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In vitro electroporation detection methods – An overview

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ABSTRACT

Exposing cells to an electric field leads to electroporation of the cell membrane which has already been explored and used in a number of applications in medicine and food biotechnology (e.g. electrochemotherapy, gene electrotransfer, extraction of biomolecules). The extent of electroporation depends on several conditions, including pulse parameters, types of cells and tissues, surrounding media, temperature etc. Each application requires a specific level of electroporation, so it must be explored in advance by employing methods for detecting electroporation. Electroporation detection is most often done by measuring increased transport of molecules across the membrane, into or out of the cell. We review here various methods of electroporation detection, together with their advantages and disadvantages. Electroporation detection can be carried out by using dyes (fluorophores or colour stains) or functional molecules, by measuring the efflux of biomolecules, by impedance measurements and voltage clamp techniques as well as by monitoring cell swelling. This review describes methods of detecting cell membrane electroporation in order to help researchers choose the most suitable ones for their specific experiments, considering available equipment and experimental conditions.

Contents

1. Introduction

When biologic cells are exposed to a pulsed electric field of sufficient amplitude, their plasma membrane permeability increases. During this increased membrane permeability, molecules that otherwise cannot enter cells can be introduced to the cell interior or, on the other hand, cellular components can leak out of the cells. This phenomenon is termed electroporation. Electroporation can be reversible or irreversible (if the electric field is too intense for the cells to recover their membrane and cell functions) [1]. From its discovery in the late fifties of the past century [2], electroporation has been the subject of decades of extensive research and investigations,

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which has led to numerous applications in medicine (such as electrochemotherapy, gene electrotransfer, cell fusion and tissue ablation) [1] and food biotechnology (such as microbial inactivation and extraction of biomolecules) [3–5].

Although electric pulses act on all the membranes in the same way – making them more permeable – the extent of electroporation is very different. This depends on various conditions: pulse parameters (amplitude, duration, pulse number and repetition rate), membrane composition, surrounding media, the orientation of cells in the tissue, temperature etc. [6]. Each application requires a specific level of electroporation (e.g., for gene transfer: enough to introduce an active compound but, at the same time, without cell death) to be fully applicable [4]. For this purpose, the extent of electroporation must be explored in advance by using at least one of the methods for detecting electroporation. Moreover, these methods enable exploration of the basics of electroporation: the spatial and temporal dynamics of membrane permeabilization [7,8], the effects of electric pulse parameters and conditions (bathing media, temperature etc.) [9], species, cell type and tissue variations [10], to estimate membrane permeabilization [11,12] and determine thresholds for reversible and irreversible electroporation [13].

Electroporation and its extent is most often determined by detecting/measuring the increased transport of molecules across the membrane [14], either import of exogenous substances into the cell [15] or leaking of cellular components out of the cell [16]. Exogenous substances must fulfil two conditions to become successful detectors of plasma membrane permeabilization: 1. they must be non-permeant for an intact cell membrane and can enter the cells only after the plasma membrane is electroporated and 2. they have to possess an intrinsic characteristic that, in combination with a specific detection method, can give information about a molecule's transport into the cell. There are numerous substances that serve as electroporation indicators: from fluorescent dyes, which are most frequently used [17–22], colour stains [23], magnetic nanoparticles [24], functional molecules such as cytotoxic bleomycin [25,26], to the largest, nucleic acids [27]. In addition to exogenous molecules and cell leakage, electroporation can also be detected by physical and chemical methods, such as conductivity and impedance measurements [28], voltage clamp methods [29] or cell swelling [30].

We review here and briefly describe different methods of electroporation detection (Fig. 1) in order to help researchers choose the most suitable ones for their particular experiments. We also highlight the advantages and disadvantages of specific method and provide references to original reports.

2. Methods of detection of plasma membrane electroporation

2.1. Transport of non-permeant exogenous substances

A plasma membrane functions as a selective barrier between the cell interior and the environment and enables a cell to maintain concentrations of solutes in the cell different from those in its environment, i.e., extracellular media. Small non-polar and uncharged polar molecules can diffuse across a lipid bilayer. On the other hand, due to the hydrophobic interior of the lipid bilayer, a plasma membrane is non-permeable for most large uncharged polar molecules and charged molecules, including ions. Transfer of these molecules across the membrane is achieved with various transport mechanisms using membrane transport proteins (carriers and channels) [31]. Some molecules enter cells by different ways of endocytosis: they are internalized by invaginations of the plasma membrane, whereby a portion of the extracellular medium containing these molecules is enclosed in endocytotic vesicles. However, for further use of these molecules, a cell has to be able to process them into a form that can escape endocytotic vesicles or be transferred to other cellular compartments [32].

Most exogenous molecules, however, lack such transport mechanisms and thus cannot cross a plasma membrane: they are either too hydrophilic or too large for simple diffusion through the lipid bilayer and are also not transported via any membrane transport proteins [33]. Such non-permeant molecules are good candidates for the detection of plasma membrane electroporation, since the application of an electric field creates hydrophilic pores in the lipid bilayer and, during electroporation, membrane permeability for these molecules is at least temporarily increased [34]. In fact, quite a number of these molecules (e.g., propidium iodide, trypan blue), which were originally widely used to determine viability (to test whether the plasma membrane has been compromised) later served as a tool for detecting membrane electroporation. However, in the case of membrane electroporation, it must be taken into account that the plasma membrane is only temporarily permeabilized and can reseal [35].

Some of these non-permeant exogenous molecules have special properties that lead to the development of detection methods that

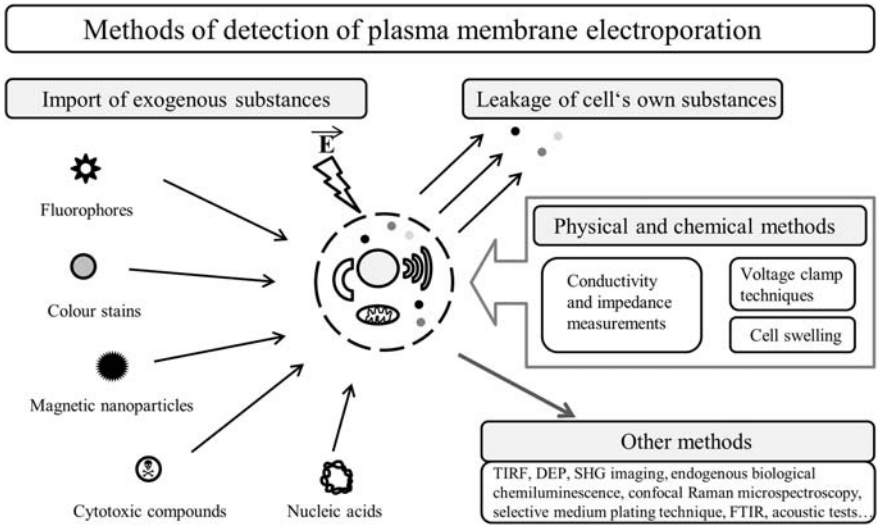

Fig. 1. Graphic outline of methods used for plasma membrane electroporation detection. Abbreviations: TIRF – total internal reflection fluorescence microscopy, DEP – dielectrophoresis, SHG – second harmonic generation, FTIR – Fourier transform infrared spectroscopy.

give us information about the location of molecules (intracellular, extracellular), hence the term "reporter molecules". Some molecules are dyes that are detected by microscopic and spectroscopic methods. Some dyes are introduced into cells in order to become visible indicators of non-permeant ions or molecules entering the cells (e.g. fura-2 for Ca^{2+}), while magnetic nanoparticles can be detected by magnetic resonance imaging (MRI).

Functional molecules, on the other hand, act differently: they have a certain biological effect on cells when they reach the cell interior and/or specific intracellular component. The extent of electroporation is thus estimated indirectly: the greater the electroporation, the more functional molecules enter the cell and the more pronounced is the observed biological effect, e.g., bleomycin, which causes cell death [25]. Among functional molecules, DNA is the most complex (and therefore discussed separately): electroporation can be determined by detecting the expression of a gene encoding a reporting protein. In this process, DNA has to be transported through the plasma membrane and reach the cell nucleus in order to gain access to transcriptional and translational mechanisms [36].

