO coating is very thin, 800-1,000Å, so that cell growth is not disturbed by the difference in height of the two surfaces, as the cells grow across the edge of the conductive coating. This offers the advantage that non-electroporated cells can be grown side by side with electroporated ones, to serve as controls. The same approach can be used for the examination of gap junctional, intercellular communication (GJIC), as described in the Video.

Gap junctions are channels connecting the interiors of adjacent cells[14]. Gap junctional, intercellular communication plays an important role in tumor formation and metastasis, while oncogenes such as Src suppress GJIC[15,16]. To examine GJIC a fluorescent dye such as Lucifer yellow (LY) is often introduced into cultured cells through microinjection or scrape-loading[17] and the diffusion of the dye into neighboring cells is microscopically observed under fluorescence illumination. These techniques however invariably cause cell damage. We now describe a technique where cells are grown on a glass slide which is partly coated with ITO[18]. An electric pulse was applied in the presence of LY (or other dyes) causing its penetration into the cells growing on the conductive part of the slide, while dye migration to the adjacent, non-electroporated cells was microscopically observed through fluorescence illumination. To avoid disturbing sensitive cells that may tend to detach from the monolayer, an assembly was designed that did not require an electrode to be placed on top of the cells to apply the electrical current[19]. This approach offers the ability to quantitate GJIC in a large number of cells, without any detectable disturbance to cellular metabolism, as indicated

by the absence of an effect on the length of the G1 phase following serum stimulation[12], increase in the levels of the fos protooncogene protein (Raptis, unpublished) or two kinases associated with cellular stress, the $p38^{hog}$ or JNK/SAPK kinase[1]. This approach made possible the examination of the link between ***levels*** of oncogene expression, transformation and GJIC[18], as well as the effect of Src and Stat3 upon GJIC in a variety of cell types, including cells freshly cultured from lung tumor specimens[20-23]. In addition, *in situ* electroporation with the setup described which lacks a top electrode has been successfully employed for the demonstration of gap junction closure upon adipocytic differentiation, although cellular attachment to the substrate is reduced at that stage[19,24].

Protocol

1. Plating the Cells in the Electroporation Chambers

1. In a laminar-flow hood, trypsinize the cells using sterile technique as usual.
   NOTE: It is very important to eliminate by centrifugation all traces of trypsin, because they may hinder spreading of the cells on the glass, hence the formation of adherens and gap junctions.
2. Pipette 1 ml of the cell suspension in the sterile electroporation chambers provided with the *in situ* electroporator (**Figure 1**), and place in a 37 ^{o}C, CO_2 incubator.
   NOTE: Cell adhesion can be improved by plating on fibronectin, collagen, poly-lysine or cell and tissue adhesive (see Materials Table).
3. When the cells have formed a confluent layer they are ready for GJIC examination.

2. Electroporation Procedure

Electroporation for GJIC examination can be conducted outside a laminar-flow hood, since it takes only a few minutes. If longer incubation times are required for a specific experiment, then it can be conducted entirely in a laminar-flow hood. In all cases, the chambers where the cells are grown must be sterile.

1. Prepare a 5 mg/ml Lucifer yellow solution: Dissolve 10 mg LY powder (provided with the electroporator) in 2 ml Calcium-free growth medium. For experiments that requires longer incubation times, filter-sterilize the solution and store at 4^{o}C.
2. Aspirate the growth medium and wash the cells with Calcium-free medium, being careful not to scratch the monolayer or dry the cells. If the cells are dried they usually have darker nuclei and pick up LY without electroporation. The effect is more pronounced in the middle of the slide which is more exposed to air drafts (**Figure 4F**).
3. With an Eppendorf pipettor, pipette the dye solution to the cells (400 µl for the whole chamber), at the edge of the chamber, being careful not to touch the cell layer.
4. Place the chamber in the holder supplied with the electroporator and apply a pulse of the appropriate strength (see Discussion).
5. Carefully aspirate some of the LY solution. It can be reused a second time for less important experiments.
6. Add Calcium-free DMEM containing 10% dialysed serum.
7. Incubate the cells for 3-5 min in the incubator to allow dye transfer through gap junctions.
   NOTE: The inclusion of dialysed serum at this point helps pore closure.
8. Wash the unincorporated dye with Calcium-free DMEM for live cell observation. Alternatively, cells can be fixed at this stage, through the addition of 4% formaldehyde to the well, then washed with PBS (phosphate-buffered saline). In all cases the washing must remove all background.
   NOTE: In preliminary experiments to determine the optimal conditions, if the voltage is too low, then it is possible to let the cells recover in the incubator for a few minutes and electroporate the same slide a second time, to save in the cost of slides.

