GÖTEBORG UNIVERSITY

Development of Electroporation and Electroinjection Methods for Single-Cell Biosensors

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AKADEMISK AVHANDLING

för filosofie doktorsexamen i kemi (examinator professor Daniel Jagner) som enligt beslut av kemiinstitutionen offentligt kommer att försvaras fredagen den 7:e juni 2002, kl 13.15 i föreläsningssal KA, Kemihuset, Chalmers Tekniska Högskola, Göteborg.

Fakultetsopponent: Professor Andrew G. Ewing,
Department of Chemistry, Pennsylvania State University, USA

Analytical and Marine Chemistry
Göteborg University
2002

Printed by Chalmers Reproservice

Göteborg 2002

ISBN 91-628-5198-5

Abstract

Three miniaturised electroporation and electroinjection methods were developed to introduce exogenous compounds into single liposomes, and single cells, as well as small populations of cells in organotypic tissues, and tissues *in vivo*.

The first method was developed for electroporation of single surface-immobilised cells using solid carbon fibre microelectrodes, 5 μm in diameter. The protocol was characterised by patch-clamp recordings and fluorescence microscopy. From transmembrane current responses, pore open times, as well as pore opening, and closing kinetics were determined. From both patch clamp, and fluorescence measurements, the threshold transmembrane potential for electroporation was determined to be ~250 mV.

A second approach for electroporation was developed based on using an electrolyte-filled capillary (EFC) made of fused silica (30 cm long, 375-μm o.d. 30-50-μm i.d.). A DC voltage pulse (square wave, duration 5-60 seconds) induced pore formation and the electroosmotic flow in the EFC delivered the cell-loading agent to the site of pore formation. The threshold transmembrane potential for electroporation was determined to be ~85 mV by patch clamp and fluorescence measurements. The method was used to introduce the DNA-intercalating dye YOYO-1 to single cell processes, single cell somas, small populations of cells in organotypic tissues, and tissues *in vivo*. The enzyme substrates fluorescein diphosphate (FDP) and casein BODIPY FL were introduced to single cells for detection of alkaline phosphatase and proteases, respectively. Detection of intracellular receptors (IP_3 and ryanodine) was performed by introduction of selective receptor agonists and blockers. Electroporation of populations of cells in microwells (100 x 100 x 45 μm) fabricated in poly (dimethylsiloxane) was demonstrated by introduction of FDP.

Third, an electroinjection method for introduction of materials into single cells and liposomes was developed. This method uses a combination of electrical and mechanical forces to penetrate the lipid bilayer membrane. A DC electric field of 10-40 V/cm, and 1-10 ms pulse duration was applied with a carbon fibre microelectrode (5 μm in diameter) in combination with an injection tip (2 μm o.d.) filled with the loading agent. Injection was performed by pressure. Small sample volumes (5-500 x 10^{-15} L) of stained DNA, and colloidal particles (30-200 nm in diameter) could be electroinjected to single liposomes and cells. Selective introduction of colloidal particles to different sub-compartments of cells was also accomplished.

The methods developed can be used to manipulate cell properties, even at the subcellular level. Manipulation, sensing, transfection, probing of intracellular pathways, phenotype profiling, and screening are examples of applications, which may have an impact on the understanding of intracellular chemistry, and for single-cell analyses. These methods might be useful in areas such as drug discovery, proteomics and for the pharmaceutical industry.

KEYWORDS; Capillary electrophoresis, single-cell, electrolyte-filled capillary, fluorescence, patch clamp, receptors, electroporation, electroinjection, intracellular proteins, liposome.

Content part A

Content part B

This thesis is based on the following publications, which are referred to in the text by their Roman numbers.

I Kerstin Nolkrantz, Cecilia Farre, Anke Brederlau, Roger I. D. Karlsson, Carrie Brennan, Peter S. Eriksson, Stephen G. Weber, Mats Sandberg, Owe Orwar, Electroporation of single cells and tissues with an electrolyte-filled capillary, *Anal. Chem.* 2001, **73** (18), 4469-4477.

II Kerstin Nolkrantz, Cecilia Farre, K. Johan Hurtig, Petra Rylander, Owe Orwar, Functional screening of intracellular proteins in single cells and in patterned cell arrays using electroporation, Accepted for publication in *Anal. Chem. 2002.*

III Frida Ryttsén, Cecilia Farre, Carrie Brennan, Stephen G. Weber, Kerstin Nolkrantz, Kenth Jardemark, Daniel Chiu, Owe Orwar, Characterization of single-cell electroporation by using patch clamp and fluorescence microscopy, *Biophys. J.* 2000, **79** (4), 1993-2001.

IV Mattias Karlsson, Kerstin Nolkrantz, Maximilian J. Davidson, Anette Strömberg, Frida Ryttsén, Björn Åkerman, Owe Orwar, Electroinjection of colloid particles and biopolymers into single unilamellar liposomes and cells for bioanalytical applications, *Anal. Chem.* 2000, **72** (23), 5857-5862.

1
Introduction

One of the most fascinating challenges of modern science is to understand how biological systems function. This represents a major challenge because of the small scale as well as the high degree of structural, and chemical complexity in these systems. For example, organelles that can be regarded as the smallest functional units within a cell, are nanoscale objects and may have a volume of only 10^{-21} L. A single differentiated eucaryotic cell contains about 10 000-20 000 different proteins, DNA, RNA, thousands of small peptides and metabolites, a multitude of inorganic ions, lipids, water, and diffusible gases such as O_2, CO_2, and NO. These different species display a rich palette of chemical properties, and a complex pattern of chemical interactions. Characterisation of genomic and proteomic maps in different organisms is of tremendous value, however, the flux of information in biological systems mainly come from chemical interactions between the different players. Understanding this flux of chemical information will be central in understanding biology, and requires ultra-sensitive analytical tools as well as methods to manipulate the biochemistry on the level of single organelles or single cells. For example, the ability to identify different proteins and follow their interactions with other substances in discrete regions of cells will take us a step closer to understanding intracellular chemistry. A central theme in the present thesis relates to development of miniaturised analytical methods for identifying proteins and chemical interactions inside cells, as well as methods to identify exogenous species acting on intracellular chemistry. In addition, development of such methods is important in drug discovery, drug development, and diagnostics.

Biological systems are, generally, studied on many cells simultaneously (bulk analysis). By working on the single-cell- or single-organelle-level, properties hidden in the ensemble average obtained from bulk analysis, can be revealed. Structural and physico-chemical studies have been performed on single cells for a long time using tools such as patch-clamp recordings (Neher and Sakmann, 1976) and electron microscopy (Pease and Porter, 1981). However, it was not until recently that chemical analysis on the level of single cells (Olefirowicz and Ewing, 1990) and single organelles (Chiu *et al.*, 1998) were performed successfully. These studies involve the use of a chemical fractionation step by capillary electrophoresis (Jorgenson and Lukacs, 1981). Capillary electrophoresis has demonstrated to be instrumental in the development towards single-cell and single-organelle analysis because of its small physical dimension, high separation efficiency, and compatibility with physiological buffers. In the studies mentioned above, as well as in related applications, biomolecules were detected using traditional techniques, such as UV absorption, fluorescence or red ox activity, based on native or acquired (through labelling schemes) physio-chemical properties of the molecules (Chiu *et al.*, 1998; Finnegan *et al.*, 1996; Fuller *et al.*, 1998; Woods *et al.*, 2001).

Miniaturised analytical methods as well as mass-spectrometry-based methods, such as, MALDI-TOF (Li *et al.*, 2000; Mann *et al.*, 2001) can identify different proteins, their concentration, and sometimes their localisation within a cell but their interactions with other components, and the rates and equilibrium constants of these interactions are, generally, not directly obtained. A complementary approach to traditional detection and analysis techniques is based on using biomolecular recognition. This biosensor concept is useful since binding of, for example, endogenous compounds and drugs to receptors, enzyme-substrate interactions, and binding of gene regulatory proteins to DNA can be studied directly, and hence give thermodynamic and kinetic information that is valuable in a biological perspective. Detection based on molecular recognition is highly selective and when intact cell biosensors are used, also a high sensitivity is obtained because the recognition event is often coupled to some sort of amplification system, such as opening of ion channels, or activation of intracellular cascade reactions (Straub *et al.*, 2001; Young *et al.*, 2000). By coupling biosensors to chemical separations, complex mixtures of *e.g.* receptor ligands can be identified after fractionation (Farre *et al.*, 2001; Luzzi *et al.*, 1998; Orwar *et al.*, 1996; Shear *et al.*, 1995).

Methods for detecting protein expression and function are central in characterising and understanding many aspects of cell biology and for drug discovery. The proteins constitute approximately 18 % of the total weight in a cell. The number of each protein contained in a cell range from a few copies to hundred thousand copies. All proteins are of central importance in supporting cell function and structure, and because of the tight regulation between different enzyme systems, and signalling pathways, a protein is often capable of imposing an effect on a large number of different reactions and interactions within a single cell. In particular, rapid, cell-based, and functional screening methods that allow characterisation of interactions between intracellularly confined proteins and small molecules would dramatically increase the target space for drug libraries. Today screening is usually performed for targets situated on the cell plasma membrane, such as receptors, ion channels, and transporters, however, these proteins represent a minority of the proteome, the rest residing inside the cell. To access the intracellular components, highly specific fluorescent probes are widely used for protein detection. Unfortunately many fluorescent probes can not enter cells spontaneously or can not easily be modified to aid in the transport over the membrane. Methods like lipofection, and electroporation has traditionally been used for introduction of exogenous substances into populations of cells, and methods based on microinjection have been used on the single-cell level. Microinjection methods, however, have some shortcomings, such as, damage of cell membranes and intracellular structures caused by mechanical impact, it is hard to perform on small cells, and is not well suited for introduction of larger particles.

Specifically, the work in this thesis relates to development of miniaturised electroporation and electroinjection methods for biosensor applications where intracellular targets are used in the recognition event. All methods rely on using focused electric fields to break the plasma membrane barrier to allow entry of exogenous compounds into the cytoplasm either through finite-lifetime membrane pores or through passage using a micropipette tip sealed to the membrane. It is demonstrated how genetic material (DNA), receptor ligands, enzyme substrates, and dyes can be introduced into single cells, single cellular processes, as well as small populations of cells in tissues with high spatial resolution. It is also demonstrated how cells can be patterned in microwell arrays for electroporation, and preliminary results are shown on a biosensor chip device that combines capillary electrophoresis and electroporation. An electroinjection method that combines electroporation and pressure-driven microinjection of single cells and liposomes held under mechanical stress for introduction of large biopolymers

and colloidal particles was also developed. It is also shown how electroporation can be used for *in vivo* electroporation of rat brain to access a small population of cells. These new methods predict a number of possible future applications, including manipulation (*e.g.* genetic and metabolic programming), sensing, probing, phenotype profiling, and high-throughput screening of biomolecules with intracellular targets, performed at the single-cell level.

This thesis combines some tools of analytical chemistry with simple physical principles, and the molecules and concepts of biology to understand and control cellular processes, to obtain new tools for detection of biomolecules, and for manipulation of biological material. Much of the current development in analytical chemistry, in particular, in instrumentation, is being focused on advanced and highly technical solutions. Here simplicity is highlighted and the hard work was actually performed by the cells used in this thesis. A future goal is to fully integrate these analytical and manipulation methods on a microchip. This will enable high-throughput screening for new drug targets, fast analysis of unknown samples in medical diagnostics and many other applications in bioanalytical chemistry, medicine, and biology.

Finally, I hope that the methods developed in this thesis will provide the readers with some food for thought, and a wish to explore these fascinating areas of research for further development.

2
Cell signalling

The human body contains about 10^{14} cells, each with a radius of 10-30 μm. If you align all these cells after each other in a straight line, they will reach from Earth to the moon! Each cell is a small but important component in a complex network where they communicate and influence each other to for example produce or release something. They are self-replicating, programmed by DNA, and by RNA the programming is translated and executed via proteins. Cell signalling regulates this process. Signalling is performed by proteins in the form of enzymes and receptors interacting with small molecules. Some of the most important signalling receptors are described in table 1. In this thesis different cell signalling systems have been used. The systems, proteins and the superfamilies they belong to are further described here together with other important signalling systems to give a better understanding of the presented results and proposed applications.

Table 1
Some properties of the four main types of receptor superfamilies.

Superfamily	Location	Effector	Coupling	Time scale	Ligand
Channel-linked	Membrane	Channel	Direct	Millisecond	ACh GABA Glutamate
G-Protein coupled	Membrane	Channel Enzyme	G-protein	Seconds	ACh Bradykinin
Kinase linked	Membrane	Enzyme	Direct Indirect	Minutes	Insulin Growth factors Cytokine
Controlling gene transcription	Intracellular	Gene-transcription	Via DNA	Hours	Oestrogen Vitamin D Thyroid

2.1 G-protein-coupled receptors

A receptor is a protein, which recognises and binds one or several other small molecules with high specificity. The binding event usually triggers a response in the cell. G-protein-coupled receptors form a large superfamily of cell-surface receptors (>1 000 members in man) that

respond to a wide range of stimuli, including light, hormones and neurotransmitters. Genes encoding this huge family of G-protein- coupled receptors occupy a hefty 3 % of our genome and about 50 % of currently available drugs on the market directly affect G-proteins. Due to the huge importance of G-proteins in biology and medicine the Nobel Prize in Physiology or Medicine in 1994 was awarded to Alfred G. Gilman and Martin Rodbell for their discovery of *"G-proteins and the role of these proteins in signal transduction in cells"*.

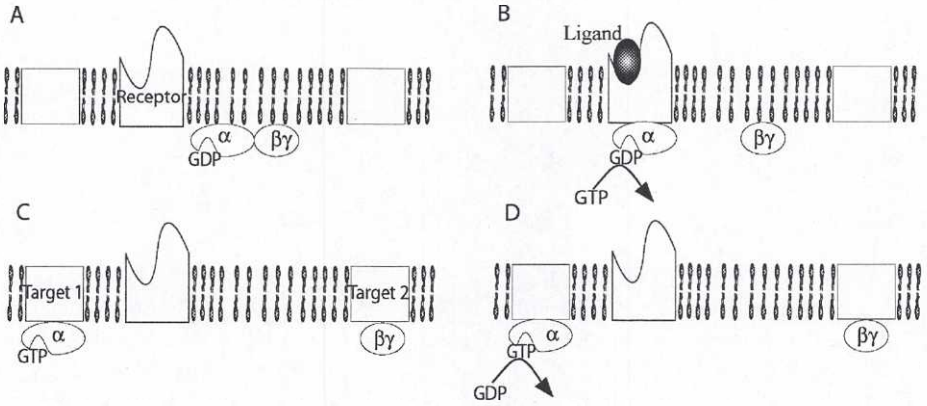

Figure 1
A) The G-protein, composed of α, β and γ subunits, binds GDP in its resting state. B) When a ligand binds to the receptor, it activates the G-protein, which converts GDP to GTP. C) The subunits separate and activate different targets. D) Some seconds later the GTP, bound to the α-subunit, is hydrolysed to GDP. The subunits recombine and the resting state of A) is resumed.

There are two main classes of G-proteins, the heterotrimeric G-proteins and the small cytoplasmic G-proteins. From now on I will refer to G-proteins as the heterotrimeric group. All G-proteins have a similar structure. They consist of seven membrane-spanning domains, an extracellular amino terminus and an intracellular carboxy terminus. G-proteins have a ligand-binding site on the extracellular side and a G-protein binding site on the cytoplasmic side. Heterotrimeric G-proteins contain three different subunits, G_α, G_β and G_γ. In the resting state, the G-protein α- unit binds guanosine diphosphate (GDP), figure 1. All three subunits are anchored to the membrane by a fatty acid chain, attached to an amino acid residue. Agonist (stimulatory molecule) occupancy of the receptor promotes the activation of the G-proteins by catalysing the exchange of GDP for GTP on the G-protein α-subunit. The α- and βγ-subunits of the G-protein dissociate from each other, and separately activate several classical effectors, including adenyl cyclases, phospholipases and ion channels. There are many different types of G-proteins, which can be linked to different receptors and effector systems. Both the receptor and the G-protein can diffuse rapidly in the plane of the plasma membrane. From this follows that every activated receptor can activate many different G-proteins, and every G-protein can effect many signalling targets. This signalling cascade serves to amplify the signal. For instance, a single receptor can sequentially activate 1 000 G-protein molecules. Some examples of available receptors, enzymes, *etc* involved in the G-protein-coupled signalling pathways are given in table 2.

Table 2
Examples of G-protein-coupled pathways.

