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Hypo- and Hyperthermal Effects on Human Lung Cancer Cell Lines

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Hypo- und Hyperthermische Effekte auf menschliche Lungenkrebszelllinien

Zusammenfassung
Diese Masterarbeit beschäftigt sich mit dem Verhalten der Ionenströme einer Zelle bei unterschiedlichen Temperaturen. Bei den in den Versuchen verwendeten Zellen handelt es sich um Lungenkrebszellen der Zelllinien A549 und H1299. Mithilfe einer automatisierten Patch-Clamp-Technik konnten einzelne Zellen extrahiert und die Ionenströme durch die Zellmembran bei unterschiedlichen Temperaturen gemessen werden.
Die geplotteten Stromspannungskurven wurden auf zwei Arten miteinander verglichen: die erste Analyse beschränkt sich auf den Vergleich gleicher Zelllinien bei unterschiedlichen Temperaturen, während die verschiedenen Zelllinien in der zweiten Analyse bei gleicher Temperatur miteinander verglichen wurden.
Als Fazit ergab sich, dass nicht nur Hyperthermie sondern auch Hypothermie einen Einfluss auf die Ionenströme der Krebszellen hat. Um genauere Aussagen über die Auswirkungen der Temperaturen auf den Ionenstrom der Zellen treffen zu können, besteht jedoch weiterer Forschungsbedarf.

Schlüsselwörter
Hyperthermie, Hypothermie, Lungenkrebs, A549, H1299, Patch-Clamp-Technik

Hypo- and Hyperthermal Effects on Human Lung Cancer Cell Lines

Abstract
This master thesis deals with the ion current characteristics of human lung cancer cell lines, especially A549 and H1299 cells which are investigated at different temperatures. With the help of an automated patch clamp technique, single cells were extracted and the ion currents through the cell membrane were measured at different temperatures.
The plotted current voltage curves were compared in two different ways: the first analysis is a comparison of similar cell lines at different temperatures, while the second compares different cell lines at similar temperatures. The conclusion of these investigations was that it is not only hyperthermia that can influence the ion current of cancer cells, but also hypothermia has an effect on the current characteristics. In order to be able to make a more precise statement, further research need to be carried out to obtain more knowledge on the influences of temperature on cancer cells.

Keywords
Hyperthermia, hypothermia, lung cancer, A549, H1299, patch clamp technique

Contents

1. Introduction

Lung cancer is still one of the most widespread forms of tumour worldwide and medical research is constantly trying to find new therapy methods to effectively fight and cure this disease and reduce cancer diagnoses. However, various risk factors such as smoking are still amongst the most important causes of origination of lung cancer. [1, 2]

Standard therapy methods for this type of cancer include, for example, the surgical removal of tumours, or radiation and chemotherapy [3]. However, none of these methods has yet seemed to completely satisfy medical needs without side effects. Thus, new curing methods are constantly under development. Hyperthermia is one of these additional methods that works with heating the human body or parts of it to certain temperatures in order to foster the apoptosis of cancer cells. There exist numerous different methods of hyperthermia such as whole body hyperthermia (which is mainly used if there already exist metastases in the patient's body) or local hyperthermia (which is applied for solid tumours). [4, 5]

At present, hyperthermia is merely being applied in combination with the standard methods listed above because its sole application has not yet proven successful. However, combining standard therapy methods with hyperthermia has so far demonstrated good results with regard to curing cancer. [6, 7]

In contrast to hyperthermia, therapeutical hypothermia is another method used in medicine. This method, however, cool the human body to a certain temperature. So far, this therapy is not being used in oncology but it has shown good results with regard to brain injuries and/or global brain ischemia as it provides good neuroprotection. [8, 9]

The overall aim of this master thesis is to investigate the effects of temperatures on A549 and H1299 human lung cancer cell lines. Therefore, measurements are carried out with an automated patch clamp technique from Nanion as well as a temperature control to examine the effects of high and low temperatures on different cell lines.

The patch clamp technique is a physiological method developed in the 1970 in order to be able to investigate the behaviour of the current running through ion channels in a cell's plasma membrane. In general, ion channels are an essential part in physiology and pathophysiology and play an important role with regard to drug targets. [10, 11]

In order to measure the ionic current flow through the membrane, the patch clamp technique uses micropipettes made of glass to aspirate a membrane patch which forms an electrical seal, rupture the patch and thereby provide access to the interior of the cell. This method is highly important in studying ion channel behaviour even though it is a very elaborate technology that requires precise micromanipulation under high power visual magnification, vibration damping as well as an experimenter with high expertise and skills. [10, 11]

In fact, constantly examining new methods for the therapy of cancer is crucial in order to be able to reduce mortality due to cancer. It has already been confirmed by research that cancer cells are more heat-sensitive than healthy cells which leads to the assumption that hyperthermia can induce cell damage – especially with regard to cancer cells. This makes hyperthermia a valuable weapon in the combat of cancer as it can successfully be combined with traditional curing methods. [4, 5]
In this connection, it seems only reasonable to also investigate other methods such as hypothermia because low temperatures might also influence cancer cells and thus bring advantages to the cure of cancer. [12]

2. Assignment

This master thesis is subdivided into two parts, one theoretical and one practical.

Theoretical part

Conforming to the title *Hypo- and hyperthermal effects on human lung cancer cell lines*, the first part mainly consists of a theoretical introduction to the fields of lung cancer and hyperthermia.

Therefore, the literature research of lung cancer focusses on the following points:

- the lung's basic form and function;
- the carcinogenesis, i.e. the process of how healthy cells transform into tumour cells;
- the different risk factors that may increase the chance to be diagnosed with lung cancer;
- the various types of lung cancer as this information is important for being able to discuss its therapy, and
- the states and therapy forms of lung carcinoma.

The second aspect that is discussed in the first part of the thesis is hyperthermia. Here, the focus is put on the issues listed below:

- What does hyperthermia mean with regard to oncology?
- What are the benefits of this therapy method?
- What happens at a biochemical level if high temperature are applied to the human body?
- What are this therapy's technical methods and physical technologies?

In contrast to hyperthermia, only a small part of the literature research focusses on hypothermia because, as mentioned above, this low temperature therapy is hardly used in oncology these days. Thus, the corresponding chapter largely focusses on the history of hypothermia as well as cellular changes occurring at low temperatures.

In addition, one chapter of the theoretical part treats the different cell lines A549 and H1299, thereby also mentioning the basics of cell membranes and the structure of the ion channel.

A detailed chapter is dedicated to the patch clamp technique because this is in fact the method used in the experiments conducted for the practical part. The two basic methods, the conventional and planar patch clamp technique, are described, whereby the second method is described in more detail as it was used in the measurements for this thesis.

Practical part

The practical part subsequently outlines and discusses the measurements with the patch clamp technique that were executed in the framework of the present thesis.
The aim of voltage-gated patch clamp measurements is to record the ion current that runs through the cell's ion channels at a defined voltage. The measurements were carried out with different cell lines (A549 and H1299, as stated above) and at various temperatures. The resulting measurement data was then collected, interpreted and filtered with the help of the software MATLAB to be able to create current profiles. Moreover, the data was used to create current voltage curves that were then plotted with MATLAB.

Two main aspects were especially important for the analysis:

- the curves with the same cell lines at different temperatures, and
- the curves with different cell lines at the same temperature.

3. Lung cancer

Tumour is a general term that defines an abnormal tissue mass that originates from a progressive increase of the body's own, mutated cells. Thereby, the regularization disturbance of genes is the main cause of this process, which is mainly influenced by progression (proliferation), cell loss (apoptosis) and differentiation of cells. Further characteristics of tumour cells are invasion, spread out and the formation of metastases in other parts of the body. [2, 1]

In general, diseases of the breathing organs are the most widespread, amongst them asthma, chronic obstructive pulmonary disease (COPD), pneumonia, pulmonary cancer and many more. For the malignant lung tumour, medicine uses the term bronchial carcinoma – in fact, more than 90 % of this form of lung cancer are the consequence of smoking tobacco. The increase of chronic lung tumour can be reduced by preventive measures, primarily with regard to the consumption of tobacco. [2, 13]
According to Statistik Austria, Austria records approximately 4 000 incidences related to lung cancer each year. In the past years, lung cancer with regard to men decreased while that concerning women increased. [14, 1]
Figure 3.1 illustrates the incidences and mortality rate of lung cancer [14].

There are two different types of tumours: the harmless or benign tumour and the malignant tumour. It is important to investigate the tumour's morphology as this indicates the behaviour. Table 3.1 shows a comparison between benign and malignant tumours. [2, 1]

Table 3.1.: Composition of benign and malignant tumour [2]

Characteristic	**benign tumour**	**malignant tumour**
growth rate	slow growth	slow to fast growth
local expansion (macroscopy)	mostly connected, good limited tumour; expansive, replacing growth	mostly bad limited tumour; invasive, destroying growth
histology	high level of differentiation (tumour tissue usually corresponds to the original)	loss of differentiation (similarities to the original tissue are lost)
metastasis	no	potentially yes

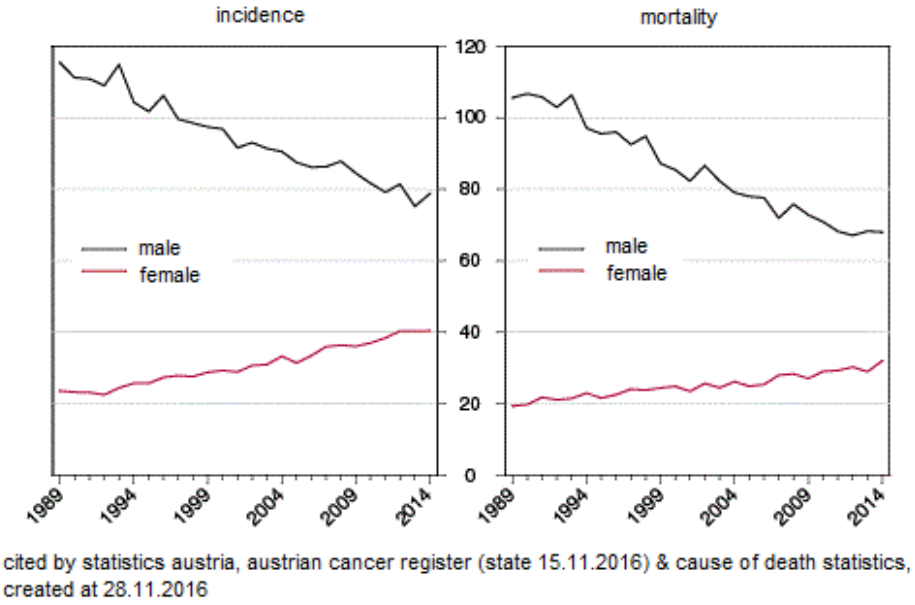

Figure 3.1.: Malignant neoplasm of the lung over a course of time (age-standardised rate of 100 000 people), the left square shows the incidences and the right the mortality of lung cancer [14]; red curve: women; black curve: men

3.1. Structure and function of the lung

The lung, as part of the respiratory system, is one of the most essential organs in the human body as it is responsible for the gas exchange, i.e. receiving oxygen from the air and releasing carbon dioxide, thereby providing the body with vital oxygen. [15, 16]

The lung has no characteristic shape but rather fills the space between the chest, diaphragm and mediastinum, with neighbouring organs engraving in the lung's surface. The lung is divided into a left and right side, whereby the right part is bigger due to the fact that the heart is located on the left. As indicated above, the lung's main function is the gas exchange, where oxygen from the air enters the blood and carbon dioxide leaves it. [15, 16]
The inhaled air passes the trachea in a tubular system to the actual exchange organ of the lung: the lung alveoli. At the bifurcation, the trachea branches into a right and left part into hollow tubes called bronchi. The latter are subdivided into increasingly smaller hollow tubes called bronchioles as far as the alveoli that form the lung's smallest part. It is at this place of the lung where the gas exchange with the blood finally takes place. [16, 2, 15, 17]
In figures 3.2 and 3.3 the structure of the lung and the bronchial tree are illustrated.

