University of Crete

Department of physics

Undergraduate Thesis

Electrophysiological recordings of single rod photoresponse

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Chapter 1

Introduction

1.1 Biophysics

As the term implies, Biophysics is an interdisciplinary science that implements theory and experimental techniques from physics, to analyze and understand biological phenomena, covering all scales of biological organization, from microscopic (molecular) to macroscopic (organismic). It is important to highlight the fact that utilizing physical instruments and devices in biological applications does not fall into the broad spectrum of Biophysics. On the contrary, quoting A.V. Hill, regarding the definition of Biophysics, it is "the study of biological function, organization, and structure by physical and physicochemical ideas and methods". [1]

1.2 Vision and Magnetoreception

The function of the visual system is not merely the focusing of light in order to create an image. The very specific properties and mechanisms of vision and perception have troubled biophysicists since the 1950's. A great example of such research, is the well known experiment on the lower limit of vision done by Hecht in 1942 in which the threshold for vision is estimated between 5-7 photons. [2]

Recent reasearch interest focuses on the perception of magnetic field by the visual system, as it is shown that a wide variety of animals can detect Earth's magnetic field and utilize that sense for navigation and orientation. It is speculated that magnetoreception is based on photoreceptor dependent mechanisms and there are indications that weak magnetic field (less than 50 μT) affect the night vision acuity. However, primary receptors involved in magnetoreception have yet to be identified and characterized. [3]

1.3 In This Thesis

Influenced by A.V. Hill's definition of biophysics, this thesis is composed of three main chapters, each of them featuring a holistic approach and interpretation of different aspects of the experiment. Chapter 2 is a brief review of the literature [4],[5], outlining theory concepts essential to clarify the importance of electrophysiology to biophysics, while also introducing the biological system in study, the retinal rod cell. Chapter 3 advances into the design of the experimental setup and the methods

utilized to conduct the experiments. Finally, chapter 4 concludes with showing the results and contemplating future perspectives.

Chapter 2

Theoretical Background

2.1 Cellular membrane structure

The cell is the fundamental biological unit of structure and function in all living organisms. Often referred to as the "building blocks of life", all cells have similarities in terms of structure and the ability to maintain a high degree of organisation, despite the very different functions different cell types perform.

As mentioned above, the cell has to maintain a high degree of organisation. In order to succeed in that, the cell must have distinct boundaries between the intracellular (cytoplasm) and the extracellular space. This barrier, surrounding the cell, is a plasma membrane, which mainly consists of a phospholipid bilayer. Phospholipids contain both hydrophobic and hydrophilic residues, a fact that, in watery environment, leads to the spontaneous arrangement of phospholipids in structures where hydrophobic residues are isolated from the surrounding water hence creating a lipid bilayer. Embedded in that substrate are other membrane constituents (proteins) with a variety of structural and cellular functions.

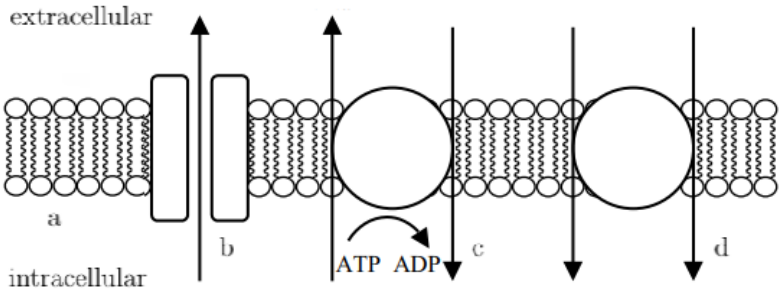


Figure 2.1: Examples of (a) membrane, (b) ionic channel, (c) pump, (d) exchanger

Cellular membrane, due to its lipid components, is essentially a delicate fluid barrier which can be affected by extreme osmotic forces originating from intracellular and extracellular medium concentration differences. Both media are mostly composed of inorganic ions, such as Na^+, K^+, Cl^-, Ca^{2+}, resulting in ion distributions for each, that are very similar regardless of cell type. This fact has enabled cells to develop certain functions to control the flow of ions across the membrane, thus accurately controlling cytoplasm's osmolarity. These functions are enabled by specialised proteins such as ionic channels, pumps and exchangers located in the membrane itself. (A discussion of these is outside the scope of this thesis).

2.2 Electrical properties

The concentration differences between cytoplasm and extracellular environment introduced before, result in concentration gradients for each ion across the membrane. Gradients in concentration induce the diffusion of ions, which from the thermodynamical point of view is a spontaneous progress, i.e. entropy increases and energy is released. The electrical potential at which a certain ion gradient is in equilibrium is described by the Nerst equation

$$E = \frac{RT}{zF} ln \frac{[ion]_{\mathrm{o}}}{[ion]_{\mathrm{i}}}$$

where E is the equilibrium potential for the ion under consideration , R is the universal gas constant, T is the temperature, z is the oxidation state of the ion, F is the Faraday constant and $[\mathrm{ion}]_{\mathrm{o}}$ and $[\mathrm{ion}]_{\mathrm{i}}$ are the extracellular and intracellular concentrations of the ion respectively. The actual membrane potential (resting potential) is the result of different ionic concentrations inside and outside the cell and more important the specific permeability of the membrane to different ions. The resting potential is described by the Goldman–Hodgkin–Katz voltage equation (GHK), which is similar to Nerst equation but the GHK equation takes into account all ions that are permeant through the membrane.