3. Microscopic Examination

1. Observe the cells under fluorescence illumination, using an inverted microscope equipped with the appropriate filter for the dye being used. For Lucifer yellow, excitation is 423nm, emission 555nm, WBV filter for a Nikon IX70 microscope.
2. Eliminate meniscus effects by placing a glass cover-slip on top of the plastic well, after filling it to the top with liquid, to examine cell morphology under phase contrast. For better pictures, the well can be detached from the slide, and the coverslip placed on the frame. For fixed cells, the well can be filled with glycerol for microscopic observation, while for live cells it must be filled with growth medium.
   NOTE: Washing and photographing is easier if the cells are fixed with formaldehyde following the transfer of the dye (step 2.7 above). If the cells are not fixed, fluorescence is eliminated from the cells within approximately 60 min depending upon the cell type, voltage and amount of LY introduced while in fixed cells fluorescence is retained for several hours. However, fluorescence fades or dissipates after O/N incubation, even in fixed cells. For this reason, photographs must be taken soon after electroporation (**Figure 2**).

4. Quantitation of Intercellular Communication

1. Photograph the cells with a 20x objective under fluorescence and phase contrast (**Figure 3**).
2. Identify and mark with a star the electroporated cells at the border with the non-electroporated area (**Figure 2**, arrow, electroporation edge).
3. Identify and mark with a dot fluorescing cells on the non-electroporated side, where the dye has transferred through gap junctions (**Figures 3A** and **3B**).
4. Divide the total number of fluorescing cells on the non-electroporated area by the number of electroporated cells along the edge (**Figures 3A** and **3B**).
   NOTE: The transfer from at least 200 contiguous electroporated border cells is calculated for each experiment. The number obtained is the GJIC value.

Representative Results

Figure 2 shows rat liver epithelial T51B cells[22], electroporated with LY and photographed under fluorescence (panel A and B), or phase-contrast (C) illumination, following fixation and washing. In panel A, the edge of the electroporated area is marked in red. The gradient of fluorescence to the right of the red line denotes transfer through gap junctions. In **Figure 3A** and **3B**, the quantitation of gap junctional communication is shown: The cells at the edge of the electroporated area were identified and marked with a star, while cells where the dye had transferred were marked with a dot. The ratio shows the average number of cells that the dye transferred into, per electroporated border cell. In this example there are 57 dots and 10 stars, *i.e.*, GJIC=5.7.

In C and D, the Signal Transducer and Activator of Transcription-3 (Stat3) has been downregulated in T51B cells through stable siRNA expression with a lentiviral vector, and this causes a reduction in GJIC, as shown by the absense of transfer[22].