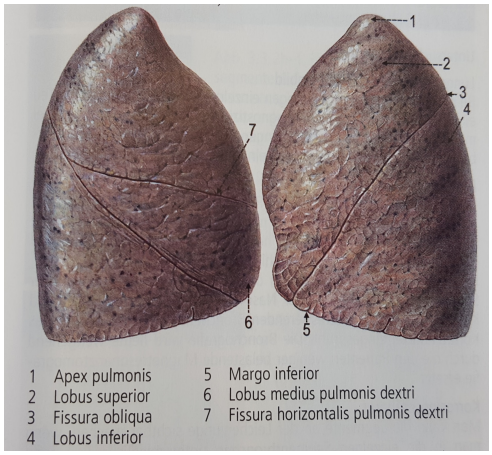

Figure 3.2.: Right and left lung, with individual segments [16]

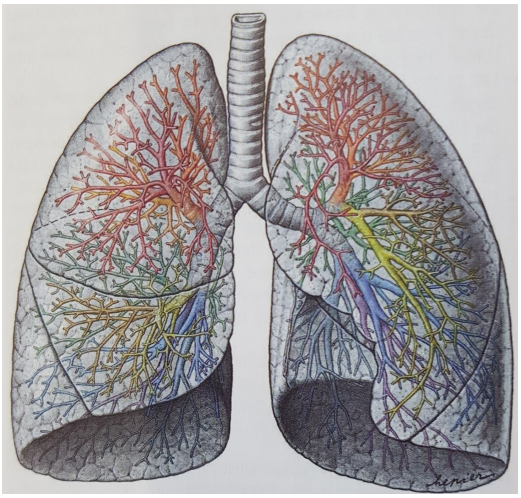

Figure 3.3.: Lungs with bronchial tree [16]
red: bronchi segmentales I - III;
yellow: bronchi segmentales IV + V;
green: bronchus segmentalis VI;
blue: bronchi segmentales VII - X.

3.2. Carcinogenesis

In general, cancer occurs due to an accumulation of somatic DNA-mutations, which lead to cell proliferation [18]. There can be several reasons why mutations arise in the first place, including random replication mistakes, an exposition to a carcinogen (e.g. asbestos, radiation, tobacco) or simply incorrect DNA repairing processes. [13, 1]
Almost all types of cancer develop from only one cell on which must be approximately 5 to 10 mutations in order to change it from a normal cell to a malignant one [13].

There are two main genes, the so called tumour suppressor gene and the oncogene, which have the most relevant antagonistic effect on tumour growth:

- Oncogenes are usually cellular genes whose expression products regulate cell proliferation, mobility and differentiation. At present, there are approximately 200 known oncogenes. In cancer cells, they mutate, leading to the malfunction of proliferation, mobility and differentiation. These genes are jointly responsible for tumour origin and progress. [2, 18]

- Tumour suppressor genes are usually also cellular genes whose gene products regulate processes such as cell growth. If the tumour suppressor gene mutates, the gene product cannot be generated which leads to a deregulated growth of tumour cells. [2, 18] At present, more than 70 tumour suppressor genes are being investigated [2].

There are several mechanisms for oncogene activation, e.g. point mutations, amplification, inversions, and chromosomal translocation. [2, 18]

Genetic mutations can occur due to substances, so called carcinogens, for example tobacco, radon etc. For more information, see sub-chapter risk factors 3.3. Carcinogens can affect the gene sequence of healthy cells and change them into a mutated tumour cell. Thereby, good properties of the formerly healthy cell are lost and make way for bad ones. Some cell mutations can also be traced back to the DNA, i.e. they are inherited from one's parents. [2, 18]

In the following, the main problems of tumour cells are summarised briefly:

- Invasion: This is an active part of tumour cells in healthy tissue which leads to the latter's destruction. [2, 1]
- Metastasis: This is the consequence of tumour cells growing in other healthy tissue. It is possible that these tumour cells infiltrate the bloodstream, the lymph channel or body cavities, thus ending up in other parts of the body and spreading there. [2, 1]
- Healthy cells have the function of apoptosis. This means that if a healthy cell mutates, it will commit suicide in order to protect other cells. In tumour cells, however, apoptosis does not function correctly due to a genetic mutation which damages it. [2]
- Healthy cells have an independent counter that counts the cell division so as to limit it. Each division shortens the end of the chromosome, the so called telomere: if it is too short, the cell division will stop. Tumour cells, however, lose this function and endlessly divide themselves. [2]

3.3. Risk factors

There are several factors that can increase the risk of lung cancer. The biggest factor is smoking: in fact, each cigarette increases the danger of being diagnosed with lung cancer. More than 90 % of malignant lung tumours can be ascribed to cigarette smoke as the latter contains approximately 7 000 chemical substances, several of them highly carcinogenic. However, cigarette smoking cannot only cause lung cancer, but it may also entail other forms of disease such as atherosclerosis, COPD or cancer in other parts of the body, including the oral cavity and the trachea. Moreover, there is also a risk of complications during pregnancy as smoking can have detrimental effects such as low birth weight of the baby or increasing the number of premature births or stillbirths. Stopping to smoke will reduce the risk but it will never be as low as with people who never smoked. [2, 13]

As has become clear over time, another reason for lung cancer that not only concerns active smokers is passive smoking. Thus, chronic passive smoking for years undoubtedly increases the risk of a bronchial carcinoma. [2, 13]

People can also be diagnosed with lung cancer because of their work, e.g. when constantly inhaling toxic substances or a lot of dust. Examples for carcinogens at the workplace are asbestos, arsenic, nickel or other aromatic hydrocarbons. [2, 13]

Another substance that fosters lung cancer is radon – a radioactive chemical element that is produced by the natural breakdown of uranium in soil, rock and water and that occurs in houses and buildings. [2, 13]

Lastly, one's personal or family genes can also cause lung cancer, for example when a family members has or has had lung cancer. [2, 13]

3.4. Types of lung cancer

Since 1999, the World Health Organization (WHO) has classified more than 50 different types of lung cancer, the following four histological forms being the most widespread ones [2, 1, 13]:

- non-small cell lung cancer
  - adenocarcinoma
  - squamous cell carcinoma
  - large cell carcinoma
- small cell lung cancer

3.4.1. Non-small cell lung cancer NSCLC

This kind of tumour is subdivided into three sub-categories:

Adenocarcinoma

This type of tumour is mostly diagnosed in non-smokers and women [13]: about 50 % occur because of the inhalation of carcinogenic substances. Adenocarcinoma is usually located in the periphery of the lungs, as illustrated in figure 3.4. It grows faster and metastasises earlier than the squamous cell carcinoma. Also, it differs from the others due to its histological form that has a glandular structure and produces mucin. [19]
Often, this type of lung cancer is only diagnosed in an advanced state because there are usually no symptoms in the first state [2].
The cell forms are highly atypical with a large vesicular nucleus and the cytoplasm is cylindrical, as shown in figure 3.5 [2].
Bronchoalveolar carcinoma also belongs to the group of adenocarcinoma and is usually located alongside the alveoli and the endobronchial system [19].

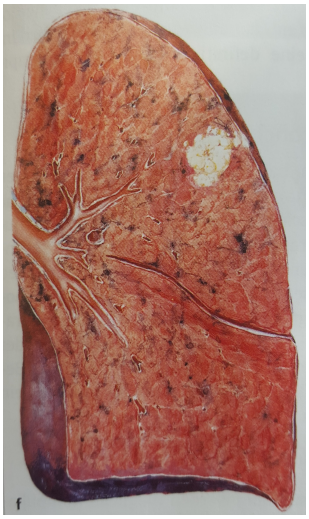

Figure 3.4.: Adenocarcinoma [19]

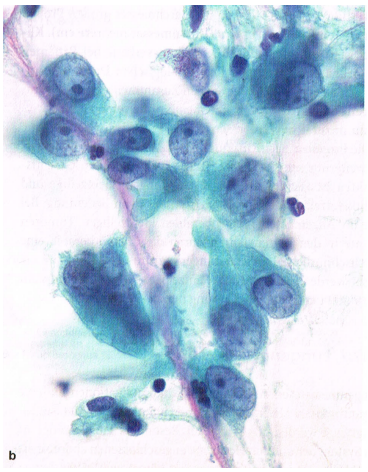

Figure 3.5.: Cells of adenocarcinoma [2]

Squamous cell carcinoma

About 40 % to 50 % of bronchial carcinoma are squamous cell carcinoma. This kind of tumour is more widespread in men than women. The biggest risk factor is, again, smoking [13], with about 85 % of this type of cancer being ascribed to the inhalation of tobacco smoke. The critical factor hereby is how long and how much a patient smokes. Other risk factors can be air pollutants or a genetic disposition. [19, 2]
In contrast to adenocarcinoma, there is a good chance of early prognosis and cure because of the rather slow growth and rare formation of metastases. The location of a squamous cell carcinoma is central, as demonstrated in figure 3.6. [19]
The cytology shows a tight association of non horny tumour cells with a polymorphic, dark coloured and rough structured nucleus, as illustrated in figure 3.7 [2].

Large cell carcinoma

About 10 % to 20 % of bronchial carcinoma are large cell carcinoma. Figure 3.8 illustrates a large cell carcinoma in the right upper lobe with involvement of lymph nodes. The cell structure and size are pleomorphic and their microscopic picture shows a big, dark coloured and striking nucleus, as displayed in figure 3.9. The red coloured structure in figure 3.9 indicates the cells' mucin production. [19]

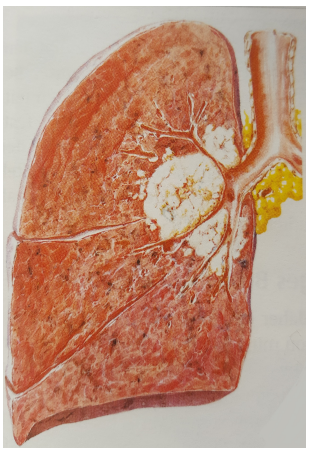

Figure 3.6.: Squamous cell carcinoma [19]

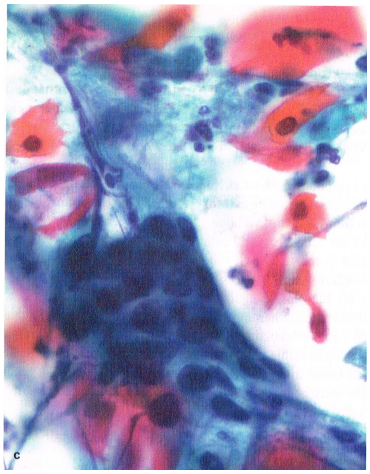

Figure 3.7.: Cells of squamous cell carcinoma [2]

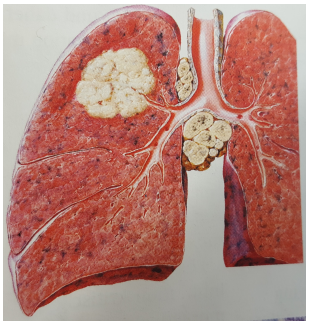

Figure 3.8.: Large cell carcinoma [19]

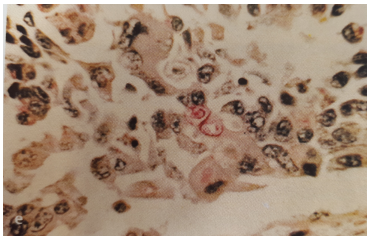

Figure 3.9.: Cells of large cell carcinoma [19]

3.4.2. Small cell lung cancer SCLC

About 20 % of lung cancer can be defined as small cell lung cancer. This type is highly aggressive and commonly grows in the lung's centre. Furthermore, with SCLC, the penetration into the tissue and metastasising hematogenes happen very quickly. The prognosis of curing this lung cancer is bad because often there are already metastases if the patient gets the diagnosis. [19, 1]
The cytology of SCLC indicates small, grouped cells with a dark coloured, rough structured and polymorphic densely populated nucleus, see figure 3.10. The tumour cells are located in very light cell bonds. [2]

Figure 3.11 represents three different types of lung cancer cells under the microscope.

