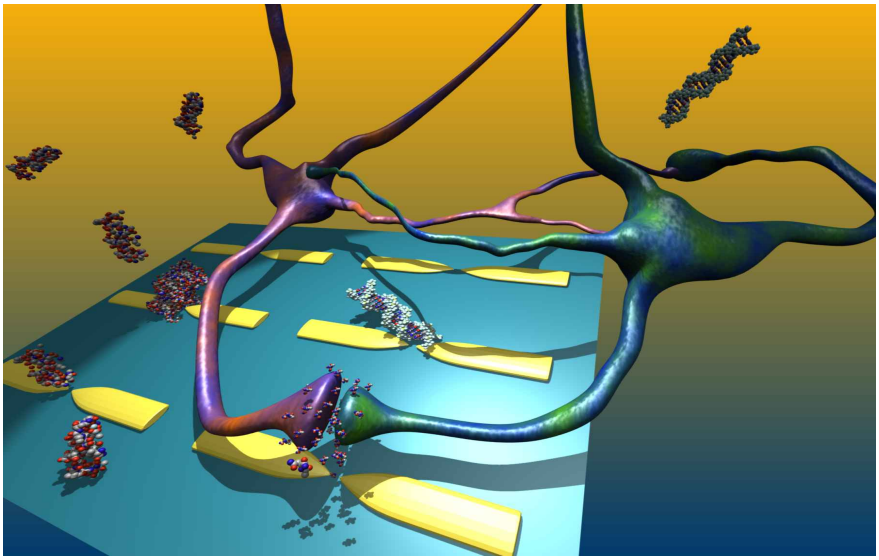
Cell-chip coupling for bioelectronic devices



V. Maybeck, S. Eick, J.F. Eschermann, B. Hofmann,
M. Jansen, U. Yegin, B. Wolfrum, and A. Offenhäusser

Institute for Bio- and Nanosystems 2 - Bioelectronics

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1 Introduction

The coupling of biologically active elements like proteins, cells, or even whole slices of tissue with microelectronic devices is of great interest to current research in the field of biosensoric systems. Merging biological systems with microelectronic technology promises to yield bio-electronic hybrid devices, which exhibit the high sensitivity and selectivity of biological detection mechanisms combined with the optimized amplification cascades of modern electronic signal acquisition and processing.

A particular exciting approach is to couple neuronal cells with chip-based electronic devices. From a fundamental research point of view, such a system allows to investigate the principals and functionality of biological information processing in a defined cellular network. The possibility of storing and processing information in neuronal networks relies on the ability to form and propagate defined changes in the cells membrane potentials by a complex interplay (defined opening and closing) of various ion channels. A depolarization of the membrane potential beyond a certain threshold leads to the triggering of an action potential, which can propagate to adjacent neuronal cells by electrical or chemical synapses. The formation of complex networks of neuronal cells with numerous synapses forms the basic infrastructure for biological information processing.

Simple metallic electrodes or microelectrodes pulled from glass capillaries have been the standard tools for the electrophysiological measurements of electrical activity of whole cells. The development of the patch-clamp technique introduced the possibility to study single ion channels in the cell membrane. Intracellular measurements enable a very good signal-to-noise ratio, but come with the disadvantages of being invasive and are limited to only a few cells. Those disadvantages can be overcome by extracellular recording with microelectronic devices like microelectrode arrays (MEAs) or field effect transistors (FETs), which can be produced in large quantities by state-of-the-art microfabrication.

This practical course gives an introduction to the following methods:

- Patch-Clamp measurements of electrically active cells

- Extracellular recording of electrical cell signals with microelectrode arrays

2 Basic Principles

2.1 The Cell

The cell is one of the most basic units of life. Each cell is at least somewhat self-contained and self-maintaining: it can take in nutrients, convert these nutrients into energy, carry out specialized functions, and most of them reproduce as necessary. For this reason the cell is divided into many compartments where each has a special purpose and therefore an optimized environment.

The cytoplasm of a cell is surrounded by a plasma membrane. It is only about 7-10 nm thick and one of its key roles is to maintain the cell potential. The basic composition and structure of the plasma (=outer) membrane is the same as that of the membranes that surround organelles and other subcellular compartments. The foundation is a phospholipid bilayer, and the membrane as a whole is often described as a fluid mosaic (compare Figure 1) – a two-dimensional fluid of freely diffusing lipids, dotted or embedded with proteins, which may function as channels or transporters across the membrane, or as receptors.