Protein class	**Variations**
Receptors	A_1 D A_2 $GABA_B$ SS VIP CCK GG α_1 β H 5HT M CGRP LHRH B SP
G-protein	G_s G_{olf} G_I G_o G_p G_T
Enzyme	AC PLC PLA_2 PDE
Second messenger	cAMP IP_3 DAG AA cGMP Ca^{2+}
Enzyme	PKA CaM-PKII PKC
Target	Ca_L Ca_T Na^+ K^+ K(Ca) K_{ACh} Cl^- I_h Enzymes Pumps Cytoskeleton Transcription factors

A_1 & A_2 = Adenosin, D = Dopamine, $GABA_B$ = GABA type B, SS = Somastostatin, VIP = vasoactive intestinal peptide & pituitary adenylate cyclase activate peptide, CCK = Cholecystokinin & gastrin, GG = Glucagone, α_1 & β = Adreno, H = Histamine, 5HT = Serotonin, M = Acethylcholine muscarine, CGRP = Amyline & adrenomedullin, LHRH = Luteinizing-hormone-releasing hormone, SP = Substance P, B = Bradykinin, G_s = Stimulate adenylate cyclase, G_{olf} = olfactory cilia, G_i = inhibit adenylate cyclase, G_o = other activators of PLC, G_p = activate PLC, G_T = Transducin, AC = adenylat cyclase, PLC = phospholipase C, PLA_2 = phospholipase A_2, PDE = cAMP phosphodiesterase, cAMP = cyclic adenosine 3, 5 monophosphate, IP_3 = inositol 1,4,5-triphosphate, DAG = diacylglycerol, AA = arachidonic acid, CGMP = cyclic guanosine monophosphate, PKA = phospholipase A, CaM-PKII = type II Ca calmodulin dependent protein kinase, PKC = phospholipase C, Ca_L = L-type Ca channel, Ca_T = T-type Ca channel, Na = Sodium, K = Potassium, K(Ca) = Big KCa channel, K_{ACh} = inward rectifying K channel, Cl = chloride, I_h = channels activated by hyperpolarisation

If the G-protein G_p is activated the $G_{\alpha p}$ will bind to the enzyme phospholipase C (PLC). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the two second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is hydrophilic and acts in the cytosol. It diffuses to the endoplasmic reticulum (ER) and binds to a ligand-gated ion channel known as the IP_3-receptor. Activation of this receptor causes release of Ca^{2+} from ER. After G-protein activation the intracellular concentration of Ca^{2+} is increased 10-100 times. The released Ca^{2+} has many different targets within the cell. One possibility is to bind to a protein called calmodulin. This complex will then activate a lot of different physiological processes. Ca^{2+} can also bind to and act directly on proteins. Calcium pumps in the plasma membrane and on ER restore the calcium level in the cytosol to remove the signal. DAG is hydrophobic and, in contrast to IP_3, remains in the membrane to activate protein kinase C (PKC). PKC will phosphorylate serine and threonine on different proteins. This class of receptors includes the angiotensin, bradykinin and vasopressin receptors.

2.2 Channel-linked receptors

Ion channels are proteins that provide a conducting, hydrophilic pathway across the hydrophobic interior of the membrane. Ion channels are present in all human cells and affect vital functions, such as, nerve transmission, muscle contraction, and cellular secretion. They generally have high transport capacity and work on the time scale of milliseconds. This is a prerequisite to be able to propagate fast electrical signals such as an action potential. Studying and understanding ion channels are important since many diseases are caused by defects in ion channel function, for example, in cystic fibrosis, a defect in a type of Cl^- channels is found.

A channel protein can as most proteins change conformation. For channel proteins this regulates pore opening and closing, so called, gating. Channel gating is induced by the sensor part, which can be changes in the transmembrane voltage (voltage-gated ion channels), binding of a ligand to a receptor (ligand-gated ion channels) or by lateral membrane tension (mechano-activated ion channels).

All ion channels have the same basic structure, figure 2. They are transmembrane proteins with several separated subunits forming the channel. The pore-forming subunits contain transmembrane α-helices. One of the subunit α-helices consists of a special set of amino acids functioning as the sensor for opening and closing. A structural change in the sensor subunit acts on these amino acids and force the helix to rotate (sliding helix) resulting in opening or closing. At the opening of the pore on the extracellular side, a selectivity filter is situated at the narrowest part of the channel. It has a certain set-up of amino acids, which functions as a filter, and by electrostatic forces attracts or repels specific ions. Selection is also made by the size of the pore.

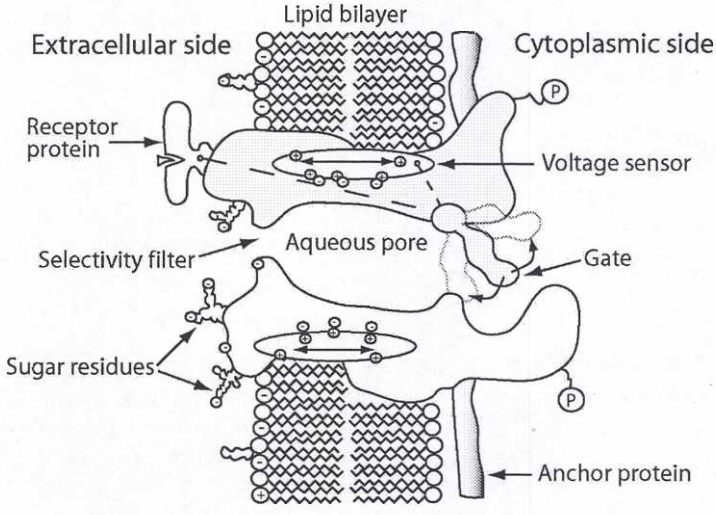

Figure 2
A schematic drawing of an ion channel displaying the functional units.

2.2.1 Voltage-gated ion channels

A voltage-gated ion channel is a transmembrane ion channel in which the permeability to ions is extremely sensitive to the transmembrane potential difference. There are different classes of voltage-gated ion channels. The important channels are named after the ion flowing through the channel, *i.e.* Na^+, K^+, Ca^{2+}, and Cl^-. They have the same structure with four subunits building up the pore. Each subunit consists of 6 membrane-spanning segments, S1-S6, where the S4 is responsible for the voltage gating. Within each class of voltage-gated ion channels there are different subtypes, which differ from each other. For example, the Ca^{2+} channel (L, N, P, Q and T type) have differences in their pharmacology, ionic selectivity, metabolic regulation and single-channel conductance, but the K^+ channels (K_a, K_v, K_{vr}, K_{vs} and K_{sr} type) on the other hand are distinguished only by their gating characteristics.

Generally, voltage-gated ion channels have steeply voltage dependent gates and shut down rapidly after repolarisation to be efficient when they transduce and produce electrical signals. For example, the Na^+ channel activates and deactivates within 0.1-1 ms. In terms of signalling this is fast. With patch clamp recordings the current over open channels can be measured and is usually in the range of 2-10 pA. This corresponds to 12-60 x 10^6 ions moving per second.

2.2.2 Ligand-gated ion channels

A ligand-gated ion channel is a transmembrane ion channel whose permeability is increased by the binding of a specific ligand. The ligand-gated ion channels belong to three different super families, the nicotine acethylcholine-γ-aminobutyric acid (nACh), the excitatory amino acid (EAA), and purine (P2X) receptors. In each superfamily, there are several different receptors. Members of the nACh superfamily are nicotinic-, $GABA_A$-, 5-hydroxytryptamine and glycine receptors. The EAA superfamily contains the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainat receptors. The P2X superfamily consists of, for instance, the purine P2X receptor gated by ATP.

Structural features are shared within this family; they all are pentamers, *i.e.* consists of five polypeptide chains spanning the membrane. Each polypeptide has four domains called M1-M4. The M2 domain is facing the centre and forms the pore thus determining the selectivity of the channel. Stimulation of the receptor results in an altered current. Generally, ligand-gated ion channels have high permeability and transport about 10^4 ions/ms. They also have high selectivity for either anions ($GABA_A$, glycine) or cations (ACh, glutamate).

2.2.2.1 IP_3 and ryanodine receptor

The two receptors responsible for release of calcium ions from intracellular stores are the inositol 1,4,5- triphosphate receptor (IP_3) and the ryanodine receptor (RyR). Both receptor families share some structural and functional characteristics. For example, both are homotetramers with six membrane-spanning helices and both are calcium ion selective channels. There are interactions between the two receptor families, but theses are poorly understood. They are activated and inhibited by different ligands while some substances affect both, but in different ways. For example, heparin and caffeine are antagonists (inhibitory molecules) of IP_3, but agonists to RyR. Both receptors are also specialised for different physiological actions.

The IP_3 receptor has four binding sites for IP_3. The interaction between IP_3 and the receptor is poorly understood, but Ca^{2+} itself plays an important role. For example, low concentrations of Ca^{2+} make the receptor sensitive to IP_3 and high concentrations inhibit it. Upon binding of IP_3 to the receptor the channel opens within milliseconds and stays open for ~4 ms. In cells the IP_3 receptor is present on endoplasmic reticulum (ER) and RyR is mainly present on the sarcoplasmic reticulum (SR). RyR is a calcium-induced calcium release receptor (CICR) with calcium as the most important ligand and transported ion. Also, cyclic adenosine diphosphate ribose (cADPr), with a similar function to IP_3, is an important ligand. The mean open time for the ryanodine receptor is ~20 ms.

The resting concentration of Ca^{2+} is 20-300 nM in cells. This level is set by ATP-dependent pumps and Na^{2+}-Ca^{2+} exchange systems at the plasma membrane and ATP-dependent pumps on the organelles, such as, the ER and SR. Upon increased intracellular Ca^{2+} concentrations these systems will act to restore the Ca^{2+} resting level.

2.3 Enzymes

Enzymes are proteins and works as catalysts, speeding up chemical reactions by up to 10^7-10^{14} times faster than non-catalysed reactions. A typical enzyme catalyses 1 000 reactions per second and some catalyse more than 10^6 reactions/second. Each enzyme is very selective and catalyse a specific reaction, but more than one substrate might be processed by the same enzyme. The selectivity depends on the structure of the active site, which is a three-dimensional region containing binding and catalytic sites. This selectivity means that thousands of different enzymes with specific tasks are needed to make the cell function properly and, so far, about 1 500 enzymes have been identified. Many intracellular transducers/messengers are enzymes. A malfunction of enzymes may cause diseases like albinism, which is due to the absence of tyrosinase, an enzyme essential for the production of cellular pigments.

The action of an enzyme lowers the activation energy of a reaction. The interaction between substrate and enzyme is hypothesised to occur according to the so-called induced fit model. The initial substrate binding to the active site will structurally distort the enzyme and the substrate. The enzymes undergo a conformational change to pull the substrate into the transition state and thereby position the reactive group in a position for catalytic reaction.

Enzyme reactions generally follow Michaelis-Menten kinetics, which describe the reversible formation of a product from a substrate through the reversible formation of an enzyme substrate complex. The kinetics is characterised by a hyperbolic relationship between initial reaction rate and the substrate concentration. The fundamental meaning of this is that the reaction rate can not increase to infinity with increased substrate concentration.

2.3.1 Regulation of enzymes

In a complex biochemical system, it is necessary to control the rate of biochemical reactions. There are different ways this can be performed and one way it is done is by enzyme inhibition. Inhibition can be either reversible or irreversible. There are two main reversible enzyme inhibition mechanisms. The most direct way is to provide a molecule, which fits into the enzyme's active site but does not react with anything there. This is called competitive inhibition since the inhibitor competes with the substrate to bind to the active site of the enzyme. Binding of the inhibitor will reduce the activity. A non-competitive inhibitor fits into a site on the

enzyme, different from the active site. When this happens, the folding of the enzyme changes a little bit, and the active site is distorted in a way which makes it a less effective catalyst or impossible for the substrate to bind.

Regulation of enzymes can also be performed allosterically and a special class of enzymes capable of this has evolved. The most common control mechanism for allosteric regulation is feed back control where the final product controls it owns synthesis. An allosteric enzyme has two states, one with high affinity for the substrate and one with low affinity. The favoured state depends on the so-called allosteric effector, which usually is a small organic molecule. The effector binds to the allosteric site on the enzyme, which not is the active site. These concepts of inhibition of enzymes can, in general sense, be applied to receptors and the action of antagonists acting on the receptor.

2.4 Networks of signalling pathways

Cell signalling does not normally work in a simple linear manner with parallel pathways that affect a single target. Instead, a signal leads to activation of a cascade of effectors with crosstalk between pathways, resulting in complex networks. At the points of cross talk between pathways within a single cell, the signal can either be integrated or split. One example of signal integrators is the group of adenylate cyclases, which receive signals from both G_s-coupled G-proteins and Ca^{2+} to produce cAMP. Since there exist different isoforms of adenlyate cyclase, which all can receive signals from different systems, the adenylate cyclases act as a complex signal-receiving interconnections (Pieroni *et al.*, 1993). An example of a signal splitter is the receptor tyrosine kinase, which directs the signal from growth factors and spread it in many different pathways (Schlessinger, 2000). From human genome sequencing it is known that 5% of the genes encode receptors, but fewer than 3% encode for kinases. This implies that individual kinases transmit signals from multiple receptors and that cells must have ways to strictly regulate the specificity in signalling (Venter *et al.*, 2001). The cascade cross talk can be stimulatory and this allows the cascade to amplify the signal. Therefore really low signals derived from single molecules can be detected. The system can also be inhibitory and regulate multiple cellular tasks. Cross talk between cells is also important and, for example, a single neuron can communicate with up to 1 000 other neurones, which also spreads the signal.

For intracellular signalling networks to function effectively, spatially restricted activity (Teruel and Meyer, 2000) is important. Compartmentalisation of the various effectors of a network inside the cell is one way to provide this. The compartmentalisation acts in many ways, for example, the compartments congregate a substrate and the enzyme that acts on it into the same compartment, or restricts the diffusion of the reagents to a specific dimension. There is also a separation of reactions in space, which allows the same molecule in the same cell to carry entirely different signals. By compartmentalisation both synthesis and degradative processes can occur simultaneously in a cell without affecting each other. Another way to obtain spatially restricted activity is to bring the signalling pathways together in complexes. There are anchoring and scaffolding proteins at specific locations within the cell. These proteins assemble different signalling proteins to achieve selective separation and specificity of the signalling pathways.

The cell synthesises many different proteins (receptors, enzymes *etc*) and must transport them to the correct site. Therefore, molecular trafficking between the compartments is important for cell signalling. Transport by diffusion, is extremely inefficient and a non-selective way to transport substances within a cell. Therefore, motor molecules are used, for example, along the actin-tubulin system (Goodson *et al.*, 1997).

The fate of a signal is dependent on the network architecture and the possibility for cross talk. By a combination of both regulatory mechanisms and desensitisation the signal will be propagated, terminated or tuned. Depending upon amplitude (dose, duration of activation), the signal is directed to evoke different responses. Thresholds to obtain a response can be set at single or multiple levels. The multilevel control can depend on the concentration of the signalling components, interactions between the signalling components and co-localisation of the interacting components. The movement of signalling proteins and high complexity with a well-optimised system enables the networks to work both spatially and temporally. This also makes them extremely complex and difficult to model.

Analytical tools cannot solve this complexity. Bioinformatic, *i.e.* the aid of computer technology to manage the information, is also needed. Some signal networks have all ready been discovered from computer simulations, *e.g.* oscillatory behaviour and bistability which is suggested to work as information processing systems and might account for memorising events (Weng *et al.*, 1999). Also virtual cells and organs, so called, *in silico* testing, are used to test the effect of new drugs.

3
Electroporation

Electroporation, or electropermeabilisation, is a method widely used in biology to manipulate cells (gene therapy, fusion of cells, insertion of proteins), introduce substances into cells (DNA, dyes, reagents) and to kill unwanted cells such as tumour cells. Basically, when an external electric field is applied over a cell membrane or synthetic cell membrane (liposome), dielectric breakdown of the membrane will occur resulting in formation of aqueous pores, or holes, in the membrane. Through these pores spontaneous transport of small molecules can be performed both into and out of the cell.

3.1 Biological membranes

Biological membranes separate the cell interiors from the outside solution and also regulate the molecular traffic across it. Without these properties life would not be possible. Besides creating the outer boundary, the membranes also divide the inside of the cell into various inner compartments, where all has specialised functions. Biological membranes can be described according to the fluid–mosaic model (Singer and Nicolson, 1972) where the membrane structure is composed of a lipid bilayer membrane containing embedded proteins. Furthermore, the plasma membrane is asymmetric with regard to the transverse and lateral distribution of phospholipids and contains domains that function as diffusion barriers, enabling accumulation of specific proteins in various locations in the membrane. Consequently, biological membranes contain regions with distinct function and composition.

Natural membranes have a large diversity in their lipid composition, for example, erythrocyte membranes consist of about 100 different types of lipids. The most frequently occurring lipid-species are the glycerophospolipids and cholesterols. Synthetic membrane vesicles (liposomes) on the other hand, often consist of a pure component or a mixture of a few lipids. In this thesis, we used cell-sized unilamellar liposomes, so called GUV´s (giant unilamellar vesicles), as a model system for the development of the electroinjection technique (paper IV). Lipid bilayer membranes are two-dimensional liquid crystals and exhibit phase behaviour. Depending on temperature, hydrostatic pressure, ionic strength and composition a lipid membrane will arrange into different phases (lamellar gel phase, lamellar liquid crystalline, hexagonal I and II). At physiological temperature the lipid bilayers in cells assume the lamellar liquid crystalline phase.

Biological fluid-state (lamellar liquid crystalline) lipid bilayer membranes behave as two-dimensional fluids. The membrane material allows lateral diffusion of lipids ($D = 10^{-8}$ to 10^{-14} cm^2/s), transversal bilayer movement (flip-flop) on the other hand is a rare event with a time constant of hours to days. Because of the very low transversal exchange, the two monolayers of the membrane can be considered to be two separate entities having the ability to relax

mechanical strain independently. From being a 2D fluid also follows that membranes have a strong resistance to area dilation. Generally, a lipid membrane can be regarded as incompressible. In fact, a lipid membrane can only be stretched 3-5 % from its tension free state before tension-induced rupture occurs. On the other hand, because the lipid membranes are very thin structures, there is very little resistance to bending deformations. Furthermore, the fluid-state membrane has zero resistance to shear deformations, *i.e.* the membrane allows in-plane shape deformations at constant area.

