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Optimisation of Electroporation for Biochemical Experiments in Live Cells

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To introduce into cells small molecules, which do not permeate the cell membrane naturally, electroporation is the fastest and most efficient technique. Although it is not completely benign, the speed at which a full population of cells can be permeated gives it a strong advantage over all other cell permeation techniques. Here we describe the potential damaging effects of electroporation and how to derive conditions which avoid these and assure its use for biochemical experiments in live cells. © 1999 Academic Press

Electroporation is a widely used technique to introduce proteins, carbohydrates, virus particles and nucleic acids in to cells. Despite this wide use, the mechanism by which electroporation causes the cell membrane to become permeable is not clearly understood and little is known about the damaging effects of electroporation in cells. It is generally thought that pores form in the membrane when cells are exposed to an electric field. Ingress of fluorescent molecules of different sizes into cells has shown that the size of the pores is directly related to the strength of the electric field [1]. Diameters, lifetimes, numbers and polar locations of electric field-induced pores have also been measured [2].

It has been suggested that lipid peroxidation may be involved in the electropermeabilisation of cell membranes [3]. Since small amounts of hydroperoxides cause local membrane disaggregation in lipid bilayers [4], it has been proposed that the production of hydroperoxides may lead to the formation of pores. A further product of electroporation induced lipid peroxidation is singlet oxygen [3]. Since free radicals are formed by this oxidative modification of the cell membrane, it is possible that oxidative damage may occur in some cellular structures. We have previously shown that electroporation can introduce breaks into DNA [1]. The number of breaks is directly related to field strength and capacitance, which, if combined, relate the number of breaks to total charge. Membrane permeability to larger molecules is increased at higher field strength but little affected by a capacitance change of a factor of two. In contrast, increasing the capacitance by this amount, has a marked effect on the number of DNA breaks induced. This implies that the length of time for which a current is applied is an important factor in DNA break induction. The relationship suggests that DNA breaks may be induced by an electrophoretic effect, however the production of free radicals could also lead to DNA breaks.

In modern biochemistry a central aim must be to examine the dynamic enzymology in the whole live cell. To do this, it is frequently necessary to introduce biochemical intermediates to label of the products of the process under study. In many cases, the cell membrane is naturally impermeable to these intermediates, for example nucleotide triphosphates. Electroporation is a very useful technique to use for the introduction of impermeable biochemical intermediates into cells. It is however necessary to ensure that the conditions of electroporation do not cause cellular disturbance which could distort the results of the experiment. Here we describe some simple assessments, which if considered together with previous findings, enable an experimenter to choose conditions of electroporation which will be valid for study of dynamic processes in live cells.

Abbreviations: PI, propidium iodide; ROS, reactive oxygen species; v, volts; μF, microfarads.

MATERIALS AND METHODS

Cell culture. HL60 cells (a human promyelocyte cell line) were maintained in culture at a concentration of (2–5) $\times$ 10^5 cells/ml in RPMI 1640 medium (Gibco-BRL, Paisley, Scotland) supplemented with 10% fetal calf serum, 100U/ml penicillin and 100μg/ml streptomycin and 2mM glutamine. The cells were washed in Puck's saline (137mM NaCl, 5.4mM $MgCl_2$, 4.2mM $NaHCO_3$, 5.5mM glucose) before being suspended in permeabilsation buffer at a concentration of 2–3 $\times$ 10^6/ml.

Permeabilisation of cells. The electroporation apparatus was constructed as described in Chu et al [5]. The permeabilisation buffer used is also described in this reference and consists of 20mM Hepes pH 7.05, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM dextrose,

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pH 7.05. A 0.9ml sample of the cell suspension was placed in a cuvette and the required voltage applied. The charge passes through the suspension via two platinum electrodes (2cm × 1cm) which are 0.4cm apart. The total charge through the cell suspension was therefore Q (coulombs) = C (capacitance in Farads) × voltage (voltage discharge across the electrodes). The $t_{½}$ for discharge of a capacitor of 1160μF is 10ms and for a capacitor of 690μF is 5.8ms.