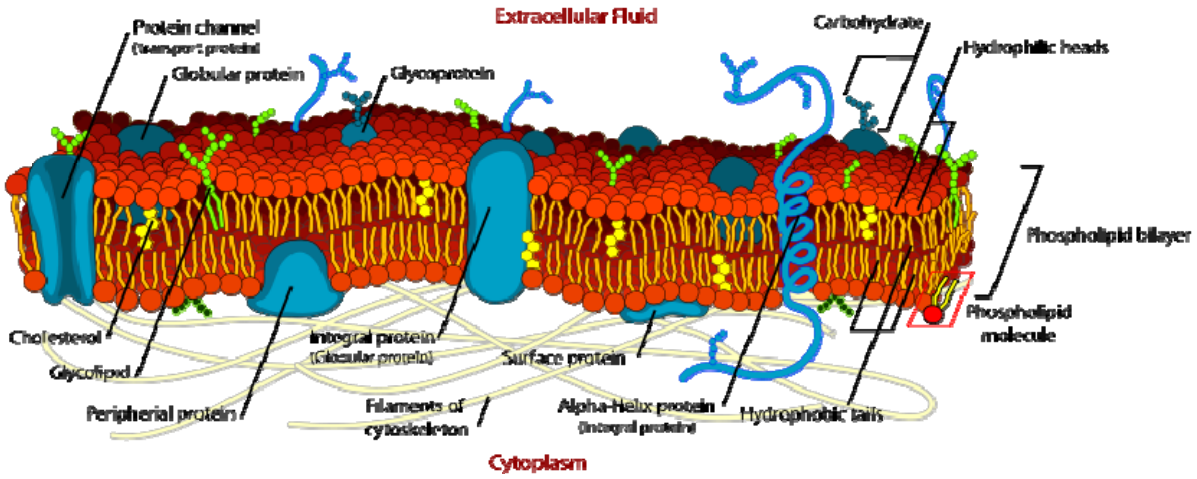


Figure 1 *Patch of a cell membrane, schematically shown [Wikimedia, 2007].*

As a lipid bilayer, the cell membrane is semi-permeable. This means that only some molecules can pass it unhindered. These molecules are either small or lipophilic. Other molecules can only pass if there are specific transport molecules involved.

Depending on the molecule, transport occurs by different mechanisms, which can be separated into those that do not consume energy in the form of ATP (passive transport) and those that do (active transport).

Passive transport is a process moving different chemical substances across membranes through diffusion. The particles are either hydrophobic (non-polar) and small polar molecules

or polar and ionic molecules, which require a transport protein to provide a channel or bind to specific molecules. Because this is a spontaneous process it decreases free energy, and increases entropy in a system. Passive transport is mainly mediated by ion channels.

Active transport typically moves molecules against their electrochemical gradient, a process that is entropically unfavourable but is driven by the hydrolysis (exothermal reaction) of ATP. This coupling can be either primary or secondary. In the primary active transport, transporters that move molecules against their electrochemical gradient, hydrolyze ATP. In the secondary active transport, transporters use energy derived from transport of another molecule in the direction of their gradient, to move other molecules in the direction against their gradient. This can be either symport (in the same direction) or antiport (in the opposite direction).

Problem: As the radius of the cell is much bigger than the thickness of the membrane, its capacity can be calculated like the one of a plate capacitor where d is the membrane thickness:

$$C = \varepsilon_r \varepsilon_0 \frac{A}{d}$$

How big is the membrane capacitance of a spherical cell (R=10µm, $\varepsilon_{r,lipid} = 3$, $\varepsilon_0 = 8{,}85 * 10^{-12}$ As / Vm, d=10 nm)?

Ion channels

Ion channels are pore-forming proteins that help to establish and control the small voltage gradient that exists across the plasma membrane of all living cells by allowing the flow of ions down their electrochemical gradient. They are present in the membranes that surround all biological cells.

An ion channel is an integral membrane protein or more typically an assembly of several proteins. Such "multi-subunit" assemblies usually involve a circular arrangement of identical or homologous proteins closely packed around a water-filled pore through the plane of the membrane or lipid bilayer. While some channels permit the passage of ions based solely on charge, the archetypal channel pore is just one or two atoms wide at its narrowest point. It conducts a specific species of ion, such as sodium or potassium, and conveys them through the membrane in a single file nearly as quickly as the ions move through free fluid. In some ion channels, passage through the pore is governed by a "gate," which may be opened or closed by chemical or electrical signals, temperature, or mechanical force, depending on the type of channel. Unlike active transport, this process does not involve any chemical energy (from ATP).

2.2 Electrical activity of cells

Cells form a membrane potential between the interior of the cell and the surrounding liquid. This potential normally has a value of -60 mV to –90 mV and is caused by an inhomogeneous distribution of ions and the different permeability of the membrane to these ions.

The active transport of potassium and sodium ions into and out of the cell, respectively, is accomplished by a number of sodium-potassium pumps scattered across the cell membrane (compare Figure 2). Each pump transports two ions of potassium into the cell for every three ions of sodium pumped out. This establishes a particular distribution of positively charged ions across the cell membrane, with more sodium present outside the cell than inside, and more potassium inside the cell than outside.

The simplest way to derive an expression for the membrane potential assumes a system in thermodynamic equilibrium. This means that the electrochemical potential inside and outside the cell is equal. Therefore, however, an unhindered exchange through the permeable membrane has to be possible.