As introduced in section 2.1, the membrane acts as an effective barrier to ions while also being selectively permeable to them through specialised proteins. Furthermore, from GHK equation arises a potential difference (voltage) across the inside and outside of the membrane. Therefore, it is clear that there is a flux of charged particles which, by definition, is current. From Ohm's law $V = IR$, the resistance quantifies membrane's property as a barrier to ionic flux. In addition, the membrane forms a capacitor due to the insulating property of the lipid bilayer and the potential difference across the membrane. The resistance and the capacitance are in parallel configuration, resulting in an equivalent RC circuit.

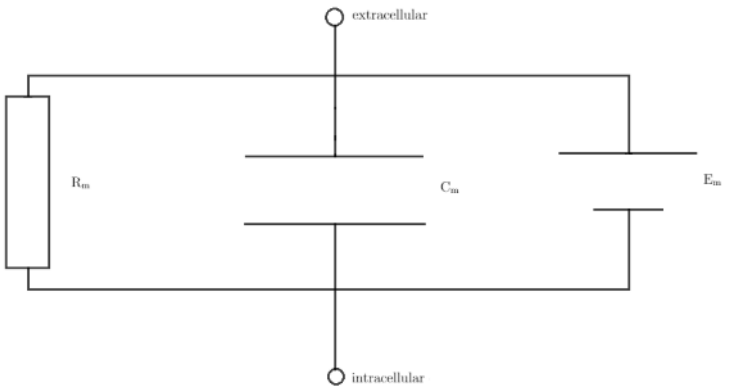


Figure 2.2: An electronic model of the cellular membrane. Membrane resistance R_m, capacitance C_m and membrane potential E_m are depicted according to their electronic symbols

Therefore, it is evident that cellular function and properties can be approached from an electrical point of view, in terms of ionic currents, which establish cellular membrane potential while expressing the physiological activity of the cell itself.

2.3 Electrophysiology

The physiological basis of cell excitability is the voltage dependent and ion-selective permeability of the cell membrane. Due to this fact, electrophysiology, which is a branch of physiology, studies the electrical activity and properties of living cells and tissues and investigates the molecular and cellular processes that take place in that level.

During most bioelectrical events, e.g. neuronal signals, the membrane potential (i.e. voltage) and the ionic flux through the membrane (i.e. current) are both changing simultaneously. In order to directly monitor the influence of membrane potential on the ionic flow across the membrane, voltage clamp technique is utilized. The principle of the voltage clamp is to use an electronic feedback circuit in order to maintain the membrane potential at a predetermined value, even in the face of permeability changes that would normally change the membrane potential. Whenever the membrane potential deviates from the predetermined value, the feedback circuit generates current and passes it into the cell in order to eliminate the deviation, thus recording a signal opposite to the ionic current. The most contemporary version of voltage clamp is the patch clamp technique, which permits independent manipulation of the elementary ionic currents and the membrane potential, thus allowing the detailed characterization of permeability changes as a function of membrane potential and time.

2.3.1 Patch Clamping

In order to achieve patch clamping measurements, a membrane patch containing several, or even a single, ion channels must be isolated electrically. In principle, thin glass micropipettes filled with electrolyte solution and sunction are utilized in this step, obtaining a tight seal and effectively isolating the patch. The ionic currents flow through the patch into the pipette and are recorded by a chlorided silver electrode connected to a highly sensitive differential amplifier. Another chlorided electrode is submerged in the bath and acts as the reference (ground) of the amplifier. The differential amplifier acts as a voltage clamp circuit, generating a compensating current that resembles the current that is flowing through the membrane to prevent alterations in the membrane potential.

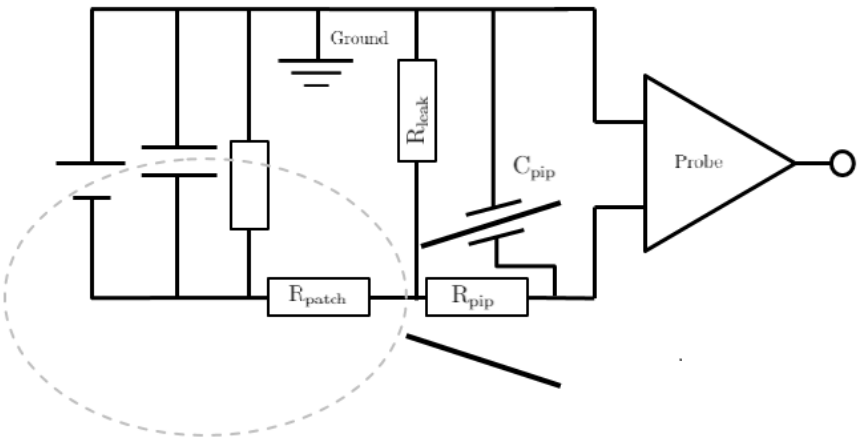


Figure 2.3: Equivalent circuit for the cell-attached patch configuration

The configuration described above is the cell-attached and constitutes the basis of all patch clamping configurations and variations, since it is non invasive and leaves the cell intact with respect to its physiological function. Nevertheless, there are some requirements for obtaining high quality measurements in the cell-attached configuration. Primarily, the resistance R_{patch} should be high compared with the resistance of the cell and the pipette resistance R_{pip}, in order to effectively monitor current flow through the patch. Furthermore, the quality of the seal to the patch, which is quantified by the resistance R_{leak}, must be in the GigaOhm range so that there is no current leakage or noise artifacts which can compromise the measurement. Lastly, the pipette capacitance C_{pip} must be well compensated due to the magnification and the high sampling rate of the patch recording.