Resealing of electroporated cells. To stimulate resealing of cells after the application of the voltage discharge, 10% fetal calf serum was added to the cells in electropermeabilisation buffer and incubated at 37°C in 5% CO_2 in air.

Annexin V and DNA staining. Early on in the cell death programme, the phosphatidylserine receptor flips from the inner membrane to the outer membrane to act as a signal to macrophages which ingest and dispose of the dying cells [6]. Annexin V is a calcium dependent phospholipid binding protein with high affinity for phosphatidylserine and thus can be used as a probe for the receptor to detect dying cells. To distinguish apoptotic from necrotic cells, a dye exclusion test can be combined with annexin staining. Propidium iodide which is a DNA binding dye, will penetrate the membrane of necrotic cells at a time when they are in a 'leaky' state in comparison to apoptotic cells in which the membrane remains intact in the initial stages of cell death [7]. Annexin-V-Fluos staining kit (Boehringer Mannheim) which consists of FTIC labelled annexin-V and propidium iodide solution was used to stain cell samples.

After exposure to electric shock, cells were incubated for the required time, centrifuged in microfuge (6500 rpm) for 1 minute and washed in PBS. The resulting pellet was resuspended in 100μl of the dual-staining solution, mixed as according to instructions and incubated in the dark for 10–15 minutes before fluorescence activated cell sorting analysis (FACS).

Externalisation of the phosphytidylserine receptor and cell death. Dual staining with annexin-V and propidium iodide can normally provide a distinction between cells which are entering apoptosis or necrosis. Electroporation however, enables propidium iodide to enter most of the cells at a field strength of 500v/cm and above. At 500v/cm, the cells reseal completely within 5 minutes (Fig. 1). If resealing takes place, then any subsequent permeability of the membrane to PI can be attributed to the membrane becoming leaky in the necrotic process. At higher field strengths (eg. 750v/cm and above) the cell membrane does not reseal efficiently and remains permeable to PI from the time of application of the electric field until cell death. In this case, staining with PI will always coincide with the annexin staining and could represent the onset of apoptosis or necrosis. Note that the field strength is not high enough in any of the experiments to allow the large annexin molecule (37kD) to gain access to the internal cellular membrane. Cell death was measured by propidium iodide and annexin staining at various times after electroporation. The levels of cell death following discharges with different capacitances are shown in Fig. 2.

Lipid peroxidation leading to cell death. High-voltage exponentially decaying electric field pulses have been reported to induce an oxidative burst in phagocytes, which can cause an influx of Ca2+ and Mg2+ ions into the cytoplasm [8]. Electroporation also induced the liberation of reactive oxygen species in mammalian cells [9]. To assess if reactive oxygen species may play a role in electroporation induced cell death, we examined the effect of ascorbate on the viability of the cells. Ascorbate is vitamin C and can reduce the cellular damage induced by ROS attack, by quenching free oxygen radicals. Figure 3 shows the effect of 200μM ascorbate on cell survival, when added just after electroporation. At a field strength of 750v/cm, where there is a large amount of cell killing, ascorbate is seen to increase cell survival by over 50%. At 500v/cm, there is almost full cell survival if a capacitance of 560μF is used. However, if the capacitance is increased to 1160μF, cell death and DNA breaks are increased (1). Addition of ascorbate (200μM) in this case, however, only reduces the cell death by 10% (data not shown). Therefore,