In reality there are special potassium channels (“leak channels") which approximately fulfil this prerequisite. Consequently, the electrical voltage across the membrane can be calculated from the NERNST-equation for K^+ ions:

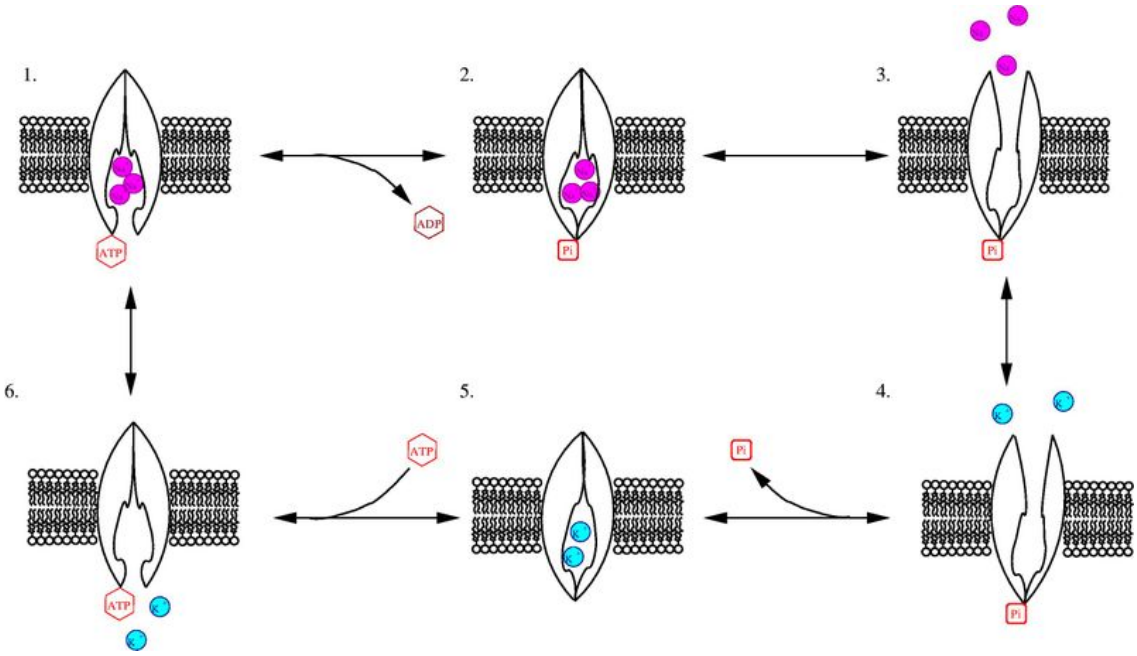


Figure 2 *Sodium-potassium-pump: This system is responsible for maintaining the concentration gradient across the cell membrane despite leak currents [Wikimedia, 2005].*

$$E_m = \frac{R \cdot T}{z \cdot F} \cdot \ln \frac{[K^+]_o}{[K^+]_i}$$

R: gas constant (8.31 J / mol K)
T: temperature
F: Faraday constant (96.5 * 10^3 C / mol)
z: charge of the ion (for

Problem: In mammalian neurons the intracellular potassium concentration is 150 mmol/l, whereas the extracellular one is 5.5 mmol/l. What is the resting potential at body temperature (37°C)? Calculate how much the resting potential depends on the temperature for this case!

GOLDMAN-equation:

The cell membrane is not only permeable for K^+-ions but also for other ions such as Na^+ and Cl^-. The Goldman equation includes all major ion permeabilities (P_x) and leads to a more exact resting potential. It is again only valid in thermodynamic equilibrium.

$$E_m = \frac{R \cdot T}{F} \cdot \ln \frac{P_K \cdot [K^+]_o + P_{Na} \cdot [Na^+]_o + P_{Cl} \cdot [Cl^-]_i}{P_K \cdot [K^+]_i + P_{Na} \cdot [Na^+]_i + P_{Cl} \cdot [Cl^-]_o}$$

A number of ion-exchanger pumps scattered across the cell membrane maintain these gradients. The sodium-potassium-pump, for example, transports two ions of potassium into the cell for every three ions of sodium pumped out (Figure 2).

Like the resting potential, neuronal action potentials depend upon the permeability of the cell membrane to sodium and potassium ions.

Problem: Calculate the resting potential at room temperature taking potassium, sodium and chloride ions into account. Compare the result with the previous problem! ($P_K=10^{-7}$ cm/s, $P_{Na}= P_{Cl} =10^{-8}$ cm/s, $[Na^+]_o= 150$ mmol/l, $[Na^+]_i= 15$ mmol/l, $[Cl^-]_o= 9$ mmol/l, $[Cl^+]_i= 125$ mmol/l)

Hodgkin-Huxley Theory

During an action potential, the membrane voltage V_M is changing over time. If the membrane voltage rises to a certain threshold, which is characteristic to each cell type, first the fast voltage-gated sodium (Na^+) channels in the cell membrane open and a positive current flows into the cell (depolarisation). With short delay slow potassium (K^+) channels open and a positive potassium current starts flowing out of the cell. At the same time, the sodium channels start closing again and the membrane potential reaches a maximum value. Subsequently, the potassium outflow leads to a decrease in the membrane potential (repolarisation). This repolarisation can even lead to a reduction of V_M to a value, which is lower than the initial resting potential V_{RP} (hyperpolarisation). Finally, the potassium channels close and the original V_M is restored.