2.4 The Rod cell

Photoreceptors are a specialized type of sensory neurons responsible for the conversion of light into biochemical signals. The vast majority of vertebrates and invertebrates get visual information using these cells. The two most common photoreceptors are the rods and the cones, whose physiology is conserved between animal species. Rods are responsible for vision in scotopic conditions (dim light), whereas cones mediate photopic vision (bright light). Therefore, it is obvious that their operation must differ in order to function in different conditions. Our case study, amphibian rod cells, have been widely used in the past for electrophysiological studies because of their large size and robustness, in contrast with photoreceptors of warm-blooded animals. [6]

2.4.1 Morphology of the rod

Rods have a very distinct structure, consisting of an elongated outer segment, an inner segment, a cell body and a synaptic terminal. The outer segment is an expanded and modified cilium, in which organelles called disks are tightly packed. Each disk, oriented perpendicular to the longitudinal axis of the outer segment, is a closed surface made of bilayer membrane, onto which various molecules and proteins are attached. A transmembrane protein (rhodopsin) densely embedded into the disc's bounding membrane, is the visual pigment of the cell. In every outer segment there are approximately 10^8-10^9 rhodopsin molecules. The inner segment contains many mitochondria responsible for generating ATP, the organic compound that provides the cell with the necessary energy to operate. The cell body primarily contains the nucleus and the synaptic terminal following the cellular body encodes and transmits signals from the rod to neurons (bipolar cells).

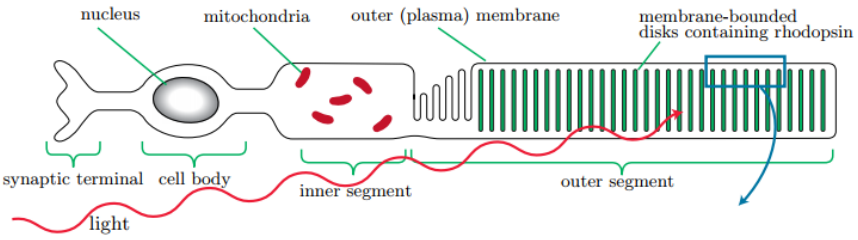


Figure 2.4: Schematic representation of a rod cell. Taken from [7].

The morphology of rod is clearly designed for increasing the probability of absorption to incident light on the longitudinal axis of the cell. Rhodopsin, the visual pigment of the rod, ultimately absorbs photons. Therefore, it is evident that the high sensitivity of the rod is defined by the spectral sensitivity of rhodopsin (max absorption at approx. 500 nm). Thus, the rod cell is able to detect and generate signal from a single "green" photon with internal quantum efficiency of 0.50.

2.4.2 Function of the rod

Being able to detect even a single photon and convert its energy to an electrochemical signal, rods should be able to amplify the acquired signal. This amplification process, from absorbing a photon to a measurable signal, is called phototransduction and is the basic function of the rod cell.

Under scotopic conditions (darkness) rods maintain an electric potential drop across their membrane utilizing specific pumps and channels. As seen in figure 2.5, sodium ions are pumped out of the cell's inner segment and potassium ions exit the cell due to their concentration gradient which overcomes the electric force exerted on them. However, in the outer segment sodium channels permit the influx of sodium ions in order to counteract the operation of the exchangers (pumps) in the inner segment. This results in a steady state, where there is a net influx of Na^+ in the outer segment, the "dark current" of 20 pA, and the resting membrane potential is about -40 mV. In the event of a photon absorption the phototransduction cascade is activated, ultimately leading to the hyperpolarization of the membrane and the modulation of the neurotransmitter release at the synaptic terminal.

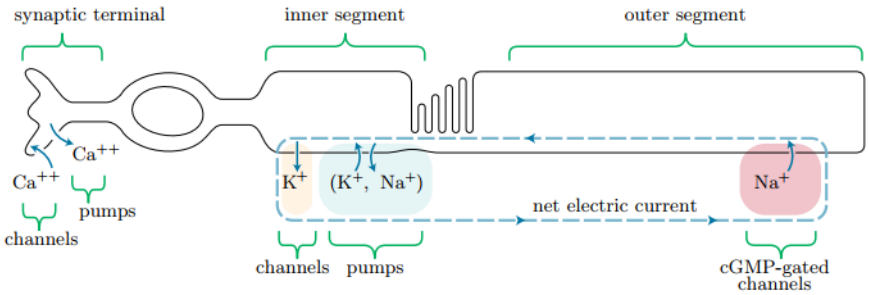


Figure 2.5: Electrophysiological function of the rod. Taken from [7].