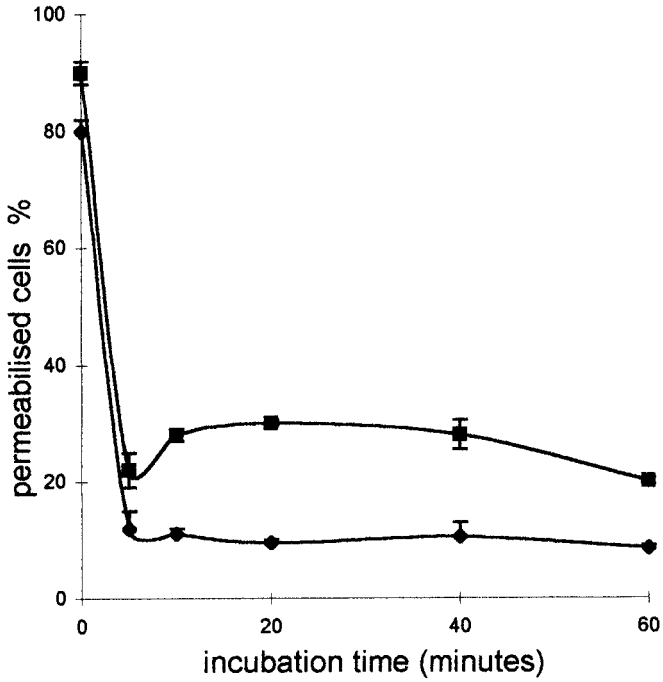

FIG. 1. Cells were electroporated at 500v/cm using 560μF capacitance (diamonds) and a 1160μF capacitance (squares). At time 0 hours, 15μM propidium iodide was present in the electroporation buffer. At later times after electroporation, following a period of incubation in buffer and serum, cells were stained with 15μM propidium iodide.

although ascorbate can clearly protect against electroporation-induced free radicals, it would not provide a useful means of improving electroporation conditions for experimental purposes, since it cannot eliminate cell death completely.

Calcium and cell death. Since the process of electroporation can cause an influx of Ca^{2+} into the cell cytoplasm, we investigated the influence of Ca^{2+} on cell death. EGTA (1mM) was added to the buffer in which the cells were electroporated. Chelating calcium ions had opposite effects at different levels of electric charge. At a field strength of 750v/cm, using a capacitance of 560μF, cell death is reduced when calcium ions have been chelated by ETGA, cell death is reduced by at least 50% (Fig. 4). At this level of electric charge (0.17coulombs) however, the total cell death is low [1]. If the level of electric charge is increased to 1160μF, using a field strength of 750v/cm which gives a total charge of 0.35 coulombs, cell death is not reduced by sequestering the calcium ions. Indeed, cell death appears to be accelerated as there is a large increase in cell death, 1 hour after electroporation when EGTA is present. Break induction and loss of cellular clonogenic potential is high at this level of charge [1].

At low levels of electric charge, the effects of electroporation may be likened to a cell stress response. It is well known that cell stress may induce apoptosis and therefore at low levels of charge, electroporation may induce apoptosis. Lipid hydroperoxides which are produced during electroporation, are known to be toxic and to increase cytosolic Ca^{2+} (10). Calcium increase in necrotic cells is always observed and occurs because of loss of membrane and organelle integrity. This allows influx of calcium from the external environment and release from intracellular stores. During apoptosis, membrane and organelle integrity are maintained, but an increase in intracellular calcium levels is often seen. This means that cells undergoing this process must actively take up or release calcium from their surroundings or internal stores. It is not fully understood what role

