Quantification of gap junctional intercellular communication

based on digital image analysis

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Abstract

Intercellular communication via gap junction channels can be quantified by several methods based on diffusion of fluorescent dyes or metabolites. Given the variation in intercellular coupling of cells, even under untreated control conditions, it is of essence to quantify the coupling between numerous cells to obtain reliable estimates of metabolic coupling. Quantification is often based on manual counting of fluorescent cells, which is both time consuming and may include some degree of subjectivity. In this report we introduce a technique based on digital image analysis and the software for the analysis is presented together with a detailed protocol in the on-line supplementary material (http://bmi.ku.dk/matlab_program/). Fluorescent dye was introduced in connexin 43-expressing C6-glioma cells by in situ electroporation and fluorescence intensity was measured in the electroporated cells and in cells receiving dye by intercellular diffusion. The analysis performed is semi-automatic and comparison with traditional cell counting shows that this method reliably determines the effect of uncoupling by several interventions. This new method of analysis yields a rapid and objective quantification process with a high degree of reproducibility.

Key words: connexin; gap junction; electroporation; dye transfer
Introduction

Gap junctions are clusters of intercellular channels consisting of connexin subunits. The gap junction channels permit the exchange of ions and small molecules up to 1 kDa in size between adjoining cells. Intercellular communication via gap junctions can be evaluated by different methods (for review see (1)), one of which is the measurement of dye diffusion from cell to cell. Dyes can be introduced in the cells by techniques such as dye injection (6), scrape loading (4) or electroporation (8). With the scrape loading technique, the dye is introduced into a large number of cells and the quantification of intercellular coupling is thus based on the diffusion of dye between many cells. The disadvantage of this technique is the physical damage caused to the cells. Dye injection may be less damaging, but the technique is time consuming and only gives rise to information from the injected cell and its immediate neighbors. Ek-Vitorin et al. showed that under untreated control conditions dye coupling exhibits large variation from cell pair to cell pair (3). This was shown in four different cell lines, and demonstrates that reliable measurement of average dye coupling requires information from many cell-cell interfaces.

In the present report gap junctional intercellular coupling was measured using the in situ electroporation system developed by Raptis et al. (8). In this method, the fluorescent dye Lucifer yellow is introduced into a large number of cells by localized electroporation in a cell monolayer. The degree of intercellular coupling was originally reported by counting the number of cells into which diffusion of dye occurred.
Counting many cells is a time consuming process and therefore we have developed a new quantification method based on digital image analysis with custom written software (see online supplementary material). The quantification is based on the measurement of fluorescence intensity in cells into which dye diffusion has occurred.
Methods

Cell culture
C6 glioma cells stably transfected with Connexin 43 (Cx43) were cultured in a humidified incubator at 37ºC and 5% CO₂ in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were grown to confluence on electroporation slides before the experiments.

Technique
In the *in situ* electroporation technique (8) the cells are grown on slides which are partly coated with a conductive layer. An electrode is placed on top of the slide and short electric pulses are applied between the electrode and the conductive surface of the slide. This results in the formation of transient pores in the membrane of the cells growing on the conductive part of the slide. Lucifer yellow enters the cells through these pores, and the dye spreads through gap junctions to adjacent cells growing on the non-conductive surface. The spread of the dye is imaged using fluorescence microscopy as described below.

Experimental procedure
The cells were rinsed with buffer (see composition below) and a drop of Lucifer yellow (10 mg/mL in buffer) was applied on the cells. The electrode was placed on top of the slide and a capacitor of 1.0 µF was charged to 5-30 V. The cells were electroporated three times by discharging the capacitor. The size of the capacitor and the number of
discharges were chosen based on a previous study of cardiomyocytes in primary culture (5).

After electroporation the slides were rinsed in buffer and for each slide, four sets of light microscopy and fluorescence images were acquired two minutes after electroporation along the border between the conductive and non-conductive layer.

**Quantification of intercellular coupling**

Figure 1A-B shows a light microscopy image and a fluorescence image acquired in the same location on the slide. A white line is drawn to mark the border between the conductive and non-conductive part of the slide. Cells growing on the conductive layer (left of the line) are electroporated and show bright fluorescence (figure 1B). The fluorescence to the right of the line is caused by diffusion of dye through gap junctions.

Three methods for quantification of intercellular coupling were compared:

**Cell counting:** Cells receiving dye by intercellular diffusion, that is stained cells in the nonelectroporated area, are counted. To obtain comparable results between slides, the number of donating cells, which are the electroporated cells on the border, is counted and the ratio between the receiving and donating cells is calculated.