Depending on the method of pulse application and uptake detection method, electroporation of a single cell or a bulk of cells in suspension can be analysed. Some of the methods require long observations of cells (e.g. DNA expression) so sterile conditions must be sustained.

The transport of charged exogenous molecules through the electroporated plasma membrane is diffusion along a concentration gradient, as well as dragging by electrophoretic force. It can be described by a one-dimensional Nernst-Planck equation [37]. The amount of molecules passing the plasma membrane is therefore an indicator of the extent of electroporation [38,39]. The transport, however, is not just simple diffusion based on an electrochemical gradient; it is influenced by a number of factors, such as temporal changes of the electric field and pores, the shape of the pores, interactions between transported molecules and pore walls and solute compositions [40]. A number of models have been proposed to describe the transport of molecules through electropores, reviewed in [41].

With the use of reporting and functional molecules, we can therefore determine the role of pulse parameters and the influence of other experimental conditions on the extent of electroporation, thresholds of reversible and irreversible electroporation, observe spatial and temporal dynamics of membrane permeabilisation, estimate pore size (diameter) and detect susceptibility differences to electroporation between species, cell types and physiological states.

However, the general behaviour of molecules used for membrane electroporation detection may be modified or changed due to a high local electric field [42–44] or membrane properties – which can affect electroporation – can be modified on their binding/interaction with the membrane [45,46].

2.1.1. Dyes

The most common way of investigating electroporation in vitro is to study the cell uptake of dyes: either fluorescent molecules (fluorophores) or colour stains (such as trypan blue).

2.1.1.1. Fluorophores. Fluorophores (or fluorescent probes) are molecules that absorb light at a certain wavelength and then re-emit it at a longer wavelength. The use of fluorescent molecules is far more prevalent than the use of colour stains. There are several advantages in the use of fluorescent probes over colour stains: fluorescent probes provide high contrast over a dark background, high sensitivity and specificity, only a small number of molecules are required for detection and, moreover, fluorophores are mostly minimally invasive compounds that can be present in living cells to provide real-time imaging without cell fixation [47]. There is a vast variety of different fluorophores, which can be used in two ways.

The first is to observe single cells under a fluorescence microscope mounted with a camera. Fast changes in the spatial and temporal distribution of molecules during and after pulse application can therefore be observed, so the plasma membrane regions in which electroporation occurred can thus be determined [48–50]. With the use of a confocal microscope the position of fluorophores can be determined very precisely [9]. In recent decades, several new techniques for fluorescence imaging (such as multiphoton excitation, total internal reflection fluorescence or TIRF, and super-resolution imaging techniques) have been developed to improve sensitivity and resolution [51]. The transport of fluorophores can be monitored by image processing and computer analysis programs such as ImageJ (Java based open source program by the National Institutes of Health NIH).

The second way of using fluorophores is to measure a large number of affected cells to get an average response of the cell population. Namely, observing a small number of individual cells under a microscope can sometimes be misleading, since the effects of the electric field can vary from cell to cell. Fluorescence changes can be measured with spectrofluorometry or microplate readers with fluorescence detection; however, large background effects must be taken into account. In contrast, the use of flow cytometry allows the determination of fluorescence individually in a large number of cells (10^4 or more) to obtain the distribution of a population response [52,53]. The disadvantage of this method, however, is the inability to track rapid molecular transport. The use of different detection methods can sometimes lead to different results, as in the case of the detection of gene electrotransfection with fluorescence microscopy and flow cytometry [54].

2.1.1.1.1. Nucleic acid binding fluorophores. Among the most frequently used nucleic acid binding fluorophores are propidium iodide (PI), ethidium bromide (EtBr), ethidium homodimer 1 (EtH) and YO-PRO®-1 Iodide (Thermo Fisher Scientific Inc., Waltham, MA, USA). There are three reasons why phenanthridinium and cyanine dyes are the most popular fluorophore family in electroporation studies: (1) they are non-permeant molecules that enter cells through an electroporated membrane, (2) after penetrating the cell they have a high affinity for binding to nucleic acids and (3) they exhibit large fluorescence enhancement when bound to nucleic acids (DNA, RNA) [55]. Electroporation detection is therefore very sensitive and cell washing is not necessary which enables a real-time monitoring of electroporation. The members of the phenanthridinium (PI, EtBr, EtH) and cyanine families (YO-PRO®-1 Iodide) are all used in a similar way: they are simply added to the medium in which cells are electroporated and analysed by microscopy, spectrofluorometry or flow cytometry soon after pulsing.

Propidium iodide (PI) is the most widely used hydrophilic phenanthridinium fluorescent molecule (molecular mass: 668 Da, the peaks of fluorescence excitation and emission are at 535 and 617 nm, respectively: EX535/EM617) and dissociates in water to a double positive charged propidium ion (which is subjected to detection) of a molecular radius of 1.5 nm (molecular mass: 542 Da), and iodide anions, although the term "PI uptake" is still nevertheless traditionally used in publications [17,56]. PI is cell impermeant and thus excluded from viable cells, so it is widely used for detecting cells with compromised membranes (i.e., determining viability) [55,57,58]. When bound to DNA, its fluorescence enhances 20- to 30-fold [59].

In electroporation studies, PI can be used in microscopic studies [7–9,11,60–63], flow cytometry [9,25,52,53,64–71], spectrofluorometry [9,72,73] or can be detected by a photomultiplier tube [8]. PI has been used to evaluate cell type variations in electroporation [53], quantification of molecule uptake [11,52], observing asymmetric transport into cells [7,9,11,61,63] and transport kinetics [8,74]. PI can be used for cell suspensions as well as for attached cells. Because of weak fluorescence when unbound to nucleic acids, PI is less suitable for erythrocyte ghosts and vesicles studies. PI has also been used in studies using multiple nanosecond (nsEP) pulses [17,56,66,75–78] and Fig. 2, although the "nanopores" that emerge after a single nsEP application are usually too small to allow PI to pass through them and so special care has to be taken in interpreting the results of electroporation by ns pulses.

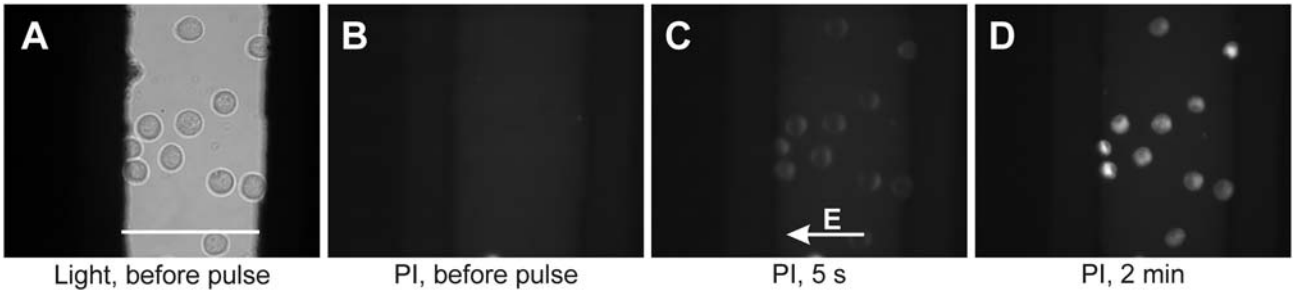

Fig. 2. Detection of electroporation with multiple nanosecond electric pulses using propidium iodide (PI). B16 F1 mouse melanoma cells were pulsed with 20 × 60 ns, 45 kV/cm, 1 Hz. Light (A) and fluorescence (B–D) microscopy were used for image acquisition. Excitation was with EX540 nm and fluorescence was collected at EM605/54 nm. A) and B): cells before pulse application, C) 5 s and D) 2 min after pulse application. In C), PI enters cells at the poles and in D), the fluorescence is markedly increased due to the binding of PI to nucleic acids. The scale bar in A) represents a 100 μm gap between the electrodes. The direction of the E field is marked in D).