Phototransduction is a multi-step proccess that utilizes a second messenger cascade to amplify the signal. When rhodopsin absorbs a photon, the chromophore 11-cis-retinal photoisomerizes to all-trans-retinal and activates the rhodopsin transforming it to metarhodopsin II (Rh*). In its activated form, Rh*, rhodopsin stimulates the G-protein transducin and catalyzes the exchange of GDP and GTP. In turn, activated transducin activates the protein phosphodiesterase and raises its catalytic rate. Phosphodiesterase binds cGMP and converts it to GMP leading to a falling concentration of cGMP. This reduction of cGMP concentration encodes the information of photon absorption and finally leads to the closure of cGMP-gated Na^+ channels located in the outer segment. Higher photon numbers activate more rhodopsins leading to more channels closing (i.e higher photocurrent), the response however is non-linear and reaches saturation at about 20000 photons absorbed per light pulse (for toad rods).

Chapter 3

Experimental Methods

In order to obtain electrophysiological measurements from rod cells, a variation of patch clamping, the sunction technique is the way to go. This recording method consists of drawing the outer segment of an isolated photoreceptor into a tight-fitting glass pipette filled with physiological extracellular solution and recording the membrane current. This method allows continuous recordings for as long as 90 minutes since it is relatively stable and noninvasive.

3.1 Ringer solution

The toad Ringer solution consists of: 111 mM NaCl, 2.5 mM KCl, 1.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM D-glucose, 3 mM HEPES. The solution is then buffered to pH 7.8 with NaOH and enriched with 5 % v/v L-15 Leibovitz. The Ringer solution maintains the rods functional and enables recordings for up to 90 minutes, at room temperature. During the experiment, the sample is continuously perfused with fresh Ringer at a flow rate of 1 mL/min. [8]

3.2 Electrode fabrication

Prior to each experiment, recording electrodes (micropipettes) must be fabricated. Using a programmable micropipette puller, borosilicate glass capillaries are heated and pulled until separation, thus creating two identical micropipettes. Configuring several parameters such as the laser output power, scanning rate and pull strength is essential in order to obtain the desired shape and tip of the micropipette.

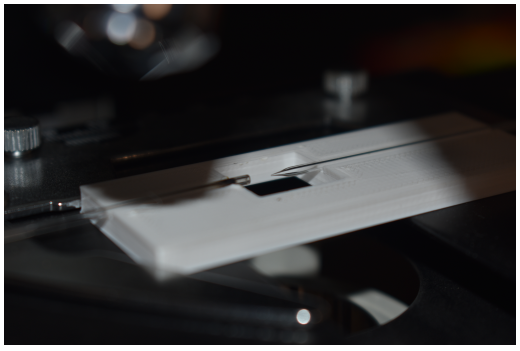


Figure 3.1: Left: Puller for micropipette fabrication. Right: Microforge

After pulling, the micropipettes are transferred to a custom-made microforge for fire-polishing the tip. The microforge was constructed using a compound microscope to visualize the pipette tip, a 3D printed base, acting as a guide for the micropipette and the capillary through which butane flame is brought close to the tip of the pipette. The butane flow is regulated so that the flame is barely seen with naked eye and can be brought close to the tip, using the translation stage of the microscope, without melting and closing the micropipette. Fire-polishing ensures that a tight seal will be possible when the outer segment is drawn into the pipette, without damaging the cell, smoothening the tip and reducing it to the desired inner diameter (approx. 8 μm).

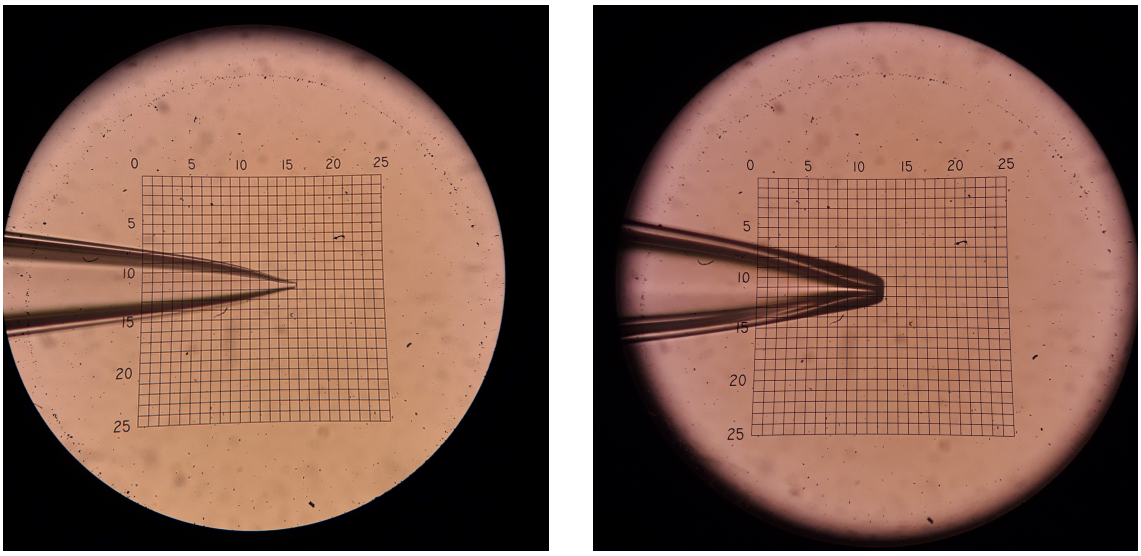


Figure 3.2: The difference between unpolished (left) and polished (right) pipette.