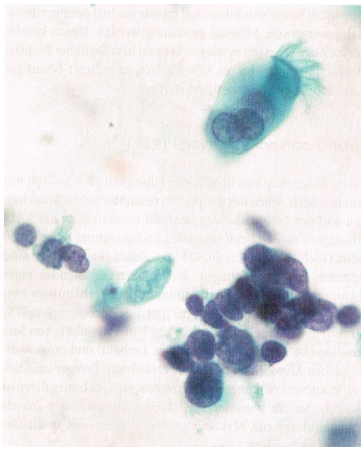

Figure 3.10.: Cells of small cell lung cancer; for the sake of comparison, a normal ciliated epithelium cell is shown above [2]

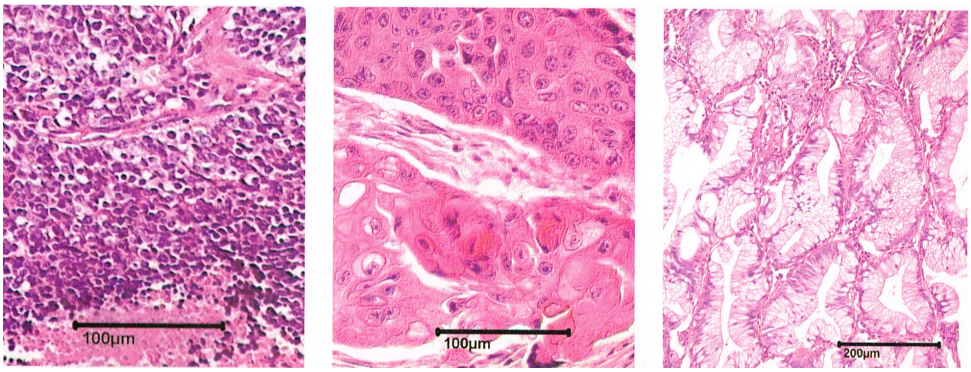

Figure 3.11.: Schema and histological examples for common differentiation patterns [2]
left: small cell lung cancer cells,
middle: squamous cell with beginning of cornification,
right: highly differentiated adenocarcinoma and atypical, cylindrical mucous generating tumour cells

3.5. States and therapy of lung cancer

The progression of the disease is usually what makes it so complicated to cure. In the relatively early states, lymph node metastases can occur, as can metastases in the liver, the skeletal system, the adrenal glands and the brain. There are several ways of classifying the tumour: one can distinguish the dignity (benign or malignant), the type, and its localisation (also of the primary tumour). [2]

The classification is very important for therapeutical and prognostic purposes because there are differences when it comes to the prediction of tumour. Also, malignant lymphomas are usually treated with chemo- and/or radiotherapy while carcinoma are normally treated with surgical therapy. [2, 13]

The most common practice for tumour classification is the so called TNM-system.
T here stands for the local spreading of the primary tumour, N for the regional lymph node metastasis and M for the hematogenous remote metastasis. [2, 1, 13]

There are different therapy methods:

- **Surgical therapy**
  The aim of surgical therapy is a complete removal of the tumour. To achieve this, it is necessary to remove the cancer including a safety distance to the tumour tissue. If there already are metastases, these must also be completely removed so as to reduce the risk of recurrence of the tumour. [3, 1]

- **Chemotherapy**
  The difference between mostly healthy and tumour cells is the latter's high cell division rate. Cytostatic drugs take advantage of this fact and inhibit the tumour-specific signalling pathways. Furthermore, they block the angiogenesis. However, cytostatics also affect healthy cells if they have a high cell division rate (as do blood producing or intestinal cells, for example). Therefore, common side effects with regard to the blood are an impairment of the patients' immune system. There are also other side effects such as nausea, diarrhoea, or loss of hair. However, the side effects of the growth regulation receptors and the angiogenesis largely vary and depend on the actual medication. To minimise them, a combination therapy of different effective cytostatic drugs is applied. [3]

- **Radiotherapy**
  The physical basis of radiotherapy is to transfer energetic beams to biological tissue by ionisation and excitation of atoms and molecules. Thereby, the energy damages the DNA as well as enzymes and parts of the membrane. Cells with a high division rate and metabolic activity are affected in particular. [3]
  There are two different types of application: teletherapy and brachytherapy [20].
  The first is the most common form: with a distance of approx. 100 cm between the tumour and the radiation source, the high energetic beams can be focussed exactly on the tumour region. By reason of this the radiation exposure of the neighbouring healthy tissue can be kept low. [3, 20]
  In contrast, in the brachytherapy, the radiation source is located in close proximity to or directly in the tumour region. Therefore, a very high local radiation can be applied with low damage to the surrounding tissue. [3, 20]

There are two forms of side effects, first, the acute complication which usually eases after 4 to 6 weeks, and, second, the chronic complication which only arises after months or years. Possible side effects are skin inflammations in the abdominal region, pulmonary fibrosis, or dysfunctions in the lymph system, e.g. lymphoedema. [3]

- **Multimodal therapy concept**
  This therapy form is a concept that uses more than one method: it is possible to apply an additional treatment before, during or after the surgical therapy.
  The aim of the so called adjuvant therapy is to eliminate cells which spread over the whole body before they can develop metastases. The second method is the neoadjuvant therapy which is applied before a surgical therapy. [3, 21]

4. Hyperthermia and Hypothermia

4.1. Hyperthermia

Hyperthermia is a method to produce artificial high body temperature in a patient's body or parts of it for medical purposes. [4, 5]
The word hyperthermia is composed of the Greek words "hyper" and "therme", which denote hyperpyrexia or excessive heat, whereby hyperpyrexia describes a fever that comes with very high body temperature. [4]

In oncology, hyperthermia is used to treat malignant diseases in combination with chemo- or radiation therapies. However, even the application of different hyperthermia models is sometimes not effective enough to cure the disease. Therefore, hyperthermia is applied in combination with other well-established therapy modes such as radio- or chemotherapy in order to improve the direct cell killing effect. [6, 5, 7]

In general, hyperthermia is used to heat the body, or parts of it, from a normal body temperature to temperatures between 40 $°C$ to 45 $°C$. Above 50 $°C$, the process is no longer called hyperthermia but thermal ablation. There are different methods to heat the body, including whole-body hyperthermia, regional deep hyperthermia or local hyperthermia, which are described in detail in chapter 4.1.4. In addition, it is important to note that healthy tissue should be kept at normal body temperature so as not to cause side effects such as burns. [22, 5]

4.1.1. Effect of hyperthermia at a biochemical level

The effects of hyperthermia at a biological level are complex and multifunctional as they largely depend on the temperature and application time, as well as on form, size and kind of tissue, the blood supply and the homogeneity of the temperature distribution. [5]

Cancer cells in the human body are more heat sensitive than healthy cells. With this knowledge in mind, hyperthermia can be used for medical treatment. In short, overheating can directly kill cancer cells and change the synthesis of RNA, DNA and proteins. Another positive effect of hyperthermia is that it leads to the impairment of the normal cell membrane function due to changing its permeability. Consequently, the overflow of proteins, also it affects the nuclear chromatin structures and leading to dissolution or even death of the tumour cells. The aim of killing such cells is to induce apoptosis. [4, 5, 7]

The tumour tissue's structure differs from healthy one. Its characteristics are, inter alia, structural disorder and disordered blood vessel patterns which originate from independent tumour growth and can form a thrombus. Other effects of the disordered structure are, for example, a high blood flow resistance or arterial and venous fistulas. These tumour properties can lead to low blood flow in the tumour tissue, a lack of oxygen and a low pH value. Whenever the tumour tissue is exposed to great heat, it is unable to supply the metabolism with essential raw materials, which results in both damage to the endothelium of blood vessels and weakness of the flexibility of erythrocytes. It can also strengthening of permeability, destruction of nuclear membranes, damage of cell organs, and in the end lead to cell death. [4, 5]

There exist numerous investigations of hyperthermic cell death in different phases of the cell cycle. These studies indicate the variations of heat and its sensitivity to cell cultures in different phases of the cell cycle. The mitotic phase has the highest heat sensitivity. If mitosis-phase cells (M-phase cells) are exposed to heat, this results in damage to their mitotic apparatus, in the end leading to inefficient mitosis and consecutive polyploidy. An elevated temperature on S-phase cells, which are also highly heat sensitive, results in chromosomal damage. In contrast to S- and M-phase cells, G1-phase cells are relatively heat resistant and do not show any microscopic damage. It can thus be concluded that hyperthermia causes a "slow mode of cell death" in S- and M-phase cells, while G1-phase cells rather follow a "rapid mode of death". This opens up a range of molecular mechanisms of cell death in the different cell cycle phases that can be induced by hyperthermia. [6, 5]

Another positive advantage of hyperthermia is the increased blood circulation of the tumour which comes with an improved oxygen supply. Here, radiation can damage the tumour's DNA-structure and the effect of radiation therapy can thus be enhanced. [6]

In hyperthermia, cells exhibit thermal tolerance if they are exposed to moderate temperatures. This phenomenon is partially based on the induction of heat-shock proteins (HSP) and other post-transitional adaptation processes such as cell cycle arrest in the G2-phase or changes in cell metabolism. HSPs are part of a heterogeneous group of molecular chaperones which are subdivided into five groups with different molecular masses and partially varying biologic functions. "In hyperthermia, HSPs are thought to be involved in the protection of cells against heat damage." The moderate heat application of tissue increases intracellular HSP-synthesis. Upon passing a certain temperature threshold, there occurs an inhibition of HSP-synthesis. The threshold temperature varies between different cell types in given experimental systems. "A lack of HSP-synthesis is associated with exponential cell death, it is generally accepted that HSPs prevent cells from lethal thermal damage". [6, 5]

4.1.2. Hyperthermia combined with conventional therapy methods

Hyperthermia and surgical therapy

In cancer therapy, a surgical operation is a very important method to remove the malignant tumour; however, only about 30 % of the patients are suitable and healthy enough to survive an operation. Nevertheless, hyperthermia in combination with a surgical treatment reaches positive results with only half the effort when compared to the application of surgery alone. [4]

There are different possibilities to combine a surgical therapy and hyperthermia [4]:

- Preoperative hyperthermia: This method is used before a surgical therapy. It can be combined either with chemotherapy, radiotherapy or hyperthermic perfusion, improves the therapeutic effects and reduces side effects.
- Intraoperative hyperthermia: This focuses on the regional thermochemotherapy and it is mostly used for advanced malignant tumours in abdominal and pelvic cavities, or for niduses, which cannot be removed by surgery. The aim is to reduce the risk of post-surgical recurrence.
- Postoperative hyperthermia: There are different postoperative methods such as thermochemo- or thermoradiotherapy to prevent the recurrence of the tumour and cure the metastases.