PI is simply added to the electroporation buffer with cells prior to electroporation in concentrations ranging from 5.1 μM [53] to 1.5 mM [64], but it is mostly used between 30 and 100 μM. The concentration is adjusted to the threshold of the detection system. The uptake of PI can be monitored in real time using a fluorescence microscope. For flow cytometry, cells must be incubated for a certain period of time, usually 5–15 min, for PI uptake and the emergence of PI fluorescence, although cells cannot be incubated with PI for longer periods of time since PI itself is toxic. On the other hand, pore resealing dynamics can be studied if PI is added at different periods of time after the exposure of cells to electric pulses [67,68,73].

PI has also been used as a tool for fast recording study of the direct interaction of fluorophore with the plasma membrane during transport through the lipid bilayer because of the enhancement of PI fluorescence when bound to membrane loci [79–81]. More precise spatial and temporal resolution of pore detection can be achieved in this way. PI has also been used in vivo for evaluation of muscle cell electroporation in mouse muscles [82].

Ethidium bromide (EtBr) is a phenanthridinium intercalator similar to PI; however, it is smaller (molecular weight: 394 Da, EX300 or EX520/EM610) and amphiphilic [83]. It also dissociates to an ethidium cation (1^+, 314 Da) and a bromide anion. Similar to PI, it exhibits >20-fold enhancement of fluorescence when bound to DNA [84]. It is a conventional dye used for nucleic acid staining, e.g., in gel electrophoresis.

EtBr is usually added to the solution in a 25 μM–1 mM concentration prior to electroporation and is mostly used for microscopic studies of temporal and spatial detection of electroporation [50,62,63,85,86]. EtBr fluorescence can also be measured spectrofluorometrically for cells in suspension [71]. It has also been used in studies with a fast camera to detect direct interaction with the plasma membrane (as in studies with PI) [79,80,87].

Ethidium homodimer 1 (EtH) is a larger dimeric molecule (molecular mass: 857 Da, EX528/EM617) from the same family as PI and EtBr. It binds strongly to DNA and it is thus used for gel electrophoresis [88]. Its fluorescence enhances 40-fold when bound to DNA [89]. EtH is used in similar protocols as PI and EtBr, for microscopic studies [63,90] and flow cytometry [90–93].

YO-PRO®-1 Iodide is a carbocyanine nucleic acid stain by Thermo Fisher Scientific Inc. (629 Da, EX495/EM509). It forms divalent cations in aqueous solution (similar to PI). When used in nsEP [15,94–96] and pulsed electromagnetic field (PEMF) [97] experiments together with PI, it has been shown that YO-PRO®-1 Iodide detects electroporation at lower electric fields than PI. Since it is somewhat smaller than PI [17], YO-PRO®-1 Iodide shows greater sensitivity in detecting electroporation of a plasma membrane, although the size of the two molecules may not be solely responsible for the difference in uptake [15,17,78,96]. Nevertheless, YO-PRO®-1 Iodide (usually used in concentrations ranging from 0.5–5 μM) has mostly been used for studying the transport of molecules after a single or multiple nsEP [39,46,98]. YO-PRO®-1 Iodide is also an indicator of the early apoptosis [99]: it can enter the cell through $P2X_7$ receptors that are related to early apoptotic phase [100,101]. In nsEP electroporation studies, this is clearly not the case since $P2X_7$ receptor inhibitors had no effect on YO-PRO®-1 Iodide uptake [96].

SYTOX® Green is another cell-impermeable cyanine dye by Thermo Fisher Scientific Inc. (600 Da, EX504/EM523) which has been used in the same way as other nucleic acid binding probes. According to the manufacturer, SYTOX® Green fluorescence enhances 500- fold on binding to nucleic acids which makes it excellent for electroporation detection. SYTOX® Green has been added to cells prior to electroporation in concentrations ranging from 23 nM to 1 μM [102–105] and detected using a plate reader [102,106] or a microscope [103–105]. Using SYTOX® Green, electroporation has been detected in microfluidic devices and micro-biochips [103–105] as well as with direct current plasma discharge devices [102]. SYTOX® Green has been used to detect resealing of the plasma membrane [102]. It has also been used to distinguish temporary electroporated cells from dead/irreversibly porated cells with the use of an additional dye 30 min after poration [103] or in combination with the MTT viability test and cell lysis [106]. The latter method can be used with any cell-impermeable nucleic-acid binding dye.

2.1.1.1.2. Small nonbinding fluorescent molecules. In addition to nucleic acid binding fluorophores some other dyes such as Lucifer yellow and calcein, are also used to study electroporation of plasma membranes. Since they do not possess the ability to enhance the intensity of their fluorescence, the cells have to be washed after applying the pulses, either by a simple replacement of the medium in which the cells were electroporated (in attached cells) or centrifuging (in cells in suspension).

Lucifer yellow (LY), a small polar non-permeant nontoxic 2^- anionic dye (522 Da, EX428/EM536) has been used in concentrations from 0.5 mM to 3.8 mM with a variety of cell lines [19,107–112]. Internalized LY has been observed under a microscope or measured spectrofluorometrically. Electroporation has to be done quickly, since LY is an indicator of fluid-phase endocytosis and accumulates in endocytotic vesicles [75,113]. Permeabilisation of such vesicles by nsEP exposure has been demonstrated by leakage of entrapped LY into the cytoplasm [75], and Fig. 3. LY has also been used in studies of transdermal transport after applying electric pulses [114]. However, it has been shown that LY fluorescence may be decreased by applying an external electric field [44], so interpreting the results must be done with great care.

Calcein is a small non-permeant highly polar 4^- charge dye (623 Da, EX495/EM515), a polyanionic fluorescein derivative that has been widely used for electroporation studies with animal/human cells in suspension [15,107,115–120] and in monolayers [121,122], as well as with algae, plant protoplasts and yeast [123–128], erythrocyte ghosts [129,130], lipid vesicles [131] and for transdermal delivery [114,132–134]. Calcein acetoxymethyl (AM) ester is a cell-permeant derivative that

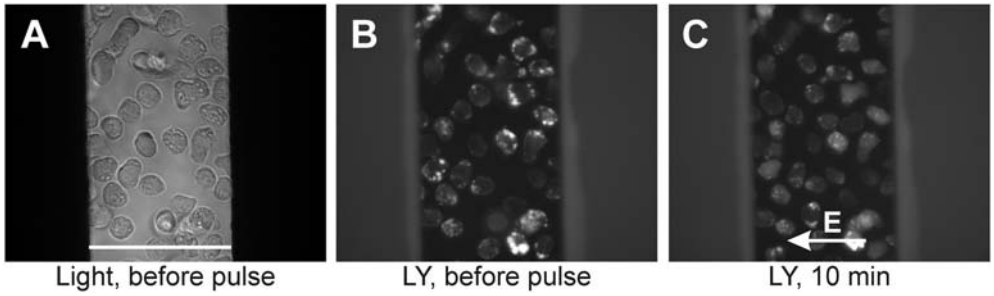

Fig. 3. Monitoring the permeabilisation of endocytotic vesicles by nanosecond electric pulses with Lucifer yellow. B16 F1 mouse melanoma cells were pulsed with 20 × 60 ns, 50 kV/cm, 1 kHz nsEP. Light (A) and fluorescence (B and C) microscopy were used for image acquisition. Lucifer yellow was excited with EX425 nm and fluorescence was collected at EM535/30 nm. Cells in A) and B) are taken before pulse application and in C) 10 min after. Before pulse application, Lucifer yellow is trapped in the endocytotic vesicles (B). After pulse application, Lucifer yellow is released from the permeabilised vesicles into the cytoplasm (C). The scale bar in A) represents a 100 μm gap between the electrodes. The direction of the E field is marked in C).

can enter the cell and the AM group is there cleaved by esterases into a non-permeant form [135]. The efflux of calcein out of the cell after applying electric pulses can thus be studied [23,129,131,136,137]. Calcein blue AM derivative has also been used in similar experiments [138]. In experiments using calcein, it is important to be aware that metal ions such as Ni^{2+}, Cu^{2+} and Fe^{3+} quench calcein fluorescence and that ions that are released from the electrodes during high-voltage electric field exposure can have a significant effect on the results [139]. Aluminium ions react with calcein and form a fluorescent complex [140].