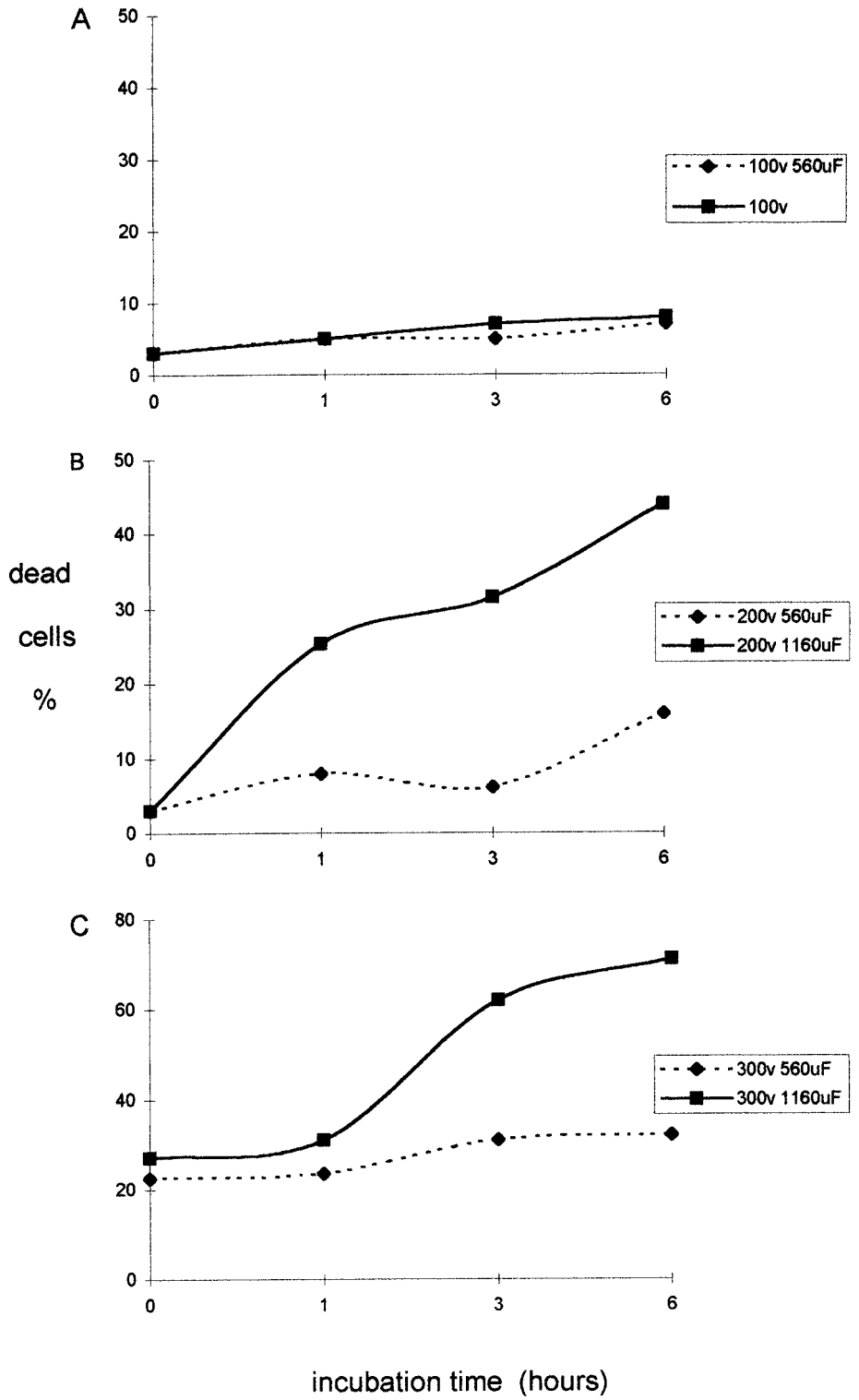

FIG. 2. Cells were stained with annexin V-FLUOS and propidium iodide following a period of incubation after electroporation. A. Cells were electroporated at 100v (250v/cm) using capacitances of 560μF and 1160μF. B. Cells were electroporated at 200v (500v/cm) using capacitances of 560μF and 1160μF. C. Cells were electroporated at 300v (750v/cm) using capacitances of 560μF and 1160μF.

calcium plays in apoptosis, but it is believed to be required for activation of certain enzymes (caspases) which are involved in the apoptotic pathway. If calcium is essential for apoptosis to occur and may be absent for necrosis to occur, the opposing results which we observe may be explained. At the lower level of electric charge, the decrease in cell death may be due to cells not being able to undergo