Hyperthermia and radiotherapy

The two main problems of cancer therapy that come with merely applying radiotherapy are poor local control and recurrence. There are, in fact, many advantages of combining hyperthermia and radiotherapy.
Radio-resistant cells are more sensitive to hyperpyrexia. Hypoxic cells, i.e. cells with a low oxygen supply, are often located in the centre of the tumour and are sensitive to heat yet resistant to radiotherapy. Radioactive rays are thus more effective on oxygen-rich cancer cells in the peripheral part of the tumour. Therefore, if hyperthermia is applied before radiotherapy, this can improve the oxygen-supply of the tumour cells and thus increase radiation sensitivity. After radiation, molecular processes like interfering sublethal cell injuries or repairing potentially lethal damages take place. If hyperthermia is applied at this stage, these effects can be restrained. [4, 5, 7]
The positive effect of combining heat and radiation depends on the temperature applied, the time interval between heat and radiation and the sequence of the treatment. It can thus be said with certainty that more investigation in this field is necessary to determine the optimal combination in clinical practice. [6]

Hyperthermia and chemotherapy

As mentioned before, hyperthermia improves the blood circulation which is positive in combination with chemotherapy because an enhanced circulation directly influences the effect of chemotherapeutic agents. In addition, this increases the therapeutic effect, reduces the chemotherapeutic drug application dosages and minimises the latter's toxic and side effects. However, the optimal dosage and composition of chemotherapeutic substances in combination with heat in clinical practice has yet to be investigated more closely. [4, 6, 5]

4.1.3. Physical technologies of hyperthermia

There are different physical technologies to heat tumour tissue to a therapeutical temperature that will entail growth obstruction and death of tumour cells. To achieve the therapeutical temperature in the tumour tissue, different methods such as mechanical vibration, infrared rays, ultrasound, microwave, and radio frequency can be used. [4]

Microwave technology

The basis of the microwave technology is the electromagnetic microwave itself: this is a radio wave with a wavelength of 1 mm to 100 cm, whose frequency is between 300 MHz to 300 GHz. The penetration depths depend on the different tissue – the higher the frequency of the microwave, the shallower the penetration. [4]

There are two distinct radiators for the local and regional hyperthermia methods: first, the external microwave radiator for superficial tumours and, second, an intracavitary microwave radiator mainly used for tumours in natural cavities such as nasopharynx, esophagus, rectum etc. One huge advantage of microwave hyperthermia is the considerably few side effects and complications which mostly only include local burning. For patients with a cardiac pacemaker, strict monitoring and specific conditions must be observed during microwave hyperthermia. [4]

It is important to note that different tissue types absorb the microwaves in a different manner. Thus, water-rich tissues (such as muscle tissue, skin or brain tissue) absorb microwaves strongly but penetrate shallowly, whereas water-poor tissue (for example fat and bones) absorbs less microwaves. However, cancer cells usually have a lot of water and microwaves can be converted into heat energy after irradiating the cancer tissues. Thereby, cancer cells are heated to a temperature of 41.4 $°C$ to 43 $°C$, which leads to damage of the synthesis of RNA and DNA and results in cell death. [4]
Heat in healthy tissues spreads out quickly because the blood circulation is faster than that of tumour tissue. Also, in cancer cells, the temperature elevates faster as blood circulation is worse. This leads to higher local temperatures in the tumour tissue and more room for damage by thermal destruction and necrosis. [4]

Radio-frequency technology

Radio frequency, also called diathermy, is a radio wave band. To heat up tumour tissue, a high-frequency electromagnetic field is used. The charged ions and molecules vibrate in this field so that the electric energy from the radio-frequency technology is converted into heat energy which then leads to a temperature increase in the affected tissues. This method is primarily used to heat deep tissues. Presently, researchers implement whole-body hyperthermia with a regional radio-frequency technology and here achieve temperatures of 39.5 $°C$ to 41.5 $°C$ for over two hours. [4]
Normal and cancer tissue can both absorb the energy of radio-frequency electromagnetic waves. The reason for the higher temperature in cancer tissue compared to healthy tissue is the low heat dispersion and its general sensibility to great heat. Therefore, cancer cells can be killed without causing damage to normal tissue. [4]
Figure 4.1 shows a radio-frequency hyperthermia machine which is used in oncology.

Figure 4.1.: Radio-frequency (RF) hyperthermia machine [4]

Ultrasonic technology

Ultrasonic waves are longitudinal waves that produce high-frequency mechanical vibration. On the basis of these sound waves, the particles of tissue cells generate movement friction that causes heat dependent of the frequency of the sound waves. Ultrasonic heating cannot be applied to tissue with air or impenetrable bone tissue as ultrasound decays fast in the air and absorbs a lot of heat in bone tissue. [4]

Laser technology

To reach a therapeutic effect, laser technology generates chemical reactions through the activation of a photosensitiser. Hence, laser radiation can directly destroy cancer by irradiation to the surface of the body or focus on the cavity surface. [4]

For the minimal invasive therapy for solid tumours called laser-induced interstitial thermotherapy (LITT), medicine uses optical fibres that are implanted into the tumour tissue. High laser energy with a specific wavelength enters the tumour tissue, photons are absorbed and the energy is transformed to heat energy so that the tissue temperature increases, resulting in the coagulation and necrosis of the tumour tissue. [4, 21]

Magnetic induction technology

In an alternating magnetic field, each metal object can generate an induction current which is called eddy current. Heat is produced when the eddy current flows in the metal. During the magnetic induction technology, a ferromagnet is located in the cancer tissue and a high-frequency electromagnetic field is set up so that the ferromagnet can induce eddy currents and heat the nearby cancer tissue. The ferromagnetic material has a Curie point, i.e. the critical temperature for the material, that helps control the heating temperature. In case the temperature in the alternating magnetic field exceeds the Curie point, the magnetism disappears completely and loses its capacity to further heat. If the temperature drops below the Curie point, the ferromagnet regains its magnetism and heats again. This technology is still being researched at the moment. [4]

4.1.4. Methods of hyperthermia

Local hyperthermia

Local hyperthermia is a method that is used in cancer therapy to apply heat to a solid tumour [23].
Here, three elevated temperature ranges are possible [23]:

- If the aim of the cancer therapy is to completely ablate the tumour, temperatures need to be within a range of 80 $°C$ when applied to the tissue.
- Temperatures between 41 $°C$ to 45 $°C$ are used when physiological effects such as cell damage without any serious injury to the healthy tissue are required.
- Additionally, there is the possibility to mimic fever-range effects by temperatures between 39 $°C$ to 41 $°C$ which do not entail damage of other tissue.

Local hyperthermia can thus be used for heating superficial and deep-seated tumours with a skin depth of 3 cm to a maximum of 5 cm, e.g. skin or superficial tumours. To use local hyperthermia for accessible tumours, one can expose the tumour to external light such as infrared light, or immerse the tumour in a water bath. [23, 22, 7, 24]

Regional deep hyperthermia

Regional deep hyperthermia is a special form of local hyperthermia that is used in special tumour centres to treat deeper seated tumours. Phase III studies [1] show good local tumour control and a better survival rate when applying a combination of regional deep hyperthermia with standard methods in contrast to using only standard therapies. [22]
Often, the tumour tissue is heated via radio frequency waves which spread in the body. However, their transfer to different tissues is not homogeneous and the reflection differs at tissue boundary surfaces. In regions with many different tissue types, the waves can be either bundled at certain positions and cause high overheating (called "hot spots"), or they can be dispersed to create so called cold spots. Both methods impair therapy efficiency. [22]
In general, there exist two options for bringing radio waves into the body. First, by irradiation with an antenna from the outside, e.g. via capacitive coupling (radiative), or, second, with the help of alternating magnetic fields which produce an electric field in the body. Both techniques use a cooling water bolus to allow optimal coupling with the patient. This water bolus is also used to cool the surface of the patient's body. Furthermore, the water inside the bolus is deionised so that radio waves cannot heat it. In this process, the choice of the used frequency is very important with regard to the radio waves' behaviour and for being able to focus them. As a general rule, the following applies: the higher the frequency of the radio waves, the shorter the wavelength. Since ions and polar particles are agitated to vibrate, radio waves produce heat in electric conductive tissue. The human body has conductive tissue with many ions located in internal organs, the muscles and in most tumour tissue. The correct value of the electrical permeability and conductibility thereby depends on the used frequency. In all this, it should be noted that hyperthermia planning allows to optimally execute the hyperthermia treatment. [22, 24]

Whole-body hyperthermia

As the name suggests, this method allows to heat the whole body and it is thus often used in combination with standard therapy methods to obtain good therapeutic effects. Moreover, this method is primarily used if metastatic cancer has spread out over the whole body. Here, the therapeutic temperature range is not standardised as there are either high temperatures between 39.5 $°C$ to 41 $°C$ (called whole-body mid- to high-temperatures) or temperatures between 38 $°C$ to 40 $°C$ (named whole-body mid-to low-temperatures) that are maintained for a longer period of time. [7, 4]

[1] Phase III studies are trials to examine the effectiveness and frequency of side effects on a large group of people. The results are collected and evaluated to determine whether practices are safe for different people. [25]

There are different ways to increase the patient's body temperature:

- Surface heating method
  During this method, the therapeutic temperature of the whole body is elevated by transferring heat through the surface of the body via radiation or conduction, e.g. by using infrared radiation, hot water baths, wrapping with thermal blankets etc. Infrared radiation is currently the most common method. [4]

- Extra-corporeal blood circulation heating method
  This method is also called perfusion hyperthermia. With the help of an extra-corporeal circulation machine, some of the patient's blood runs through arteriovenous shunts in the heat exchanger where the blood is heated. Afterwards, it is pumped back to the region that is to be internally heated. There are two possibilities to use this method: either with drugs for a chemotherapy, or without drugs. Without drugs, temperatures are about 43 $°C$, while in combination with drugs, the adjusted temperatures for perfusion are lower so as to avoid drug toxicity. Currently, this method is only rarely used because of the expensive equipment and elaborate requirements. [4, 7]

- Biological pyrogen method
  For this method, the patients take drugs to increase the body temperature. This method's risk should not be underestimated because the temperature cannot be controlled, which is why this method is only rarely used nowadays. [4]

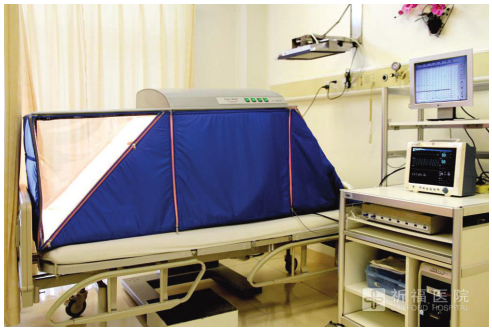

Figure 4.2.: Whole-body hyperthermia machine [4]

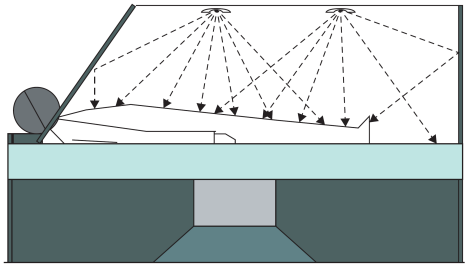

Figure 4.3.: Schema of a whole-body hyperthermia with infrared [4]

Interstitial hyperthermia

Interstitial hyperthermia is used if a very small part of the body is to be heated. The heat-generating applicators, e.g. radio frequency antenna, are implanted into the body which allows to heat small tumours. This form is a thermal ablation because it can cause high temperatures in tumour tissue but will not affect healthy one. [22]

4.2. Hypothermia

In contrast to hyperthermia, hypothermia is a method to cool the human body to a certain temperature. During heart and vascular surgeries in circulatory arrest, hypothermia is used for neuroprotection during cerebral ischemia. Comparted to hyperthermia, it is far less researched in oncology. [8, 9]

In 1815, James Arnott observed that local freezing of malignant breast tissue with a mixture of salt and crushed ice led to a halt in tumour growth. In addition, the American neurosurgeon Temple Fay made observations by sampling human cell cultures: he found out that healthy cells are more tolerant in low temperature environments than tumour cells. He then determined the critical temperature limit for the division of cancerous cells at approx. 19.7 $°C$. He was also the first to investigate hypothermia with patients whilst transferring them into a state of stable hypothermia to increase the effectiveness of radiotherapy. However, most patients whose body temperature dropped below 24 $°C$ got heavy cardiac arrhythmia and ventricular fibrillation. Thus, the investigation of hypothermia in cancer therapy lost its appeal but was paid more attention in heart and vascular surgery. In 1950, Wilfred G. Bigelow presumed that hypothermia resulted in a decreased metabolism activity and, hence, an decrease of oxygen consumption. This formed the basis to stop heart activity and separate the heart from the body circulation, which was confirmed with experiments on dogs. Only three years later, in 1953, John Gibbson presented the first heart-lung machine with an integrated heat exchanger. Nowadays, hypothermia is a standard method for protecting organs during ischemia at circulatory arrest. [12]
Currently, the influence of hypothermia on the progression and regression of tumours is unclear – a fact which shows that additional investigation and studies are necessary [12].