Fluorescein itself and other fluorescein derivatives apart from calcein (fluorescein isothiocyanate, fluorescein diphosphate and fluorescein diacetate) have not often been used for membrane electroporation detection [141–145]. The reasons for the more frequent use of calcein than other fluorescein derivatives is probably its higher cell retention and the insensitivity of its fluorescence to pH in the physiological range [146] but may also be due to the historical use of fluorophore in electroporation laboratories.

2.1.1.1.3. Large fluorescent molecules and particles. Dextrans are water-soluble polysaccharides – polymers of glucose with high molecular weight (>1000 Da). The linear backbone consists of α-1,6 linked glucopyranosyl repeating units and branches with α-1,2, α-1,3 and α-1,4 linkage. Different chain lengths and side branching of dextrans lead to different molecular weights (3–2000 kDa). They have low toxicity and immunogenicity and are biologically inert. Dextrans can be conjugated to fluorophores, which are then detected with fluorescence microscopy, flow cytometry or spectrofluorometers.

Dextrans have a small electrical charge and are therefore relatively unaffected directly by an electric field [52,53]. Because of their variable molecular weight and size, dextrans of different molecular weight (or in combination with other indicator molecules) have often been used in electroporation studies to estimate the size of pores, the effects of pulse parameters on pore size, spatial distribution and pore dynamics [18,49,73,119,129,144,147–151]. Smaller dextrans enter cells more efficiently than larger ones, depending on the size of the pores [144,148,150,151]. Dextrans have also been used to study intracellular traffic of molecules introduced into cells by electroporation [152]. In addition to in vitro studies, dextrans have also been used to study transdermal delivery [153–155] and in vivo blood vessel permeability [156,157]. Fluorescein isothiocyanate (FITC)-dextrans of 4–2000 kDa have mostly been used in electroporation studies.

Quantum dots (QD) are semiconductor crystals of nanometer size (typically between 2 and 6 nm), which fluoresce at narrow discrete wavelengths depending on their size. They are photostable, have a high quantum yield and a large Stokes shift (i.e., the difference between absorption and emission maxima), are resistant to quenching, with proper surface modifications they are water-soluble, biocompatible and can be conjugated to biomolecules, and therefore have a practical use in biomedical applications for fluorescence imaging [158–160]. However, QD may exhibit some toxicity, mostly due to their core constituents (cadmium, zinc, tellurium, selenium) [158]. Quantum dots enter cells via endocytosis and remain trapped inside the endocytotic vesicles. Several biochemical and physical methods have been developed for the delivery of QD into cell cytoplasm [158,159,161], electroporation being one of them [21,159,162–166], meaning that they can also be used for electroporation detection. Because of the larger size QD delivery requires a high pulse intensity and duration [165] and the delivery is slower than that of, e.g., propidium iodide [21]. Aggregation of QD in cytoplasm after electroporation is another problem [162–164,167]; however, it can be overcome by coating the QD surface (e.g., with dihydrolipoic acid-sulfo betaine) to prevent interaction between nanoparticles and/or cytoplasmic components [164]. QD have also been delivered to cell cytoplasm by nanochannel electroporation [166].

In addition to quantum dots, other fluorescent nanoparticles have been used to detect electroporation, e.g., fluorescently labelled magnetic nanoparticles [168], dendrimers conjugated with fluorophores [169], silica nanoparticles with fluorescent dyes incorporated into the core [170]. Rhodamine B labelled multiwalled carbon nanotubes have also been transported to cells [166]. However, they have been shown to increase the electroporation extent – it has been said that they function as "lightning rods" [171,172] and, as such, may affect electroporation by their presence.

2.1.1.1.4. Ions and fluorescent ion indicators. The influx of ions through a permeabilised plasma membrane can also be an indicator of electroporation. The most convenient way of detecting electroporation by ions is to observe the influx of ions that are present in the cytoplasm at very low concentrations (such as calcium – due to its signalling role; free calcium in cytoplasm is kept at concentrations of 100 nM or below [173]) or is not present at all (thallium). In these cases, cells are first loaded with a fluorescent indicator which changes its fluorescence on ion binding. Cell-permeable acetoxymethyl (AM) ester forms of indicators (e.g. fura-2 AM) are able to diffuse passively into cells and they are cleaved in the cytosol by intracellular esterases to membrane-impermeant dyes [135]. Ions are much smaller than other fluorescent molecules, so they can be used for detecting smaller "pores", especially in the case of nsEP, which provoke "nanopores" that are too small to allow fluorescent dyes such as PI to pass across the plasma membrane [17] so these ion indicators can be considered very sensitive. However, due to their high sensitivity in detecting smaller "pores", the results cannot always be related to membrane permeability changes with larger molecules such as DNA.

Fura-2, fluo-3, calcium green and indo-1 have been used as indicators for Ca^{2+} concentrations. The possible influx of Ca^{2+} ions from the cell exterior into the cell after applying electric pulses can thus easily be detected by the use of these indicators, together with a fluorescence microscope [20,63,174–177], Fig. 4, or spectrofluorometer [178].

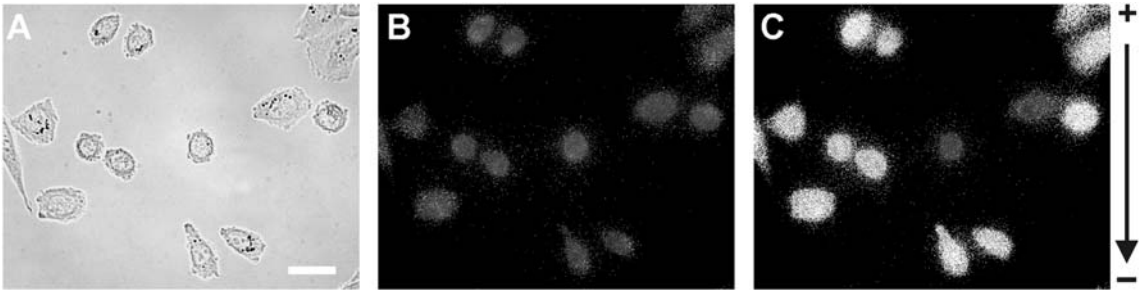

Fig. 4. Monitoring electroporation with fura-2. (A) CHO cells—bright field. (B) Ratio of fluorescence (F345/F385) for cells in control (non-porated cells). (C) Cells 1 min after electroporation with a 250 V/cm, 10 ms pulse. Brighter cells were electroporated. Arrow denotes the field direction. Bar represents 20 μm. Source: © 2011 IEEE. Reprinted, with permission, from Pucihar, G., Krmelj, J., Reberšek, M., Napotnik, T.B., and Miklavčič, D. (2011). Equivalent pulse parameters for electroporation. IEEE Trans. Biomed. Eng. 58, 3279–3288 [20].

Some indicators shift their excitation (fura-2) or emission (indo-1) spectra on ion binding. They can thus be used in ratiometric measurements. With ratiometric measurements, variations and artefacts due to different concentrations of the dye in different cell regions or cells (uneven loading), in the thickness of a specimen, and also due to the temporal dynamics of the dye (photobleaching, leakage) can be overcome or minimized [179]. In ratiometric measurements, the fluorescence intensity ratio at peak excitation/emission wavelengths for bound and unbound indicators is calculated. In the case of fura-2, two excitation wavelengths are used (EX340 and EX380) and the emission is measured at around EM520 for both excitation wavelengths. In the case of indo-1, the excitation is single (EX335) and the ratio between the fluorescence intensities at two emissions (EX405 and EM485) is determined [180].