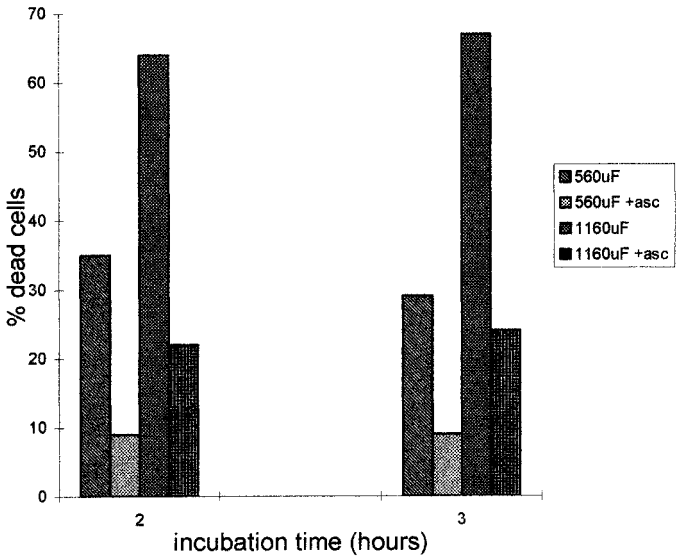

FIG. 3. Cells were electroporated at 750v/cm using a capacitance of 560μF or 1160μF, incubated in the presence or absence of ascorbate for 2 and 3 hours, then stained with Annexin-V-FLUOS and propidium iodide. Bar 1, 560 μF, no ascorbate. Bar 2, 560μF 200μM ascorbate. Bar 3, 1160μF, no ascorbate. Bar 4, 1160μF, 200μM ascorbate.

apoptosis in the absence of calcium ions, which activate the caspases. At the higher level of electric charge, where no decrease in cell death was seen in the presence of EGTA, the cells are induced to die by necrosis and so removal of calcium had no effect. We have detected apoptotic cleavage of nuclear lamins in cells which were electroporated a moderate field strengths and can therefore confirm that electroporation may induce apoptosis [unpublished observations]. Calcium may moderate this response, but adding EGTA to the premeabilisation buffer would not sufficiently improve the electroporation conditions and increase cell viability for biochemical experiments.

DISCUSSION

We now understand several of the factors which can lead to a reduction of viability in electroporated cells. These are DNA breaks, the production of reactive oxygen species and a small effect from fluctuation in calcium levels. Addition of quenchers of reactive oxygen species or calcium chelators to the electropermeabilisation buffer can reduce the toxicity of the electric field effects on cells, but the improvement in cell viability is not substantial enough to make these measures essential to optimise electroporation for live cell experiments. The best approach is to find the electroporation conditions ie. field strength and voltage, which allow maximum permeability to the molecule of interest while maintaining maximum viability of the cells. Since cell staining by propidium iodide can assess, in turn, cell permeability, resealing and subsequently cell death, the best approach is to add this dye at various times following electroporation as described in the materials and methods section. If analysed by FACS, a profile will be obtained, which, as in Fig. 5, illustrates a phase of membrane permeability, membrane resealing and subsequent onset of cell death. This profile, since it reflects the incidence of DNA damage, cell death [1] and nuclear lamin disruption by electroporation [unpublished observations], can be used to assess the integrity of electroporated cells for experimental purposes.

To allow entrance of molecules of greater than 600 Da, the electric field strength may have to be increased to enhance membrane permeability while the capaci-