4.2.1. Cellular changes in hypothermia

There are different cellular reactions of mammal cells during low temperature exposure [12, 26]:

- slowdown of the cell cycle, included extension of the G1-phase;
- inhibition of transcription and translation which results in a general slowdown of protein biosynthesis;
- denaturation and misfolding of proteins;
- changes of the membrane permeability with an intracellular accumulation of Na^+ and H^+

It depends on the temperature and the exposition time whether cellular apoptosis which leads to necrosis during hypothermia can be induced [12, 26].

In the pathophysiological field, a fundamental part for the prevention of neuronal cell death is halting early gene expression and excitatory neurotransmitters. Furthermore, therapeutical hypothermia is applied to stabilise the blood-brain-barrier and lower cerebral edema by decreasing permeability to inflammatory cytokines and potentially harmful substances such as free radicals and thrombin. [8, 27]

4.2.2. Cooling methods

There are different aspects to consider when applying hypothermia to a patient [8]:

- the mode of cooling and ease application;
- the safety and tolerability of the patient;
- the speed until the target temperature are reached;
- the time period of the cooling;
- the monitoring for complications

The ideal method for hypothermia has yet to be determined but three phases of cooling can already be distinguished: induction, maintenance and re-warming [8].

There are furthermore two methods of using hypothermia that are described in detail below: surface cooling and endovascular cooling.

Surface cooling
For many years, surface cooling has been used to treat fever. Later, it was used with therapeutical hypothermia for studies and patients with strokes, cardiac arrest and neonatal asphyxia. In fact, this method has two benefits: first, it does not require advanced equipment and, second, the operators do not need experience regarding the risks of central venous catheter placement because this is not needed here. However, there are also disadvantages as external cooling is slower than endovascular cooling and needs specific substances to prevent discomfort and shivering when dropping below body temperatures of 35 $°C$. It must also be observed that cooling of the skin surface induces vasoconstriction and decreases heat exchange in cooled patients. The problem of vasoconstriction is that it leads to surpassing the target temperature and control deficiencies during the re-warming phase. Surface cooling can also include other methods such as convective air blankets, water mattresses, alcohol bathing, cooling jackets and ice packing. [8, 26, 9, 27]

Endovascular cooling
Endovascular cooling is an invasive, practicable and safe technique that uses catheters with antithrombotic coatings. In contrast to the above-mentioned method, the catheter insertion requires knowledge and training. Also, it needs to be kept in mind that larger patients are more difficult to cool to a certain target temperature than lighter or older patients. Still, there are numerous advantages of this method: a shorter time period until the target temperature is reached and a precise temperature control which avoids accidental re-warming, inadvertent rise in intracranial pressure and cerebral edema are only some of them. Possible disadvantages are pneumonia, cardiac arrhythmia, thrombocytopenia and vascular dissection. [8, 9, 27]

4.2.3. Therapeutic uses

Hypothermia is used for anoxic brain injuries and global brain ischemia to provide neuroprotection. Therefore, it is used with cardiac arrest patients and neonatal hypoxic-ischemic encephalopathy. The cooling process should be initiated as soon as possible [27] to reach optimal neuroprotection and edema blocking effects. Animal models show that longer hypothermia treatment provides better neuroprotection in contrast to shorter treatments that have only transient neuroprotection. [8, 26]

Concluding, it can be said that therapeutical hypothermia improves neurological outcomes and reduces mortality in patients with global cerebral ischemia or cardiac arrest as well as in newborns with hypoxic-ischemic encephalopathy. For an effective treatment, the two factors of optimal induction and safe rewarming are crucial. [8, 27]

5. Method

5.1. Basis of the cell membrane and the membrane potential

The cell membrane surrounds the whole cell and separates the extracellular from the intracellular space. It shows a three-layered structure of two hydrophilic parts and one intermediate lipophilic part. Figure 5.1 shows the schematic construction of a phospholipid molecule. The cell membrane consists of a bilayer of phospholipids, whereby the lipophilic parts are arranged towards each other, whereas the hydrophilic parts are located at the inner and outer side of the cell membrane, as can be seen in figure 5.2. The extracellular sides are covered by a fine layer of polysaccharides, which are called glycocalyx and serve the cells to differentiate if it is endogenous or exogenous. [28, 29, 30]

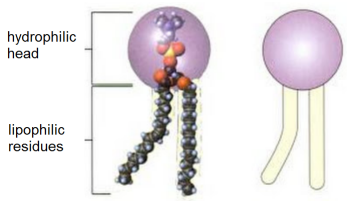

Figure 5.1.: Schematic construction of the cell membrane, which consists of a hydrophilic head structure and an lipophilic tail structure [30]

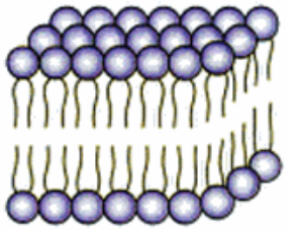

Figure 5.2.: Basic structure of the cell membrane [30]

For the required interaction between the two spaces there are transport proteins embedded in the membrane for the passive and active transport of particles through the cell membrane. [28, 29, 15]

Inside a cell, there is the cytoplasm and ions which mainly consists of positively charged potassium ions (K^+) and negatively charged proteins, amino acids, phosphate and many more. In contrast, in the extracellular space there are more positively charged sodium (Na^+) and negatively charged chloride (Cl^-) ions. [29, 28]
Table 5.1 shows a distribution of the main ions in the extra- and intracellular space.

Table 5.1.: Intracellular and extracellular distribution of the main ions [29]

Ion	**Intracellular range (mM)**	**Extracellular range (mM)**
Na^+	5 - 20	130 - 160
K^+	130 - 160	4 - 8
Ca^2+	50 - 1000 nM[2]	$1,2$ - 4
Mg^2+	10 - 20	1 - 5
Cl^-	1 - 60	100 - 140
$HCO_3{}^-$	1 - 3	20 - 30

The easiest form of transporting particles through the cell membrane is the passive transport mechanism. Particles lead to some temperature-dependent autonomous movements (also called Brownian movements) throughout a solvent, which results in a uniform distribution of the substances. However, if there is a concentration difference between two spaces, this results in a transport of particles from the place of higher to that of lower concentration. This transport mechanism is called diffusion. It should be noted, that it does not matter whether the transport penetrates a membrane or not. Therefore, the concentration gradient between two sides is the driving force for the diffusion, and diffusion thus balances differences in concentration. [31, 32, 15]

Due to the differing distribution of charged particles (ions) in the extracellular media and the cytoplasm, there is a concentration gradient (chemical potential) between the two sides that results in a diffusion from ions through the membrane from higher to lower concentration. On the basis of the diffusion there also occurs an electrical potential between the cell membrane as positively charged ions penetrate the membrane, which would cause the cell's interior to be negatively charged. At a certain membrane potential, the chemical and electrical potential will be equally strong. [31, 32, 15]

At the end of the 19th century, Walther Nernst formulated the related laws for the balance between the electrical and chemical potential. Thermodynamically speaking, diffusion is a spontaneous process as the arrangement in the system decreases, which entails an increase in entropy [29]. This implies that diffusion releases energy which is expressed as

$$\Delta G = -RT \ln \frac{[ion]_o}{[ion]_i} \tag{5.1}$$

where ΔG is the (Gibbs) energy released by the diffusion of ions through the membrane, R is the universal gas constant ($8.31\ Jmol^{-1}\ K^1$), T is the absolute temperature measured in Kelvin and $[ion]_o$ and $[ion]_i$ are the extracellular and intracellular ion concentrations. [29, 31]

[2] Given as nanomolar [29]

For example, if the plasma membrane is permeable for positively charged potassium ions, this results in a movement of ions from the intracellular to the extracellular space driven by the driving force of the chemical potential that exists because of the concentration gradients of the two sides. Therefore, the interior of the cell would be negative compared to the exterior, which attracts the potassium ions back inside the cell. [29, 31]
The responsible electrical energy can be quantified as

$$\Delta G = -EzF \tag{5.2}$$

where E is the potential across the membrane, z is the oxidation state of the ion under consideration, and F is the Faraday constant ($9.65\ x10^4 Cmol^{-1}$). [29]

The movement of potassium ions through the membrane will continue until the electrical force (that is responsible for the diffusion) is equal in size but opposite the diffusion energy. [29, 31, 15, 33]
This is the situation where no net movement of ions penetrating the membrane occurs and the two forces are in an equilibrium and quantified as

$$-RT \ln \frac{[ion]_o}{[ion]_i} = -EzF \tag{5.3}$$

To calculate the electrical potential at which a certain ion gradient is in a state of equilibrium, the above equation can be rearranged to match the Nernst equation:

$$E = \frac{RT}{zF} \ln \frac{[ion]_o}{[ion]_i}\ s \tag{5.4}$$

where E is the equilibrium potential for the distinct ion (for potassium it is named E_K) and typically is between -80 to $-90\ mV$. In reality, the membrane potential is more positively charged than E_K in the range of -50 to $-80\ mV$ because the plasma membrane in rest is also permeable for other ions. [29]
With regard to muscle or nerve cells, it can be assumed that the membrane potential is determined by the permeability of potassium and sodium ions. Therefore, a potential value between the equilibrium potential of potassium (-90 mV) and sodium (+60 mV) is expected. As the potassium permeability in rest is higher than the sodium permeability, the resting potential of muscle or nerve cells is between -70 to $-90\ mV$, which is in the range of the potassium equilibrium potential. This means that the resting membrane potential of muscle and nerve cells is basically the potassium equilibrium potential E_K. [31]

The maintenance of the membrane potential needs different transport mechanisms:

- **Ion channels:** Tubular protein complexes which are important for the passive transport of electrically charged particles through the membrane. [29, 28]

- **Pumps:** This is an active transport mechanism to transfer molecules through the membrane against their concentration gradient by consumption of ATP. For example, the Na^+/K^+ pump transports two potassium ions to the intracellular space and three sodium ions to the extracellular space against their concentration gradients in order to build up and maintain the electrical potential. [29, 28, 31]

- **Transporters:** This is a method to transport molecules across the membrane without forming a pore. [29]

- **Co-transporters or exchangers:** They are also called secondary active transporters and work like a pump. A transporter is able to carry molecules against their concentration gradient but the energy is derived from the diffusion of other molecules, normally Na^+ ions. It is called co-transporter if the sodium ions move in the same direction as the transported molecules, otherwise it is named exchanger. [29, 31]

- **Electrogenic:** "If the transported molecules are charged, the activity of the transporter might result in a net influx or efflux of charge, influencing the membrane potential" [29].

In figure 5.3, three different transport mechanism are represented.