Importantly, the lipid and protein composition defines the function and materials properties of a biological membrane. The perhaps most important feature of a biological membrane is selective permeability. Generally, charged species can not pass the membrane, while polar solutes pass to a varying degree. For example, oxygen molecules, ethanol, and urea can diffuse across the membrane rapidly, while glycerol and glucose diffuse slowly. Ionic substances generally need pores, channels (*e.g.* voltage-gated ion channels, ligand-gated ion channels) or transporters (*e.g.* ATPases, red ox coupled transporters such as cytocrome c) to be transported across the membrane.

In cell membranes negatively charged lipids are predominantly located in the bilayer leaflet facing the cytosol. This give rises to a charge gradient, which has influences on the electrophysiology of the membrane and intracellular signalling. The lipid bilayer in biological membranes is typically less than 5 nm thick and has low dielectric constants (K_m ~2-3). Consequently, lipid membranes are excellent capacitors (conductivity ~1 $\mu F/cm^2$). The transmembrane potential (potential at the inner side of the membrane relative to the potential at the outside of the membrane), V_m, of a cell is normally –20 to –200 mV. When an external electric field is applied, V_m changes and at a critical value, dielectric breakdown of the membrane will occur. When short pulses (μs- ms) are applied, dielectric breakdown of the membrane has been found to occur when the critical value of 0.5- 1.5 V is reached (Weaver, 1993). These values are independent of the method used and cell type. For longer pulses (> ms) 200-300 mV is sufficient (Akinlaja and Sachs, 1998; Zimmermann and Niel, 1996). The transmembrane voltage, V_m, of a spherical cell can be calculated according to

$$V_m = 1.5 r_{cell} E_{appl} \cos\theta \left[1 - e^{(-t/\tau_m)}\right] \qquad \text{Equation 1}$$

Where r_{cell} is the radius of the cell, E_{appl} is the applied electric field strength, θ the angle between the axis of the applied electric field and the site on the cell membrane at which V_m is calculated, t is the duration of the pulse and τ_m is the membrane charging time (Cole, 1968). If $t >> \tau_m$, equation 1 can be simplified to

$$V_m = 1.5 r_{cell} E_{appl} \qquad \text{Equation 2}$$

The membrane charging time, τ_m, depends on the radius of the cell, the capacitance of the membrane C_m and the resistivities of the intra- and extracellular medium, ρ_{int} and ρ_{ext}, according to

$$\tau_m = r_{cell} C_m (\rho_{int} + 0.5\rho_{ext}) \qquad \text{Equation 3}$$

3.2 Formation of pores in electroporation

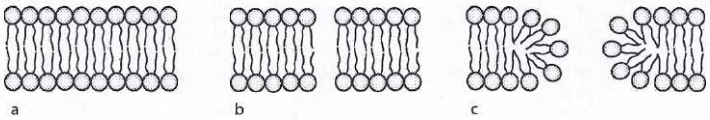

Figure 3
Hypothetical structure of a pore formed by electroporation. An intact lipid membrane (a) spontaneously forms a hydrophobic pore (b) due to defects in the lipid membrane. During the influence of an external applied electrical field the hydrophobic pores are transformed into a hydrophilic pore (c).

In a typical cell membrane, small defects are constantly formed due to thermal motion in the lipid membrane. It is believed that these defects spontaneously convert to hydrophobic pores, figure 3. In a hydrophobic pore a structural rearrangement in the water phase close to the lipid phase takes place. When two hydrophobic surfaces come into close proximity these layers will overlap and thereby lower the interfacial tension of the hydrocarbon tails facing the hydrophilic environment (Israelachvili and Pashley, 1984). As a result of this, the energy needed to form hydrophobic pores decreases and the probability for their existence increases. The probability for formation of hydrophobic pores in the absence of an external electric field is low, but increases when the external electric field is applied. The lifetime of a hydrophobic pore is on the same time scale as lipid fluctuations. As the pore dilates, this interaction decreases and the pore edge will diminish. At r_* (r = 0.3- 0.5 nm), figure 4, the transformation from a hydrophobic to a hydrophilic pore (figure 4, stage c) becomes more energetically favourable, and proceeds as follows. The lipids at the pore lining reorientate to point their head groups into the pore channel and thereby a hydrophilic pore is formed. To form a pore and build up the edge of the pore at a small radius ($r_{pore} << h$, h = is the overall thickness of the lipid bilayer membrane and, r_{pore} = pore radius) requires a lot of energy. This is seen as an energy barrier in figure 4, stage a. The energy barrier is contributed to by several factors: deformation and rearrangement of the lipids inside the pore require energy and hydration interactions will increase the pore energy by repulsive forces.

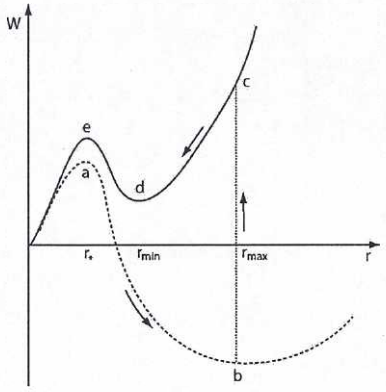

Figure 4
Pore energy, W, as function of pore radius, r, in the absence (upper curve) and presence (lower curve) of an external applied electric field.

The energy required to form a hydrophobic pore, E_{HO}, is

$$E_{HO} = 2\pi r_{pore}\sigma_{HO} \qquad \text{Equation 4}$$

Where r_{pore} is the radius of the hydrophobic pore and σ_{HO} the interfacial tension between the bare hydrocarbon tails and water molecules (Glaser *et al.*, 1988). The energy needed to form an hydrophilic pore, E_{HI}, can be described by the following equation

$$E_{HI} = 2\pi\gamma r_{pore} - \pi\sigma_{memb} r_{pore}^{2} \qquad \text{Equation 5}$$

Where r_{pore} is the pore radius at the narrowest part, γ the line tension, which is the energy per unit length of the membrane contour at the pore edge describing the energy needed to build up the edge of the pore, and σ_{memb} the membrane surface tension, *i.e.* area energy (Glaser *et al.*, 1988). This equation tells us that if the radius of the pore is smaller than γ/σ_{memb} the pore will reseal again since the first term in equation 5, the edge energy of the pore, is dominating. If the surface tension dominates, *i.e.* the second term in equation 5, the pore will not reseal (Wilhelm *et al.*, 1993). It has been concluded that the magnitude of the transmembrane potential, which follows from the external applied field, is the only factor governing the dielectric breakdown of membranes (Kinosita and Tsong, 1977b). The time required to form pores is the time it takes for the charge separation over the membrane (V_m) to reach the critical value for dielectric breakdown, equation 2. The time constants for formation of a hydrophobic pore, k_{HO}, is 10 μs and for hydrophilic pore, k_{HI}, 100 μs (Neumann *et al.*, 1992).

The uptake of molecules during electroporation has been reported to be asymmetric (Teruel and Meyer, 1997). Most often positively charged ions to a larger extent enter at the anode facing part of the cell and vice versa.

Electroporation affects not only lipids but also proteins (Tsong, 1991). Voltage-gated ion channels present in the plasma membrane have a gating potential in the range of 50 mV. This is smaller than the potential needed for breakdown of the membrane, and therefore the ion channels will open before the critical value of V_m is reached. Channel opening itself is not powerful enough to abolish the membrane potential. Since a much higher current is induced though the open channels than they are designed to conduct, electroporation possibly causes irreversible denaturation of these proteins, but reversible electroporation of proteins is also possible.

3.3 Pore expansion in electroporation

Expansion of pores occurs when the external applied field is present, since this lowers the pore energy. The applied field (intensity and duration) influences the number, size and conductivity through the pores. Also, the ionic strength of the medium affect the pore size (Kinosita and Tsong, 1977a; Kinosita and Tsong, 1977b).

The size of hydrophobic pore fluctuates, resulting in a population of pores with different sizes. Chang and Reese showed by electron microscopy that the hydrophilic pores had a diameter within 20-40 nm at 2 ms after the pulse was applied. Pores expanded to a maximum of 120 nm, with an average of 40 nm, at 1.7 s after the pulse (Chang and Reese, 1990). Others have reported average diameter of 1 nm (Kinosita and Tsong, 1977a), 0.6-1 nm (Glaser *et al.*, 1988)

and 0.7-1.2 nm (Neumann *et al.*, 1998). A pore can interact with molecules like membrane proteins or cellular structures (cytoskeleton) and thereby form pores with longer lifetimes, so called metastable pores (Teissie and Rols, 1992; Weaver, 1993). This could explain the large pores (120 nm) reported by Chang. Also substances in the surrounding medium can affect the pores. For example, the presence of polyethylene glycol (PEG) during electroporation increased membrane permeability due to enlarged, PEG-stabilised pores (Hood and Stachow, 1992). Liquid-flow through the pore might also cause the pore to expand. The total porated area of a cell plasma membrane is reported to be 2% (Neumann *et al.*, 1998).

3.4 Resealing of membrane in electroporation

The resealing of pores after electroporation is illustrated in figure 4 (Saulis *et al.*, 1991). At the end of the pulse the pore radius is at a maximum (figure 4, stage b). As the pulse is terminated the pore energy increase within $\leq 1\mu s$, figure 4, stage c. A restoring force decreases the radius of the pore. This takes place in two steps, one initial fast phase followed by a slower phase. After the slow phase a local minimum of the pore energy is reached with r_{min} = 0.5-1.5 nm (figure 4, stage d). The lifetime is quite long (>10 minutes) since there is an energy barrier to over-come (figure 4, stage e), but not all pores need this long time to reseal. The barrier is caused by the lipid molecules, which are radially, exposed with their hydrophilic part into the aqueous pore. This will contribute to the energy barrier by electrostatic repulsion and the energy of the deformation of the lipid molecules, *i.e.* the same barrier as for formation of pores. In the final phase the cell membrane reseals completely.

The time for resealing after electroporation has been reported by many and varies from 0.1 ms to 2.8 h (Weaver, 1994), but most researchers find it to be in the μs to ms range (Kinosita *et al.*, 1992), which we also obtained in paper III. One reason for the large variation is due to the differences in methods used to determine the resealing time and another might be due to the structure of the pores produced. It is also known that the process of resealing is temperature dependent.

Why do pores not grow infinitely (irreversible pore formation) instead of resealing (reversible pore formation)? One reason for this might be the mechanical properties of the membrane, which will hinder rapid growth. Or is there an additional energy barrier to be overcome for irreversible pore formation to proceed? It has been shown that the rate of pore expansion is lower at decreased transmembrane voltages (Barnett and Weaver, 1991, Glaser *et al.*, 1988). It has been shown how size and duration of the applied field decides if reversible or irreversible pore formation occurs (Benz and Zimmermann, 1980; Benz and Zimmermann, 1981).

Pore resealing refers to the restoration of the original high resistance and low permeability of the cell membrane, but it does not mean that the structure of the membrane is restored. Conformational changes in membrane proteins and enzymes and asymmetry in the phospholipid layer require minutes to hours to fully repair.

3.5 Transport through pores

The proposed mechanisms for transport through the formed pores are electrophoresis, electroosmosis, diffusion and endocytosis. During the pulse (μs-ms) the main mechanism are electrophoresis and electroosmosis, and after the pulse (ms-s), diffusion is the leading mechanism (Prausnitz *et al.*, 1995). Endocytosis is referred to as a secondary effect of electroporation and might therefore not be the main reason for transport.

Diffusion through pores might be hindered by mechanisms like size exclusion (sieving) and electrostatic exclusion (Born energy repulsion) with lower dielectric constant of the membrane interior or interaction with charged head groups within the pore (Weaver, 1993). In general, size, shape and charge of the compound to be introduced to the cell can be expected to play an important role regarding its transport.

Rols and Teissie performed a study on how different parameters of the applied electric field (pulse strength, duration number, frequency) affected transport over the membrane (Rols and Teissie, 1998). They saw a difference between small- and macromolecules, which depended mainly on the lifetime of the state of transfer, *i.e.* the open time of the pore. In the case of macromolecules the duration plays a major role, but has no significant role for small molecules.

The amount of material entering the cells during electroporation can vary greatly. Different groups have reported the intracellular concentration to be between 2.0 to 37 % of the external concentration surrounding the cells (Canatella *et al.*, 2001; Gift and Weaver, 1995).

In paper III the pulse is short and electroosmotic and electrophoresis will have a small impact for the transport into cells and diffusion is the main mechanism. In paper I and II where an EFC and longer pulses (5-60 seconds) are used factors, such as, as electroosmosis and electrophoresis are more important.

3.6 Electroporation in membranes under tension stress

Because of the fluid nature of biological membranes, closed membrane structures such as cells and lipid vesicles adopt shapes that represent a minimum in surface free energy and thus also represent a minimum in lateral membrane tension. Accordingly, when the surface energy of the membrane is increased (*e.g.* by adhesion or pressurisation by application of mechanical strain), the lateral membrane tension will increase. If this tension is sufficiently high, pores can spontaneously form in order to increase the area of the membrane (Sandre *et al.*, 1999). Consequently, the applied electrical field needed for membrane permeabilisation may be substantially reduced when a mechanical force is applied onto a closed membrane structure. Needhamn *et al.* showed that electrical fields established over lipid bilayer membranes impose an electrocompressive mechanical stress σ_e, acting on the lipid membrane (Needham and Hochmuth, 1989). This force works normal to the plane of the membrane and leads to a decrease in membrane thickness.

If assuming that a lipid membrane behaves as a capacitor, then the electro-compressive force is proportional to the voltage drop, V_m, over the membrane and thus to the strength of the applied electric field. This can be calculated according to

$$\sigma_e = \frac{1}{2}\varepsilon\varepsilon_0 \cdot \left(\frac{V_m}{h_e}\right)^2 \qquad \text{Equation 6}$$

Where ε, is the relative dielectric constant and ε_0, is the permitivity and h_e is the dielectric thickness of the membrane. The differential overall mechanical work dW, done on the lipid membrane is then the sum of the electrocompressive stress σ_e, and the isotropic membrane tension $\bar{T}$, controlled by the amount of mechanical strain applied to the membrane according to

$$dW = \left[\bar{T} + \frac{1}{2}\varepsilon\varepsilon_0\left(\frac{V_m}{h_e}\right)^2 \cdot h\right]dA \qquad \text{Equation 7}$$

Where h is the overall thickness of the lipid bilayer membrane, and dA is the change in membrane area. Consequently, when a mechanical strain is applied to a membrane vesicle, the transmembrane potential needed to achieve permeabilisation can be significantly reduced.

Akinlaja *et al.* tested the theory proposed by Needhamn *et al.* on cells in a series of experiments where cells were mechanically strained by using aspiration micropipettes (Akinlaja and Sachs, 1998). They observed (by patch-clamp registrations) that the voltage needed for membrane breakdown was inversely proportional to the applied membrane tension for short voltage pulses (50 µs), all in agreement with Needham *et al.* For long pulses (> 100 ms), however, the breakdown appeared to be tension independent. In addition, Akinlaja *et al.* noted that a lower voltage was needed to achieve membrane breakdown for longer pulses than for short pulses. Based on these observations, they believe there are different mechanisms for pore formation with varying pulse length.

Interestingly, this approach for membrane permeabilisation may be even less invasive than conventional electroporation since lower electric fields can be used, minimizing the risk of unwanted electrochemical reactions at the membrane surface of a cell or a liposome. In paper I and II, the electroosmotic flow from the fused silica capillary could potentially generate a hydrostatic pressure acting on the cells, giving rise to a mechanical strain promoting low voltage membrane breakdown. In paper IV the relation between tension and electroporation is more obvious. We showed how it was possible to insert micropipettes into cells and giant vesicles by mechanically straining the membrane before applying a voltage. If electroporation was performed without applying a mechanical destabilization tension it was not possible to insert the micropipette, figure 5.

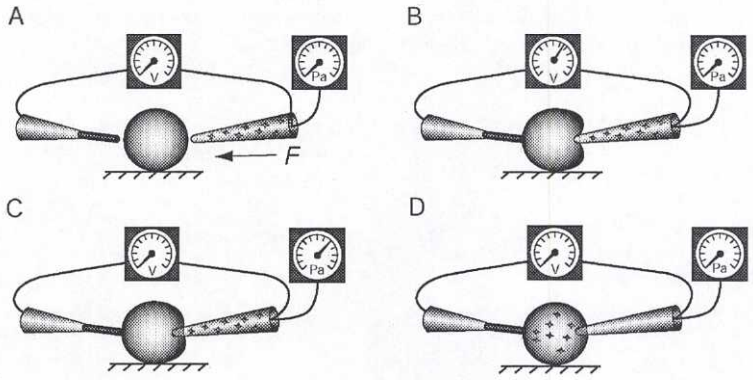

Figure 5
A) Electroinjection is performed by applying mechanical pressure over a biological membrane by moving the injection tip (a glass micropipette equipped with a Pt electrode, o.d. 2 µm) into the object towards the carbon fibre microelectrode (5 µm in diameter). B) An electric pulse is applied, which destabilise the membrane. C) The injection tip penetrates the membrane and by applying pressure over the injection tip injection D) is performed. The loading-agent contained in the injection tip is introduced to the cell or liposome.