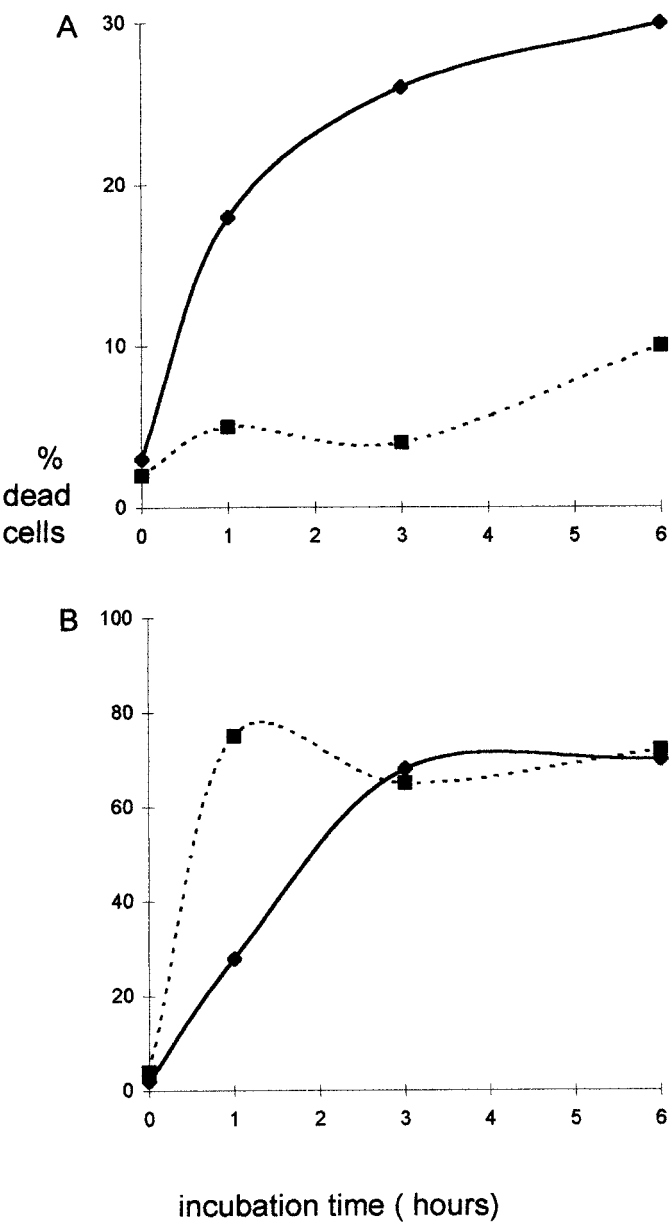

FIG. 4. Cells were electroporated at 750v/cm using a capacitance of 560μF (A) and 1160μF (B), in the absence of EGTA (diamond symbols) and the presence of 1mM EGTA. Following incubation cells were stained with Annexin-V-FLUOS and propidium iodide.

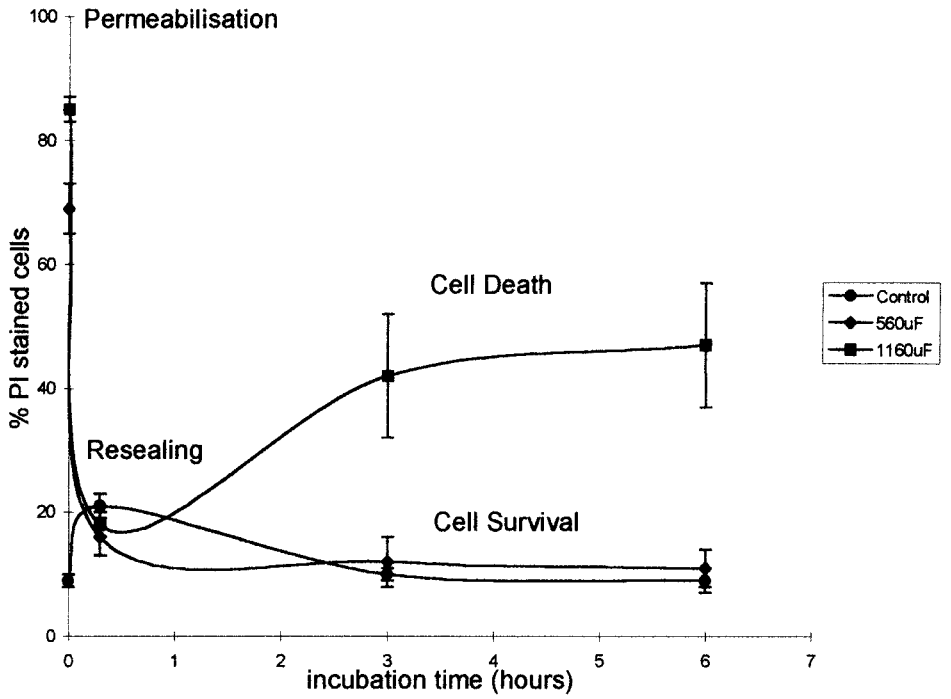

FIG. 5. Cells were electroporated at 500v/cm using a capacitance of 560μF (diamonds) or 1160μF (squares), and stained with 15μM propidium iodide following incubation. A control cell profile is represented by circles.

tance is reduced to maintain cell viability. Pulsed radio-frequency electric fields [11] may allow greater cell survival than the exponential decay pulse used in our experiments. The conditions chosen may be tested for resealing and viability by the time profile of the propidium iodide staining. Such studies can also be applied to assess the effectiveness of the new therapeutic technique of electrochemotherapy, which may enhance the uptake of drugs and their toxicity in tumours cells.

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