Two chlorided silver (Ag/AgCl) wires, obtained with chemical chloriding, act as the recording and reference electrode. The recording electrode is mounted on the probe (holder) and into the Ringer filled micropipette. The reference electrode is immersed into the bath chamber containing the rods and effectively grounds the solution.

3.3 Electrophysiology Rig

The main experimental setup is the electrophysiology rig. The core of the rig is an inverted optical microscope placed on a vibration isolation table. The microscope is equipped with an IR-camera for visualization of the sample and IR-filter, so that the intergrated halogen lamp does not stimulate the cells. The recording chamber (bath) was custom built from acrylic glass, featuring the bath and two additional chambers, which are used as input and output reservoirs to the perfusion system. The perfusion system includes a peristaltic pump and 2 I-V infusion lines connected to the recording chamber, ensuring the replenishing of the Ringer (1 mL/min) in it and fixing it at a constant level avoiding overflow. The recording chamber is mounted on a holder and a translation stage enabling the visualization of the entire bath chamber, when searching for a cell. The probe mentioned in the previous section, is connected to the pre-amplifier via BNC and this setup, the recording headstage,

is mounted on motorized piezo micromanipulator so that the suction of the cell is possible. Another manipulator is utilized in order to target the light stimulus from an optical fiber, directly onto the outer segment of the rod when recording. The whole rig is electrically isolated from the environment with a Faraday cage in order to minimize noise. Lastly, the setup is also in a light tight enclosure and the only visualization of the setup is through an IR-camera and LEDs mounted on it, used to monitor the setup during the experiment.

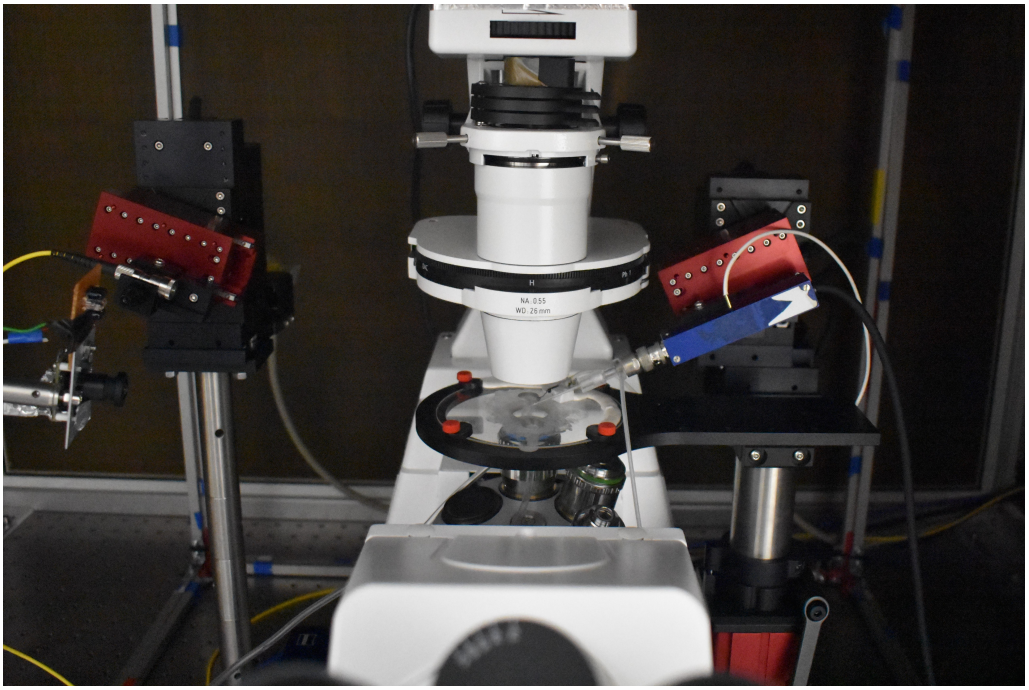


Figure 3.3: The electrophysiological setup as described in section 3.3 .

3.4 Optical setup

The collimated beam of a continuous (CW) diode-pumped solid state laser, wavelength of 532 nm and power 0.9 mW, passes through an acousto-optic modulator (AOM) in order to obtain the first diffracted beam. The beam is attenuated by two polarizers reflected by a mirror of a piezo controlled mount and is coupled to a multi-mode optical fiber which delivers the light stimulus to the cell. The light pulse duration as well as the intensity are computer controlled. The duration of the light stimulus can be manipulated by changing the duration of the TTL pulse, sent to the RF generator, which triggers the AOM. The intensity of the stimulus is controlled by applying voltage to the piezo controlled mirror, resulting in a less optimized coupling and less power delivered to the cell.