3.7 Experimental set-ups for single-cell electroporation

Electroporation is usually performed on cell suspensions in a cuvette (bulk electroporation). Equipment for electroporation of smaller number of cells (suspended or adherent) has also been developed. For miniaturised electroporation both equipment with solid electrodes (paper III), figure 6 A-B, electrolyte-filled capillaries (paper I and II), figure 6 C-D, or micropipettes (Haas *et al.*, 2001; Rae and Levis, 2001) has been used for single-cell electroporation. Generally, homogenous electric fields are applied, but when working with single cells the electrodes have the same size or are smaller than the cell and an inhomogeneous field is obtained. In this thesis two different methods for single-cell electroporation are presented. One uses short pulses, paper III, and the other long pulses, paper I and II. It has been proposed that the mechanism for electroporation between high field/short pulse and low field/long pulse is fundamentally different (Akinlaja and Sachs, 1998), see section 3.6.

Okino and co-workers published the first study of *in vivo* electroporation in 1987 (Okino and Mohri, 1987) and during the last years the number of publications has grown enormously. Applications for this technology are delivery of cancer chemotherapeutics (Singh and Dwivedi, 1999), transdermal drug delivery (Prausnitz, 1999), vaccination (Misra *et al.*, 1999) and gene therapy (Matthews *et al.*, 1995). Generally, the substance to be introduced into the tissue is microinjected into the desired region before electroporation is performed. The electrodes used are most commonly needle electrodes or plate (caliper) electrodes. Generally, electrodes larger than 0.5 mm are used. Either two parallel electrodes are used or multiple electrodes arranged either in rows (arrays or single row) or in a circle. The pulse length used for *in vivo* experiments is generally longer (100 µs-50 ms) than for *in vitro* electroporation and most often a train of pulses is used. The electroporation model described above may not be completely adequate for *in vivo* electroporation since the milieu is very different from *in vitro*. Cells within a natural tissue *in vivo* are usually organised in a tightly compact 3-D structure, the

extracellular fluid surrounding the cells is not homogenous and the structured tissue is far more complex than *in vitro* conditions, to name a few examples.

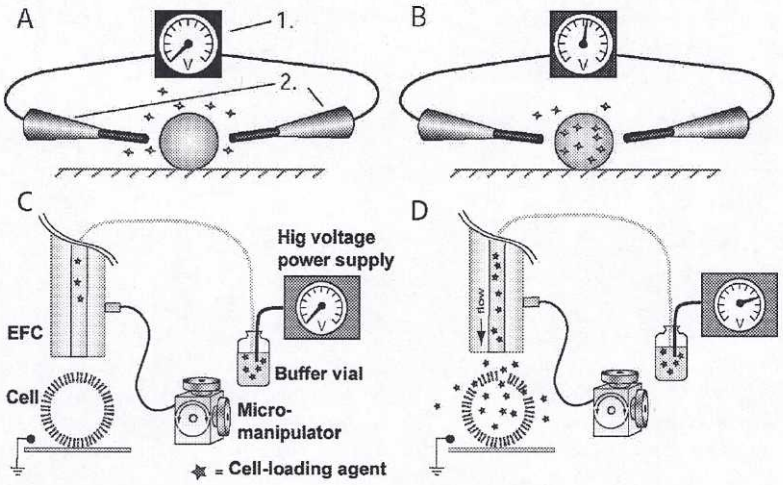

Figure 6
The different experimental set-ups for single-cell electroporation developed in this thesis. A) Two carbon fibre microelectrodes, 5 μm in diameter, (2) are positioned on each side of the cell. The angle between the electrodes is 180° and the distance between the electrode and the cell 2-5 μm. B) Square wave millisecond long DC voltage pulses (1) were applied to perform electroporation. The cell-loading agent is kept in the surrounding media. C) The set-up for the electroporation with an electrolyte-filled capillary (EFC). The cell-loading agents are supplemented to the electrolyte of the EFC and therefore not present in the surrounding buffer. D) When a pulse is applied, pores are formed and the sample is delivered by the electroosmotic flow to the site of pore formation.

3.8 Alternatives to electroporation and electroinjection

There are alternative approaches to electroporation and electroinjection to introduce foreign substances into cells. There are purely mechanical methods (microinjection, pressure mediated) as well as chemical-mediated approaches (DEAE-dextran, calcium phosphate, artificial lipids, proteins, polymers) (Luo and Saltzman, 2000). The conventional way of performing microinjection techniques is by the so-called stab injection technique where the injection needle is inserted by mechanical force facilitated by the use of a sharp "cutting" tip. This technique can be somewhat harmful to the cell since a high impact rate is required to insert the tip, which might damage intracellular components of the cell. These methods vary in efficiency and toxicity. Compared to these methods electroporation has the advantage of being non-invasive, non-chemical, easy to perform, and with a higher loading efficiency. Furthermore, the electric field is generally non-toxic, can be applied to various types of cells, and can be performed *in vivo*. In a related method called optoporation, a pulsed laser beam is used to permeabilise the cell membrane (Kurata *et al.*, 1986). Unfortunately, pore-mediated transport induced by optoporation is low, limiting the efficiency of the method (Soughayer *et al.*, 2000).

Optoporation has been used to load cells in confined spaces, such as, on microchips. Another method is particle bombardment of the target cell with colloidal metal particles coated with the cell-loading agent, usually DNA (gene gun biolistics), in which the particles are shot into the cell by brute force (O'Brien *et al.*, 2001).

4
Analytical methods

To determine the structure and activity of a substance has always been an essential task for chemists. In order to identify an unknown compound and/or establish its chemical composition, systematic methods are required which are based on specific reagents, suitable reactions and accurate weighting. In analytical chemistry, separation and detection have always been intimately connected. Separation serves no purpose if one cannot detect or identify the individual components in a mixture. On the other hand, detection can give detailed analytical information without any previous separation, but the selectivity required to identify and quantify a single compound in a mixture generally limits the approach.

4.1 Capillary electrophoresis

In this thesis, a capillary electrophoresis (CE) set-up is used (paper I and II) for electroporation and the electroosmotic flow delivers the sample to the cell surface. CE is a separation method based on the differential migration rates of charged particles under the influence of an electric field in a conducting media. There are different modes of CE (capillary zone electrophoresis, micellar electrokinetic chromatography, capillary gel electrophoresis, capillary electrochromatography, capillary isoelectric focusing, capillary isotachophoresis). In this work only capillary zone electrophoresis is used, so from now on capillary zone electrophoresis will be referred to as capillary electrophoresis (CE). Usually, fused silica capillaries are used. At a pH higher than 1.5, the capillary wall is deprotonated and $Si\text{-}O^-$ groups obtained. The positive ions in the buffer will be attracted to the negative charge at the capillary wall and an electric double layer between the wall and the buffer is obtained, the so-called Stern layer. When voltage is applied over the capillary, the field induces the positive ions at the Stern layer to migrate, causing the solution in the capillary to move in the direction of the negative electrode. This is called electroosmotic flow (EOF). The electrophoretic mobility, *i.e.* the movement of a charged particle, μ_{ep}, can be expressed as;

$$\mu_{ep}=q_i(6\pi\eta r_i)^{-1} \qquad \text{Equation 8}$$

Where η is the viscosity of the separation medium, and r_i and q_i are the radius and charge of the migrating particle. The electroosmotic mobility, μ_{eo} can be expressed as;

$$\mu_{eo}= \varepsilon\zeta(4\pi\eta)^{-1} \qquad \text{Equation 9}$$

Where ε is the dielectric constant of the separation medium and ζ the zeta potential, which is the potential difference between the Stern layer and the separation medium.

The net migration rate of the analyte depends on both the electoosmotic and electrophoretic mobility, and is defined as

$$\upsilon_{net} = \mu_{ep}\mu_{eo} E \qquad \text{Equation 10}$$

Where E is the electric field strength.

4.1.1 CE for single-cell analysis

Single-cell analysis is not an easy task. Consider this: a cell can express 10 000 different proteins simultaneously, with the total amount of protein reaching 5 fmol (10^{-15}). The average expression level per protein will then be in the zmol (10^{-21}) range. It follows that identification of intracellular components demands exquisite separation and detection methods. The main separation methods are CE, gas chromatography (GC) and liquid chromatography (LC). In many analytical aspects it is an advantage to use CE instead of traditional analytical techniques, such as, GC, HPLC, thin-layer chromatography (TLC) and slab gel electrophoresis. The main advantage of CE is that it can be applied to a wide range of compounds using the same set-up, and often the same capillary. In addition, it has the highest resolving power of the available liquid separation techniques. The required sample size is small (10^{-9} L) which is beneficial when working with many biological applications, *e.g.* single cell analysis. In single-cell and single-organelle analysis the obtainable sample volume is per definition the volume of the cell itself (~10^{-12} to 10^{-21} L), and most often only one sample can be obtained. Standard methods such as LC and GC would require dilution of a cell sample by at least 10^6 times, and even micro LC will still need 100-1 000 fold dilution, which can not be compensated for by, for example, good detectors. In CE, the sample dilution required is negligible because the capillary internal diameter can be chosen to match the size of the cell. CE also provides high separation speed, in some cases in the ms range. In contrast to most separation techniques, CE is also compatible with the biological environment. CE can also easily be coupled to other techniques, such as microdialysis sampling, competitive immunoassays, fibre optic sensors and molecularly imprinted polymers. To this date, very few hyphenated techniques in which CE is coupled to a biosensor have been proposed. One example is the whole-cell-based sensor described in this thesis where CE is coupled on-line to a living cell. It is known from work on electrochemical detection in CE that the high-voltage applied over an EFC will create an electric field at the capillary outlet (Lu and Cassidy, 1994). We show how the electric field at the outlet of a CE capillary can be used for electroporation, and thereby for detection by intracellular proteins in cells as well as for manipulation of cells *in vivo* and *in vitro*.

4.1.2 Microfabrication

CE and LC separations can easily be miniaturised and integrated on microchip platforms. Initially, microfabricated systems were developed mainly to perform separation combined with pre- and postcolumn derivatisation reactions. Today the concept of microfabrication includes much more. Method development now aims to integrate separation, detection and reagent handling steps (dilution, mixing, and incubation) and integration of pumps, filters, gratings and detectors on the same device. Another topic is array formation for parallel analysis, or multitasking. In paper II, we propose how one of our methods, the EFC electroporation, can be used in an array format. The advantage of this over conventional systems is a higher sample throughput, reduced reagent consumption, the ability to perform multiple operations

simultaneously on one device, and improved reproducibility, all ultimately resulting in low cost for production and analysis. Generally, a lab-on-a-chip provides automation of highly repetitive laboratory tasks by replacing cumbersome equipment with miniaturised microfluidic assay chemistry, and provides ultra-sensitive detection methodologies at significantly lower cost per assay than traditional methods and in a significantly smaller amount of space.

The forces used in microfabricated devices to create a flow are mainly pressure, in the form of micropumps, and electrokinetic forces. For CE, the fluid flow is controlled by electrokinetic mechanisms. One of the first microchip with an analytical system was developed in 1979 and consisted of a gas chromatograph on a silicon chip (Terry *et al.*, 1979). Manz demonstrated the first miniaturised device with electrokinetic pumping capability in 1990 (Manz *et al.*, 1990). Fabrication of these devices included photolithographic etching in glass or quartz and the use of polymers. Polymer chips are made both in rigid polymers such as acrylics, and flexible polymers such as poly (dimethylsiloxane), a material that is used in contact lenses. One advantage of the polymer design is that chips are cheap to produce and can be re-used.

The detection modes used on microchips are not new, but integrating them on a chip presents a new challenge. The most common on-chip detection modes are based on fluorescence and electrochemistry. Chemiluminescence, Raman, mass spectrometry and refractive index are also applicable.

Many different applications have been developed for chip-based analysis, *e.g.* DNA separation, immunoassays, polymerase chain reactions (PCR), peptide mapping and cell sorting. A few systems are available on the market, for instance LabCard™ by Aclara and GeneChip® by Affymetric. Multiplexing on-chip makes it possible to perform several analyses simultaneously. The breakthrough for microcapillary array electrophoresis (μCAE) was performed by Mathies and colleagues (Woolley and Mathies, 1994; Woolley *et al.*, 1997). A wide variety of μCAE systems have been developed since then.

Miniaturisation is not only about shrinking size and reducing cost. It also makes it possible to use physical processes, like diffusional mixing, which can not be performed or are impractical to perform in conventional size instrumentation. It also allows placing analytical systems in remote or hostile environments.

4.1.3 Fabrication of a chip-integrated biosensor

We developed a microfluidic system made out of poly (dimethylsiloxane), PDMS, with integrated electrodes for CE separation (Hurtig, 2001). Our vision was that the developed chip would function as a biosensor for intracellular targets. A single cell or a population of cells is positioned just outside the separation channel in a cell chamber. A residual electric field is obtained at the end of the separation channel, as for the EFC system, which is described in paper I and II. This electric field electroporates the cell, allowing the separated sample components in the capillary to enter the cell interior and react with different intracellular targets, such as, enzymes and receptors. By the use of fluorescent probes and substrates, detection and screening of specific intracellular proteins can be performed.

Fabrication of the channel system in PDMS was done with the same procedure as for the microwells in paper II. Briefly, a master was fabricated with lithography techniques. Moulding

the polymer and curing it in an oven produced a replica of the master pattern. Replica moulding (McDonald *et al.*, 2000) was used for construction of the microfluidic channels instead of sandwich moulding as for the microwells. Photolithography, metal evaporation and lift-off were used to fabricate the electrode pads.

In untreated PDMS, the EOF is negligible compared to a fused silica CE capillary. By treatment in oxygen plasma, EOF can be obtained since silanol groups are formed at the surface, which are ionisable. Unfortunately the surface degrades over time (Ren *et al.*, 2001), resulting in a limited time of use.

Electrodes were integrated on the glass substrate as pads to apply the voltage needed for CE separation. One set of pads was positioned in the fluidic channels (figure 7). These pads were connected to pads at the periphery of the PDMS slab. To the peripheral pads, wires were soldered on and connected to the high voltage power supply. The PDMS structure was aligned over the glass and covalently sealed to it by treatment with oxygen plasma, creating a single unit. In this configuration, one glass wall and three PDMS walls form each channel. A cell chamber is located downstream from the separation channel and, importantly, out of range of the electric field.

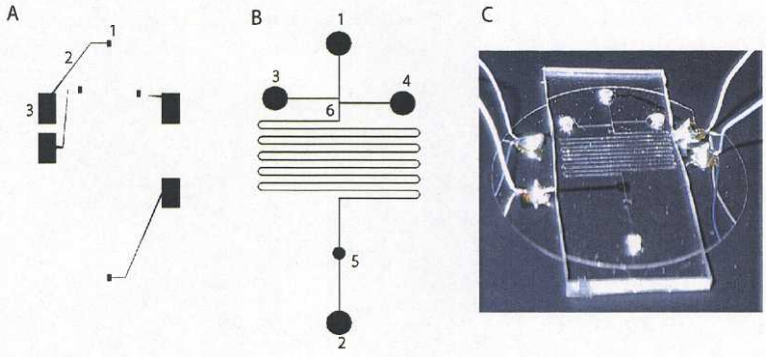

Figure 7
Microfluidic devices for use with single-cell biosensors. A) Configuration of the electrode pads on the glass substrate, where (1) designates pads in fluidic channels, (2) the connection to (3) the peripheral pads. B) The design of the channel system where (1) is buffer reservoir, (2) waste1, (3) sample (4) waste2 (5) the cell chamber and (6) the injection cross. C) Shows the assembled microchip where the PDMS slab is aligned on the glass surface with integrated electrodes.

At this time, the major result is the actual design and fabrication of the electrodes and channel system, and that after treatment of the PDMS with oxygen plasma, we were able to obtain EOF. We determined how EOF degraded with time, due to degeneration of the silanol groups of the PDMS in the channel walls. EOF could be sustained for at least 5 hours, but had seriously deteriorated after 12 hours. Concerning cell viability, we were able to grow and maintain cells for 7 days on the chip. Longer times of testing were judged to be unnecessary.

4.2 Fluorescence

In this thesis fluorescence is used for detection. Fluorescence is the property of some atoms and molecules to absorb light of a particular wavelength and reemit it at a longer wavelength. When a molecule is subjected to light, different processes can occur within the molecule. The Jablonski diagram, figure 8, displays some different photophysical processes a molecule can undergo. Absorption is a process in which a photon is taken up, exciting the molecule electronically from the S_0 (the ground state) to S_1, the first excited state. Absorption only occurs when the energy of the incoming photon matches the energy difference between the ground state and the excited state. The absorption process occurs within a time scale of 10 femtoseconds (10^{-15}s). Since the excited state is unstable, it will sooner or later return to the ground state via a relaxation process, either by a radiative or non-radiative process. The radiative processes include fluorescence and phosphorescence, fluorescence being most common. This is due to that phosphorescence is a spin forbidden transition from T_1 to S_0 and has low probability of occurrence. Fluorescence is the emission of photons due to an S_1-S_0 transition. The excited electronic state lasts for approximately 10^{-5} - 10^{-8}s. Internal conversion (IC) is a non-radiative process in which the energy gained from the excitation is lost by collision with solvent molecules or by internal vibration. ISC stands for inter system crossing and refers to the conversion of an excited singlet into an excited triplet state. That state can be converted to the singlet ground state in the form of phosphorescence. Another non-radiative process is quenching. This is, for example, due to collision or complexation with solvent molecules and will diminish the fluorescence. The observed fluorescence is the sum of all radiative and non-radiative processes.