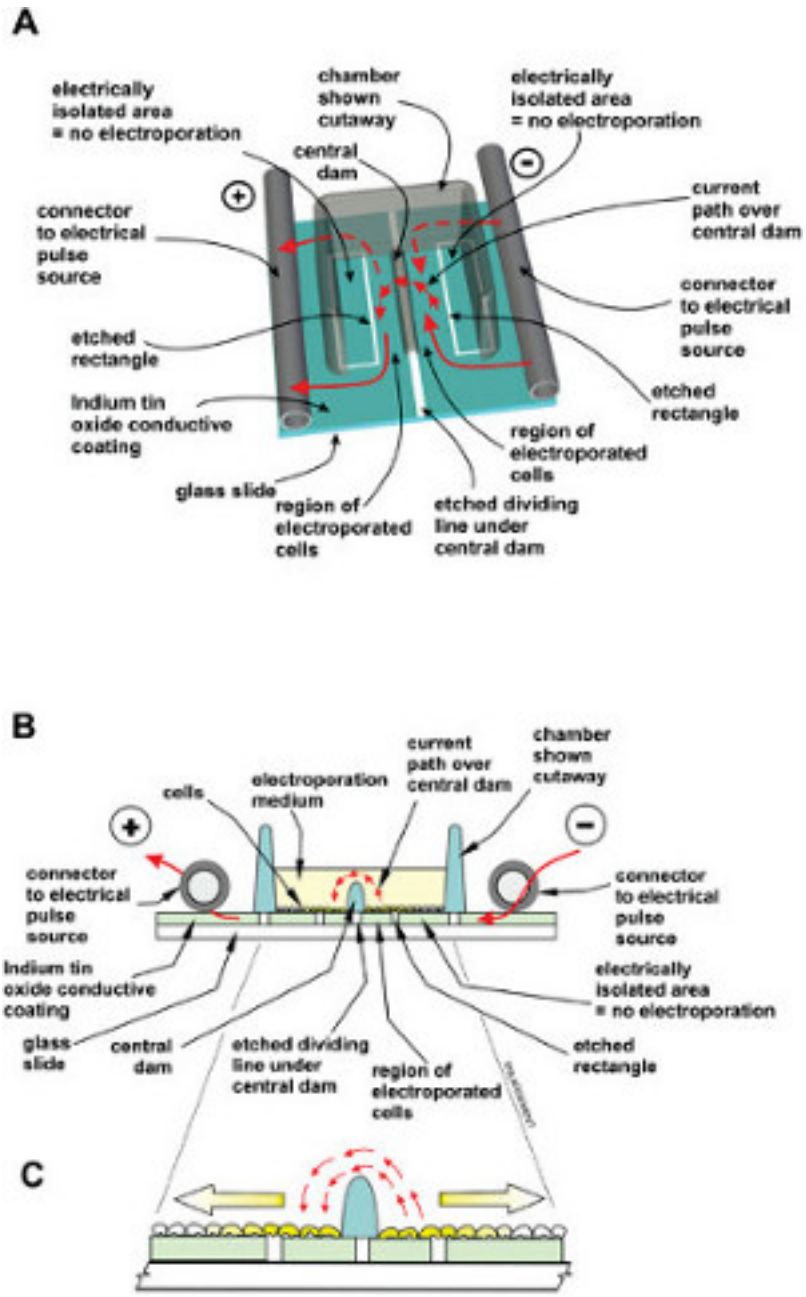

Figure 1. Electroporation electrode and slide assembly. (A) Top view: Cells are grown on a glass slide, coated with conductive and transparent indium-tin oxide (ITO). A plastic chamber is bonded onto the slide, to form a container for the cells and LY. The coating is laser-etched in a straight line in the middle, essentially forming two electrodes. A dam is used to divert the current upwards, thus creating a sharp transition in electric field intensity. To provide non-conductive areas, the ITO is also etched in two rectangles. Current (red arrows) from the pulse generator flows from each contact point to the other, via a conductive highway between the rectangles, spreading in a direction parallel to the middle barrier, then over the dam through the medium to the other side, electroporating the cells in areas parallel to the dam. For clarity, the front part of the chamber is removed. **(B)** Side view: The slide with the cells growing on the ITO coated (light green) and etched, bare glass regions are shown. When the electroporation medium containing LY is added to the chamber to a level above the height of the dam then an electrical path between the electrodes (+) and (-), and the cells growing in this area, is formed. Note that the ITO layer is shown with exaggerated thickness for clarity although its actual thickness (800 A) is much less than the thickness of the cells. **(C)** The area of electroporated and non electroporated cells is shown enlarged. The dye is dense near the dam where all cells were electroporated and fades across the electroporated edge as the concentration weakens through multiple gap junction transfers. Large arrows show the direction of dye transfer through gap junctions. Note that the size and thickness of the cells are exaggerated for clarity. Please click here to view a larger version of this figure.