**Length constant and ratio measurements:**

Fluorescence intensity is measured and analyzed digitally. The calculation of the spread of the dye is done using these images. A fluorescence profile is generated by a routine written in MatLab (Mathworks Inc., Massachusetts, USA). The position of the white line is set to zero in each horizontal line and the fluorescence intensity for each position relative to position zero is summed across all horizontal lines. This procedure results in
the generation of a profile showing the average fluorescence intensity for each position. A typical profile is shown in figure 1C, which represent the sum of all horizontal lines in the image presented in figure 1B. The profile shows the high intensity in the electroporated cells (negative position values) and decaying intensity with increasing distance from the line located at position zero.

**Length constant:** The decay constant is determined by fitting the following equation to the profile in Origin 6.0 (Microcal Software Inc. Northhampton, MA, USA):

\[ I(x) = I_0 + A_1 \cdot e^{-\frac{x-x_0}{k}}, \]

where \( I(x) \) denotes fluorescence intensity in position \( x \), \( I_0, A_1 \) and \( x_0 \) are constants and \( k \) is the decay constant.

**Ratio measurement:** Intercellular coupling is quantified as the ratio of the mean intensity in a 100 pixel region starting 20 pixels right of the line (figure 1C, region B, “spread”) divided by the mean intensity in a 15 pixel region immediately behind the line (region A, “uptake”) to allow comparison between experiments with different levels of dye loading. In all presented data 1 pixel corresponds to 0.7 µm. Since some electroporated cells can extend into the area where the spread is calculated, the spread is measured at a certain distance from the line to avoid interpreting this fluorescence as a result of dye diffusion. The distance was chosen to be 20 pixels from the line, where the detected dye transfer via gap junctions was largest relative to the unspecific contribution. The data on which this conclusion was reached, are presented in supplementary figure 1.
**Materials**

C6 glioma cells stably transfected with Connexin 43 were kindly provided by Zealand Pharma (Glostrup, Denmark). DMEM, FBS, penicillin, and streptomycin were from Invitrogen, Carlsbad, CA, USA. Lucifer yellow, carbenoxolone, octanol and Cx43Gap27 were from Sigma-Aldrich (St. Louis, MO, USA). The sequence of the Cx43 targeting gap mimetic peptide Cx43Gap27 was SRPTEKTIFII.

The electroporation buffer contained: 136 mM NaCl, 4 mM KCl, 10 mM HEPES, 0.5 mM NaH₂PO₄, 1 mM EGTA, 0.8 mM MgCl₂, 5 mM glucose, 10% FBS, pH adjusted to 7.4 with NaOH. EGTA was added to the buffer to avoid calcium overload during electroporation.

The electrode (EPIZAP Small Electrode LE, model 33), the electroporation slides (Cat. no. 4318) and the capacitor (EPIZAP™ EZ-17 Pulse Generator, Model 17039621) were from Ask Science products Inc. (Kingston, Ontario, Canada). Cells were imaged using a Leica DMRE microscope equipped with a 10X/0.3 objective and an I3 filter cube (Leica, Heidelberg, Germany). Images were captured with a MicroMAX camera and WinXTest software (Princeton Instruments, Trenton, NJ, USA).
Results and Discussion

In this report we have compared three different methods for analyzing intercellular coupling after \textit{in situ} electroporation. When using electroporation for the measurement of physiological parameters such as dye coupling, care must be taken to optimize conditions to allow the formation of pores in the plasma membrane, while causing minimal damage to the cells. This was demonstrated in a recent study where dye coupling measured by electroporation (low damage) was compared to dye coupling measured by scrape loading (high damage). Data showed that electroporation resulted in a significantly higher spread of dye and the authors concluded that intracellular signals initiated by cell damage inhibited gap junctional communication (2).

Pore formation results in dye uptake which can be determined from the intensity profile after electroporation (figure 1C). The uptake is measured as the mean intensity in region A, and represents the efficacy of the dye loading during electroporation. To determine the optimal electroporation intensity with C6 cells, we measured the dye uptake at electroporation intensities ranging from 5 to 30 V (applied as 3 discharges of a 1 \( \mu \)F capacitor). Figure 2A shows that the uptake of Lucifer yellow is very low at voltages up to 10 V, but hereafter increases with increasing voltage. Cell death was measured by the trypan blue exclusion assay 15 minutes after electroporation, and no cell death was induced by electroporation even at 30 V (data not shown). For this study we chose to electroporate at 20 V which resulted in low mortality combined with a reproducible and high uptake of Lucifer yellow. Damage induced by electroporation may also induce morphological changes in the treated cells (7). To test for this possibility the cell cultures were investigated by DIC microscopy. No morphological changes could be detected at
the electroporation intensity used (20 V). A representative image is presented in supplementary figure 2.