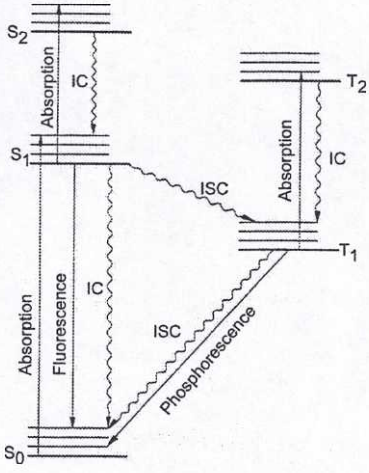

Figure 8
A Jablonski diagram in which S_0 is the singlet ground state, S_1 an S_2 excited singlet states, and T_1 and T_2 excited triplet states. The wavy lines describe non-radiative deactivation processes where IC signifies internal conversion and ISC inter system crossing. Every electronic state (S_0, S_1, S_2, T_1, T_2) is superimposed by vibrational states. Relaxation occurs to the lowest vibrational state in each electronic level.

In condensed phases, the emission spectrum is always shifted toward a longer wavelength (lower energy) relative to the excitation spectrum. The difference in wavelength between the

emission peak and the excitation peak is known as the Stokes shift. This shift in wavelength represents the energy dissipated during the lifetime of the excited state before the fluorescent light is emitted.

τ_R is the radiative lifetime describing the average relaxation time of the excited state assuming deactivation only by fluorescence. The fluorescent lifetime, τ_F, is described by all deactivation processes taken in account, by:

$$\tau_F = \frac{1}{k_F + k_{IC} + k_{ISC}} \qquad \text{Equation 11}$$

Where k_F, k_{IC} and k_{ISC} are the rate constants for fluorescence, internal conversion and inter system crossing, respectively. Another important factor for fluorescence measurements is the fluorescent quantum yield, ϕ_F, which is the ratio of fluorescing molecules to photon-absorbing molecules. It can be expressed in terms of rate constants as

$$\phi_F = \frac{k_F}{k_F + k_{IC} + k_{ISC}} \qquad \text{Equation 12}$$

The fluorescence intensity depends on the fluorescent quantum yield. At lower irradiances a linear relationship is found between the incident light, I_0, and fluorescent intensity, I_F, according to

$$I_F = 2.303 \cdot \phi_F \cdot K \cdot I_0 \cdot \varepsilon_{abs} \cdot c \cdot l \qquad \text{Equation 13}$$

Where ε_{abs} is the molar absorptivity, c the analyte concentration, K denotes the light collection efficiency, ϕ_F the fluorescent quantum yield and l the optical pathlength. This allows quantitative measurements.

4.2.1 Fluorescent probes

A fluorescent probe is a fluorophore designed to localise within a specific region of biological systems such as single cells or respond to a specific stimulus (Johnson, 1998). The fluorescent probes used within this thesis are tabulated in table 3. There are endogenous fluorophores such as NADH (ex 350 nm, em 460 nm), porphyrins (ex 400 nm, em 610/675 nm) and tryptophan (ex 275 nm, em 350 nm), which can be used as indicators of cellular processes. Such intrinsic probes are generally rare and instead synthetic probes are utilised. A fluorescent probe usually consists of a fluorophore and a targeting group, which is used to bind the probe to the target molecule by a reaction. Examples of this are fluorescent lipid analogues and reporter ligands. Another approach is to couple the fluorophore and target group in such a way that binding to a target ion or molecule alters the fluorescence properties of the probe. Examples of this kind of probe are fluo-3, which increases its fluorescence quantum yield upon binding Ca^{2+}, and fura-2, which experiences a spectral shift upon binding Ca^{2+}. Fluorescent probes are not restricted to detection via binding to a target. They can also be sensitive to the environment. There are membrane potential probes where the electronic structure of the probe, and thereby the fluorescence, is sensitive to the surrounding electric field. pH indicators, *e.g.* BCECF and SNARF, exhibit a spectral shift when the pH is changed (Haugland, 1996).

Fluorescent probes for enzymes are generally non-fluorescent substrates that form fluorescent products in an enzyme catalysed reaction. They are available for a wide range of enzymes such as glycosidases, peptidases, phosphatases and peroxidases. Probes for membranes are usually amphiphiles, with a lipophilic anchor part and a charged fluorophore. The aliphatic tails of the probe insert into the membrane, while the fluorophore remains at the membrane surface. One example is dialkylcarbocyanine probes, *e.g.* DiO, which is widely used for labelling cells for tracing applications (Haugland, 1996).

Another system for labelling is the green fluorescent protein (GFP) of the chemiluminiscent jellyfish *Aequorea victoria*. It has been used as a tool to monitor and localise gene expression, proteins, and molecular interactions in cells, imaging of intracellular protein localisation and transport. Several mutants of GFP have been developed to have different emission maxima and to be expressed at specific intracellular locations. This allows monitoring of second messenger generation, lipid metabolite production, and so on. The different GFP-based constructs mainly reflect conformational or environmental changes, but GFP has also been used to construct entirely new types of probes, *e.g.* the cameleons (Miyawaki *et al.*, 1997). They consist of a blue/cyano-emitting mutant of GFP (the acceptor), calmodulin (CaM), the calmodulin-binding peptide M13 and a green/yellow emitting GFP (the donor). The CaM and M13 unit act as separators for the two GFP units. Upon binding of Ca^{2+} to the M13 unit, the CaM and M13 will interact and the structure compress. The donor and acceptor will get closer to each other and the fluorescence resonance energy transfer (FRET) between the two GFP units will increase. FRET is a distance-dependent excited state interaction in which emission of one fluorohore is coupled to the excitation of another. The donor and acceptor molecules are separated and upon conformational changes they are brought together and start to fluoresce. By mutations of CaM, the affinity for Ca^{2+} can be altered from 10^{-8} to 10^{-2} M. They can also be transported to specific regions of the cell for measurement of local Ca^{2+} signals. Similar probes are designed to monitor ATP, GTP, and cAMP.

Semi-conductor nano-crystals (cadmium, selenide, and cadmium sulfide) can also be used as fluorescent probes (Bruchez *et al.*, 1998). The nano-crystals consist of a core crystal with a shell of another crystal, and finally a shell of silica. The silica makes the probe water-soluble and provides a chemically modifiable surface to which, for example, proteins can be bonded, *e.g.* avidin, and thereby interactions with biological samples can be controlled. The wavelength is tuneable and has a spectral range from 0.4 - 2 μm. They also have long fluorescence life times.

Molecular beacons are DNA probes (Tyagi and Kramer, 1996). The probe has the form of a hairpin and is made of single-stranded oligonucleotides, *i.e.* the loop of the hairpin. The hairpin stem consists of a probe sequence. At one end a fluorophore is covalently attached and at the other end a quencher is covalently attached. In the absence of the target, the stem of the hairpin holds the fluorophore close to the quencher and the probe is non-fluorescent. When the oligonucleotide complementary to the one in the probe is present, the molecular beacon will unfold and the two oligonucleotides hybridise. This will lead to separation of the quencher and the fluorophore and the fluorophore starts to fluoresce when excited. Molecular beacons have been used for real time monitoring of PCR, RNA detection in living cells, protein studies and genetic analysis. They can also be used as DNA biosensors.

Table 3
Data for some of the dyes and labels used in this thesis.

Fluorescent probe	**K_D Ca^{2+} (nM)**	**ε x 10^{-3} (cm^{-1} M^{-1})**	**ϕ_F**	**λ_{abs} (nm)**	**λ_{em} (nm)**	**Application**	**Comment**
Fluorescein		90	0.9200	490	514	Reactive label	pKa 6.4-6.5
6-carboxyrhodamine 6G		102	0.4500	524	550	Reactive label	
Fluo-3	400	83	0.0051	506	526	Ca^{2+} indicator	Low Ca^{2+}
		78	0.1830	506	526		High Ca^{2+}
Fura-2	135	33	0.2300	335	512	Ca^{2+} indicator	Low Ca^{2+}
		27	0.4900	360	505		High Ca^{2+}
Oregon Green	170	88	0.9600	496	524	Reactive label	
YOYO-1		98.9	0.5200	491	509	DNA and RNA probe	
Propidium iodide		6.4	0.0900	536	623	DNA probe	
BODIPY®-FL		91	0.4900	505	513	Reactive label	
DiO		149		485	501	Membrane probe	
GFP native			0.8000	476	508	Protein label	

K_D is the dissociation constant, ε the molar extinction coefficient, ϕ_F the quantum yield, λ_{abs} the maximum absorption wavelength and λ_{em} the maximum emission wavelength. All data from Molecular Probes, Inc.

It is obvious from above that there are a lot of different probes for different purposes available. With the developed fluorescent probes of today it is easier to do fluorescent investigations on the single cell level. The future challenge lies in the consequent combination of fluorescence detection techniques with procedure to handle, manipulate and analyse molecules at the micro and nanometer level.

4.2.2 Fluorescence microscopy

Fluorescence microscopy is an analytical technique, which allows the user to look inside cells and tissues. The basic principle of analysis is to deliver excitation energy to the fluorescent species in the specimen and to separate the much weaker fluorescence light from the brighter excitation light. Thus, only the emission light reaches the eye or a detector. The resulting fluorescent areas shine against a dark background with sufficient contrast to permit detection. For conventional fluorescence microscopy, the image has a depth of field of 2-3 μm. A cell attached to a surface is about 1-2 μm high, so the whole depth of the specimen, in this case a cell is typically observed.

In this work a microscope equipped for epifluorescence, which means that the specimen is illuminated and observed from below, was used. We used two different illumination and detection set-ups. In paper I and II, a 150 W xenon lamp provided light for excitation and a photomultiplier tube (PMT) was used for detection. In paper I-IV we used a laser for excitation and a charged-coupled device (CCD) camera for detection. A xenon lamp has the advantage of supplying light over a broad range of wavelengths. A laser on the other hand gives a single wavelength, but higher excitation intensity.

An improvement of conventional fluorescence microscopy is confocal fluorescence microscopy (Minsky, 1988). Here two pinholes are inserted in the optical path. One in front of the image plane and the other at the detection side. This will restrict illumination to the focal plane instead of the whole specimen. The depth of field is about 0.5 μm thick and has a probe volume in the range of 5×10^{-16} L. Importantly, all light outside the focal plane is excluded which drastically reduces background noise as well as the light intensity reaching the detector. To compensate for the reduced signal intensity it is therefore common to use lasers in combination with confocal fluorescent microscopy to compensate for this. Another advantage is that scanning can be performed to obtain a 3-dimensional image of the specimen. In this thesis, confocal fluorescence microscopy has been used for detecting the fluorescence after electroporation in organotypic tissues and *in vivo*, paper I.

4.2.3 Laser-induced fluorescence

Laser-induced fluorescence was used in paper I-IV. Compared to other light sources, a laser has the advantages of high power, directionality and temporal coherence. It also has a higher intensity of the excitation light, which according to equation 11, will give increased fluorescence intensity as well. This leads to high sensitivity and selectivity. Depending on the purpose, various laser types are used. In analytical chemistry, laser-induced fluorescence is known as a powerful technique for concentration determination of solutes. Here the narrow bandwidth of a laser is of great advantage. It is also easy to focus the laser beam to a small spot without losing any energy, a phenomenon leading to a high intensity excitation source necessary for attaining a low detection limit. The detection limit obtained by LIF is typical 2-5 orders of magnitude lower than for lamp-based fluorescence detection. If the analyte of interest

does not fluoresce, one derivatises the analyte using a suitable fluorescent or fluorogenic reagent. Laser-induced fluorescence detection is mostly used in capillary electrophoresis and liquid chromatography, where, for example, derivatised amino acids and peptides are separated and detected, and in flow cytometry.

4.3 Patch Clamp

In this thesis patch clamp is used in paper I-III to characterise the current over cell membranes during electroporation. Patch clamp can measure electric currents in the pico-ampere (10^{-12} A) range. A glass pipette containing a conductive solution works as an electrode, which is coupled to an operational amplifier. The pipette (1-5 μm tip diameter) is brought in contact with a part of the cell membrane and will adhere tightly by the application of a moderately reduced pressure, *i.e.* it forms a seal. The resistance of the seal should be in the range of 10^9 Ω, a so-called giga seal. A high seal resistance is necessary to reduce background noise. A poor seal results in a large leak current between the membrane and the pipette and thereby give rise to noise, which will disturb the recording. A voltage is applied over the pipette to keep the membrane potential at a constant level, *i.e.* it is clamped.

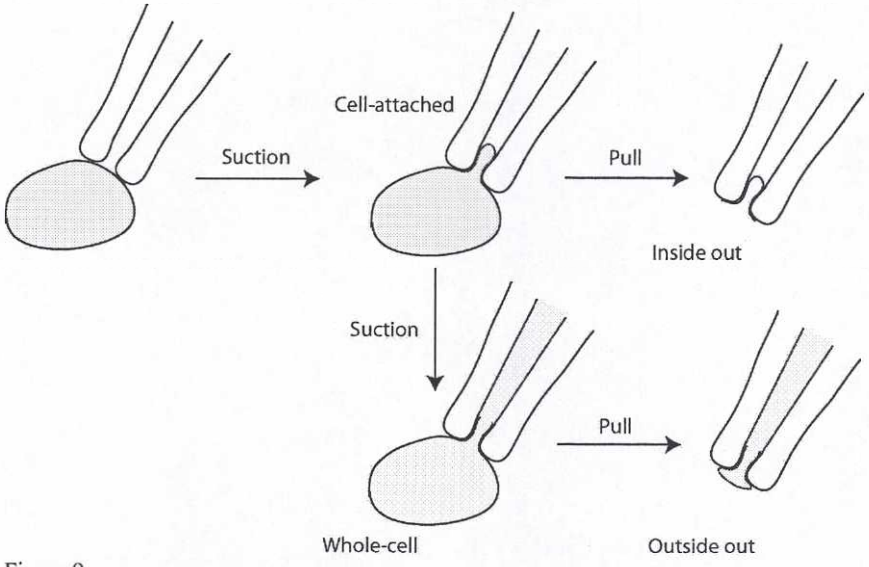

Figure 9
The different patch clamp-recording configurations.

Depending on the experimental problem, different recording configurations are used (figure 9). The first configuration, where the pipettes adhere tightly to the membrane, is called "cell-attached". From the cell-attached configuration, applying pressure via suction ruptures the patch and the "whole-cell" configuration is obtained. Here the whole population of ion channels from the entire cell membrane is studied. Following this, the pipette can be withdrawn from the cell. The membrane will break and reseal so that the outer cell membrane leaflet will

come to face the bath solution, and therefore this configuration is called "out-side out". Now single channels can be studied. From the cell-attached configuration one can also withdraw the pipette and a patch of the membrane will follow. This is called the "inside-out" configuration since the inner part of the membrane faces the bath solution. In this recording configuration single channels are recorded and the effect of intracellular components on the channel can easily be studied. The current from a single channel is in the order of 1 pA (10^{-12}), from a whole cell 1 nA (10^{-9}) and an axon 1 μA (10^{-6}).

From a single current trace, a lot of information can be extracted. The amplitude of the current single-channel reveals information about the identity of the channel. Changes in membrane area, revealed by capacitance measurements in the whole cell configuration, can be used to follow endo- and exocytotic events. Conductance measurements in the whole-cell configuration describe the population of ion channels, and the nature of the current is often specific for the ion channel type. This make is an excellent analytical tool and it has been widely used to study and describe ion channels. The time resolution of patch clamp is in the μs range making it possible to study electroporation events, which was our purpose.

5
Combining biology and analytical methods

In the area of cell analysis there is a need to develop old and new technology to proceed the development and to find out how cells function and communicate. This is an analytical challenge since cells are extremely complex. To mention a few properties, a single cell contains about 10 000 proteins, consumes 10 000 000 molecules of ATP every second, has thousands of organelles, and is not in chemical equilibrium. Since cells are small and the sample volume is limited. From this follows that it is hard to extract a sample and most often not more than one sample can be extracted from each cell. For biological materials many different analytical approaches can be used and generally there are two areas, bimolecular analysis and biosensors. Bimolecular analysis and biosensors are not competitive techniques, rather supplementing each other.

5.1 Biomolecular analysis

A wide range of biochemical and analytical techniques are available, but new analytical tools are needed for characterising and probing complex biological samples. In this overview of bioanalytical systems, I will emphasise bioanalysis of the content of single cells. The interest in performing single-cell analysis is explained by the fact that every single cell in a single biological tissue has an individual protein profile and function. Population analysis, *i.e.* a measurement in which the result is contributed to by thousands of cells, will provide an average answer but will not reveal details about cell-to-cell variations. Therefore it is in some instances preferable to do single cell analysis than to look at populations.

Within the area of electrochemistry, the development of microelectrodes has made it possible to do sophisticated analysis at the single-cell level from which zeptomole (10^{-21}) amount of analytes can be detected (Wightman, 1981). For example, exocytotic events of single chromaffin, mast, pheochromocytoma and pancreatic β-cells was measured by carbon fibre microelectrodes (Finnegan *et al.*, 1996). The electrode was positioned above the cell surface and a stimulating pipette was positioned at the cell. With this technique it was possible to detect cathecoleamines, histamine, serotonin and insulin. Electrochemical methods can also be combined with other techniques. Logman *et al.* performed microdialysis and electrochemical measurements *in vivo* simultaneously (Logman *et al.*, 2000). One big advantage with electroanalytical techniques is the minimal number of interferences since only a few molecules are easily oxidised or reduced in biological systems. CE can be coupled on-line with electrochemical detection. One problem with this combination is the interference between the high voltage from the CE capillary and the working electrode. By isolating the electric field this can be overcome.