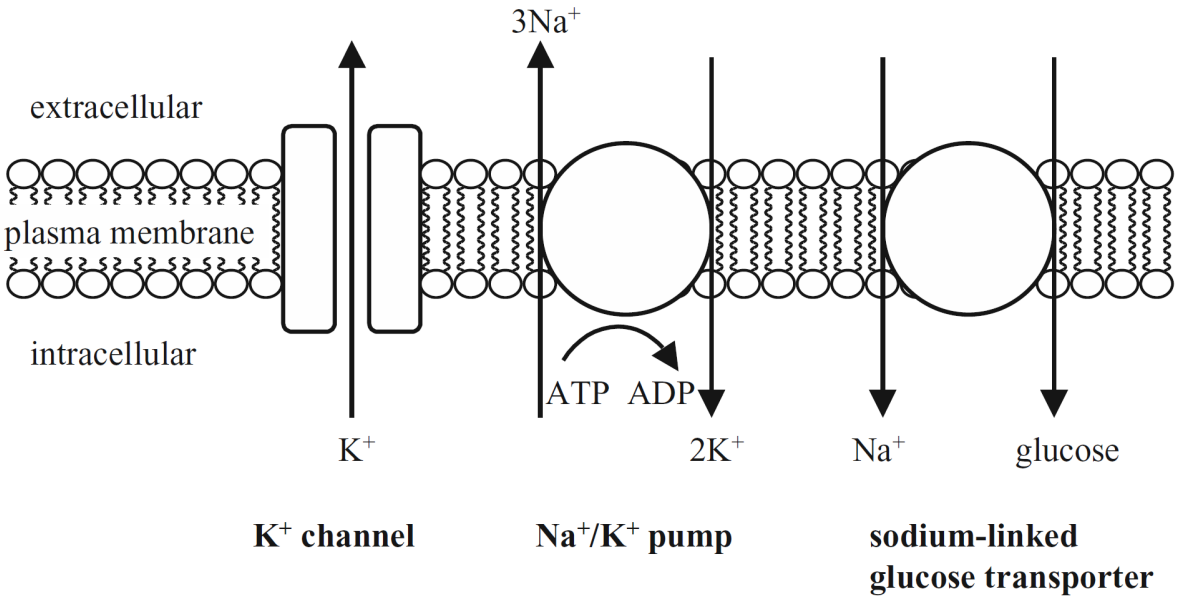

Figure 5.3.: A K^+ ion channel, a Na^+/K^+ pump and a co-transporter or exchanger [29]

Ion channels

Ion channels are tubular protein complexes that span the cell membrane. These channels are aqueous pores that are mainly faced by hydrophilic amino acid residues and connect the extracellular and intracellular medium. They have the ability to change the protein conformation which results in the pore closing with the help of a gating mechanism. It depends on the ion channel when the protein conformation changes and the pore closes. [29]

There are three different ion species [29, 31]:

- **Voltage-dependent channels:** These have voltage sensors and open and close depending on the membrane potential.
- **Ligand-gated channels:** An external factor, for example a neurotransmitter or a hormone, can connect to the channel and open or close it.
- **Second messenger operated channels:** This describes an intracellular factor such as calcium ions or activated G protein subunits that open or close the channel.

It is important to note that these channels are not mutually exclusive as there also exist voltage gated calcium dependent potassium channels. [29, 31]

Electrical properties of the cell membrane

The phospholipid bilayer of the cell separates the extracellular from the intracellular space, which establishes an effective barrier for the exchange of charged particles through the membrane. The membrane forms an electrical insulator between the exterior and the cytoplasm conductor because the two solutions are conductive for ions. [29, 34]
Moreover, there are integrated transport proteins for ions in the cell membrane, which means that the membrane is not a perfect insulator but is in fact permeable for some particles. Due to the permeability with regard to ions, there is a finite resistor (membrane resistance R_m). [29, 34]
The different charge between the extracellular and intracellular space forms a membrane potential. If this completely depends on potassium ions, the membrane potential is equal to the potassium equilibrium potential E_K. "At this potential the force by which the electrical field is pulling potassium ions inward is exactly the same as the diffusion force pushing potassium ions outward.[29]" If the membrane potential is not equal to the potassium equilibrium potential, there will be a net movement of potassium ions through the membrane. This movements can be expressed in current i.e. charged movement $Q(t)$ per unit of time

$$I = \frac{dQ}{dt} \tag{5.5}$$

where I is the ion flow through the membrane in Ampere, and dQ/dt is the change in charge per time.

The ion current flow through the membrane can be determined by the driving force and the membrane resistance. In detail, the driving force is the difference between the membrane potential E_m and the equilibrium potential. In this case, the equilibrium potential is the potassium equilibrium potential. Thus, the further the membrane potential E_m is away from the equilibrium potassium potential, the greater is the imbalance between the electrical and the diffusion force. This results in a higher potassium ion flow through the membrane, which is proportional to the driving force. However, the potassium ion flow is also limited to the membrane resistance, which means if fewer potassium channels are open, fewer ions can flow through the membrane. [29]
The ion current through the membrane is inversely proportional to the membrane resistance R_m, which can be expressed as a special case of Ohm's law

$$I_K = \frac{E_m - E_K}{R_{M,K}} \tag{5.6}$$

where I_K is the current of potassium ions passing through the membrane (in A), $E_m - E_K$ is the driving force (in V), the imbalance between the membrane potential and the potassium equilibrium potential, and $R_{M,K}$ is the membrane resistance for potassium (in Ω).
In the case of I_{leak}, the ions flowing through the membrane are almost equal and the equilibrium potential is "neutral" so that any variances from the resting potential will result in a leak current that can be quantified as

$$I_{leak} = \frac{E_m - E_{rm}}{R_{M,leak}} \tag{5.7}$$

where $E_m - E_{rm}$ is the deviation from the resting membrane potential. [29]

Membrane capacitance
As mentioned above, the cell membrane and the extracellular and intracellular media form a capacitor so that it is possible to store charge [34]. The two sides exert an electromagnetic field across the membrane which attracts charged particles. This means an accumulation of ions near the cell membrane as the negatively charged particles in the interior will attract those positively charged from the outside and vice versa. [29] How many charge Q can be stored is described as follows:

$$Q = E_m C \tag{5.8}$$

Here, Q is the stored charge, E_m is the membrane resting potential (in V), i.e. the potential difference across the membrane, and C is the membrane capacitance (in F). "This equation shows that capacitance is a measure of the capacity of the membrane to store charge at a given potential. [29]"

The physical dimension of the membrane and the membrane thickness are also important to determine the capacitance. The higher the dimension of the membrane, the more charge can be accumulated. Therefore, the membrane surface area is proportional to the capacitance. [29]

The membrane thickness, on the other hand, is inversely proportional to the capacitance: the greater the distance between the two sides, the weaker the electromagnetic field that attracts the ions to either side of the cell membrane. [34] However, the thickness of the lipid bilayer in all cells does not vary much which is why this parameter does not strongly influence the capacitance [29].
Another parameter which the capacitance depends on is the characteristic of the insulating material between the two conductive sides [34]. The properties of the membrane, which refers to the capacitance, is almost similar in all living cells and summarised as dielectric constant or ϵ_r. [29]
The capacitance C can be described as follows:

$$C = \frac{A\epsilon_r}{d} \tag{5.9}$$

Here, A is the membrane surface area, ϵ_r is the dielectric constant for the cell membrane and d is the membrane thickness.

Electric model of a cell membrane
The plasma membrane of an undamaged cell can be illustrated as an electronic model with the membrane resistance R_m, the membrane capacitance C_m and the membrane potential E_m, as shown in figure 5.4. Here, C_m represents the insulator on which the particles are stored while R_m limits the ion current running through the membrane. [29]

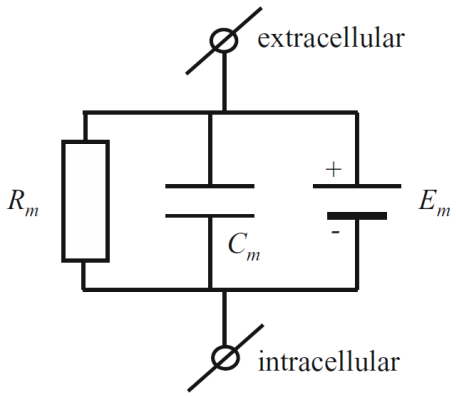

Figure 5.4.: Electric model of a cell membrane of an undamaged cell. R_m represents the membrane resistance, C_m is the membrane capacitance and E_m the membrane potential illustrated with the appropriate electronic symbols [29]

5.2. Cell cultures

5.2.1. A549 cells

In 1972, Giard et al. researched the A549 cell line by removal and culturing of cancerous lung tissue in the explanted tumour of a 58-year old Caucasian male. The cell line is an adenocarcinomic alveolar basal epithelial cell that is hypo triploid with a modal chromosome number of 66 occurring in 24 % of the cells. Modal chromosomal numbers of 64 and 67 with higher ploidies usually arise only rarely. [35]
The morphology of the epithelial lung cancer cells is squamous and they grow adherently, e.g. as a monolayer, in vivo. Characteristically, this cell type diffuses water, electrolytes and other substances across the surface of the alveoli. [35]

"When cultured in vitro, these cells become a monolayer and adhere to the surface of the culture flask. In addition, A549 cells may be grown as a suspension. These cells are also important because they make lecithin and have high levels of fatty acids, important for maintaining membrane phospholipids. A549 cells are an important model for drug metabolism and are also used as a host for gene transfection" [35].

Figure 5.5 shows the A549 cells under the microscope, while the second illustration (figure 5.6) depicts the cells' detachment with Trypsin [3].

The A549 cells for the present measurements are supplied by the Center for Medical Research (ZMF) Graz. The cells for the first measurement series from 04.07.2016 had a passage number of 50 while those from the second measurement series from 14.11.2016 had one of 6.

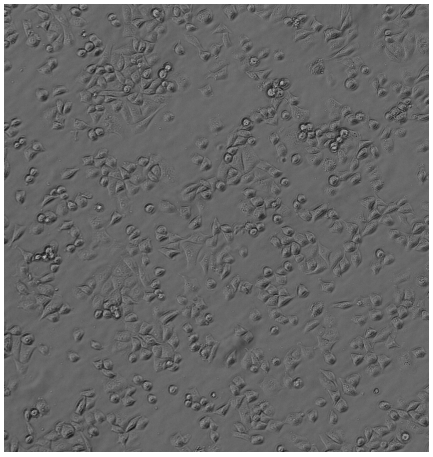

Figure 5.5.: A549 cells under the microscope

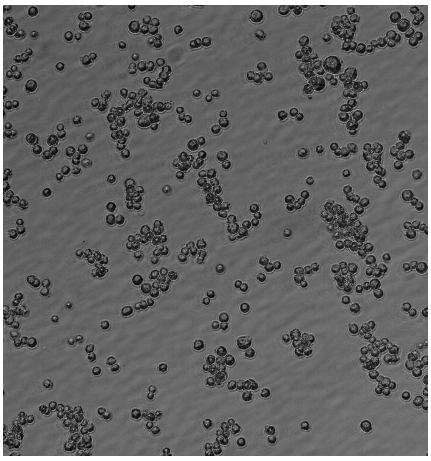

Figure 5.6.: A549 cells after detachment with trypsin

[3] Trypsinisation is a gentle method to detach adherent growing cells from the culture vessel for further cultivation. [36]

Ion channels in A549 cells

In A549 cells, endogenous K^+ channels are essential for stabilizing the membrane voltage and maintaining the driving force for the electrogenic transport of Na^+ and Cl^- ions. Another notable function of the potassium channels is the release of K^+ into secreted fluids. These lung cancer cells typically consist of voltage-gated potassium channels or ATP- or Ca^{2+}-dependent ion channels. Cl^- channels are also ubiquitously expressed in lung cells and are essential for the functionality of this epithelial tissue. However, there is currently little knowledge about the molecular nature of their Cl^- channels; it is only known that the cystic fibrosis transmembrane regulator (CFTR) is the most dominant Cl^- channel in the lung. [37]

5.2.2. H1299 cells

H1299 cells are also lung epithelial cells, as treated by A.F. Gazdar, H.K. Oie, J.D. Minna. In this case, the patient was a 43-year old Caucasian male with a primary non-small cell lung cancer who had received prior radiation therapy. The cell line is established from a lymph node metastasis of the lung. [38]

"These cells stain positive for keratin and vimentin but are negative for neurofilament triplet protein. The cells have a homozygous partial deletion of the p53 protein, and lack expression of p53 protein. The cells produce neuromedin B" [38].

For the present measurements, cells were also provided from ZMF Graz. Figure 5.7 shows the H1299 cells under the microscope at low and high density.