As seen in figure 1B, cells outside the electroporated area was also stained, presumably by transfer of dye through gap junctions. To test this assumption, Texas red conjugated dextran, which has a molecular weight of 10 kDa and therefore can not permeate gap junctions, was electroporated into Cx43 transduced C6 cells. In these experiments no transfer of dye into cells outside the conductive area of the slide was observed (example shown in supplementary figure 3). As a further control Lucifer Yellow was electroporated into native C6 cells which lacks connexins, and, thus, are not coupled by gap junctions. In the native C6 cells we only observed a low number of stained cells outside the electroporated area (0.3 recipient cells per donor cell, for further discussion of these images see below).

To allow comparison between different experiments we calculated the ratio between the spread of dye (region B in figure 1C) and the level of dye uptake in the electroporated cells (region A in figure 1C). It is a prerequisite for comparison of the ratio calculations that a linear relationship exists between the level of dye spread and the level of dye uptake. Figure 2B shows the spread as a function of the uptake and a reasonable linear correlation exists (\(R^2=0.86, P<0.0001\)). We therefore proceeded to calculate the ratios for untreated control conditions and for three different uncoupling conditions using carbenoxolone (100 µM, 30 min preincubation), \(^{43}\)Gap27-peptide (300 µM, 4 h preincubation) or octanol (2 mM, 10 min preincubation). Furthermore, we electroporated native C6 cells in the presence and absence of carbenoxolone (100 µM, 30 min preincubation).
Figure 3 shows the results of analyzing the same data using (A), cell counting, (B) fitting a length constant to the fluorescence profiles, or (C) the ratio method. With all three methods of analysis, the lowest value of dye spread was observed in native C6 cells (Cx deficient) and in cells (native and transduced with Cx43) treated with carbenoxolone. The residual spread likely is due to cells that have processes into the electroporated area, and/or cells that have been electroporated, and thus have taken up dye, as a result of an uneven distribution of the electrical field in the border zone. The low level of uptake under these conditions can therefore be considered unspecific, and, thus, the minimal values reflect a total uncoupling of the cells. The specific dye transfer due to gap junction coupling of the cells can therefore by quantified by the excess uptake relative to these minimal values. Accordingly, the axes on the three graphs were adjusted to maximize the range between untreated control and the lowest values.

Octanol and $^{43}$Gap27-peptide reduced coupling less potently than carbenoxolone, irrespective of the analysis method used. The difference in uncoupling efficacy observed could be due to suboptimal concentration and/or pre-treatment time with octanol and $^{43}$Gap27-peptide. For carbenoxolone both concentration and pre-treatment time was chosen based on a study on cardiomyocytes in primary culture where 100 µM resulted in maximal uncoupling (5). However, we did not attempt to optimize the concentration and the pre-treatment time for octanol and $^{43}$Gap27-peptide. What is important in the present context is the fact that with all three analysis methods, the ranking between uncouplers (carbenoxolone>octanol>$^{43}$Gap27-peptide) was preserved, although variation in the absolute differences between the experimental conditions occurred. The ratio and length constant methods, which both rely on measurement of the amount of dye transferred,
were quantitatively closer. However at present it is unknown which measure best reflects the actual intercellular permeability in the monolayers.

To further evaluate the effectiveness of the ratio method, the time course of dye spread was investigated. Cells were electroporated and images were captured after 2, 5, 10 and 15 minutes. The majority of dye transfer occurred within the first 2 minutes (Figure 4). This is to be expected, since the gradients are largest just after electroporation, dye is lost due to leak and/or that Lucifer Yellow to some extent binds to intracellular proteins. The three different methods differed in their ability to detect diffusion of dye over time. The length constant and ratio method both showed values that increased significantly after the 2 minute time point, whereas the cell counting method failed to demonstrate an increase in the number of recipient cells. The reason for this failure is likely that factors like donor and recipient cell intensity are not taken into account when counting cells. This increases the variation and makes the method less able to detect small differences.

In terms of time spent during analysis, cell counting was by far the most time consuming method. For the data analysis presented 11771 donor cells on the border and 16184 recipient cells were counted. The ratio calculations and fitting of length constants were considerably less time consuming than cell counting, and the calculation of the ratios was the most straightforward method requiring only activation of the Matlab application.

We propose that an easy and objective way to analyze dye coupling data is to determine the ratio between the dye spread into the non-electroporated cells and the intensity of dye in the electroporated cells (uptake). Because the spread is linearly correlated to the uptake, dividing the spread by the uptake allows comparison of experiments with different levels of dye loading. In experiments using either dye injection or
electroporation, cells with high uptake will donate dye to more recipient cells even if the underlying permeability is the same. This is not taken into account when counting cells, and may therefore introduce bias, or decrease the signal to noise ratio. The ratio method can in its current form be used to analyze data from electroporation studies, where data from many cells need to be analyzed.