Most measurements are done in a qualitative manner, although the concentration of free cytosolic Ca^{2+} can be determined quantitatively by in situ calibrations with solutions of known ion concentration (in combination with chelators) and ionophores for manipulating intracellular Ca^{2+} concentrations [181].

However, in experiments in which electroporation of a plasma membrane is determined by Ca^{2+} uptake, one must always be aware of the possibility that Ca^{2+} is also released from internal cellular stores, especially in the case of nsEP use [92,174,182]. Moreover, more complex Ca^{2+} pathways, such as calcium-induced calcium release [183,184] and store-operated (capacitive) calcium entry [178], can contribute to Ca^{2+} elevation in cells, as well as the activation of voltage-sensitive calcium channels in the plasma membrane [185–187].

To avoid false detection of plasma membrane electroporation, thallium ions that are not present in cells in any detectable concentrations are used instead of Ca^{2+}. The detection of Tl^{+} influx is thus always related to plasma membrane permeabilisation. The detection technique for plasma membrane permeabilisation using the Tl^{+} indicator FluxOR™ (Thermo Fisher Scientific Inc.) was first introduced by Pakhomov and colleagues [17,188]. Thallium ions are especially suitable for detecting nsEP "nanopores", since they are even smaller than Ca^{2+} (Tl^{+}: 0.392 nm, Ca^{2+}: 0.462 nm) [17].

2.1.1.1.5. Annexin V and phosphatidylserine externalization. Annexin V is a vascular protein that binds to membrane phospholipid phosphatidylserine (PS). PS is only present in the inner leaflet of the plasma membrane but it is translocated to outer layer of the membrane during apoptosis. Fluorescently labelled annexin V (mostly FITC-annexin V) is therefore widely used as a tool for detecting PS externalization in cells and hence serves as an assay for apoptosis [189]. However, Vernier and co-workers hypothesized that PS can translocate from the inner to the outer leaflet of plasma membranes along the walls of electropores that emerge during electroporation, since PS translocation occurs very rapidly (within seconds) after pulse application [22,96]. PS externalization detection with FITC-annexin V has thus been used for detection of electroporation, especially after nsEP application [188,190,191]. Since PS externalization occurs at voltages below dye uptake (PI, YO-PRO®-1 Iodide), it is considered to be a sensitive method for electroporation detection [96,188,190]. FITC-annexin V is added to the electroporation solution prior to or immediately after applying electric pulses and analysed under fluorescence microscope [188,191] or by flow cytometer [22,190]. PS externalization and detection >20 min after EP can, however, be a sign of apoptosis rather than membrane permeabilisation [192].

2.1.1.2. Colour stains. Colour stains are not used to detect plasma membrane electroporation as often as fluorescent dyes. Trypan blue has mostly been used in experiments, although other colour stains have also been used in a few studies, e.g., phenosafranine [193,194] and erythrosine B [195]. Detection of colour stain influx through electropores can be done by simple light microscopy [23,195–197] or spectrophotometrically [194].

2.1.1.2.1. Trypan blue. Trypan blue is a diazo dye with a molecular weight of 961 Da. It has already been widely used for more than a hundred years as a vital stain to determine cell viability, since it is excluded by most living cells with intact membranes. It enters cells with compromised membranes and stains cellular structures blue [198], especially nuclei [196], so it can also be used for detecting plasma membrane electroporation. Trypan blue is added to media prior to [23,172,197,199,200] or immediately after (within minutes) electroporation [196,201], as well as at different times after applying electric pulses, to monitor plasma membrane resealing [200,201]. It has been used to test pulse parameter effects (duration, field strength, pulse number) on electroporation [196,197,199,201,202], to detect the site of electroporation and observe dye influx in single cells on an electroporation chip [23] and to determine the role of electroporation in cell fusion [201]. Trypan blue has also been used as a fluorescent molecule: it emits red fluorescence (EM 605 nm) on activation by green light (EX 550 nm) [203]. The disadvantages of trypan blue are low sensitivity and contrast (compared to fluorescent probes), cytotoxicity in longer incubations, and it is not suitable for nsEP experiments.

2.1.2. Magnetic nanoparticles

Iron, manganese and gadolinium are chemical elements with paramagnetic properties and therefore have a strong effect on a local magnetic field. They can be used in the form of 35–1000 nm nanoparticles with a magnetic core and coated with various substances, such as dextran, PEG, polystyrene or silica. Magnetic nanoparticles can be additionally coated with a variety of molecules (e.g., DNA, fluorescent molecules) that can be delivered to cells alongside. Iron oxide particles are most widely used and can be obtained commercially (Feridex, Bayer HealthCare Pharmaceuticals, NJ, USA; Endorem, Guerbet, Villepinte, France), as reviewed in [204]. The paramagnetic properties of such particles can also be exploited for detection and separation. Cells loaded with magnetic nanoparticles can be traced with MRI, various staining protocols or magnetic force microscopy (MFM) or can be separated from nonlabelled ones in a magnetic field.

There have been some attempts to use electroporation to deliver magnetic nanoparticles to cells in vitro. Magnetic nanoparticles have been detected with DAB-enhanced Prussian blue staining for iron [205–208], particle surface related fluorescent molecules [24] and mRNA gene expression [209], as well as by MRI [24,205,206,208,210] and MFM [211]. Cells labelled with magnetic nanoparticles have mostly been later transplanted and monitored by MRI in vivo [24,205,207,208, 210], although electroporation in the presence of magnetic nanoparticles has also been done in vivo [212]. MRI contrast agent – chelate containing gadolinium (Gd-DOTA, Dotarem, Guerbet, Aulnay-sous-Bois, France) has also been used as an electroporation test in vivo [213, 214]. However, with the use of gadolinium, it is necessary to be aware that it has been shown that Gd^{3+} ions cause a decrease of electroporation yield [45,46].

Most papers report that magnetic nanoparticles remain in endosomal compartments after electroporation. In the process termed magnetoelectroporation, magnetic nanoparticles are transported to cells by macropinocytosis, not through the pores in the membrane that emerge from exposing cells to an electric field [205–208]. However, Tachibana reported nanoparticle localization in the cytoplasm [24].

The possibility of designing a microfluidic device that uses electroporation, electrophoresis and magnetophoresis for dragging DNA-labelled magnetic nanoparticles into cells, and later magnetic separation of successfully loaded cells, has been studied [215].

2.1.3. Functional molecules

2.1.3.1. Cytotoxic compounds. Among functional molecules, cytotoxic compounds are most widely used for electroporation detection due to their use in electrochemotherapy. Electrochemotherapy combines non-permeant cytotoxic drugs with electric pulses that locally – in tumours – permeabilise cell membranes. Chemotherapeutics (such as bleomycin and cisplatin) enter tumour cells and kill them. In such a way, electrochemotherapy increases the effectiveness of chemotherapeutics at the site of action and decreases adverse systemic side effects. Electrochemotherapy is now a widely used method for local cancer treatment [216]. The cytotoxic effect is dependent on pulse parameters [25] so cytotoxic compounds can be used indirectly, as a tool to investigate the extent of electroporation in vitro and in vivo. The cytotoxic effect on cells can be estimated by cytotoxicity tests, such as clonogenic assay, dye exclusion tests (using trypan blue or PI), MTT or MTS tests.

Bleomycin (BLM) is a non-permeant 1.5 kDa glycopeptide. Electroporation permeabilises the plasma membrane and allows BLM uptake: BLM causes DNA fragmentation immediately after reaching the cell interior: a few hundred molecules of BLM are already enough to kill the electroporated cell [217,218]. BLM cytotoxicity has been used to detect electroporation in a number of studies in vitro [25,26,219–225] and in vivo [226].

Other cytotoxic drugs, such as cisplatin [227–229] can also be used in the same manner as BLM to detect electroporation, although the factor of increased cytotoxicity after electroporation is not as high as it is in the case of bleomycin [227]. Cisplatin, however, is easily determined in cells and tissues by atomic absorption spectroscopy or inductively coupled plasma mass spectrometry [230–232] and can thus be used as a very sensitive probe for membrane electroporation.