3.5 Cell preparation

All manipulations with animals are in accordance with the protocol approved by the Research Ethics Committee. Adult male toads (Bufotes Viridis) are dark-adapted overnight and are euthanized by decapitation under dim red light. The eyeballs are

enucleated using scissors and transferred to a petri dish containing ringer solution. The eyes are then transferred under a stereoscope with infrared illumination, where the retina is to be isolated. In order to do so, the eyeballs are hemisected and the cornea, lens and the vitreous are removed. Afterwards, the retina is essentially peeled from the pigment epithelium layer and is cut into pieces to release individual rod cells. Except for initial decapitation and enucleation, the procedure and the experiment is carried out without visible light. [9]

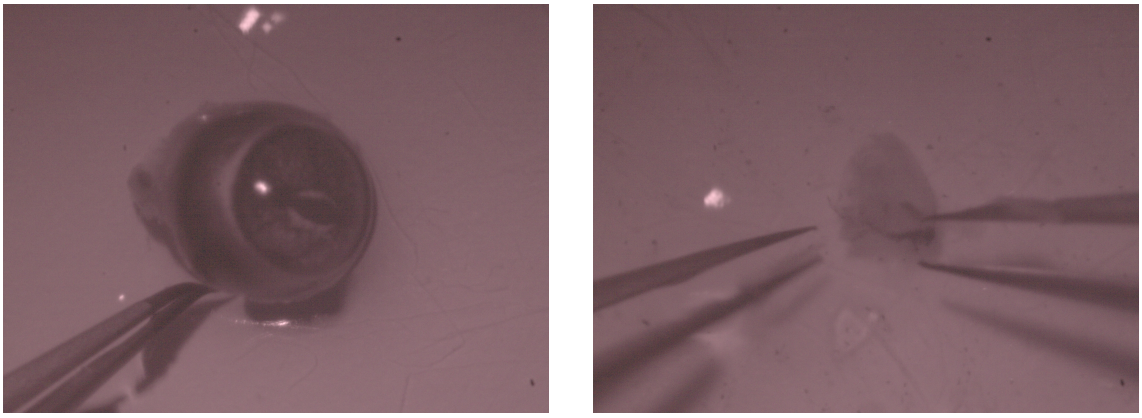


Figure 3.4: Left: Enucleated eye. Right: Isolated retina.

3.6 Recording configuration

Following the successful isolation of the retina, the pieces are transferred to the the rig and into the chamber with a Pasteur pipette. The perfusion of fresh Ringer is paused until the slices and cells settle down to the bottom of the chamber, usually in about 4 minutes. Meanwhile, the resistance of the micropipette is checked (about 800kOhm) to confirm that a optimal seal is possible, and noise tests take place to ensure that the chlorination of the electrodes is sufficient. Afterwards, the chamber is scanned until finding an isolated rod with intact outer and inner segment, visualized through the microscope IR-camera, with appropriate size (about 70x8 μm).

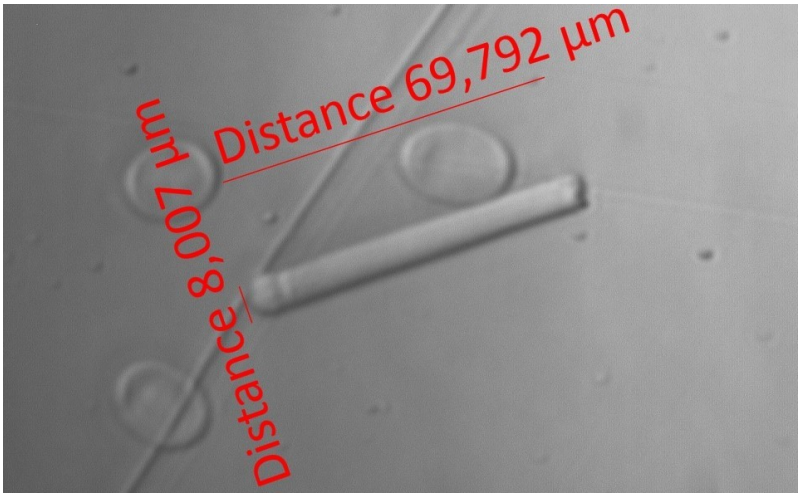


Figure 3.5: A typical example of an intact and functional rod cell.

With the assistance of suction through the micropipette, the outer segment of the rod is drawn into the pipette and when stabilized the seal is checked. If the resistance is greater than 5 Mohm (about 10x the pipette resistance), the seal is good and the capacitance artifacts are compensated with appropriate settings within the amplifier's programm. This is the configuration needed to proceed to the next step, which is the recording of the photocurrent of the rod. [10]