We conclude that the ratio method yields results comparable to other methods used to evaluate dye coupling, but has the advantage of having a higher analysis throughput.

**Perspectives and Significance**

In most tissues, cells are coupled by gap junctions, and there is a growing interest in determining the functional and regulatory aspects of this coupling. In the present paper we present an easy and automated method for quantifying cell-cell coupling in cell cultures. It allows a high throughput, and because of its automated nature, has a high degree of objectivity. The latter is important, since it reduces the risk of bias. The method allows faster and better assessment of cell-cell coupling than existing method, and could therefore contribute to rapid progress in the field of gap junction mediated cell-cell coupling.

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Competing interests statement

The authors declare no competing interests.


Figure legends:

Figure 1: Measurement of intercellular coupling by electroporation with Lucifer yellow. Panel (A): Light microscopy image showing C6 cells growing on an EPIZAP slide. The white line indicates the border between the conductive (left) and non-conductive (right) surface. Scale bar: 100 µm. Panel (B): Fluorescence image taken at the same location as in Panel (A). Cells growing on the conductive layer are electroporated and show bright fluorescence. The fluorescence to the right of the line is caused by diffusion of dye through gap junctions. Scale bar: 100 µm. Panel (C): Image profile used for quantification of intercellular coupling. The border position was set to zero in each horizontal line in the image. The fluorescence was summed for each position and plotted in the graph. The ratio between the average intensity in region A and the average intensity in region B was used to calculate the relative coupling. One pixel corresponds to 0.7 µm.

Figure 2: Determinants of dye uptake and dye spread. Panel (A): The uptake of Lucifer Yellow as a function of electroporation voltage. Data are displayed as mean ± SEM (n = 5-7). Even at the highest voltage no acute cell death was observed (data not shown). Panel (B): A plot of the spread of dye into neighboring cells as a function of the dye uptake of the electroporated cells. The line shows a linear fit to the data (I(spread) = a * I uptake + b, R^2=0.86, P<0.0001).
**Figure 3:** Comparison of different methods of analysis.

Untreated control cells and cells preincubated with either carbenoxolone (100 µM, 30 min preincubation), \(^{43}\text{Gap27-peptide (300 µM, 4 h preincubation) or octanol (2 mM, 10 min preincubation)}\) were electroporated and dye coupling was evaluated by either (A) cell counting, (B) fitting a length constant to the fluorescence profile, or (C) the described novel ratio method. The dye coupling in untreated control native C6 cells and native C6 cells preincubated with carbenoxolone (100 µM, 30 min preincubation) was also determined by the three methods of analysis (A-C). Data are displayed as mean ± SEM (n = 7-11).

**Figure 4:** Time profile of dye transfer determined by different methods of analysis.

Dye coupling was measured at 2, 5, 10 and 15 minutes after electroporation by either (A) cell counting, (B) fitting a length constant to the fluorescence profile, or (C) the described novel ratio method. Data are displayed as mean ± SEM (n = 5-6).

**Supplementary figure 1:** Effect of varying the distance from the line to the measurement area. Fluorescence was measured in a 100 pixel zone as described in the methods section. The distance from the border line between the electroporated and non-electroporated area was varied from 1 to 50 pixels in experiments in untreated control cells and in cells exposed to carbenoxolone (100 µmol/L, 30 min preincubation). Panel (A): A plot of the spread as a function of distance. The carbenoxolone sensitive fluorescence (the difference between the curves) is caused by gap junctional communication. Data are displayed as
mean ± SEM (n = 10-11) Panel (B): A plot of the signal to background ratio as a function of distance, where the signal is the carbenoxolone sensitive signal and the background the carbenoxolone insensitive signal. The highest ratio was obtained at a 20 pixel distance, where the balance between the loss of signal and reduction of background was optimal. One pixel corresponds to 0.7 µm.

**Supplementary figure 2:** Representative DIC image of Cx43-expressing C6 cells after electroporation (3 times 20 V at 1 µF). The white line indicates the border between the conductive (left) and non-conductive (right) surface. No signs of nuclear condensations, membrane blebs or other morphological abnormalities were observed in a total of 6 electroporations. Scale bar: 100 µm.

**Supplementary figure 3:** Texas-red conjugated dextran (10 kDa) does not enter cells outside the electroporation area. Texas-red conjugated dextran (40 mg/ml) was electroporated into Cx43-expressing C6 cells (3 times 20V at 1 µF) and clear labeling was observed in the cells growing on the conductive surface. The image is representative of 4 experiments. Scale bar: 100 µm.