2.1.4. DNA and RNA

Nucleic acids can also be introduced in vitro and in vivo to cells by using electroporation: foreign genes can in this way be transferred to cells via gene electrotransfer [233,234]. DNA and RNA in connection with fluorescence can serve as detectors for electroporation by directly observing fluorescently labelled DNA transport into the cell [27, 235–237] or monitoring expressed proteins derived from transferred DNA, e.g., fluorescent proteins such as green fluorescent protein or GFP [65,238,239], luciferase activity [240], using selective media [234], evaluating the biological effect of the protein [241] or silencing a target gene in the case of siRNA [242]. DNA (or RNA) can thus be a reporter candidate for electroporation detection [243]. However, DNA uptake is a very complex process and is not always easily achievable. There are a few basic steps of gene electrotransfer: 1. plasma membrane electroporation, 2. DNA-plasma membrane interaction, 3. translocation of DNA across a plasma membrane, 4. migration of DNA toward and into the nucleus and 5. gene expression [244]. Only if all the steps are achieved is gene electrotransfer successful. DNA is a large molecule and its transport across an electroporated plasma membrane (whether through large pores, by electrophoresis or by various endocytotic pathways) is still under investigation [36].

When the expression of transferred genes, such as GFP (EX396 or 475/EM509), is used for electroporation detection, at least 24 h is required for full expression of the protein (Fig. 5). Sterile work is therefore required in such experiments.

2.2. Cell's own ions/molecules: leaking out of the cell

Due to electroporation, plasma membrane permeability increases and not only exogenous substances are transferred into the cell interior but the cell's own components, ions and molecules such as ATP, leak out of the cell to the cell's surroundings [245], which has led to numerous applications in the food industry for the extraction of juices and other valuable compounds, as reviewed in [3–5]. Electroporation can also be detected by detecting the efflux of cell constituents.

2.2.1. Extraction of biomolecules

Electroporation can also be detected by the efflux of biomolecules (e.g. proteins, nucleic acids, pigments, lipids and sugars) from cells into the surrounding medium [3–5].

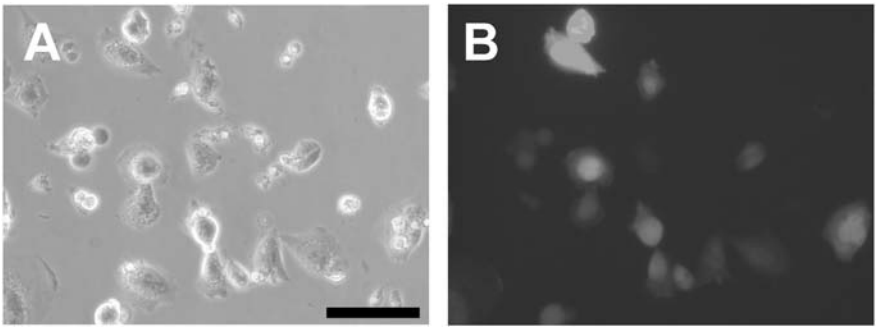

Fig. 5. Gene electrotransfer of a green fluorescent protein (GFP) encoding plasmid: CHO cells that were successfully electrotransfected were detected with a fluorescence microscope. With light microscopy (A), all the cells are visible, whereas with fluorescence microscopy (B) only transfected cells are visible. GFP was excited with EX488 nm and fluorescence was collected at EM525/50 nm. Cells were pulsed with 8 × 5 ms, 700 V/cm, 1 Hz and allowed 24 h for gene expression. The scale bar in A) represents 50 μm.

Overall protein release after electric pulse application has been monitored spectrophotometrically in bacteria [246], yeast [16,247] and microalgae [248]. Moreover, the release of active protein enzymes such as glutathione reductase, alcohol dehydrogenase, 3-phosphoglycerate kinase, hexokinase and proteases has also been detected spectrophotometrically [16,247,249]. The efflux of specific intracellular proteins has also been monitored by fluorescence detection [250,251] or polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting [248,250,252].

During electroporation, nucleic acids (plasmid DNA and RNA) are released from bacterial cells and can be detected by anion exchange chromatography or gel electrophoresis [250,253].

Various other compounds such as pigments (e.g., chlorophyll *a* and b, carotenoids) [254,255], oils [256], carbohydrates, proteins and phenolics [257] can be extracted from microalgae in the process of electroporation.

Electroporation can also lead to electroextraction of components from plant tissues, such as fruit [258,259] and vegetables [259,260], reviewed in [5,261]. These detection methods can however be cell/tissue type specific, or at least dependent, so they may not necessarily be transferable to other cell/tissue types. Other drawbacks of these methods are that additional detection methods are required, they can be time consuming and are not necessarily very sensitive.

2.3. Physical and chemical methods

2.3.1. Conductivity and impedance measurements

Electroporation causes cell membrane permeabilisation and an immediate increase in membrane conductivity and, consequently, affects the measured impedance of the cell suspension or tissue (it is decreased). Measuring the passive electrical properties of cell membranes can therefore be used for electroporation detection [262,263].

Impedance is a complex ratio of the voltage to current in an alternating current circuit and it takes into account the contribution of both resistive and capacitive components. From impedance measurements we can extract both the conductivity and resistivity of the sample. Impedance measurements are performed by inducing a known current flow through the sample while measuring the resulting voltage, or vice versa [262], see Fig. 6.

During an electroporation pulse, the conductivity can be estimated by dynamic conductivity measurements from simple current-voltage recordings [220,264–266]. It has been shown that a rapid increase in conductivity of cell suspensions due to the formation of pores is followed by a slow increase in conductivity [264,267]. The conductivity increase due to electroporation can be best seen in dense cell suspensions and tissues in which cells represent a large part of the sample volume and the resolution of current measurements is increased [264,265]. Before and after electroporation, the electrical properties of cells can also be determined by applying an alternating current signal at a specific frequency, and the voltage is measured [152,268]. At lower frequencies, the membrane conductivity increase due to electroporation causes a drop in impedance magnitude and allows current to flow through the electroporated cell. At high frequencies, current already flows freely across the membrane in non-porated cells. High frequencies are therefore not suitable for electroporation detection, and frequencies below 10 kHz are used to detect electroporation [262]. Some studies have performed multifrequency measurements (electrical impedance spectroscopy, EIS). However, this method is less suitable for recording fast changes in membrane properties since it requires a long measuring time compared to an EP pulse [28,269,270]. Electroporation can also