The Hodgkin-Huxley Theory [Hodgkin, 1952] describes the behaviour of ion channels in the membrane of an electrically active cell during an action potential by an equivalent circuit (Figure 3). The capacitance of the cell membrane is described by the specific capacitance c_M. The specific conductances g^{Na} and g^K for the sodium and potassium ions are related to the ion current densities i^i_m, the membrane potential V_m and to the Nernst-potential E_i of the ion species i:

$$g^i = \frac{i_M^i}{V_M - E_i}$$

All other currents are summed up to a leakage current and described by a voltage-independent resistor with a specific conductance g^L and a Nernst-potential E^L. The resulting ion current density across the membrane can then be described as the sum of all current densities:

$$i_M = c_M \frac{dV_M}{dt} + g_{Na}(t)(V_M - E_{Na}) + g_K(t)(V_M - E_K) + g_L(t)(V_M - E_L)$$

The first element is the capacitive current across the membrane, which can be caused by a quick rise of the membrane potential. The other parts are the ion current densities for each ion type and the leakage current. However, the ion conductances of real cells are time dependent. In the Hodgkin-Huxley Theory those conductances are described by the Hodgkin-Huxley equations, which are based on empirical values for the conductances of the different ion types [Hille, 1992].

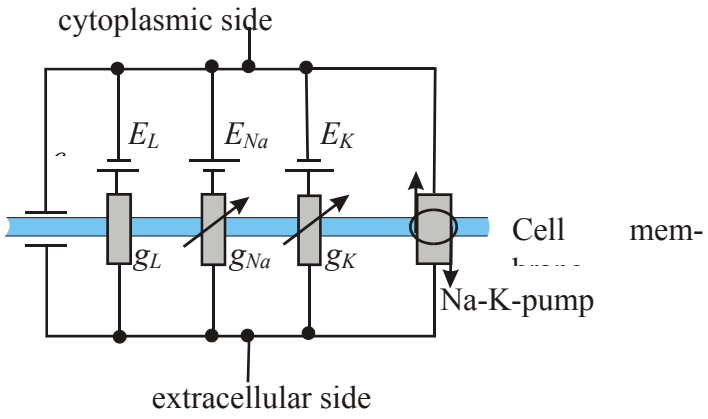


Figure 3 *Equivalent circuit derived from the Hudgekin-Huxley-Theory for a neuronal cell membrane. The properties are described with the Nernst-potential of the ions E_i, with the specific ion conductances g^i of the voltage-gated ion channels and with the specific membrane capacitance c_M. Current densities of other ions and currents caused by leaks in the membrane are summed up to a voltage-independent leakage conductance g^L. The ion concentration gradient is strongly influenced by the Na^+-K^+ ion pump.*

2.3 Excitable Cells

All cells form a membrane potential but only nerve- and muscle-cells have the ability to rapidly change this potential. For this reason they are called excitable (electrogenic) cells. Beside the passive ion channels they also have gated channels such as voltage dependent or ligand-dependent ion-channels. These channels can either increase the voltage gradient (hyperpolarisation) or decrease it (depolarisation).

Neurons

The most important function of nerve cells is the signal propagation by action potentials. An action potential is a short but distinct change of the membrane potential which travels along the neuron and also can be transmitted to other neurons via synapses.

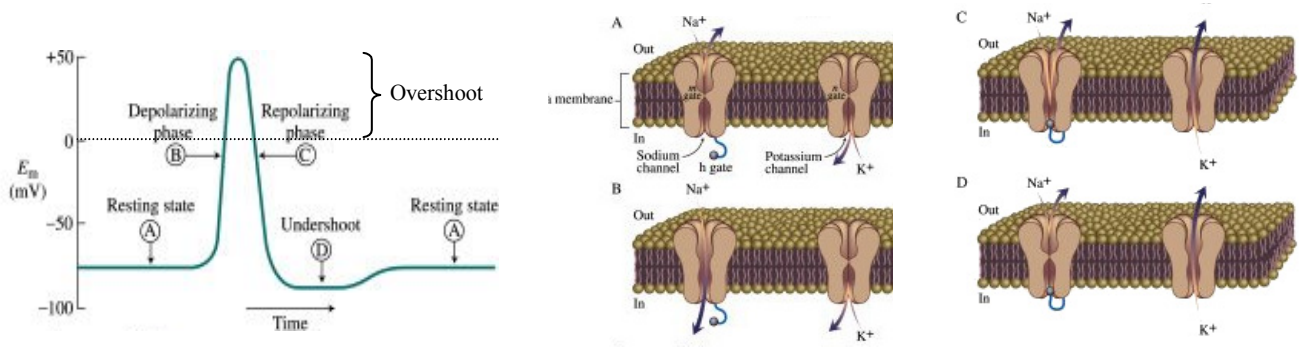


Figure 4 *Phases during the creation of an action potential [Matthews, 2001].*

An action potential can be divided into the following parts (Figure 4):

A: At the resting potential some potassium leak channels are open but the voltage-gated sodium channels are closed. The potassium concentration gradient creates a negative membrane potential.