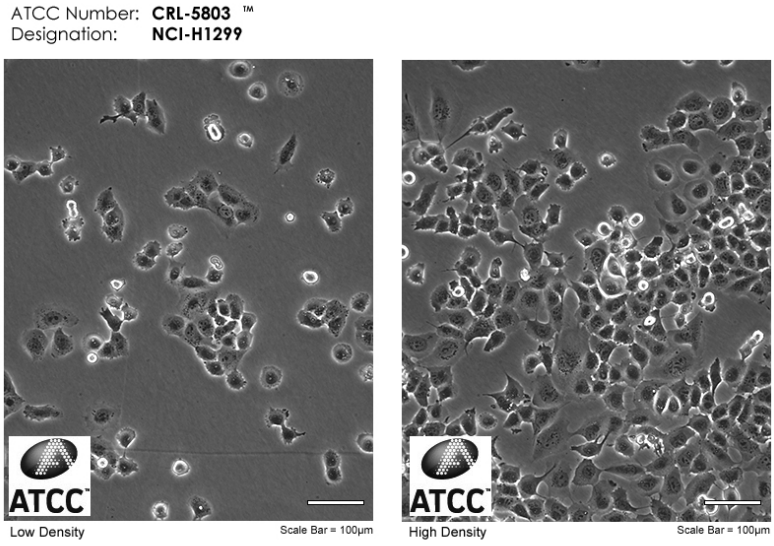

Figure 5.7.: H1299 cells under the microscope [38]

5.3. Patch clamp technique

The patch clamp technique developed by Bert Sarkmann and Erwin Neher in the late 1970s is a method used for electrophysiological and biophysiological research to study single or multiple ion channels in cells. It allows to form $G\Omega$ seals, i.e. a resistance between the cell's membrane and the glass pipette, to record ion movements that go through the ion channels. [11, 29, 39, 40]
This method has several benefits such as a high throughput, a high resolution, good precision and automation. The field of application is wide since the ion current is an important regulation and transport mechanism for tissue and thus not only essential for excitable cells. To investigate a wide field of biophysical research and drug development, different configurations are practicable and they are described in more detail in chapter 5.3.1. [39, 41, 11]

Referring to the name of this technique, "patch" denotes a small piece of cell membrane and "clamp" means an electro-technical connotation. The recorded membrane potential changes demonstrate the activity of ion channels that reflect the membrane reaction to ions leaving or entering the cell. [29]

In principle, two patch clamp methods are possible: first, the so called voltage clamp and, second, the current clamp method. Voltage clamp here refers to forcing a constant voltage on a membrane patch, where the resulting current can be measured and recorded. In contrast to that, the rarely used current clamp method imposes a constant current on the membrane patch to measure the voltage across the patch. [40, 42, 29, 43]
Current patch clamping is used for a useful quality check of the patch and the cell. By using the whole-cell mode, it is possible to measure the effective membrane potential if a brief switch to current clamp with a not-injected current (I = 0) is applied when access to the cytoplasm was obtained. Also, it can record synaptic potentials using the current clamp method. [29, 42, 40, 43]
By using the voltage clamp method, the operator has the opportunity to change the command voltage over time, either stepwise or by using complicated waveforms, and study specific ion channel characteristics [29].

Figure 5.8 shows the simplified electric circuit of a voltage clamp measurement consisting of an electronic feedback system with an amplifier and a feedback resistor $R_{feedback}$. In general, the measured potential is compared to that set by the user (command voltage, holding potential). Each difference of the recorded potential from the command voltage is immediately corrected by injecting a compensatory current into the system. This compensatory current is then an exact representation (albeit inverse in sign) of the ion flow over the cell membrane under investigation. [29, 41]

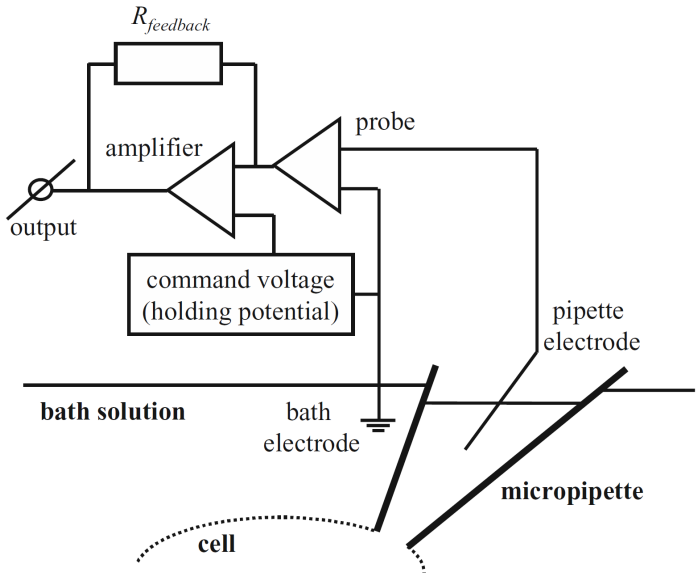

Figure 5.8.: Voltage clamp principle for a whole-cell configuration [29]

Furthermore, two methods for using the patch clamp technique can be applied, first, the so called conventional and, second, the planar patch clamp technique, both being describe in detail in chapters 5.3.3 and 5.3.4. Usually, if the term patch clamp technique is used, this is referred to as the conventional method. [11, 44]

5.3.1. Configuration

Depending on the research interests, different configurations for the investigation of cells with the patch clamp technique are practicable. For example, one can investigate the activity of all ion channels in a cell or only individual ones, and it is also possible during the recording to modify the fluid on the extra- or intracellular side of the cell. [29, 40, 11] In figure 5.9, all configuration methods are represented, while table 5.2 shows the advantages and disadvantages of different patch clamp modes.

Cell-attached configuration
Each configuration starts with the cell-attached mode. The pipette is moved towards the membrane and suction is applied at the back of the pipette to form a strong connection. A tight (high resistance) seal between the pipette and the cell membrane is established without damaging the cell. This configuration is used to record the current of single ion channels in the tiny patch of the membrane. It is thus the best "physiological" mode and the simplest one to study the single ion channels. [40, 29, 11]

Table 5.2.: Distinction of different patch clamp configurations [29]

Configuration	Use	Advantages	Disadvantages
Cell-attached patch	Single-channel recording	Cytosolic side intact (physiological) Easy to obtain	Exact patch potential unknown No easy superfusion possible
Outside-out patch	Single-channel recording	Extracellular side can be superfused Cytosolic environment is controlled	Washout of cytosolic factors Disruption of cytoskeletal structure
Inside-out patch	Single-channel recording	Cytosolic side can be superfused Extracellular environment is controlled	Bath solution must be replaced by intracellular solution Relatively difficult to obtain Disruption of cytoskeletal structure
Whole-cell	Macro-current recording	Quick assertion of ion channel populations Cytosolic environment is controlled Extracellular side can be superfused	Washout of cytosolic factors

Whole-cell configuration
This configuration is the most common mode used for recording electrical potentials and currents from the entire cell. It is started with the cell-attached configuration and, if the gigaseal has been created, strong suction is briefly applied to the membrane. The membrane patch is disrupted and comes into direct electrical contact with the solution inside the pipette and the cytoplasm of the cell. To directly record the membrane potential, the patch electrode is located on one side of the cell membrane and the ground electrode on the other side, as depicted in figure 5.8. To create a "physiological" condition, it is necessary to fill the pipette with an ionic composition that is similar to cytoplasm. [40, 29, 11]

Inside-out patch
The inside-out configuration measures single channel activities with the advantage of being able to change the medium that is exposed to the intracellular surface. In this method, the current of the small membrane patch and not that of the whole cell is measured. To apply this mode, the patch pipette is first attached to the cell membrane, thereby forming a gigaseal, and then excised from the cell by abruptly removing the pipette from the cell, which forms a vesicle. By exposing the cell vesicle to the air by briefly lifting the pipette from the bath solution, it breaks out and the inside of the cell is exposed to the bath solution. [40, 29, 11]

Outside-out patch

This configuration is necessary to study the characteristics of ion channels when isolated from the cell and to investigate the effects of extracellular factors on the channels by changing the bath solution. First, it needs a whole-cell configuration. Afterwards, the pipette is slowly moved away from the cell which forms two pieces of membrane that are then reconnected to form a small vesicular structure with the intracellular side facing the pipette solution. To conduct this experiment under "physiological" conditions, the pipette solution must be similar to the intracellular ionic environment. [40, 29, 11]

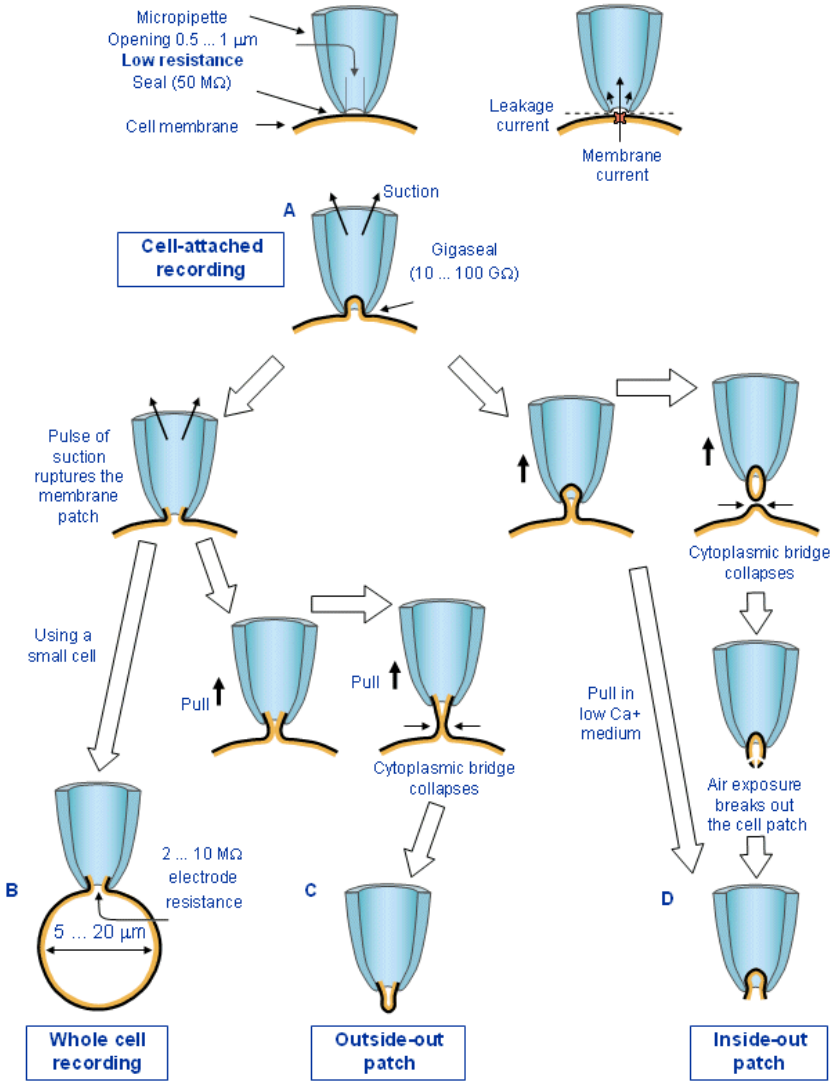

Figure 5.9.: Different possibilities for patch clamp configuration [33]

5.3.2. Description of the electrical concept of the patch clamp technique

Intracellular recording

To understand the electrical concept of the patch clamp technique, the intracellular recording is described first. This is a simple electrophysiological method used for puncturing the cell's plasma membrane. There, direct measurements of the membrane potential are possible because one electrode builds a direct contact with the cytoplasm of the cell, while the other is located in the bath solution on the other side of the plasma membrane. The tip of the micro glass pipette is very small (in fact, it measures tens of nanometres) which limits the pipette's electrical conductivity (in a relatively high pipette resistance) and the elution of cytoplasm by the pipette liquid. There is an electrical circuit between the pipette electrode and the cytoplasm due to the penetration of the cell by the glass pipette. Therefore, the potential difference of the bath and the pipette electrode represent the membrane potential. [29]