Fluorescence is a tool that has been used for a long time as a detection technique for biological samples. It is widely used for detection in LC and CE, but can also be used for imaging purposes. Single-molecule investigations, scanning confocal and multiphoton microscopy can give 3D pictures of cells and spatial information of the distribution of intracellular components, even within individual organelles. Fluorescence microscopy is a complement to other analytical techniques such as MS, LC, CE and NMR (Yeung, 1999). Also bio- and chemiluminescence can be used. For example, chemiluminiscence was used to monitor ATP release from astrocytes with detectability down to 10^{-8} M (Wang *et al.*, 2000). Direct measurement of cytochrome c distribution in single cells and release from mitochondria to cytosol in cells cultured on a microchip was performed with a scanning thermal lens microscope (Tamaki *et al.*, 2002). Another microscopic technique of great importance is the scanning electron microscope (SEM), which has been used since the 1950s to analyse cells. It gives information about topography and morphology.

Traditional separation techniques have been miniaturised for use in single cell analysis. In liquid chromatography (LC), either packed or open columns are used for separation. An example is separation and detection of serotonin and dopamine in single snail neurones (Kennedy and Jorgenson, 1989). Also, epinephrine and norepinephrine could be separated with micro column LC (Cooper *et al.*, 1994). CE is an important separation technique with growing applicability and it is complementary to LC and GC. There are areas where CE is clearly the method of choice, *e.g.* single cell and cellular structure analysis. Laser-induced fluorescence is the most common detection principle coupled to CE and might be used to detect a single molecule. By combining the sensitivity of LIF and the possibility to analyse ultrasmall sample volumes, neurotransmitters in single secretatory vesicles could be detected (Chiu *et al.*, 1998). The protein content of single cells can be studied by injecting the cell on the capillary followed by injection of a fluorescent label. In the capillary, the cell is lysed and the proteins labelled (Hu *et al.*, 2001; Zhang *et al.*, 2000). The sample does not have to be derivatised before analysis when native fluorescence from trytophane-containing polypeptides (Paquette *et al.*, 1998) or aromatic monoamines, flavins, adenosine and guanosine-nucleotide analogues is detected (Fuller *et al.*, 1998). Also electrochemical detection is widely applied in CE. To mention a few applications, sensitive analysis of serotonin and dopamine in cytoplasm of single invertebrate neuronal cells was performed with CE and electrochemical detection (Olefirowicz and Ewing, 1990). In this work sampling was performed by directly inserting an etched capillary, functioning as a microinjector, into a single nerve cell and sampling of cytoplasm was performed. With small internal diameter capillaries (nm range) and sampling volume in the low fL range Woods *et al.* showed how bioanalytes in the microenvironment of single cells could be detected by electrochemical techniques (Woods *et al.*, 2001). Other interesting areas for CE applications are peptide mapping, where separation and detection of cleaved proteins or polypeptides is performed to identify proteins, monitor post-translational modifications and structure elucidation.

The development of different ionisation methods has made mass spectrometry (MS) applicable to an increased area of biological samples. The combination of high performance liquid chromatography with tandem mass spectrometry (LC-MS-MS) is a very powerful tool, surpassing spectroscopic and electrochemical detection techniques. LC-MS-MS offers specificity, speed, good precision and accuracy and is applicable to almost every type of matrix. Also, matrix-assisted laser desorption ionisation coupled to mass spectrometry (MALDI-MS) is an important tool which expanded the use of MS for analysis of biological samples. It has a soft ionisation and is well suited for analytes with higher mass like peptides

and proteins. It has been used for peptide identification and characterisation of post-translational modifications in single cells (Li *et al.*, 2000) and to study processing of pheromones in single cells (Garden *et al.*, 1998). To get richer information CE can be performed with multiple detection modes. Sweedler and co-workers radio labelled single cells and separated them by CE (Page *et al.*, 2002). The effluent was collected in nanovials and splitted to radionucleotide and mass spectrometry detection. Detection limits in the zmol range was obtained. Single organelle peptide analysis can been done and it has been shown how the peptide content in 1-2 μm diameter secretory vesicles can be analysed (Rubakhin *et al.*, 2000). This is probably the smallest sample to be profiled by MALDI MS so far. Combining CE with MS is still rare due to the problem with the interface. The high electric field from the CE separation must be isolated from the ionisation potential without diluting the sample.

5.2 Biosensors

In complex mixtures (urine, blood) the analyte of interest is usually present in very small amounts. This means that discrimination of the analyte from the sample matrix is a critical step. Biosensors provide a direct solution to this need. This is explained by the high selectivity and sensitivity, which can be obtained by biological material. The principle for recognition is based on molecular recognition. The selectivity and sensitivity is directly dependent upon the properties of the biorecognition element. By increased selectivity and sensitivity it might be possible to eliminate separation or any pre-treatment of the sample before analysis. This will save time and also reduce cost. Another reason for developing biosensors is to develop analytical systems where the analytical environment is not changed from the natural environment of the analyte. Thereby biological relevant information about the analyte is obtained as well.

A widely used definition of the term biosensor is "The direct spatial combination of biological recognition and transduction into an electrical signal". Biosensors can be said to contain two parts, one molecular recognition component, and an energy-transducing component. The molecular recognition can be divided into enzymes, antibodies, whole cells and cellular structures, nucleic acids, and biomimetic receptors (synthetic receptors mimicing the recognition properties of biological molecules). Examples of energy-transducing modes are, in general, all the existing analytical techniques like electrochemical (amperometric, potentiometric), optical (absorbance, fluorescence, NIR, Raman), mass sensitive (quarts microbalance, acoustic plate model), thermal (adiabatic, heat conducting) and some others (biomagnetic, scanning electron microscope). A biosensor can be classified upon the molecular recognition element (*e.g.* an enzyme sensor), the transducing element (*e.g.* an optical sensor) or the analyte (*e.g.* a glucose sensor). I have chosen to divide them according to their molecular recognition element and here I will place emphasis on whole-cell biosensors.

5.2.1 Whole-cell biosensors

In this type of biosensor, intact living cells are used. Specific components of the cell are used for molecular recognition, usually protein systems. Cell-based detection offers many applicable detector systems (ligand-receptor, enzyme-substrate, and antigen-antibody) all present within the same cell, figure 10. Those detection systems are based on the molecular recognition between the analyte and the protein, which confers great selectivity and sensitivity due to low K_D (dissociation constant) values in the nM-μM range. The cell-based detector has the ability to perform simultaneous measurements of several systems within a single cell, and detection of

biologically significant concentrations is possible thanks to intracellular amplification and transduction pathways. Cell detectors also retain many key regulatory pathways that are difficult or impossible to reconstitute *in vitro*, and have the capacity of constant renewal of the sensing element. Furthermore, a cell-based detector can be tailored for a specific application. This is due to the existence of numerous cell-lines with different properties and molecular recognition elements as well as techniques for genetic manipulation of their protein-expression. They are generally more tolerant than purified components. Biologically relevant information about the analyte is obtained since they are sustained in their natural environment.

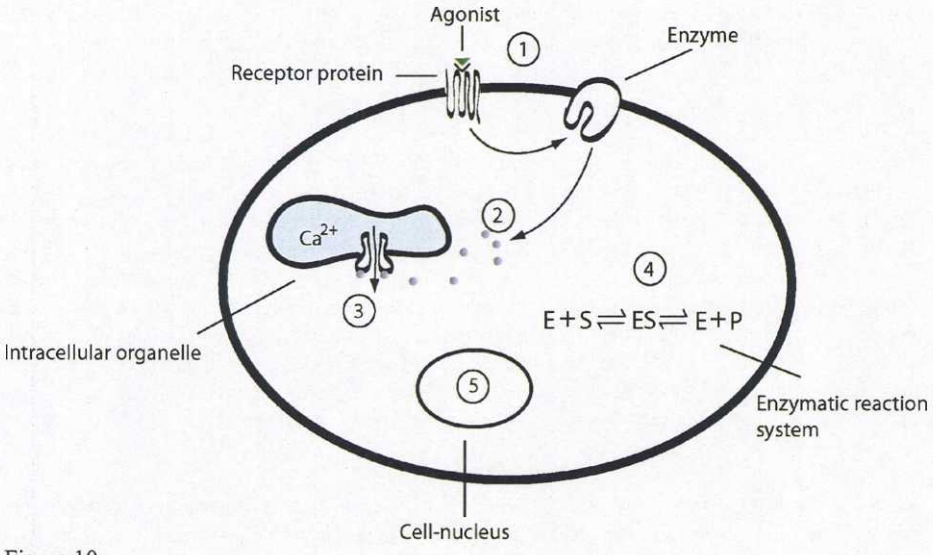

Figure 10
Within a cell there are several proteins at various sites and locations that can be used for detection and probing of various ligands. Receptors bound to the cell plasma membrane are most often transmembrane proteins (1) and the activation of such receptors can be measured using different probing strategies. Upon stimulation of such receptors, a transmembrane signal may trigger a cascade of events including (2) release of messengers such as inositol 1,4,5-triphosphate or cAMP, (3) changes in intracellular free cytosolic calcium ion concentration, (4) induction of enzymatic activity and (5) altered gene expression. At all generic levels, but not in all signalling systems, commercial markers are available for fluorescent labelling and targeting.

Many of the early whole-cell biosensors used bacterial or algae cells and were based on electrochemical transduction. The biochemical oxygen demand biosensor (BOD) is one example of this (Karube *et al.*, 1977). Immobilised bacteria or yeast is used to measure the quality of water. A Clark- type oxygen electrode measures the oxygen consumption of the bacteria. Another well established branch of whole-cell biosensors is to measure metabolic products from the cell. For example, the light-addressable potentiometric sensors (LAPS) are used to detect different cell affecting agents in the extracellular solution. The cell is placed in a flow chamber with a potentiometric sensor, which measures acidic metabolites, *i.e.* protons (pH). Chemical stimulation by chemotherapeutic drugs has been detected this way (Parce *et*

al., 1989). With the development in microbiology, another type of whole-cell sensors is developed where cells are engineered to report specific activity. HEK293 cells were cultured on an oxidised silicon chip coated with collagen IV (Straub *et al.*, 2001) and recombinant maxi K_{Ca} channels were expressed in the cell. Upon activation the ion current gave rise to a voltage between the chip and the cell, which evoked an electronic response in the chip. Another elegant system is the CANARY (Cellular Analysis and Notification of Antigen Risks and Yields) system where a B cell line was engineered to express a membrane form of antibody as well as the aequorine gene from *Aequorea victoria* (Young *et al.*, 2000). The binding of an analyte to the antibody resulted in an increase in Ca^{2+}, which triggers aequorin bioluminescence. The cells were patterned on a glass surface, but can also be positioned in flow chambers and integrated on microfluidic devices. This shows how manipulation of a biological system can be used to obtain better detection properties.

For high-throughput screening cells can be cultivated in 96 well plates. By labelling the cells with a marker, *e.g.* fluorescence, followed by addition of the ligands, the effect on the cells can be detected. The fluorescence imaging plate reader (FliPR) instrument (Molecular Devices, Sunnyvale, CA, USA) is one example where 96 well plates are used in combination with liquid handling and kinetic detection by a laser and a CCD camera.

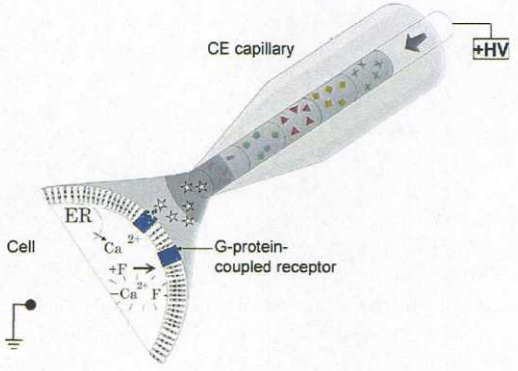

Figure 11
The set-up for single cell biosensor coupled to capillary electrophoresis with fluorescence detection. The effluent from the CE capillary is directed to a cell. By different molecular recognition elements, the cell will respond to biological active substances in the sample eluting from the capillary.

Biosensors can be coupled to separation and this makes them even more powerful. A cell-based biosensor coupled to capillary electrophoresis (SCB/CE) was developed by Shear and co-workers (Shear *et al.*, 1995). The effluent from the CE separation capillary was directed onto a single cell. As depicted in figure 11, the analytes (*e.g.* bradykinin, ATP, acethylcholine) stimulated release of Ca^{2+} into the cytosol. Since Ca^{2+} is a key cellular messenger it is therefore suitable as readout for a variety of targets. The detector, a PC-12 cell, was stained with the dye fluo-3, which selectively binds Ca^{2+}, and the response was quantified by changes in fluorescence. By use of selective antagonists for specific receptor subtypes, the SCB/CE system was used to screen unknown agonists. In addition, the cell biosensor system could

detect endogenous bradykinin in whole cell lysates and identification of degradation products of bradykinin in plasma (Fishman *et al.*, 1996; Fishman *et al.*, 1995). The same concept can be used with patch clamp for detection instead of fluorescence (Shear *et al.*, 1995). An oocyte from *xenopus leavis* was injected with $5HT_1$ mRNA encoding for the serotonin $5HT_1$ receptor. The oocyt expressed the receptor and the receptor was used for detection. To avoid desensitisation of the ion channel an excellent system for using patch clamp where the detector cell was superfused by a pulsed flow of buffer was developed (Farre *et al.*, 2001).

The concept of the SCB/CE was further developed. Luzzi *et al.* made it possible to use intracellular receptors for detection by incubating the detector cell with digitonin to permeabilise the cell before analysis (Luzzi *et al.*, 1998). Thereby the effluent from the capillary could reach the cytoplasmic domain of the cell membrane and interact with different signalling systems. They demonstrated how IP_3 could be quantified by use of the internal standard method as well as determining the K_D value of the intracellular IP_3 receptor (Luzzi *et al.*, 2000). In paper I and II a biosensor set-up for detection of intracellular targets is presented in which access the intracellular targets is provided by electroporation. The intracellular messenger IP_3 was detected after electroporation of the cell with the EFC approach. This concept was expanded for detection of specific agonists, receptors and enzymes in paper II.

Except for single cell also networks or tissue can be used where the response from a whole population of cells is used. Networks of neuronal cells are able to detect signals of different amplitudes, analyse the temporal and spatial components of a signal and simultaneously process signals from several systems. Cell can be grown on microelectrode arrays. When exposed to neurochemicals the change in membrane potential during an action potential is measured. One example is neuronal cells cultured on substrate integrated thin film microelectrode arrays (MEAs), which formed a network. By the electrode array the neural activity was measured at multiple sites in the network. By electrical stimulation (Maeda *et al.*, 1998) and chemical stimulation (Gross *et al.*, 1997; Keefer *et al.*, 2001) the response of the neuronal network was analysed. Recently a system responding to mechanical stimuli is developed (Heal *et al.*, 2001). Also organotypic tissue from the brain grown on silicon based chips with arrays of electrodes and used for the same purpose as above (Jahnsen *et al.*, 1999). Another type of tissue biosensor might be developed from the experiments performed in this thesis and paper I. It shows how electroporation can be performed in tissue.

6
Summary of papers

This thesis is based on the development of three electroporation and electroinjection methods for single-cell biosensors.

6.1 Paper I and II

In which we show how the residual electric field at the outlet of an electrolyte-filled capillary (EFC) can be used to permeabilise cells (paper I) and how this method can be used for probing, phenotype profiling and sensing of intracellular proteins on the single cell level as well as in population of cells grown in microwells (paper II).

When applying an electric potential across a conventional fused silica capillary, here referred to as an electrolyte-filled capillary (EFC), a residual electric field is created at the outlet. This field is, typically small but can be made large enough to be used in electroporation. The EFC function as an electrode (anode), and a Pt wire was used as the counter electrode (cathode). Since the EFC is hollow, the substance to be introduced to the cell is kept within the EFC. As a square wave DC-pulse (5-60 seconds, 80-400 V/cm) is applied, pore formation will be initiated as well as an electroosmotic flow delivering the cell-loading agent to the site of pore formation at the cellular membrane. From patch clamp and fluorescence measurements the threshold potential for dielectric breakdown of the membrane was calculated to be approximately 85 mV.

In paper I we showed how this set-up could be used for electroporation of single cellular processes, single cell somas, as well as in multidimensional networks of small populations of cells in organotypic tissues and tissues *in vivo*. This was demonstrated by introduction of the dye YOYO-1. It was also demonstrated how this electroporation technique could be used to introduce the receptor agonist inositol 1,4,5-triphosphate to the cytoplasmic space of single cells. In paper II, we further demonstrated how the concept could be applied for screening of receptors by introducing receptor-specific agonists (IP_3 and cADPr) as well as blockers (ruthenium red) of receptors, figure 12. Introduction of fluorogenic enzyme substrates into cells allowed detection of target proteins (proteases and alkaline phosphatase). Also, by performing electroporation within defined populations of cells grown in microwells, the number of cells affected by locally applied electroporation was determined as a function of the applied voltage and the size of the EFC. This shows how the developed electroporation method with an EFC can be used together with cells and function as a single-cell biosensor for intracellular targets.