B: A local membrane depolarization caused by an excitatory stimulus results in the opening of some of the voltage-gated sodium channels in the neuron cell surface. Sodium ions diffuse in through the channels along their electrochemical gradient. Being positively charged, they elicit a reversal in the potential difference across the membrane from negative-inside to positive-inside. Initially, the inward movement of sodium ions is also favoured by the negative-inside membrane potential. As sodium ions enter and the membrane potential becomes less negative, more sodium channels open, causing an even greater influx of sodium ions. This is an example of positive feedback. As more sodium channels open, the sodium current dominates over the potassium leak current and the membrane potential becomes positive inside.

C: Once a membrane potential of around +40 mV has been established, voltage-sensitive inactivation gates of the sodium channels, sensitive to the now positive membrane potential gradient, close (so further influx of sodium is prevented). While this occurs, the voltage-sensitive activation gates on the voltage-gated potassium channels begin to open. As voltage-gated potassium channels open there is a large outward movement of potassium ions driven by the potassium concentration gradient and initially favoured by the positive-inside electrical gradient. As potassium ions diffuse out, this movement of positive charge causes a reversal of the membrane potential to negative-inside and repolarisation of the neuron back towards the large negative-inside resting potential.

D: The large outward current of potassium ions through the voltage-gated potassium channels causes the temporary undershoot of the electrical gradient, with the inside of the neuron being even more negative (relative to the outside) than the usual resting potential. This is

called hyperpolarization. The voltage-sensitive inactivation gates on the potassium channels now close and the continual movement of potassium through potassium leak channels again dominates the membrane potential. Sodium-potassium pumps continue to pump sodium ions out and potassium ions in, preventing any long-term loss of the ion gradients. The resting potential of -70 mV is re-established and the neuron is said to be repolarised.

HL-1 Cells

In an attempt to create cells that can be passaged and retain the characteristics of adult cardiac muscle cells, Claycomb et al. successfully derived a cell line (HL-1) from a mouse cardiomyocyte tumor cell lineage in 1998 [Claycomb 1998]. The resulting cells maintained their contractile activity and could be passaged in culture as well as recovered from frozen stocks. Since these cells are relatively easy to culture and show spontaneous electrical activity, the HL-1 cell line is an ideal model system for cell-chip communication experiments. After a few days in culture, confluent layers of HL-1 cells exhibit synchronous beating and propagation of action potentials. These action potentials can be monitored using extracellular recording devices such as microelectrode arrays. Since the electrophysiological properties of HL-1 cells are very similar to those of primary cardiomyocytes [Sartini 2002, White 2004], first attempts have been made to develop portable cell-based biosensor systems capable of monitoring effects of biochemical agents on the cardiac function of the cells [DeBusschere 2001, Gilchrist 2001].

3 Experimental Techniques

3.1 Electrophysiological Measurements with Patch-Clamp Electrodes

The patch-clamp technique is an experimental method to determine the electrical behaviour of biological membranes. It has been developed in 1976 by Erwin Neher and Bert Sakmann who were awarded the Nobel Prize for this invention in 1991. Roughly, the setup is based on an electrolyte filled glass pipette with an electrode inside. By applying vacuum to the pipette the membrane can be manipulated and subsequently characterized.

Four patch clamp configurations are possible (Figure 5):

- Cell attached: Here the pipette is attached to the membrane until a seal of $R_{seal} > 1$ Gohm forms. Now the small patch under the pipette is accessible for measurement. With this configuration single channels can be characterized.
- Whole-Cell: After sealing, the patch of membrane under the pipette is ruptured by gentle underpressure or a voltage pulse. Now the interior of the pipette becomes continuous with the cytoplasm of the cell. This arrangement allows measurements of electrical potentials and currents from the entire cell. This configuration also allows diffusion driven exchange between the pipette and the cytoplasm, producing a convenient way to inject substances into the interior of a “patched” cell

- Inside-Out: Simply retracting a pipette that is in the cell-attached configuration causes a small vesicle of membrane to remain attached to the pipette. By exposing the tip of the pipette to air, the vesicle opens to yield a small patch of membrane with its (former) intracellular surface exposed. This arrangement allows the measurement of single-channel currents with the added benefit of making it possible to change the medium to which the intracellular surface of the membrane is exposed.
- Outside-Out: If the pipette is retracted while it is in the whole-cell configuration, a membrane patch is produced that has its extracellular surface exposed. This arrangement is optimal for studying how channel activity is influenced by extracellular chemical signals, such as neurotransmitters.

For our experiments we use the whole cell configuration.

The patch is performed as described in the lab manual handed out later.

Voltage-Clamp:

During voltage-clamp experiments the membrane potential is controlled and the currents over the membrane are measured (Figure 6 A)

By variation of the membrane potential, voltage-gated channels can be opened and the flowing current can be measured. The recorded current-trace consists of different inward (Na^+, Ca^{2+}) as well as outward (K^+) ionic currents. By definition, inward currents are negative and outward currents positive.

The ion-specific currents can be measured in pharmacological experiments. TTX (Tetradotoxin, poison of Japanese blowfish) specifically inhibits Na^+-channels, whereas K^+-channels can be blocked by TEA (Tetraethylammonium).