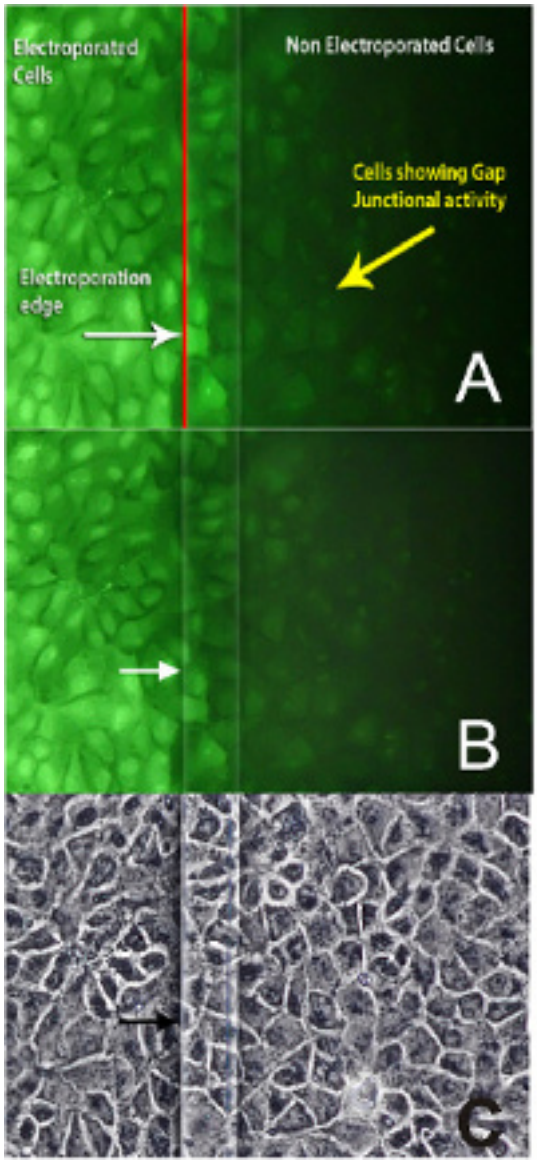

Figure 2. Electroporation of T51B, rat liver epithelial cells. LY was electroporated into T51B rat liver epithelial cells (mild, 12 volts). Following a 5 min. incubation, cells were photographed under fluorescence **(A, B)** or phase contrast **(C)** illumination. Note the extensive transfer through gap junctions, shown by the gradient of fluorescence, extending from the edge of the electroporated area (arrows, red line in **A**). At the same time, there is no visible damage to the cells, as seen under phase-contrast **(C)**. Please click here to view a larger version of this figure.