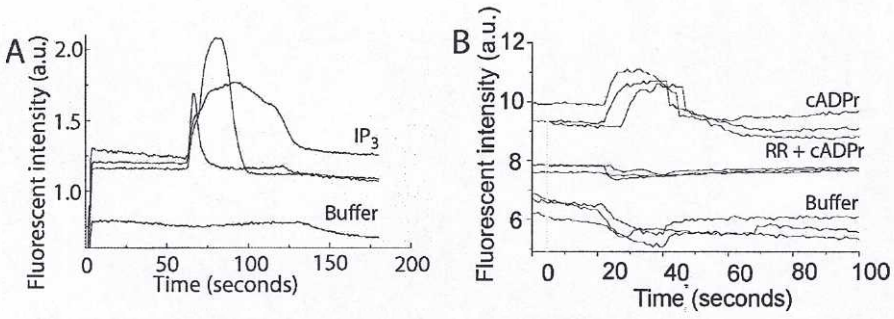

Figure 12
Detection of intracellular receptors in single cells by electroporation. A) Detection of the intracellular receptor inositol 1,4,5-triphosphate (IP_3), upper trace, after introduction of IP_3 by electroporation with the EFC. B) In a similar way cADPr was introduced for detection of the ryanodine receptor, upper trace. By coelectroporation of ruthenium red (RR), and cADPr the receptor response was blocked, middle trace.

Applications of electroporation with an EFC include, phenotype profiling, sensing, and screening. Manipulation of cell contents as well as fusion of cells to each other can also be performed with this technique (data not shown).

The main advantage of the EFC technique is that only the selected target cell is electroporated and subjected to the cell-loading agent. Additionally, because of the small diameter and large aspect ratio of the EFC, it can be used to access deep lying structures in tissues with minimal tissue trauma. The dimensions of the EFC tip can be varied from low nm to hundreds of micrometers and thereby the size of the target area is easily varied.

6.2 Paper III

In which we characterise the electroporation performed by two solid microelectrodes.

The method developed by Lundqvist *et al.* was characterised by using patch clamp and fluorescence microscopy measurements (Lundqvist *et al.*, 1998).

The field loss due to interfacial reactions between the electrode surfaces and the buffer was determined and corrected for in these experiments. By stepwise withdrawing a patch-clamped cell out of the electric field, the spatial distribution of the electric field was investigated. At a distance of approximately 30 μm from the focus of the field, the electric field is too low to cause dielectric breakdown of the membrane, figure 13. From this it can be concluded that the electric field is very focused. The threshold potential for electroporation was determined by fluorescence measurements to be 3.0 V, and by patch clamp recordings to 2.5 V (V_m = 250 mV) applied over the electrodes. With increased applied voltage, the conductance over the membrane increased, finally reaching an upper limit where the cell membrane was irreversibly ruptured. The time constants for pore formation ranged between 0-50 ms and the time constants for pore resealing ranged from 10-620 ms (figure 13). Both processes were

dependent upon applied voltage. From the patch clamp traces in figure 13, it can be seen how the current becomes increasingly noisier with higher voltages. This might be due to presence of low-conducting metastable pores. All these observations are in agreement with previously published data.

This electropration method has the advantage of being mild towards the target cell, and is characterised by a high success rate. The focused electric field allows for high target selectivity, regarding the electroporation process, and permits electroporation of small structures within the cell. Unfortunately the cell-loading agent has to be kept in the surrounding medium and may therefore affect the rest of the cell population. Also, the size of the compound to be introduced is limited to the size of the formed pores, which are around 250 nm wide.

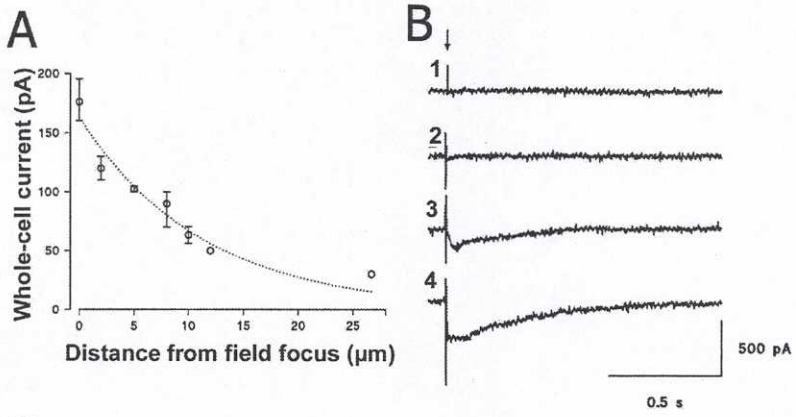

Figure 13
A) The total voltage drop was measured by voltage-clamping the cell and moving it out of focus from the electric field. Voltage pulses of the same amplitude were used for all distances. At a distance of approximately 30 μm from the focus of the field the cell is not affected. B) Patch clamp traces, where 1) corresponds to 2.7 kV/cm, 2) 3.2, 3) 3.8 and 4) 4.3

6.3 Paper IV

In which we introduce a new method for microinjection of larger particles to cells by a combination of electrical and mechanical forces.

A method for electroinjection of large biopolymers and colloidal particles into single liposomes (10-20 μm), and single cells was developed.

Fluorescein, fluorescent latex spheres (30 nm), small liposomes (100 nm), and YOYO-1-labelled T7-phage DNA (R_G = 0.56 μm) were injected with a success rate > 95 % to liposomes. The volumes injected were between approximately 50 - 500 fL. Since liposomes are confined containers with a defined volume they can function as small test tubes. This was demonstrated by injection of T2-phage DNA (R_G = 1.1 μm) followed by injection of YOYO-1 to the same liposome. The DNA and the dye interacted to form a fluorescent complex. The

time scale for mixing was 0.1-1 second at T = 20°C. This also demonstrated that multiple injections could be performed in the same liposome without leakage. The technique can be applied to introduce organelles and other cell structures into a liposome in order to create an artificial cell model system. Reactions can be performed in the confined containers for the purpose of derivatisation, study of reactions, and so on.

Using the same technique, individual PC12 cells were injected with fluorescein, and T7 phage DNA. The injected material could be directed to specific locations within the cell, which was demonstrated by selective introduction of YOYO-1-stained DNA either to the cytoplasm or to the nucleus. Since the developed technique is milder in the treatment of the cells than conventional stab injection, it could be used in similar areas of research. Genes and organelles could be introduced to manipulate cell identity and function. Different kinds of sensor molecules or colloidal probes could be inserted for direct monitoring of intracellular events or for labelling of cell components prior to separation and detection. Compared to other injection techniques, this method allows injection of larger particles, which is a great advantage. Unfortunately the injection volume is hard to control, and there is an upper volume limit defined by the target structure.

7
Conclusion and future outlook

In this thesis, three different techniques for manipulating the genetic, metabolic, and synthetic composition of single cells were developed. Biosensor applications of these experimental platforms were demonstrated where intracellular proteins were used as recognition elements. In addition, the following five main areas of applications and further development are identified.

Manipulation of the contents of cells and liposomes by introduction of substances and particles is useful for studying biological processes and chemical reactions. For example, biological receptors can be introduced into liposome membranes, thereby engineering the liposome to mimic cells to study specific cellular systems. Also, colloidal metal particles can be introduced into cells for SERS applications (Chourpa *et al.*, 1996), and colloidal sensors such as PEBBLES can be administered to single cells to report on local cellular chemistry (Clark *et al.*, 1999). In the future, microelectrodes and electrolyte-filled capillaries will probably be miniaturised to nanometer dimensions, allowing selective manipulation of single organelles within a cell. Manipulation can be performed both *in vivo* and *in vitro* and not only for the purpose of basic research but also for medicinal purposes. One can, for example, speculate on the use of electroinjection for nuclear transfer or tissue-directed gene delivery using electroporation with EFC's. By delivery of genes, for instance, encoding ion channels or receptors, it might be possible to treat some diseases related to defects in these systems. Furthermore, drugs can be rapidly delivered to cells *in vivo*. The high spatial selectivity makes it possible to target small populations of cells without affecting cells in the neighbourhood. This capability is particularly interesting in highly organised tissues such as brain tissue. In the case of depletion of tumours, the drug may be delivered and electroporated into the tumour cells, possibly in combination with high potentials that would assist in killing the tumour cells.

Sensing can be performed where an EFC is used to separate analytes in a sample, or simply to direct an analyte to the target without separation. Receptors in the plasma membrane and intracellular membranes, as well as soluble intracellular enzymes, of the sensor cell can be used for detection. The method can be used for sensing of unknown analytes in a complex sample and to discover new biologically active compounds. By the use of stimulators and blockers, the pharmacology and kinetics of different molecular systems can be studied.

At the time of writing, this sensor system is qualitative, but can most likely be developed into a quantitative system. Most receptor systems have a concentration-dependent response. This dose-response curve is within a region linear, and can be used for calibration.

To fully implement the concept of cell-based intracellular sensing into a routine analysis concept, it is necessary to work with stable systems. Detector cells can be tailored by genetic engineering protocols to overexpress the molecular recognition element and increasing the signal. Also, the kinetics of the intracellular cascades can be manipulated for a faster response. The use of artificial systems such as liposomes with inserted receptors in the membranes could provide an alternative to cells. Furthermore, it is necessary to miniaturise and simplify the instrumental set up to make it user-friendly. Integrating all the required equipment such as electrodes, separation capillaries, and cell chambers on microchips can do this.

Probing can be performed by all three methods by introduction of dyes, substrates, ligands and reaction initiators of any kind. One may map subsequent changes in morphology, events during cell-to-cell communication, subcellular compartmentalisation and trafficking, all within a living tissue. Substrates for metabolic pathways can be injected to study and understand the fate of particular metabolic pathways. Studies can be performed both *in vitro* and *in vivo*. *In vivo* electroporation can also be combined with sampling by microdialysis. Sampling can be performed simultaneously with selective electrical or chemical stimulation of a single or a small population of cells.

Phenotype profiling is, in principle, the opposite of sensing and can be performed both by the electroporation and electroinjection techniques. Here the cell-loading agent is known, but the composition of the cell is unknown. A selective substrate, *e.g.* a receptor agonist, is introduced to determine the presence of a component within the cell. This is useful for research focusing on looking at changes in the proteome as a function of different factors, and stimuli *i.e.* how protein expression is affected by the milieu and physico-chemical cues. Also, when new cells lines are developed or tumours are investigated, it is useful to be able to follow the development of a phenotype with time. A more practical use is in medical diagnostics to look for disease markers or biomarkers.

Screening is the way sensing, probing and phenotyping is automated and performed on a large number of cells in a short period of time. There is an increasing need in biotechnology for small simple devices that are capable of investigating the pharmacological properties of drugs acting on cells rapidly. Coupling of techniques might be one way to do it. All three techniques developed in this thesis can be miniaturised and combined in different chip-based set-ups. There is also a need to perform parallel analysis to improve the efficiency of the analysis. We have shown how cells can be patterned and grown in PDMS membranes. This concept can be further developed into a parallel system and the vision is, of course, that the system will be fully integrated on a chip and used for high-throughput screening. For example, an array of cells can be either patterned in microwells contained in PDMS membranes or cells can be grown in 96-well plates and an array of EFC's can be constructed matching the cell array. By having one cell line, and different substances in each EFC, such a platform can be used for screening of intracellular receptor ligands or for new ligands binding to G-proteins newly discovered by the HUGO project.

Intracellular protein systems represent interesting new application areas for drug screening. Today there are many different methods available for screening of proteins on the cell membrane surface, but not so many for intracellular targets, especially not in intact cells. It was demonstrated in the present thesis how we could access the cytoplasmic domain and to a certain extent control which area to target within the cell. With further development, as described above, high-throughput screening of intracellular systems should be possible.

8
Acknowledgement

First of all I would like to thank my supervisor Professor Owe Orwar for your passion for science of an evangelist, and never-ending steam of ideas during those years. Thanks for letting me be a part of it! Professor Mats Sandberg for scientific advice and support, and Professor Daniel Jagner for support during those years. A big hug to Anne Fabrot, who put me in contact with Owe, and so got the show on the road. All former and present members of the Orwar research group and all collaborators during those years. You all made this journey pleasant and joyful!

Everybody at AMK for making a fantastic working environment during the years. Especially Kenneth and particular for help with drilling 1600 holes in a stupid table where unfortunately not a single of those holes never ever during those 5 years have been used. Leo for nice songs and the "true" boss of the department, Ingrid (always a smile present and answers to ANY questions).

Göran Ljungkvist who helped me taking my first steps "in the real world". I learned a lot from you!

My mentor, Hillevi Mattson, who expanded my view in many ways. Thanks for your support and all fruitful discussions.

All friends all over Sweden (especially Trollhättan, Strömsund, Umeå and Göteborg) and abroad (Amsterdam). I am grateful to all of you for being such good friends over the years though the long physical distance between us. I can't imagine a life be whiteout you!

Finally, I would like to give my deepest and greatest gratitude to the most important persons in my life. Mamma, pappa, Mats and Frida, thanks a lot for your endless support and encouragement and for trying to understand what I do.

Scholarship and contributions from Wilhem och Martina Lundgrens Vetenskapsfond, Stiftelsen Bengt Lundkvists Minne, Gunvor och Josef Ahners Stiftelse, Adlebertska stipendiefonden, Paul och Marie Berghaus donations fond, Rådman och Fru Ernst Collianders Stiftelese, Kungliga och Hvitfeldska Stiftelsen, Kriewskys stiftelse, Karl och Annie Leons Minnesfond, Fredrika Bremer förbundet, Naturvetenskapliga fakulteten vid Göteborgs Universitet and Svenska Kemistsamfundet is greatly acknowledged. This work was supported by the Swedish Natural Science Research Council (NFR) and Swedish Foundation of Strategic Research (SSF).

9
References

Akinlaja, J., and Sachs, F. (1998). The breakdown of cell membranes by electrical and mechanical stress. *Biophys. J.* ***75***(1), 247-254.

Barnett, A., and Weaver, J. C. (1991). Electroporation: a unified, quantitative theory of reversible electric breakdown and rupture. *Bioelectrochem. Bioenerg. (J. Electroanal. Chem.)*, ***25***(2), 163-182.

Benz, R., and Zimmermann, U. (1980). Pulse-length dependence of the electrical breakdown in lipid bilayer membranes. *Biochim. Biophys. Acta* ***597***(3), 637-642.

Benz, R., and Zimmermann, U. (1981). The resealing process of lipid bilayers after reversible electrical breakdown. *Biochim. Biophys. Acta* ***640***(1), 169-178.

Bruchez, M., Jr., Moronne, M., Gin, P., Weiss, S., and Alivisatos, A. P. (1998). Semiconductor nanocrystals as fluorescent biological labels. *Science* ***281***(5385), 2013-2016.

Canatella, P. J., Karr, J. F., Petros, J. A., and Prausnitz, M. R. (2001). Quantitative study of electroporation-mediated molecular uptake and cell viability. *Biophys. J.* ***80***(2), 755-764.

Chang, D. C., and Reese, T. S. (1990). Changes in membrane structure induced by electroporation as revealed by rapid-freezing electron microscopy. *Biophys. J.* ***58***(1), 1-12.

Chiu, D. T., Lillard, S. J., Scheller, R. H., Zare, R. N., Rodriguez-Cruz, S. E., Williams, E. R., Orwar, O., Sandberg, M., and Lundqvist, J. A. (1998). Probing single secretory vesicles with capillary electrophoresis. *Science* ***279***(5354), 1190-1193.

Chourpa, I., Morjani, H., Riou, J. F., and Manfait, M. (1996). Intracellular molecular interactions of antitumor drug amsacrine (m- AMSA) as revealed by surface-enhanced Raman spectroscopy. *FEBS Lett* ***397***(1), 61-64.

Clark, H. A., Kopelman, R., Tjalkens, R., and Philbert, M. A. (1999). Optical nanosensors for chemical analysis inside single living cells. 2. Sensors for pH and calcium and the intracellular application of PEBBLE sensors. *Anal. Chem.* ***71***(21), 4837-4843.

Cole, K. S. (1968). Membranes, ions and impulses: A chapter of classical biophysics. In Biophysics series (University of Berkeley Press), pp. 12-18.

Cooper, B. R., Wightman, R. M., and Jorgenson, J. W. (1994). Quantitation of epinephrine and norepinephrine secretion from individual adrenal medullary cells by microcolumn high-performance liquid chromatography. *J. Chromatogr. B Biomed. Appl.* ***653***(1), 25-34.

Farre, C., Sjöberg, A., Jardemark, K., Jacobson, I., and Orwar, O. (2001). Screening of ion channel receptor agonists using capillary electrophoresis-patch clamp detection with resensitized detector cells. *Anal. Chem.* ***73***(6), 1228-1233.

Finnegan, J. M., Pihel, K., Cahill, P. S., Huang, L., Zerby, S. E., Ewing, A. G., Kennedy, R. T., and Wightman, R. M. (1996). Vesicular quantal size measured by amperometry at chromaffin, mast, pheochromocytoma, and pancreatic beta-cells. *J. Neurochem.* ***66***(5), 1914-1923.

Fishman, H. A., Orwar, O., Allbritton, N. L., Modi, B. P., Shear, J. B., Scheller, R. H., and Zare, R. N. (1996). Cell-to-cell scanning in capillary electrophoresis. *Anal. Chem.* ***68***(7), 1181-1186.

Fishman, H. A., Orwar, O., Scheller, R. H., and Zare, R. N. (1995). Identification of receptor ligands and receptor subtypes using antagonists in a capillary electrophoresis single-cell biosensor separation system. *Proc. Natl. Acad. Sci. USA* ***92***(17), 7877-7881.

Fuller, R. R., Moroz, L. L., Gillette, R., and Sweedler, J. V. (1998). Single neuron analysis by capillary electrophoresis with fluorescence spectroscopy. *Neuron* ***20***(2), 173-181.

Garden, R. W., Shippy, S. A., Li, L., Moroz, T. P., and Sweedler, J. V. (1998). Proteolytic processing of the Aplysia egg-laying hormone prohormone. *Proc. Natl. Acad. Sci. USA* ***95***(7), 3972-3977.