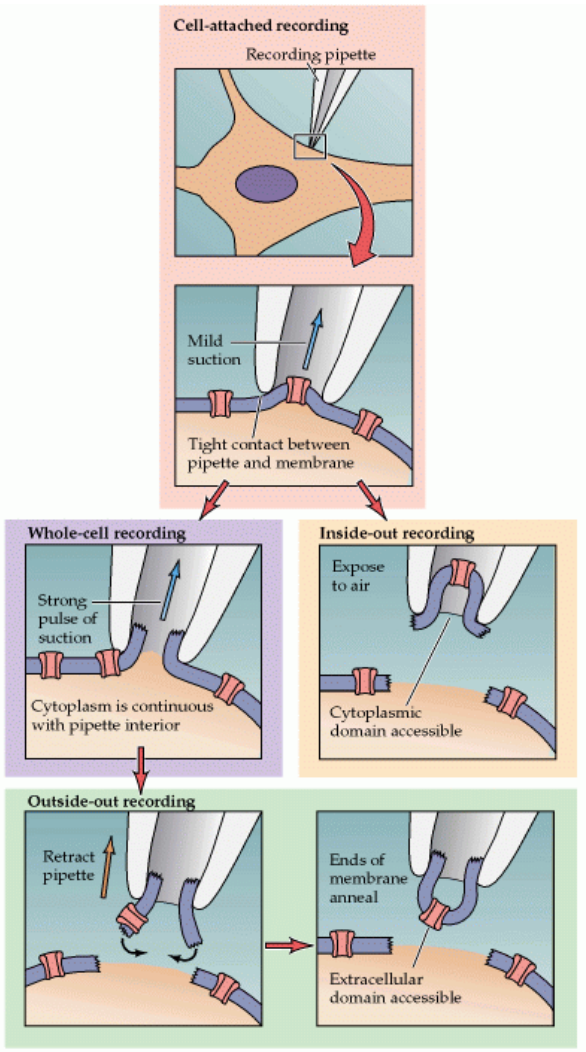


Figure 5 *Overview of the different patch clamp configurations [Williams, 2001].*

Current-Clamp:

Here, in contrary to the voltage clamp mode, the current over the membrane is kept at a distinct value and changes in membrane potential are measured (Figure 6 B) This gives access to the characterization of electrophysiological excitation of the cell. The cell is for example depolarized by increasing currents and after reaching a certain threshold voltage it is possible to evoke action potentials in electrically active cells.