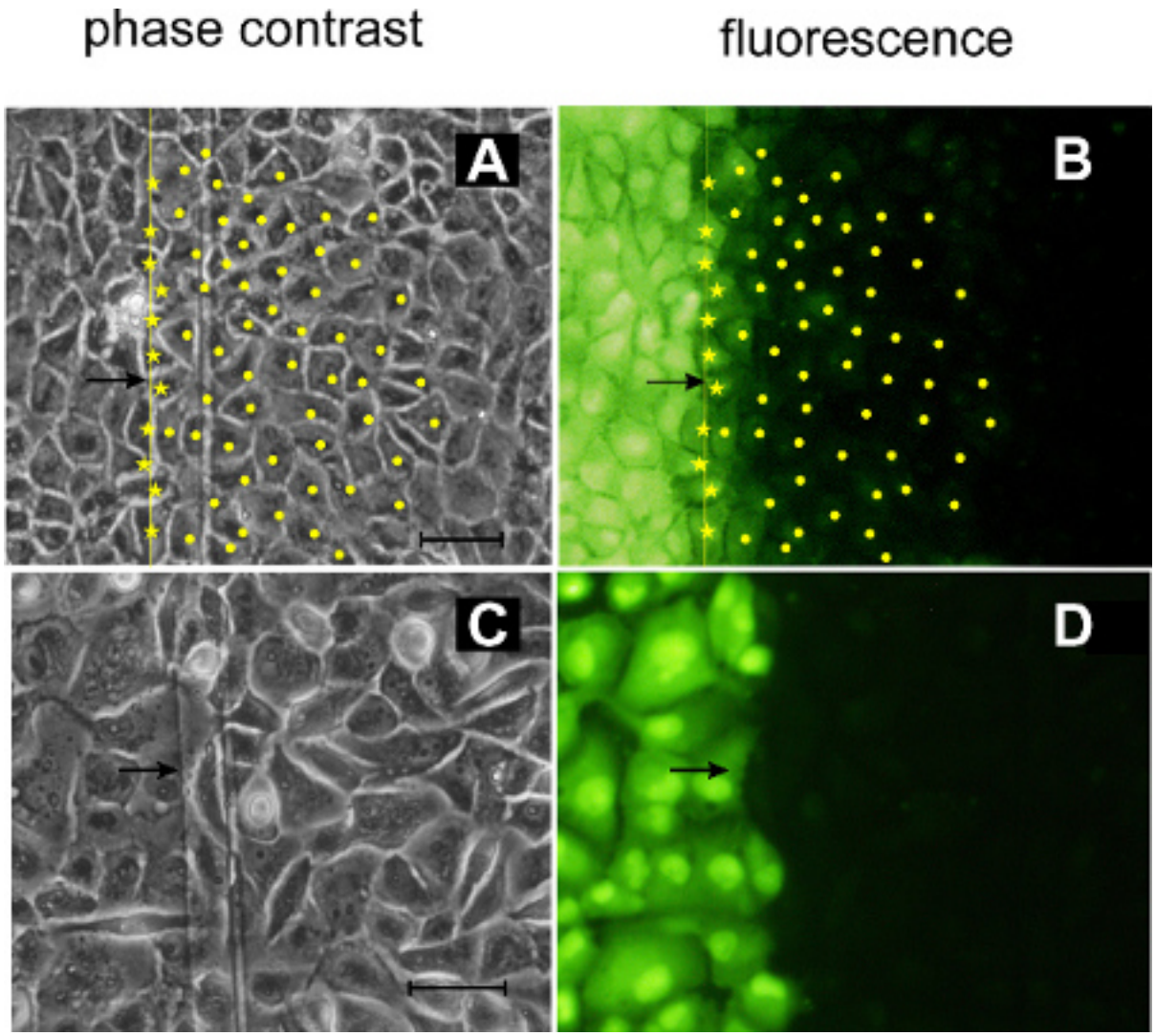

Figure 3. Quantitation of gap junction transfer. The cells at the edge of the electroporated area that picked up LY by electroporation (donors of LY to the non-electroporated cells) were marked with a star. Cells where LY transferred into through gap junctions were marked with a dot. In **(A)** and **(B)**, there are 57 dots and 10 stars, *i.e.,* GJIC=5.7. Cells in **(C)** and **(D)** do not have gap junctions, as shown by the absence of a gradient of fluorescence. Arrows point to the edge of the electroporated area. Bar= 100 µm. Please click here to view a larger version of this figure.