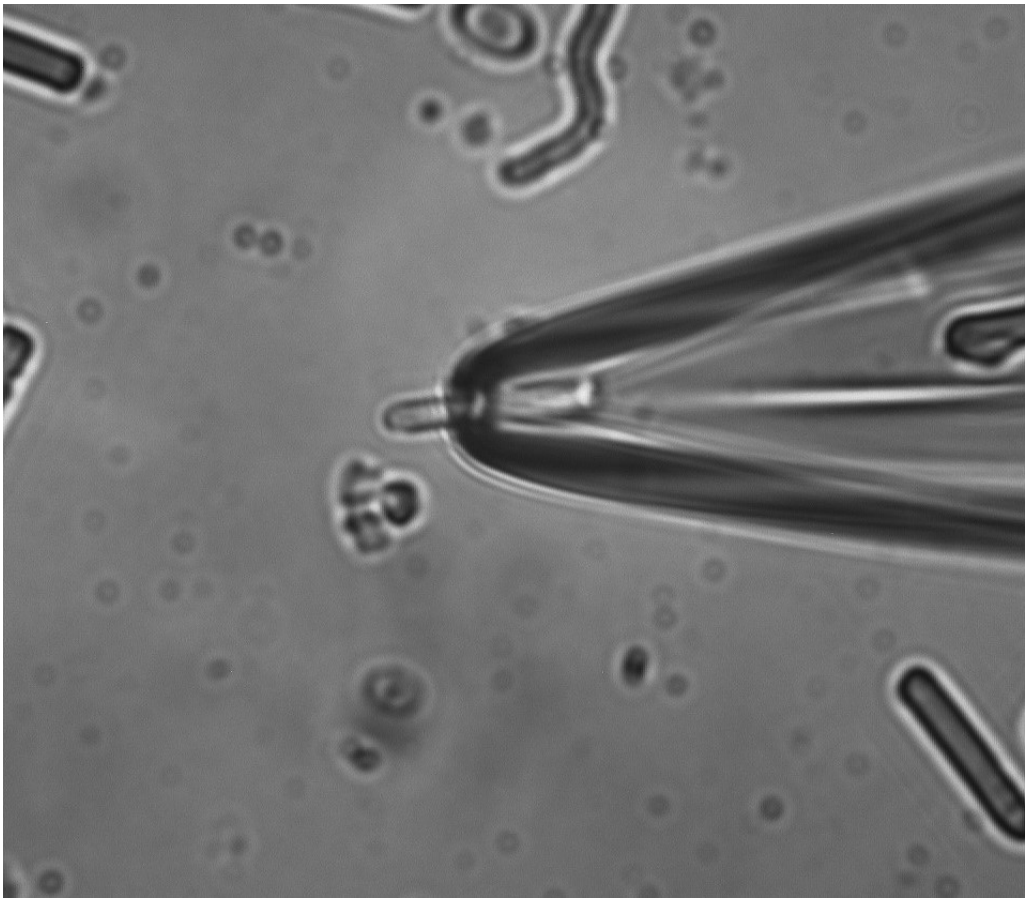


Figure 3.6: An intact rod cell with the outer segment drawn into the pipette prior to recordings.

Chapter 4

Results

All electrical measurements are recorded through the headstage, which is a pre-amplifier, and are then transmitted to the patch-clamp amplifier (HEKA EPC-10). The amplifier first filters the raw analog data through an analog 6-pole Bessel filter with cut-off frequency of 10kHz, substantially reducing high-frequency noise. Afterwards, the signal is digitized and displayed in the integrated digital oscilloscope within the amplifier's software. Through the software (Patchmaster) the entire amplifier's hardware is configured and controlled, according to the requirements of the recording. In addition, the protocol of the desired recording can be encoded and saved in the software, thus enabling consistent recordings with the exact same characteristics.

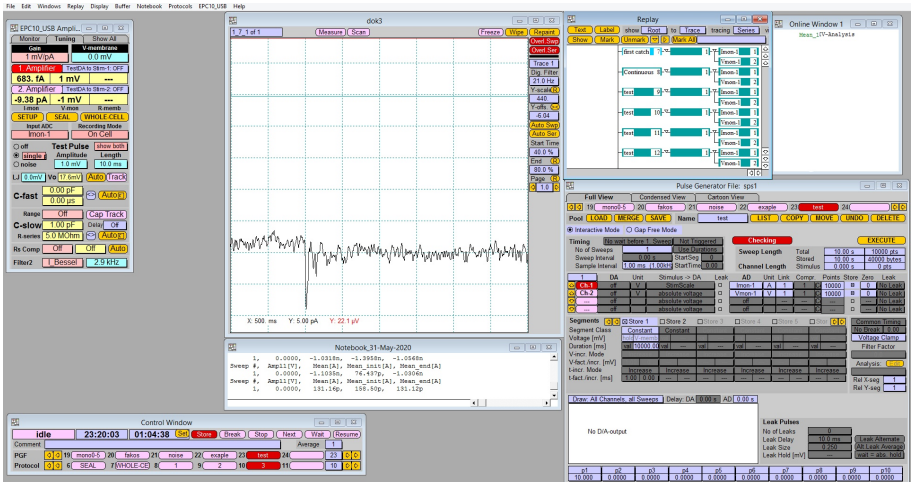


Figure 4.1: The interface of Patchmaster used for monitoring and controlling different parameters of the recording.

The same protocol was used to obtain the recordings presented later on in this chapter. The sampling rate of the recording is 1 kHz and the data are also filtered by a second analog Bessel filter (-3dB at 3kHz). The amplifier is connected to the RF-genarator of the AOM, so that during a recording the control of the light stimulus can be maintained through the computer, by choosing the duration of the ttl-pulse that triggers the RF-generator, producing a light pulse delivered on the cell.

4.1 Noise

The maximum photocurrent response of toad rods is about 20 pA. In order to observe such currents, the background noise of the experimental setup must be considerably smaller. The optimization of the electrophysiology rig in order to reduce the "intristic" noise is imperative and leads to high precision measurements. The background noise of the setup has been measured with an open pipette in the bath (800 kOhm). A signal to noise ratio of about 40 that is achieved is sufficient for observing the photoresponse of rods.