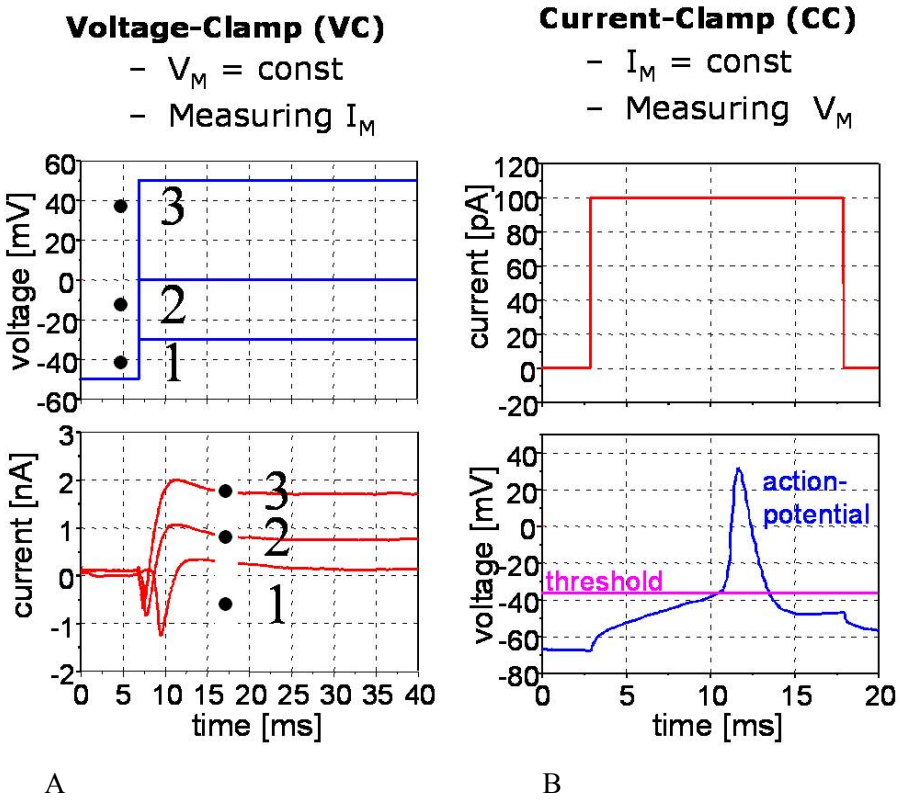


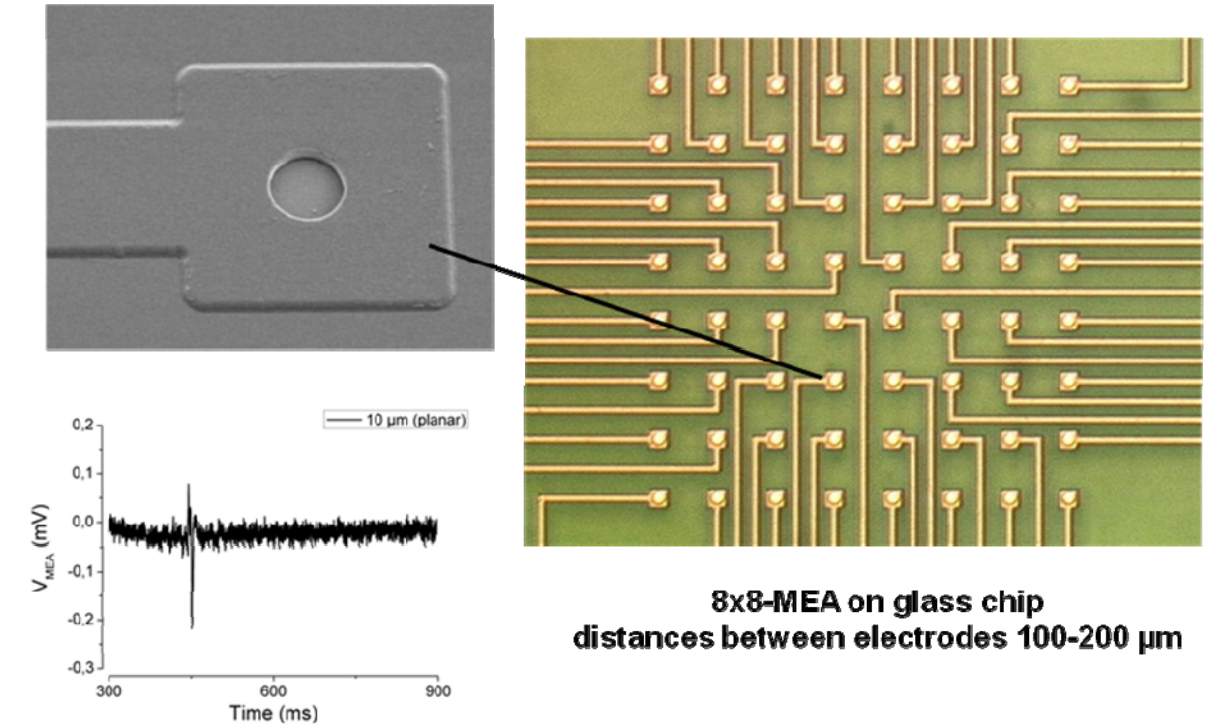
Figure 6 *In the whole-cell configuration, either the membrane potential (A) or the current over the membrane (B) are controlled. The upper graph shows the voltage (A) or current (B) pulse protocol and the lower graph the respective reaction of the cell, schematically shown.*

3.2 Extracellular Recording of Cell Signals

In order to combine electrically active cells and microelectronic devices for the extracellular recording of cell signals, it is necessary to acquire knowledge about the structure and functionality of cells as well as sensor devices like microelectrode arrays (MEAs). The following section will explain the basic functionality of MEAs, which will be used to record cellular signals from an HL-1 cell culture growing on-chip. Subsequently, an equivalent circuit for the description of the electrical properties of the cell and a simple model for the cell-sensor-coupling is given.

Metal microelectrode arrays:

Planar metal microelectrode arrays consist of feed lines and active electrode sites that are fabricated on a non-conducting substrate, usually glass or silicon/silicon oxide.



The diameters of the electrodes used for cellular recordings are typically in the range of 10 µm, with an inter-electrode distance of around 100 µm. Such electrode systems have been used to study the activity of cultured neuronal networks or brain slices [Rutten 2004]. This approach is especially attractive when signals from several cells in a network are to be investigated simultaneously, since multiple recordings are very difficult to implement with the conventional patch clamp technique. The microelectrodes are connected to an amplifier system and can detect a voltage drop in the cleft between cell and electrode interface.

An advantage of microelectrode arrays over other planar sensing devices is the relatively simple fabrication procedure, which includes only two lithography steps. First a metal layer, typically gold or platinum, is deposited on a planar substrate and structured by optical lithography into sensor spots, contact lines, and bond pads. The whole chip is then passivated against the electrolyte by an insulating layer, and only the bond pads and sensor spots are opened by another lithography and an etch process [Eick, 2009].

The essential prerequisites for high-quality recordings of cellular activity are a high seal resistance in the cleft between cell and electrode and a good electronic coupling (low impedance) at the electrode interface. The latter can be improved by modifying the metal interface for example with titanium nitride or iridium oxide, materials which exhibit a high specific capacitance. To ensure tight binding of cells to the surface, the substrate is usually coated by adhesion promoting molecules like polylysine.

Bioelectronic Coupling: The Point Contact Model

For the modelling of extracellular signals in cell-transistor-coupling experiments a simple equivalent circuit (*Point Contact Model*) is applied (Figure 7). This model was first introduced by Regehr et al. [Regehr, 1989] and turned out to be sufficient to describe the extracel-

lular signal shapes qualitatively. It can also be extended to coupling of cells to other solid state devices like planar microelectrodes by including Faradaic currents at the interface.