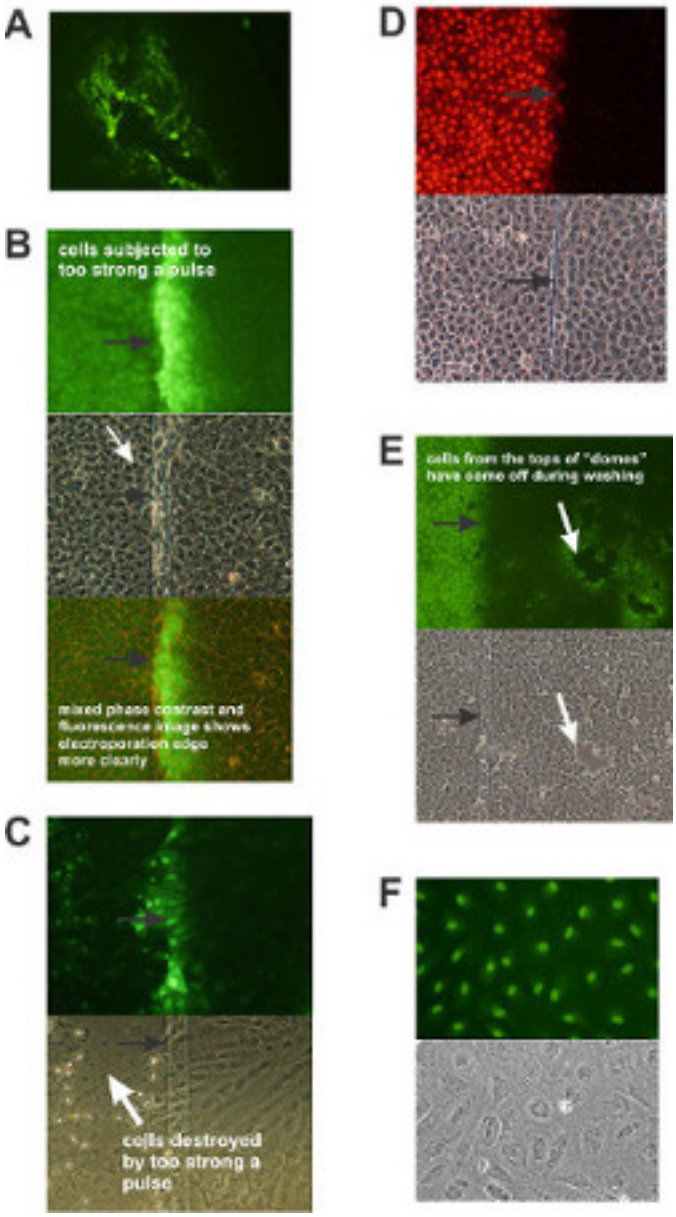

Figure 4. (A) T51B cells that have been damaged by scraping during manipulation. These cells may pick up LY even without electroporation and may transfer it to their neighbors through gap junctions. **(B)** and **(C)** Cells damaged by a pulse that is too strong. **(B)** T51B rat liver epithelial cells were electroporated at mild setting, 20 volts. The cells on the left of the etched line (arrow) have been damaged by the electroporation. In this case the cells did not detach, however a close look at the phase contrast photograph (middle panel, white arrow) shows the cells to have dark nuclei, while they fluoresce very weakly, if at all (top panel). However, transfer through gap junctions is evident, to the cells on the right side that are not electroporated. The bottom photograph was made by combining UV and phase contrast illumination, to facilitate viewing of the edge of electroporation. **(C)** T51B rat liver epithelial cells electroporated at medium setting, 30 volts. The cells on the left of the etched line have been severely damaged by the pulse. Note how the cells have come off and do not fluoresce. Some survivors around the edge have picked up LY and appear to be passing it on to their neighbours to the right via gap junctions. **(D)** T51B cells electroporated with 10µg/ml Propidium iodide. Note the intense staining of the nuclei. **(E)** Domes of epithelial cells. T51B cells electroporated at mild setting, 15 Volts. Note the even electroporation of cells at the left. However, when confluent, T51B cells form domes that may come off during manipulation and washing, and the surrounding cells may take up LY. Note the extensive dye transfer. **(F)** Cells that have dried may pick up LY without electroporation.The growth medium was removed from T51B cells and cells were left in a laminar-flow hood for 30 min. LY was subsequently added to the cells for 1 min and cells were subsequently fixed, washed and photographed under fluorescence (top panel) or phase-contrast (bottom panel) illumination. Please click here to view a larger version of this figure.