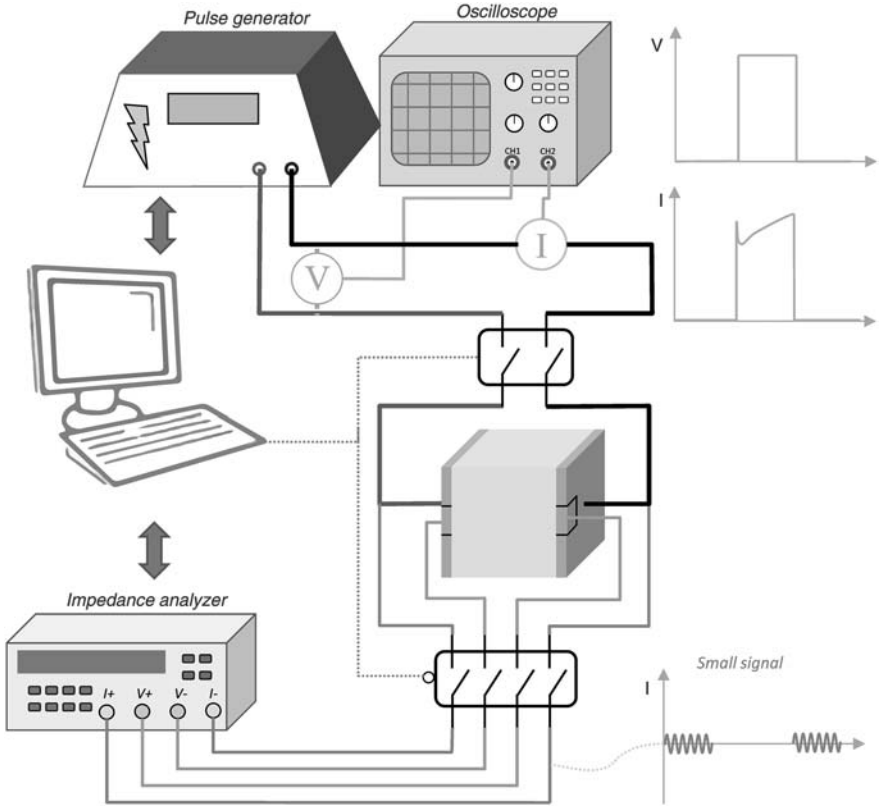

Fig. 6. Schematic presentation of common setup in electroporation applications for measuring the conductivity before and after the pulse using a small signal with a tetrapolar configuration. Source: © 2016 Springer International Publishing. Castellví, Q., Mercadal, B., and Ivorra, A. (2016). Assessment of Electroporation by Electrical Impedance Methods. In Handbook of Electroporation, D. Miklavcic, ed. (Springer International Publishing), pp. 1–20 [262]. Reprinted with the permission of Springer International Publishing AG.

be monitored by time domain methods such as time domain reflectometry (TDR) [271,272] and time domain dielectric spectroscopy (TDDS) [273,274] in which the reflected signal that contains information on the electrical parameters is measured and converted into the frequency domain by a Fourier transform. Electrical impedance tomography (EIT) [275] and magnetic resonance electrical impedance tomography (MREIT) [276] can be used for spatial conductivity distribution. Electroporation can be also monitored by microwave dielectric spectroscopy [277].

However, after the electroporation pulse, impedance measurements are affected not only by membrane conductivity changes due to electroporation but also by leakage of intracellular contents (ions) into the surrounding medium (resulting in a conductivity increase), Joule heating in a highly conductive medium (conductivity increase) and by cell swelling (conductivity decrease) [220,265]. These processes all influence impedance measurements and mask the membrane conductivity increase due to electroporation. This can lead to erroneous results of the extent of electroporation and difficulties in electroporation detection.

2.3.2. Voltage clamp techniques

Voltage clamp techniques enable direct measurements of membrane currents by clamping a constant voltage across the cell membrane and simultaneously measuring the transmembrane current in single cells or membrane patches. Changes in the membrane conductance can thus be observed [278]. By applying a ramp or rectangular voltage steps to the membrane before and after the electroporation pulse, the breakdown threshold and membrane conductance change can be monitored [279,280]. Electroporation-induced changes in membrane conductance can be evaluated from current-voltage (I-V) curves before and after electroporation. It is one of the fastest (with μs resolution) and most informative methods of analysing changes in membrane permeability [279,281].

vVoltage clamp techniques were initially developed as a tool for monitoring changes in currents through ion channels and utilises clamping at a physiological voltage range, usually from around −60 to +40 mV [282]. Electric pulses for electroporation are therefore defined as supra-physiological membrane potential pulses, with a magnitude of 300 mV or higher [283,284]. This poses a significant problem, since such high voltages can damage the voltage clamp amplifier and cause saturation or recording artefacts, and additional electrodes for delivering the EP pulse are needed, especially in the case of short (ns) pulses of higher electric fields (tens of kV/cm) [281].

In addition to planar lipid bilayer experiments [285–288], various methods of voltage clamp techniques have so far been used for studying electroporation in vitro. A classical, two-electrode voltage clamp technique (a double vaseline gap modification) has been used to observe the effects of electric pulses on skeletal muscle cell membrane both during and after exposure to the electrical pulse and moreover, it enabled pulse-induced electroporation (or "leakage") current to be distinguished from ionic channel currents [278,279,283]. In these experiments, transmembrane potential threshold for EP, a heterogeneous population of pores, two-phase resealing dynamics and the amplitude and duration effect on EP were observed.

In most other experiments, a patch clamp technique in various configurations (cell-attached, outside-out, and whole-cell) has been used [10,76,188,286,289–299]. The advantage of a patch clamp over a classical voltage clamp is the use of a single electrode (in a glass micropipette) instead of two (in a two-electrode voltage clamp), which makes measurements simpler, and, moreover, it provides the ability to measure transmembrane currents at small patches of membrane. However, since the distribution of pores is highly dependent on the spatial position in an electric field [1,34] whole-cell configuration recording currents over the membrane of the entire cell is advantageous. Patch clamp techniques have been used to determine thresholds of EP and irreversible EP, kinetics of pore expansion and resealing [293,294],

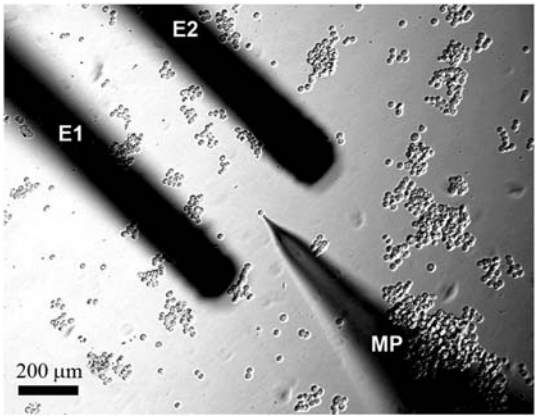

Fig. 7. Patch clamp recording in studies with nanosecond electric pulses: Nanosecond pulsed electric field (nsPEF) exposure of individual cells attached to a glass cover slip. NsPEF was delivered by a pair of electrodes (E1, E2) made of 125-μm diameter tungsten rod. These electrodes were placed symmetrically on the sides of the selected cell (center). Cells chosen for exposure or sham exposure were situated out of large cell clusters and showed no visible signs of damage or deterioration. A glass micropipette (MP) for patch-clamp recording was brought in contact with the exposed cell after nsPEF exposure. Source: © 2007 Elsevier. Reprinted from Pakhomov, A.G., Shevin, R., White, J.A., Kolb, J.F., Pakhomova, O.N., Joshi, R.P., and Schoenbach, K.H. (2007). Membrane permeabilization and cell damage by ultrashort electric field shocks. Arch. Biochem. Biophys. 465, 109–118 [76], with the permission of Elsevier.

differences in EP in cell types [10,76,286], EP in plants [296–298], characteristics of nanopores after nsEP [10,29,76,188,290–292], the inhibition effect by electric field reversal using nanosecond electric field oscillations [289] and poration by subnanosecond electric pulses [295]. In nsEP studies, high voltages of the porating pulse can damage the patch clamp setup or tight, gigaohmic seal between the cell membrane and the micropipette ("gigaseal"), all of which can lead to the production of artefacts in the recording currents [10,281]. This can be avoided by establishing the patch after nsEP pulsing [10,29,76,290], and Fig. 7, however, when using slightly lower voltages than usual in nsEP studies, the gigaseal can withstand pulsing and effective patch clamp recording is possible [29,188,291,292].

2.3.3. Cell swelling

One of the physico-chemical effects of electric pulses, cell swelling, can also be used to detect electroporation. Swelling occurs due to the osmotic imbalance generated by the leakage of ions and small molecules, which is followed by water influx, which is the primary cause of cell swelling [30,56,300]. There are four main stages of cell swelling after electric pulse application: 1. electroporation opens pores that allow the passage of small ions; 2. equilibration of intra- and extracellular Na^+, K^+, Cl^- ion concentrations by diffusion through pores or other structures; 3. during equilibration, osmotically driven water influx to the cell induces cell swelling; 4. when ion concentration gradients reach zero, water influx decreases until pores close. Ions afterwards re-establish concentration gradients or, if this is no longer possible, the cell ruptures [30]. Swelling occurs rapidly after plasma membrane electroporation [30,301] but if the cell survives swelling, cell size is regulated back to initial values by the extrusion of osmolytes in less than ten minutes [301]. Nevertheless, swelling has been proven to be a reliable method of determining the electroporation extent [30,56,104,220,301–303], even for nanosecond pulses that open pores too small for dye molecules such as propidium iodide [30,56]. The method is simple and only requires use of light microscopy. Swelling has been reported to be dependent on pulse field intensity [104,220,302,303] and other pulse parameters such as number and repetition rate [30]. Swelling after electric pulse application is strongly dependent on buffer osmolarity: it is most prominent in a hypotonic buffer [300,301,304]. Moreover, by observation of swelling in buffers containing different sugars and PEGs, electropore size can be determined [56].