In a first approach it is assumed that all currents are flowing through one common point in the cleft between the cell and the recording element. The important central parameter is the junction potential $V_J(t)$ in the cleft between cell and sensing interface.

If a patch pipette is attached to the cell, the voltage $V_M(t)$ of its inner part can be controlled by the patch-clamp amplifier. If the cell is in addition attached to the microelectrode, the electrical signals can be measured intra- and extracellularly at the same time and the resulting shapes can be compared. The cleft can be described by the specific conductance g_J, which is defined as the global seal conductance per junction area A^{JM}. The membrane of the cell is divided into a free part and the attached part with the respective capacitances c_{FM} and c_{JM} (typical value $c_M = 1\ \mu F/cm^2$). The membrane contains various channels with time- and voltage-dependent and –independent specific ion conductances g^i and with electrochemical driving forces E^i. In the Point Contact Model these channels are modelled by Hodgkin-Huxley elements [Hodgkin, 1952]. The ion channels in the contact region may have different opening properties or different densities which can be modelled by scaling factors X^i:

$$g^i_{JM} = X^i\ g^i$$

The specific capacitance of the gate is c_{JG}.

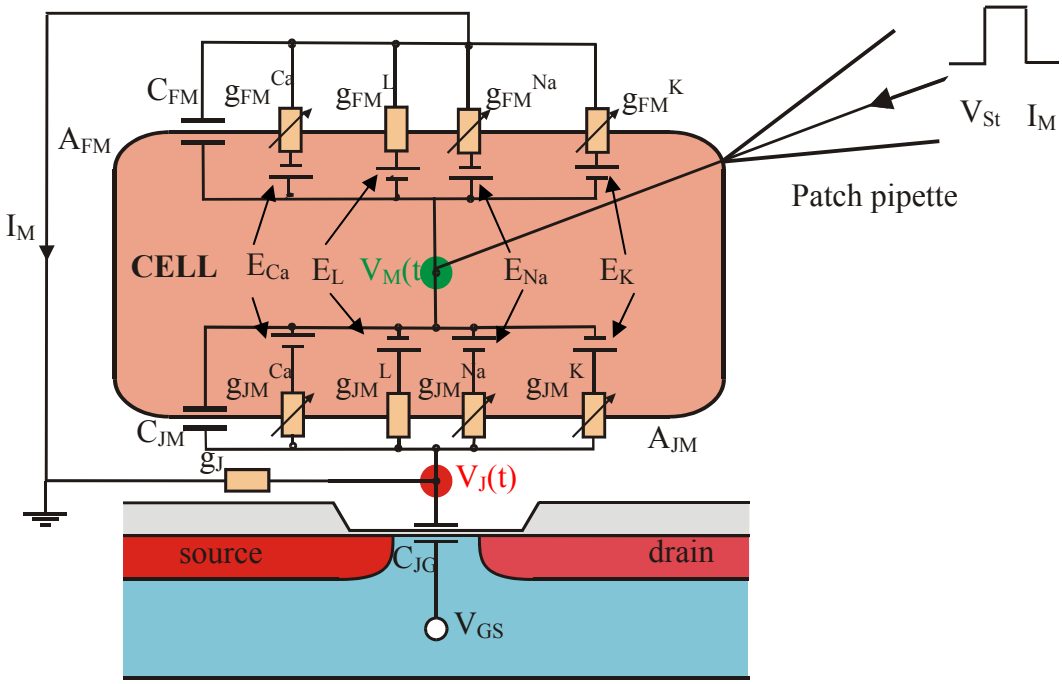


Figure 7 *Point Contact Model for the description of the extracellular coupling of an electrical active cell with a field effect transistor.*

Using Kirchhoff's laws it is possible to calculate $V_J(t)$ in the contact area between cell and transistor gate:

$$c_{JG}\frac{dV_J}{dt}+g_J V_J = c_M\frac{d\left(V_M-V_J\right)}{dt}+\sum_i g_{JM}^i\left(V_M-V_J-E_i\right)$$

It is possible to simplify this equation by the following approximations:

(1) The capacitive current through the gate or electrode can be neglected in comparison to the current through the junction:

$$c_{JG}\frac{dV_J}{dt} << g_J V_J$$

(2) The voltage across the attached membrane is similar to the intracellular voltage:

$$V_M - V_J \approx V_M .$$

(3) It can be assumed that the ion concentrations in the cleft are not changing with respect to the bulk electrolyte, implying unchanged electrochemical driving forces E^i.

This will result in the following equation:

$$g_J V_J = c_M\frac{dV_M}{dt}+\sum_i g_{JM}^i\left(V_M-E^i\right)= c_M\frac{dV_M}{dt}+\sum_i X^i g^i\left(V_M-E^i\right)$$

Thus, it is possible to simulate and describe the extracellular signal $V_J(t)$ for a known course of $V_M(t)$, if the electrophysiological parameters (c_{FM}, c_{JM}, g^i, X^i, E^i) of the examined cell type are given. Therefore, the parameters of model cells in combination with real input data from the patch-clamp experiment are usually used for this purpose.

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