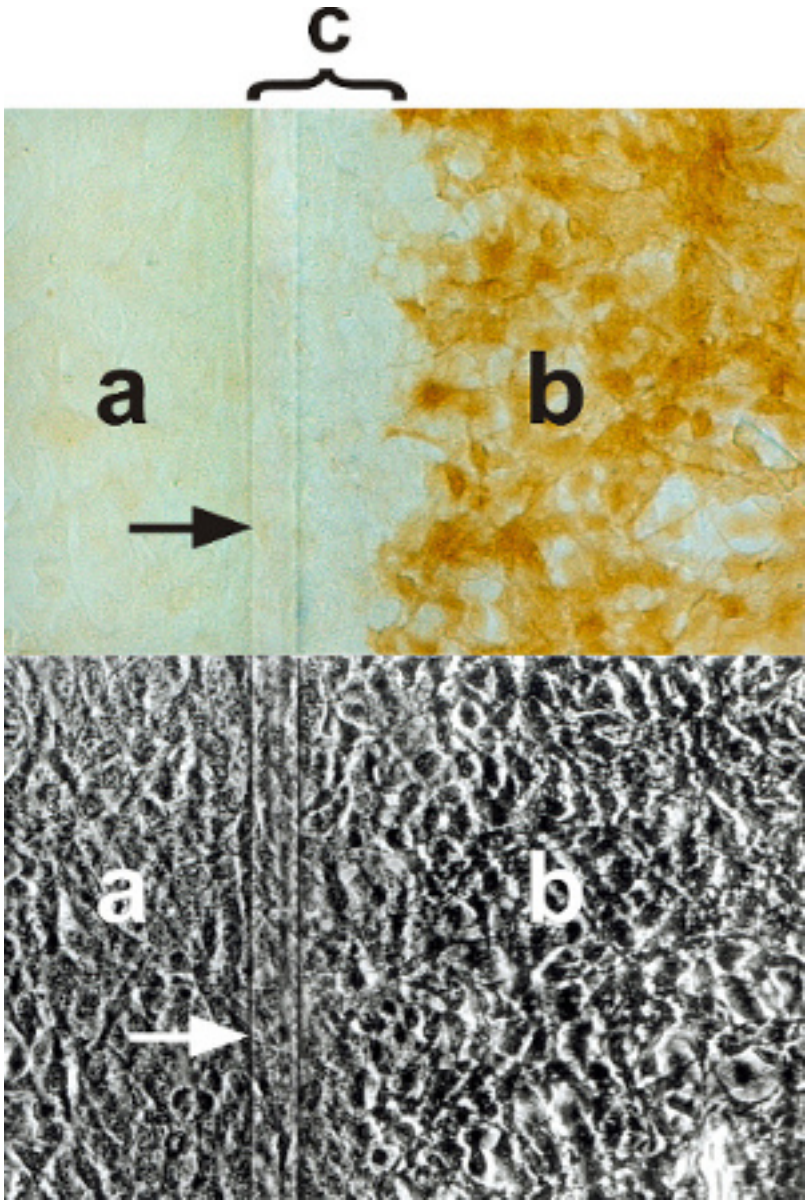

Figure 5. The Grb2-SH2 blocking peptide inhibits EGF-mediated Erk activation in intact, living cells. Combination of GJIC and signal transduction studies. Following ligand binding, a number of growth factor receptors such as the Epidermal Growth Factor Receptor (EGFR) are trans-phosphorylated on specific tyrosine residues. These constitute docking sites for the Src-homology-2 domains of signal transducers such as Grb2, which is bound to the GTP/GDP exchange factor ***sos***, which is thus brought to the membrane and activates ***Ras***. This results in activation of the Raf/Mek/Erk cascade and phosphorylation of Erk at a ^{P}TE^{P}Y sequence. Therefore, probing with specific phospho-Erk antibodies can be a measure of activation of the EGF receptor. Following fixation, cells are probed with an anti-pErk antibody (Cell Signalling), followed by a biotin-coupled secondary antibody, streptavidin-Horse-raddish peroxidase and the Diaminobenzidine substrate, which gives a brown color[29]. In this experiment, a phosphopeptide blocking the Grb2-SH2 domain (PVPE^{p}YINQS) was introduced by *in situ* electroporation into NIH 3T3 cells growing in electroporation chambers and growth-arrested in spent medium. 5 min after pulse application, cells were stimulated with EGF for 5 min, fixed, probed for activated phospho-Erk1/2 by immunoperoxidase stainingand cells from the same field photographed under brightfield (top) or phase contrast (bottom) illumination. Note that the Grb2-SH2 blocking peptide dramatically reduced the EGF signal, *i.e.*, Erk phosphorylation (area ***a***). The inhibition of the signal extends into ~3-4 rows of adjacent cells in the non-electroporated area (squiggly bracket), likely due to movement of the 1123 Da peptide through gap junctions. This finding constitutes compelling evidence that the observed inhibition must be due to the peptide, rather than an artifact of electroporation, since cells in this area did not receive any current, but acquired the peptide from their neighbors. At the same time, there is no detectable effect upon cell morphology as shown by phase contrast. Arrow points to the edge of the electroporated area. Magnification: 240x. For more details and controls see reference[29]. Please click here to view a larger version of this figure.

Discussion

Critical steps in the protocol

Electroporated material
The purity of the material to be electroporated is very important. If the cells are very flat, then higher concentrations of the tracking dye must be used (up to 20 mg/ml for LY) and in such cases, purity is even more important than in cells with a more spherical shape[1]. Besides LY a large variety of other dyes or nonpermeant molecules have been employed, such as a series of Alexa dyes to use as probes for channels consisting of different connexins[25]. However, dyes such as Ethidium Bromide or Propidium Iodide are intercalated in the DNA so that the nuclei fluoresce very strongly, which makes the quantitation of dye transfer more difficult (**Figure 4D**). The tracking dyes must be prepared and washes conducted in Calcium-free growth medium, because a Calcium influx may interrupt junctional communication, as well as reduce cell viability. All solutions must be kept at 37°C prior to electroporation, which facilitates pore closure and viability[1].

Determination of the optimal voltage and capacitance
Electrical field strength is a critical parameter for cell permeation, as well as viability. The application of multiple pulses has been shown to offer better results in terms of permeation and cell viability than a single pulse. The Insitu Porator apparatus of Cell Projects has three settings for capacitance and pulse number: Mild (10 pulses, 10 µs apart, 0.1 sec between pulses), Medium (20 pulses, 80 µs apart, 0.2 sec between pulses) and Strong (50 pulses, 120 µs apart, 0.5 sec between pulses), while the Voltage can be finely controlled independently (2-45V). In fact, flat cells require lower voltages than transformed ones, cells in a clump or cells with a small adhesion area to the substratum (*e.g.*, insect Sf9 cells), possibly due to the larger amounts of current passing through an extended cell that is in contact with a larger conductive area. For further details on this subject, please see[1].