Gift, E. A., and Weaver, J. C. (1995). Observation of extremely heterogeneous electroporative molecular uptake by Saccharomyces cerevisiae which changes with electric field pulse amplitude. *Biochim. Biophys. Acta* ***1234***(1), 52-62.

Glaser, R. W., Leikin, S. L., Chernomordik, L. V., Pastushenko, V. F., and Sokirko, A. I. (1988). Reversible electrical breakdown of lipid bilayers: formation and evolution of pores. *Biochim. Biophys. Acta* ***940***(2), 275-287.

Goodson, H. V., Valetti, C., and Kreis, T. E. (1997). Motors and membrane traffic. *Curr. Opin. Cell Biol.* ***9***(1), 18-28.

Gross, G. W., Harsch, A., Rhoades, B. K., and Gopel, W. (1997). Odor, drug and toxin analysis with neuronal networks in vitro: extracellular array recording of network responses. *Biosens. Bioelectron.* ***12***(5), 373-393.

Haas, K., Sin, W. C., Javaherian, A., Li, Z., and Cline, H. T. (2001). Single-cell electroporation for gene transfer in vivo. *Neuron* ***29***(3), 583-591.

Haugland, R. P. (1996). Handbook of fluorescent probes and research chemicals, Sixth edition edn (Eugene, OR, Molecular Probes, Inc.).

Heal, R. D., Rogers, A. T., Lunt, G. G., Pointer, S. A., and Parsons, A. T. (2001). Development of a neuronal pressure sensor. *Biosens. Bioelectron.* ***16***(9-12), 905-909.

Hood, M. T., and Stachow, C. (1992). Influence of polyethylene glycol on the size of *S. pombe* electropores. *Appl. Environ. Microbiol.* ***58***(4), 1201-1206.

Hu, S., Lee, R., Zhang, Z., Krylov, S. N., and Dovichi, N. J. (2001). Protein analysis of an individual Caenorhabditis elegans single-cell embryo by capillary electrophoresis. *J. Chromatogr. B Biomed. Sci. Appl.* ***752***(2), 307-310.

Hurtig, J. (2001) Development of a cellular biosensor microchip with integrated electrodes and capillary electrophoresis, Master thesis, Linköping Institue of Technology, Linköping.

Israelachvili, J. N., and Pashley, R. M. (1984). Measurement of the hydrophobic interaction between two hydrophobic surfaces in aqueous electrolyte solutions. *J. Coll. Interface Sci.* ***98***500-514.

Jahnsen, H., Kristensen, B. W., Thiebaud, P., Noraberg, J., Jakobsen, B., Bove, M., Martinoia, S., Koudelka-Hep, M., Grattarola, M., and Zimmer, J. (1999). Coupling of organotypic brain slice cultures to silicon-based arrays of electrodes. *Methods* ***18***(2), 160-172.

Johnson, I. (1998). Fluorescet probes for living cells. *Histochem. J.* ***30***(3), 123-140.

Jorgenson, J. W., and Lukacs, K. D. (1981). Free-zone electrophoresis in glass capillaries. *Clin. Chem.* ***27***(9), 1551-1553.

Karube, I., Matsunaga, T., Mitsuda, S., and Suzuki, S. (1977). Microbial electrode BOD sensors. *Biotechnol. Bioeng.* ***19***(10), 1535-1547.

Keefer, E. W., Gramowski, A., Stenger, D. A., Pancrazio, J. J., and Gross, G. W. (2001). Characterization of acute neurotoxic effects of trimethylolpropane phosphate via neuronal network biosensors. *Biosens. Bioelectron.* ***16***(7-8), 513-525.

Kennedy, R. T., and Jorgenson, J. W. (1989). Quantitative analysis of individual neurons by open tubular liquid chromatography with voltammetric detection. *Anal. Chem.* ***61***(5), 436-441.

Kinosita, K., Jr., and Tsong, T. Y. (1977a). Formation and resealing of pores of controlled sizes in human erythrocyte membrane. *Nature* ***268***(5619), 438-441.

Kinosita, K., Jr., and Tsong, T. Y. (1977b). Voltage-induced pore formation and hemolysis of human erythrocytes. *Biochim. Biophys. Acta* ***471***(2), 227-242.

Kinosita, K. J., Hibino, M., Itoh, H., Shigemori, M., Hirano, K., Kirino, Y., and Hayakawa, T. (1992). Events of membrane electroporation visualized on a timescale from microseconds to seconds. In Guide to electroporation and electrofusion, D. C. Chang, B. M. Chassy, J. A. Saunders, and A. E. Sowers, eds. (Academic Press, Inc., California), pp. 31-46.

Kurata, S., Tsukakoshi, M., Kasuya, T., and Ikawa, Y. (1986). The laser method for efficient introduction of foreign DNA into cultured cells. *Exp. Cell Res.* ***162***(2), 372-378.

Li, L., Garden, R. W., and Sweedler, J. V. (2000). Single-cell MALDI: a new tool for direct peptide profiling. *Trends Biotechnol.* ***18***(4), 151-160.

Logman, M. J., Budygin, E. A., Gainetdinov, R. R., and Wightman, R. M. (2000). Quantitation of in vivo measurements with carbon fiber microelectrodes. *J. Neurosci. Methods* ***95***(2), 95-102.

Lu, W., and Cassidy, M. (1994). Background noise in capillary electrophoretic amperometric detection. *Anal. Chem.* ***66***(2), 200-204.

Lundqvist, J. A., Sahlin, F., Åberg, M. A., Strömberg, A., Eriksson, P. S., and Orwar, O. (1998). Altering the biochemical state of individual cultured cells and organelles with ultramicroelectrodes. *Proc. Natl. Acad. Sci. USA* ***95***(18), 10356-10360.

Luo, D., and Saltzman, W. M. (2000). Synthetic DNA delivery systems. *Nat. Biotechnol.* ***18***(1), 33-37.

Luzzi, V., Murtazina, D., and Allbritton, N. L. (2000). Characterization of a biological detector cell for quantitation of inositol 1,4,5-trisphosphate. *Anal. Biochem.* ***277***(2), 221-227.

Luzzi, V., Sims, C. E., Soughayer, J. S., and Allbritton, N. L. (1998). The physiologic concentration of inositol 1,4,5-trisphosphate in the oocytes of Xenopus laevis. *J. Biol. Chem.* ***273***(44), 28657-28662.

Maeda, E., Kuroda, Y., Robinson, H. P., and Kawana, A. (1998). Modification of parallel activity elicited by propagating bursts in developing networks of rat cortical neurones. *Eur. J. Neurosci.* ***10***(2), 488-496.

Mann, M., Hendrickson, R. C., and Pandey, A. (2001). Analysis of proteins and proteomes by mass spectrometry. *Annu. Rev. Biochem.* ***70***437-473.

Manz, A., Graber, N., and Widmer, H. M. (1990). Miniaturized total chemical analysis systems: a novel concept for chemical sensing. *Sens. Actuators B* ***1***(1-6), 244-248.

Matthews, K. E., Dev, S. B., Toneguzzo, F., and Keating, A. (1995). Electroporation for gene therapy. In Methods Mol. Biol., J. A. Nickoloff, ed. (Totowa, NJ, Humana Press Inc.), pp. 273-280.

McDonald, J. C., Duffy, D. C., Anderson, J. R., Chiu, D. T., Wu, H., Schueller, O. J., and Whitesides, G. M. (2000). Fabrication of microfluidic systems in poly(dimethylsiloxane). *Electrophoresis* ***21***(1), 27-40.

Minsky, M. (1988). Memoir on inventing the confocal scanning microscope. *Scanning* ***10***(4), 128-138.

Misra, A., Ganga, S., and Upadhyay, P. (1999). Needle-free, non-adjuvanted skin immunization by electroporation- enhanced transdermal delivery of diphtheria toxoid and a candidate peptide vaccine against hepatitis B virus. *Vaccine* ***18***(5-6), 517-523.

Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997). Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* ***388***(6645), 882-887.

Needham, D., and Hochmuth, R. M. (1989). Electro-mechanical permeabilization of lipid vesicles. Role of membrane tension and compressibility. *Biophys. J.* ***55***(5), 1001-1009.

Neher, E., and Sakmann, B. (1976). Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* ***260***(5554), 799-802.

Neumann, E., Toensing, K., Kakorin, S., Budde, P., and Frey, J. (1998). Mechanism of electroporative dye uptake by mouse B cells. *Biophys. J.* ***74***(1), 98-108.

Neumann, E. A., Sprafke, E., Boldt, E., and Wolf, H. (1992). Biophysical considerations of membrane electroporation. In Guide to electroporation and electrofusion, D. C. Chang, B. M. Chassy, J. A. Saunders, and A. E. Sowers, eds. (Academic Press, Inc., California), pp. 77-90.

O'Brien, J. A., Holt, M., Whiteside, G., Lummis, S. C., and Hastings, M. H. (2001). Modifications to the hand-held Gene Gun: improvements for in vitro biolistic transfection of organotypic neuronal tissue. *J. Neurosci. Methods* ***112***(1), 57-64.

Okino, M., and Mohri, H. (1987). Effects of a high-voltage electrical impulse and an anticancer drug on in vivo growing tumors. *Jpn. J. Cancer Res.* ***78***(12), 1319-1321.

Olefirowicz, T. M., and Ewing, A. G. (1990). Capillary electrophoresis in 2 and 5 microns diameter capillaries: application to cytoplasmic analysis. *Anal. Chem.* ***62***(17), 1872-1876.

Orwar, O., Jardemark, K., Jacobson, I., Moscho, A., Fishman, H. A., Scheller, R. H., and Zare, R. N. (1996). Patch-clamp detection of neurotransmitters in capillary electrophoresis. *Science* ***272***(5269), 1779-1782.

Page, J. S., Rubakhin, S. S., and Sweedler, J. V. (2002). Single-neuron analysis using CE combined with MALDI MS and radionuclide detection. *Anal. Chem.* ***74***(3), 497-503.

Paquette, D. M., Sing, R., Banks, P. R., and Waldron, K. C. (1998). Capillary electrophoresis with laser-induced native fluorescence detection for profiling body fluids. *J. Chromatogr. B Biomed. Sci. Appl.* ***714***(1), 47-57.

Parce, J. W., Owicki, J. C., Kercso, K. M., Sigal, G. B., Wada, H. G., Muir, V. C., Bousse, L. J., Ross, K. L., Sikic, B. I., and McConnell, H. M. (1989). Detection of cell-affecting agents with a silicon biosensor. *Science* ***246***(4927), 243-247.

Pease, D. C., and Porter, K. R. (1981). Electron microscopy and ultramicrotomy. *J. Cell. Biol.* ***91***(3 Pt 2), 287s-292s.

Pieroni, J. P., Jacobowitz, O., Chen, J., and Iyengar, R. (1993). Signal recognition and integration by Gs-stimulated adenyl cyclases. *Curr. Opin. Neurobiol.* ***3***(3), 345-351.

Prausnitz, M. R. (1999). A practical assessment of transdermal drug delivery by skin electroporation. *Adv. Drug. Deliv. Rev.* ***35***(1), 61-76.

Prausnitz, M. R., Corbett, J. D., Gimm, J. A., Golan, D. E., Langer, R., and Weaver, J. C. (1995). Millisecond measurement of transport during and after an electroporation pulse. *Biophys. J.* ***68***(5), 1864-1870.

Rae, J. L., and Levis, R. A. (2001). Single-cell electroporation. *Pflügers Archiv* ***DOI 10.1007/s00424-001-0753-1***.

Ren, X., Bachman, M., Sims, C., Li, G. P., and Allbritton, N. (2001). Electroosmotic properties of microfluidic channels composed of poly(dimethylsiloxane). *J. Chromatogr. B Biomed. Sci. Appl.* ***762***(2), 117-125.

Rols, M.-P., and Teissie, J. (1998). Electropermeabilization of mammalian cells to macromolecules:control by pulse duration. *Biophys. J.* ***75***(3), 1415-1423.

Rubakhin, S. S., Garden, R. W., Fuller, R. R., and Sweedler, J. V. (2000). Measuring the peptides in individual organelles with mass spectrometry. *Nat. Biotechnol.* ***18***(2), 172-175.

Sandre, O., Moreaux, L., and Brochard-Wyart, F. (1999). Dynamics of transient pores in stretched vesicles. *Proc. Natl. Acad. Sci. U S A* ***96***(19), 10591-10596.

Saulis, G., Venslauskas, M. S., and Naktinis, J. (1991). Kinetics of pore resealing in cell membranes after electroporation. *Bioelectrochem. Bioenerg. (J. Electroanal. Chem.)*, ***26***(1), 1-13.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell* ***103***(2), 211-225.

Shear, J. B., Fishman, H. A., Allbritton, N. L., Garigan, D., Zare, R. N., and Scheller, R. H. (1995). Single cells as biosensors for chemical separations. *Science* ***267***(5194), 74-77.

Singer, S. J., and Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Science* ***175***(23), 720-731.

Singh, B. N., and Dwivedi, C. (1999). Antitumor drug delivery by tissue electroporation. *Anticancer Drugs.* ***10***(2), 139-146.

Soughayer, J. S., Krasieva, T., Jacobson, S. C., Ramsey, J. M., Tromberg, B. J., and Allbritton, N. L. (2000). Characterization of cellular optoporation with distance. *Anal. Chem.* ***72***(6), 1342-1347.

Straub, B., Meyer, E., and Fromherz, P. (2001). Recombinant maxi-K channels on transistor, a prototype of iono- electronic interfacing. *Nat. Biotechnol.* ***19***(2), 121-124.

Tamaki, E., Sato, K., Tokeshi, M., Sato, K., Aihara, M., and Kitamori, T. (2002). Single-cell analysis by scanning thermal lens microscope with a microchip: direct monitoring of cytochrome c distrubution during apoptosis process. *Anal. Chem.* ***74***(7), 1560-1564.

Teissie, J., and Rols, M. P. (1992). Interfacial membrane alteration associated with electropermeabilization and electrofusion. In Guide to electroporation and electrofusion, D. C. Chang, B. M. Chassy, J. A. Saunders, and A. E. Sowers, eds. (Academic Press, Inc., California), pp. 139-154.

Terry, S. C., Jerman, J. H., and Angell, J. B. (1979). A gas chromatographic air analyzer fabricated on silicon wafer. *IEEE Trans. Electron. Devices* ***26***(12), 880-886.

Teruel, M. N., and Meyer, T. (1997). Electroporation-induced formation of individual calcium entry sites in the cell body and processes of adherent cells. *Biophys. J.* ***73***(4), 1785-1796.

Teruel, M. N., and Meyer, T. (2000). Translocation and reversible localization of signaling proteins: a dynamic future for signal transduction. *Cell* ***103***(2), 181-184.

Tsong, T. Y. (1991). Electroporation of cell membranes. *Biophys. J.* ***60***(2), 297-306.

Tyagi, S., and Kramer, F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* ***14***(3), 303-308.

Wang, Z., Haydon, P. G., and Yeung, E. S. (2000). Direct observation of calcium-independent intercellular ATP signaling in astrocytes. *Anal. Chem.* ***72***(9), 2001-2007.

Weaver (1994). Theory of electroporation. In Biomembrane Electrochemistry, M. Blank, and I. Vodyanoy, eds. (Washington D.C., American Chemical Society), pp. 447-470.

Weaver, J. C. (1993). Electroporation: a general phenomenon for manipulating cells and tissues. *J. Cell. Biochem.* ***51***(4), 426-435.

Weng, G., Bhalla, U. S., and Iyengar, R. (1999). Complexity in biological signaling systems. *Science* ***284***(5411), 92-96.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., *et al.* (2001). The sequence of the human genome. *Science* ***291***(5507), 1304-1351.

Wightman, R. M. (1981). Microvoltametric electrodes. *Anal. Chem.* ***53***(9), A1125-1134.

Wilhelm, C., Winterhalter, M., Zimmermann, U., and Benz, R. (1993). Kinetics of pore size during irreversible electrical breakdown of lipid bilayer membranes. *Biophys. J.* ***64***(1), 121-128.

Woods, L. A., Roddy, T. P., Paxon, T. L., and Ewing, A. G. (2001). Electrophoresis in nanometer inner diameter capillaries with electrochemical detection. *Anal. Chem.* ***73***(15), 3687-3690.

Woolley, A. T., and Mathies, R. A. (1994). Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips. *Proc. Natl. Acad. Sci. USA* ***91***(24), 11348-11352.

Woolley, A. T., Sensabaugh, G. F., and Mathies, R. A. (1997). High-speed DNA genotyping using microfabricated capillary array electrophoresis chips. *Anal. Chem.* ***69***(11), 2181-2186.

Yeung, E. S. (1999). Study of single cells by using capillary electrophoresis and native fluorescence detection. *J. Chromatogr. A* ***830***(2), 243-262.

Young, A. M., Rider, T. H., Petrovick, M. S., Rundell, A. E., Chen, J., and Hollis, M. A. (2000). Biosensing based on B cells transfected with aequorin. Paper presented at: World sixth conference on biosensors (San Diego, USA).

Zhang, Z., Krylov, S., Arriaga, E. A., Polakowski, R., and Dovichi, N. J. (2000). One-dimensional protein analysis of an HT29 human colon adenocarcinoma cell. *Anal. Chem.* *72*(2), 318-322.

Zimmermann, U., and Niel, G. A. (1996). Electromanipulation of Cells (Boca Raton, CRC Press LLC).

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ISBN: 91-628-5198-5