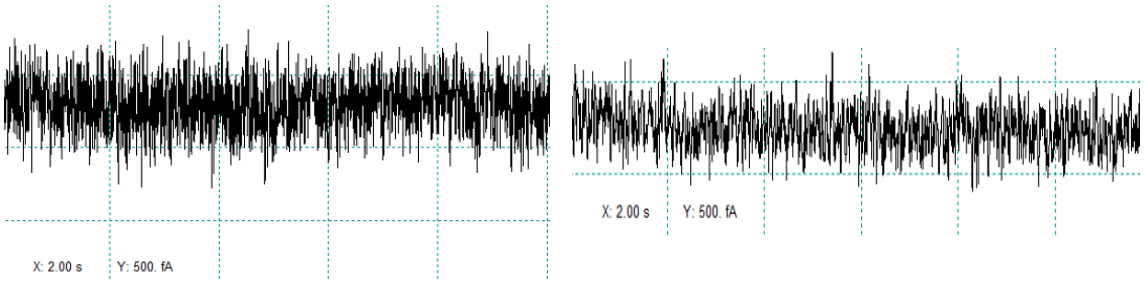


Figure 4.2: Recordings of the background noise.

4.2 Recordings of photoresponse

The photoresponse of rods to bright light flashes of 20 milisecond and of saturating intensity, delivering to the cell about half a milion photons, is recorded in order to check the reproducibility of the results. The photoresponses are about 12 pA with slight variations, due to the fact that the photoresponse is polarization dependent in the transverse configuration (incident light transverse to the rod axis) and due to bleaching. In figure 4.3, the signal is depicted according to the convention of the amplifier, whereas in 4.4 the recordings are inverted so that they better reflect the closure of the Na^+ channels.

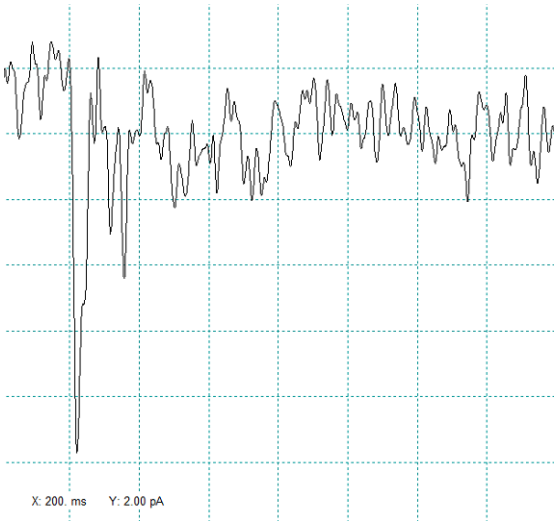
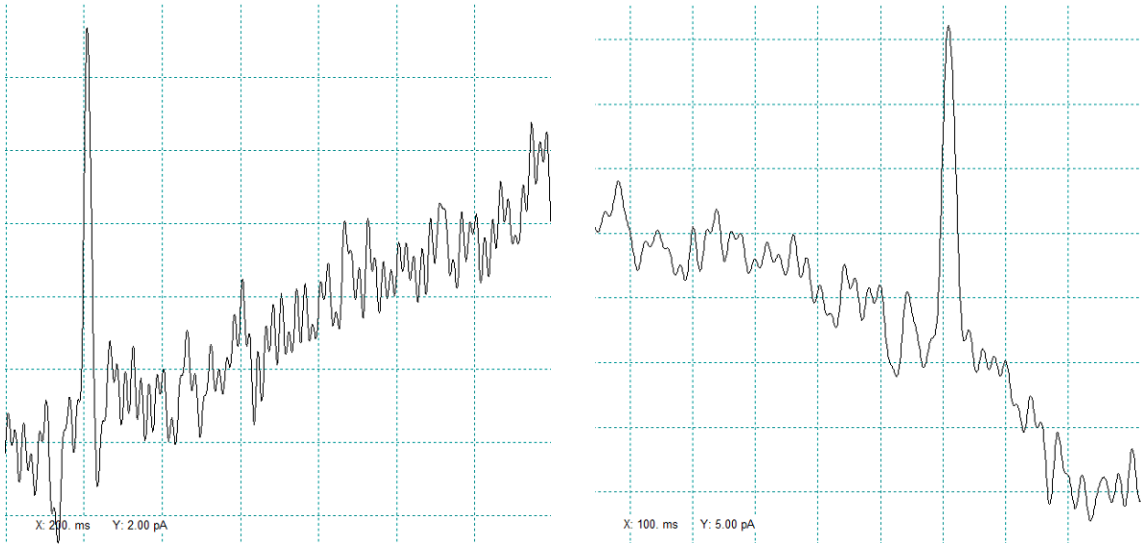


Figure 4.3: Photoresponse of the rod.

In the following figure, the linear drifts are caused by the degraded chlorided electrodes. In addition, the oscillations in the recordings are inevitable, since they are introduced by the peristaltic pump used to refresh the Ringer solution. Nevertheless, reproduction of the photoresponses is possible as expected.



(a) (b)

Figure 4.4: Photoresponses of different amplitudes. In (a) same as in figure 4.3. In (b) maximum photoresponse approx. 20pA is recorded.

4.3 Future perspectives

In summary, we have achieved to consistently record photoresponses from rod cells. In the future, we hope to further improve the experimental setup, to obtain photoresponses for different photon numbers and ultimately to investigate other biophysical processes that take place in the rods.

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