Potential problems and troubleshooting

If the cells have been scraped during the manipulations, they may pick up the dye and fluoresce without electroporation (**Figure 4A**). The amount of energy delivered to the cells affects the efficiency of electroporation. If the pulse is too weak (*i.e.*, too low voltage and pulse settings), then the cells appear normal under phase contrast but they do not fluoresce, except perhaps for certain cells that may be dead or have been scraped during manipulation (**Figure 4A**). If the pulse is too strong, under phase contrast cells appear to have very dark nuclei (**Figure 4B**), but may retain some LY, and even allow some dye transfer to neighboring cells. At even higher pulses the cells are destroyed (**Figure 4C**). Such cells are lysed, therefore they do not retain LY and fluoresce very weakly, if at all. Certain epithelial cell lines such as T51B form dome structures which may come off during medium changes. The surrounding cells may then fluoresce as the dye may be transferred through gap junctions (**Figure 4E**). Examples of optimal electroporation conditions for representative lines are shown in **Table 1**.

Advantages over other techniques and significance

DNA introduction
There is a large number of transfection protocols, such as Calcium-phosphate co-precipitation[26] or different kinds of liposomes. However, they may affect the cell in subtle ways that may be very important, especially for signal transduction studies. For example, the tyr-705 phosphorylation of the signal transducer and activator of transcription-3 (Stat3) which correlates with its transcriptional activity is dramatically increased by the procedure of Calcium-phosphate transfection even in the absence of DNA. This could be due to the fact that the precipitate alters cell to cell adhesion and cadherin engagement, a known Stat3 activator[27]. Certain liposomes had a similar but less pronounced effect, while electroporation or retroviral infection did not affect Stat3 levels[6].

The introduction of peptides
A common method is the construction of a fusion peptide with sequences that cross the cell membrane, such as derived from the HIV-tat gene. However, the transfer through the membrane is a relatively slow process and signal inhibition is not as efficient. For the study of the sequence of events occurring following receptor activation by a ligand, the ability for instant introduction of a peptide, peptidomimetic or other compound offers an important advantage. Electroporation of a cell-permeable peptide to accelerate its uptake is not possible, likely because the lipophilic peptide is embedded into the cell membrane, as revealed with FITC-coupled peptides (Raptis, unpublished). It is interesting to note that the inhibition of Erk activation by EGF through the use of a cell permeable peptide blocking the Grb2-SH2 domain for example, was only partial[28], while electroporation of the same peptide achieved a complete inhibition of the signal[29] (**Figure 5**). Other techniques, based on liposomes are also in existence. However, they do not allow the examination of untreated cells side by side with treated ones, in conjunction with gap junction studies, which can offer the strongest possible demonstration that the signal inhibition must be due to the material being introduced (**Figure 5**, squiggly bracket, c).

Gap junction studies
Microinjection of dyes or scrape-loading are commonly employed, but they invariably introduce cellular damage. Another technique, fluorescence recovery after photobleaching is not invasive but it requires expensive equipment, and few cells can be examined at a time, while the formation of phototoxic substances may be an issue. The parachute assay consists of pre-loading cells with the dye calcein AM (green), then letting the cells adhere onto a cell layer, as gap junctions form within 15 min-3 hrs. A large number of cells can be treated in this way, but the ability of the cells to form gap junctions in this assay varies enormously with the cell type[30].

Limitations

The voltage required is higher for the introduction of molecules of larger size and electrical charges. At the high voltages required for the introduction of large plasmids, there may be some cell death. The relative voltages required for different molecules have been described previously[9,12]. Although we describe the electroporation using a specific electroporation apparatus, it is possible for someone experienced with

electrical appliances to make it using the detailed description in[19], or make a simpler setup with a top electrode above the cells, and etch with acids[1].

Future directions

Software have been developed to precisely quantify GJIC[31]. A further improvement is the development of quantum dots that fluoresce only once they are in the cell, so that there is no need to wash the unincorporated dye. This avoids the stress of washing, while the process can be observed in live, not fixed cells in real time. The introduction of peptides to interrupt signaling pathways is a powerful approach for the *in vivo* assessment of the relevance of interactions identified using purified components. The examination of the potential of different peptides to inhibit a specific pathway is the first important step in the development of peptidomimetic drugs, for the rational treatment of neoplasia.

Other comments

The slides can be washed with Extran-1000 solution using a small paintbrush to reach the ITO surface in the well, and rinsed with water. If the cells have been fixed on the slide, it may be necessary to remove traces of protein with trypsin or other proteolytic enzymes first. Sodium Dodecyl Sulphate containing detergents must be avoided. Washed slides can be sterilised with peroxide gas. However, it is very important to avoid breaking the seal of the well onto the slide because if any LY solution leaks in the holder it may cause a short circuit.

Disclosures

The corresponding author is the inventor in a patent held by Queen's University, which has been licensed to Cell Projects Inc. The company or the University had no influence upon the contents of the paper.

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