2.4. Other methods

Other methods have also been used to detect electroporation in various cells and tissues. Through the decades of research in the field of electroporation, scientists have striven to visualize pores that emerge after electric pulse application. Since the pores are mostly smaller than the resolution of light microscopy, there have been a few attempts to observe the pores with electron microscopy. However, volcano shaped openings in freeze-fractured samples of erythrocytes [305] were later argued to be experimental artefacts – haemolysis pores induced by a secondary effect of cell swelling [306]. Other studies have revealed areas with rough structure on the membranes of melanoma xenografts

Table 1
Overview of all the methods with their advantages and drawbacks, together with some references in the last column.

Detection method	Compounds	Advantages	Drawbacks	References
Nucleic acid binding fluorophores	Propidium iodide, ethidium bromide, ethidium homodimer-1, YO-PRO®-1 Iodide, SYTOX® Green	• Significant fluorescence enhancement upon binding to DNA • No need to wash before detection • Suitable for fluorescence microscopy (real-time monitoring in individual cells), microplate readers, and flow cytometry (for average population response) • Also in vivo experiments.	• Toxic, long incubations have to be avoided due to cytotoxicity • Not suitable for detecting nanopores after nsEP application • Not the most sensitive	[7,8,15,17,50,53,68,81,96,188]
Small non-binding fluorescent molecules	Lucifer yellow, calcein	• Suitable for spectrofluorometers and fluorescence microscopy (after washing) • For transdermal delivery • AM ester form for studying efflux of the dye out of the cell • Detection of vesicle poration after nsEP	• No fluorescence enhancement • Washing of cells before detection needed • Time consuming – not for real-time observations • Leakage due to incomplete resealing of the membrane in the working step or dilution due to adding medium to soon • Possible quenching with metal ions	[15,75,108,109,114,116,123]
Dextrans	3–2000 kDa dextrans, labelled with fluorophores	• Suitable for fluorescence microscopy, flow cytometry and spectrofluorometry • For transdermal delivery • For estimating pore size and threshold as a function of molecular size	• Not suitable for detecting nanopores after nsEP application	[49,52,119,144,151,155,156]
Quantum dots and fluorescent nanoparticles		• High photostability, quantum yield and a large Stokes shift • Resistant to quenching • Suitable for fluorescence microscopy	• May be toxic • Aggregation in cytoplasm after EP • Increased pulse duration and intensity required for delivery	[21,162–164]
Ions and fluorescent ion indicators	Fura-2, fluo-3, calcium green, indo-1, FluxOR™	• Very sensitive, also suitable for nsEP experiments (especially Tl^+) • Ratiometric measurements •Suitable for fluorescence microscopy, or spectrofluorometry	• Ca^{2+} can also be released from internal stores or pass the plasma membrane via calcium channels or more complex pathways, which can all contribute to false positive results • Not directly related to membrane permeability for larger molecules of interest	[20,63,174,175,188]
Annexin V	FITC-annexin V	• Sensitive • Also suitable for nsEP experiments • For fluorescence microscopy and flow cytometry	• With long incubations might overlap with apoptosis detection	[22,96,188,190,191]
Colour stains	Trypan blue, phenosafranine, erythrosine B	• Can be used with simple light microscopy	• Low sensitivity and contrast • Toxic, long incubations have to be avoided due to cytotoxicity • Not suitable for detecting nanopores after nsEP application	[23,197,199–201]
Magnetic nanoparticles		• After delivery of magnetic nanoparticles, cells survive and can be transplanted to organisms • In vivo detection possible	• Require additional staining protocols or the use of magnetic resonance imaging or magnetic force microscopy	[24,205,206,208,211]
Cytotoxic compounds	Bleomycin, cisplatin	• Sensitive • Can be used in vitro and in vivo	• Time consuming, indirect observation of electroporation • Toxic	[25,26,220,222,224]
DNA and RNA		• Suitable for fluorescence microscopy and flow cytometry • In vitro and in vivo	• Time consuming • Require sterile work when detected by GFP expression • Not sensitive (gene electrotransfer is a complex process, depending on different conditions and not easy to achieve)	[27,234,235,238,242]
Extraction of biomolecules	Proteins, nucleic acids, pigments, lipids, sugars	• Biological species-dependent (can be an advantage or drawback)	• Not sensitive • Requires additional detecting methods • Time consuming • Biological species- dependent	[246,249,256,259,260]
Conductivity and impedance measurements		• In vitro and in vivo	• Not specific • Not directly related to membrane permeability for larger molecules of interest	[152,220,264,265,271,273]
Voltage clamp techniques		• Very sensitive • With high time resolution • Single cell observation	• Require equipment for electrophysiology and a skilled expert • Only single cell observation	[10,278,279,286,296]
Cell swelling		• Can be used with simple light microscopy • Very sensitive • Also suitable for nsEP experiments	• Strongly dependent on experimental buffers • Cell volume regulation mechanisms/dynamics need to be considered	[30,56,300,301,303,304]

and erythrocytes without pore-like craters [307], or concave-shaped pores in irreversible electroporation ablated tissues [308]. However, the techniques for sample preparation in electron microscopy are very aggressive and can affect metastable structures in a cell membrane [309], leading to possible artefacts. Recently, using total internal reflection fluorescence microscopy (TIRF), it was shown to be possible to detect and visualize individual electropores in planar droplet interface layers in real time by detecting the fluorescent signal proportional to the flux of calcium [12] or potassium ions [310] flowing through a pore (by exploiting optical single-channel recording). Pore dynamics, stochastic appearance and disappearance of pores, diffusion through pores, interaction between electropores, and fluctuations in pore radius have been estimated with TIRF. However, there are still limitations to this method: direct measurement of pore diameter is not possible since the detected cloud of ions is larger than the pore itself, and diffraction and time resolution limit the detection of events on a smaller time and space scale [12,310].

Another goal in electroporation studies has been to separate electroporated cells from non-electroporated. This can be done by the use of dielectrophoresis (DEP) in microfluidic devices [13,311–313]. Electroporated cells change their geometrical and electric properties, which has a strong influence on the behaviour of cells when exposed to DEP. This allows separation of non-porated cells from porated ones and even reversibly- and irreversibly-porated cells [13] by choosing the proper frequency for DEP.

There are many other methods of detecting electroporation but they are not often used, such as second harmonic generation imaging [314,315], ultra-weak light emission (endogenous biological chemiluminescence) detection for assessment of electroporation-induced lipid peroxidation [316,317] or confocal Raman microspectroscopy to investigate the effect of pulsed electric fields on the chemical composition of membrane proteins and lipids [318,319]. Some methods are avoided for safety reasons, such as cell uptake of radioactive isotopes [320]. Several other techniques have been specifically developed or modified for detecting electroporation in microorganisms such as the selective medium plating technique or Fourier transform infrared spectroscopy (FTIR), and food tissues, such as acoustic tests, and are more thoroughly described elsewhere [321,322]. A concise overview of all the methods is given in Table 1.

3. Conclusion

The number of researchers contributing to the development of the field of electroporation is increasing every year. They search for new applications, seek to optimize process parameters and to elucidate the mechanisms of this phenomenon. However, most studies begin with the detection of electroporation and/or determination of the extent of membrane electroporation in specific conditions. This can be done by using one of the numerous methods of detection that have been developed over the decades of EP research. However, they all have advantages and disadvantages, considering the available equipment and experimental conditions used. This review describes methods for the detection of membrane electroporation in a way that is intended to help scientists to choose the most suitable one for their specific studies.

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