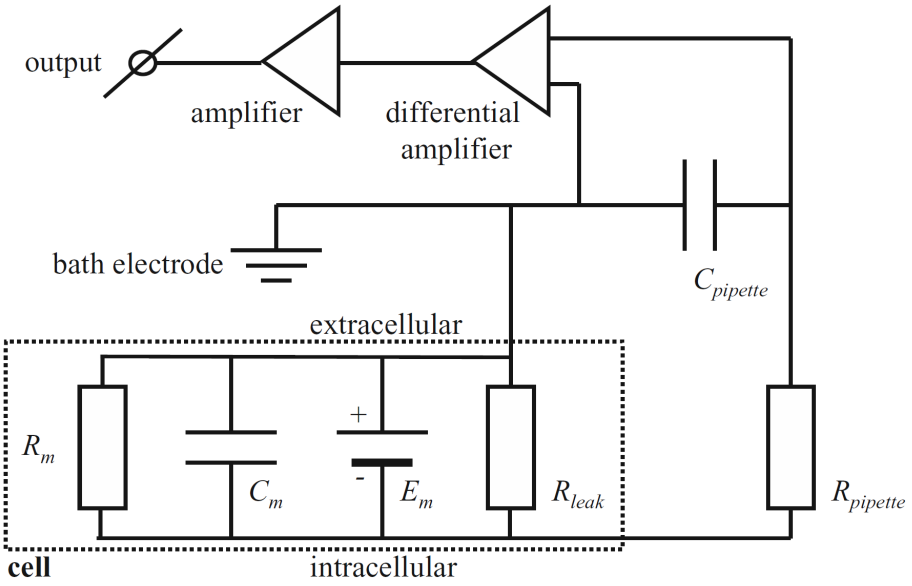

Figure 5.10.: Equivalent circuit of an intracellular recording [29]

The following factors need to be considered:

Pipette resistance
The small size of the pipette tip causes a resistance which is minimised by using a highly conductive solution ($2-3$ $MKCl$) to fill the glass pipette and form a connection to the metal junction leading to the probe. [29]

"The membrane potential [...] is distributed over the pipette resistance and the input of the differential amplifier. The differential amplifier records the voltage difference between two inputs. In practice the differential amplifier is housed separately from the main amplifier [...] and is usually referred to as a 'probe', although strictly speaking the 'probe' is the physical housing that contains the differential amplifier and other circuits. According to Kirchhoff's voltage law, the greatest voltage drop in a series circuit will be over the highest resistance, so if the input resistance of the probe is very high compared with the pipette resistance, then the probe will 'see' most of the membrane potential. Modern probe resistances are very high indeed ($> 1\ G\Omega$)", as shown in figure 5.11. [29]

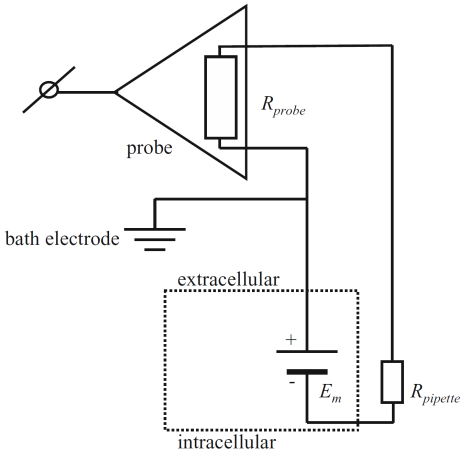

Figure 5.11.: Simplified equivalent circuit of an intracellular recording [29]

Pipette capacitance
It is important that the pipette is made of glass since this forms a capacity between the bath solution and the pipette solution. Capacities delay potential changes such as action potentials which, in practice, can be distorted. Many amplifiers have circuits inside that allow the introduction of negative capacities to avoid this. The user can set these circuits by means of a "pipette capacitance" controller. However, one must pay attention to overcompensation of the circuit because the circuit becomes hypersensitive to potential changes and will easily oscillate. [29]

Leak resistance $\mathbf{R}_{leak}$
The leak resistance is introduced due to the cell damage by the micro pipette: this produces a short circuit from the cytosol to the ground of the bath electrode, see figure 5.12. The smaller the cell damage, the higher the R_{leak}. If there is a large whole in the cell due to the puncturing, the R_{leak} is low and there is considerable load on the membrane potential that the cell might not be able to sustain. [29]

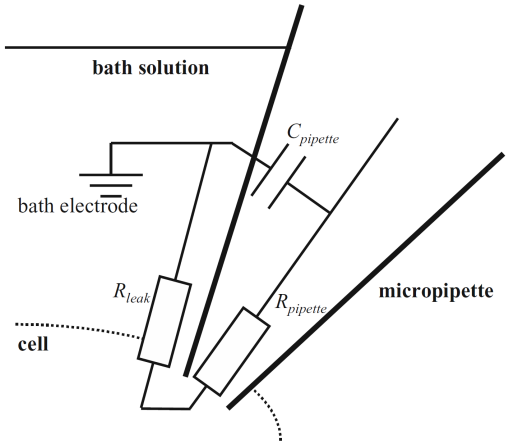

Figure 5.12.: Detailed cell-pipette system [29]

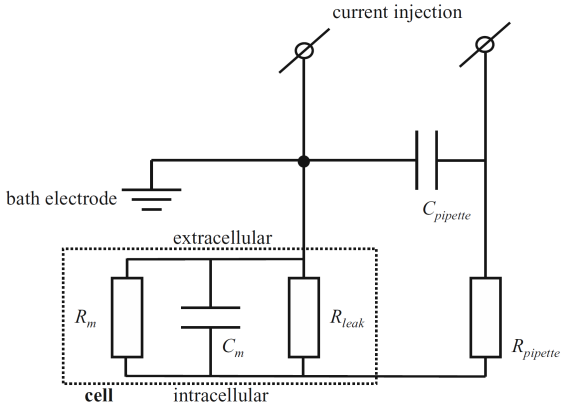

Figure 5.13.: Circuit for estimating the membrane resistance and the capacity [29]

Current injection
The resulting voltage deflection after injecting a small current through the pipette into the cell can be used to estimate the membrane resistance R_m and the capacity C_m, as shown in figure 5.13 for the relevant circuit. [29]

Equivalent circuit for the cell-attached mode

Figure 5.14 illustrates the equivalent circuit for the cell-attached configuration.
In this mode, there is a series circuit of the membrane resistance R_m, the resistance of the membrane patch R_{patch} and the pipette resistance $R_{pipette}$. In this case, the membrane patch resistance R_{patch} is high compared to the membrane resistance R_m and the pipette resistance $R_{pipette}$. The highest resistance in a series circuit limits the current flow. Thus, the circuit effectively observes current flows through the patch and any ion channels in it. [29]

The leak resistance R_{leak} indicates the quality of the seal between the glass of the micro pipette and the cell membrane. If the only parallel R_{leak} of the circuit is high, the seal is good and no significant current will leak. The current passing through R_{patch} has two pathways to ground: either across the probe or across the R_{leak}. Hence, R_{leak} should be much higher than the current input resistance of the probe. Furthermore, a low R_{leak} significantly increases noise, which is why resistance should be in the range of 10 $G\Omega$. [29]

The relevant capacities are the pipette capacity $C_{pipette}$ and the low patch capacity C_{patch} (the latter is not shown in figure 5.14 as it is very small). $C_{pipette}$ must be well-adjusted because high magnification and a fast time scale often required in single channel recording can enhance the capacitive artefacts. The whole-cell membrane capacity C_m is not so important because R_m is normally much lower than R_{patch} and R_m effectively short-circuits C_m, which means that the circuit does not see C_m. [29]

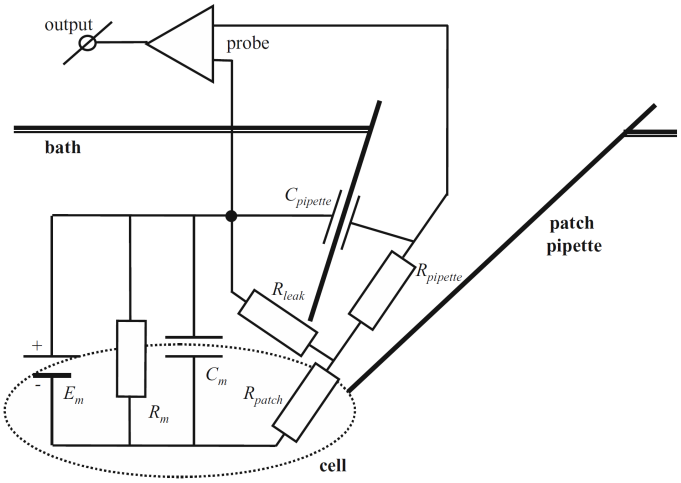

Figure 5.14.: Equivalent circuit for the cell-attached configuration [29]

Equivalent circuit for the whole-cell mode

The whole-cell configuration is achieved by rupturing the membrane patch under the pipette in a cell-attached mode. At the electrical visual, R_{patch} becomes very low and is usually renamed to R_{access}. Thereby, the pipette electrode has direct electrical contact with the cytoplasm of the cell. [29, 43]

In this mode, the series circuit consists of the pipette resistance $R_{pipette}$, the access resistance R_{access} and the membrane resistance R_m. R_m is the largest current-limiting resistor and this mode thus allows an observation of the current through the membrane resistor and the monitoring of the sum of ion currents of each ion channel in the cell. Also, here the parallel R_{leak} should be very high to obtain a good seal between the cell and the glass pipette as well as minimise the risk of short-circuiting of the membrane current. [29, 43]

Contrary to the cell-attached mode, the membrane capacity C_m plays an important role in the whole-cell mode because it influences the voltage clamp time characteristics. Each potential change will be delayed as there is a significant RC circuit consisting of R_{access}, $R_{pipette}$ and C_m. [29]

Figure 5.15 shows the equivalent circuit for the whole-cell mode.

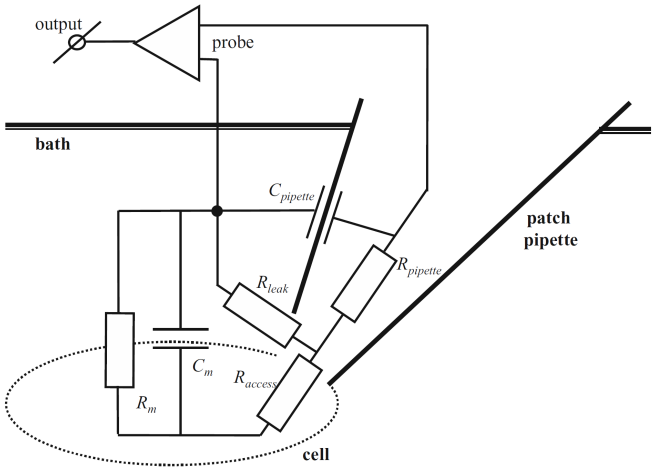

Figure 5.15.: Equivalent circuit for the whole-cell configuration [29]

5.3.3. Conventional patch clamp technique

The basic elements of the conventional patch clamp technique are as follows:

- "a platform with minimal mechanical interference" [29];
- "a microscope for visualisation of the preparation" [29];
- "manipulators to position the micropipette" [29];
- "electronics to perform stimulation, recording and analysis in an electrically clean environment" [29].

It is necessary to place the set on a location where vibration is prevented to avoid changes in the micromanipulator's mechanism or bad seals. The table on which the patch clamp system is set should have dampening properties to compensate the residual transfer of vibration. This table is a so called anti-vibration table, as shown in figure 5.16. [29, 43]

All metal objects around the configuration should connect to the electrical "ground" or "earth" for the purpose of effectively smoothing noise by short-circuiting it through an ultralow resistance route. Ideally, the ground should be a large metal pole lodged deep into the earth, with only the electronics and shielding connected to it. Furthermore, shielding in the form of a grounded metal cage, the so called Faraday cage, is necessary. This cage expands over the whole worktop and is used to avoid electromagnetic interferences. Figure 5.17 shows such a cage. This Faraday cage is usually home-made from aluminium or copper mesh and sits on a frame with legs separated from the table to avoid mechanical interferences. [29, 43]

Figure 5.16.: Anti vibrate table for the conventional patch clamp system [29]

Figure 5.17.: Faraday cage for the whole worktop [29]

A high quality microscope and micromanipulators are other important elements to establish a tight seal with the cell membrane. With a microscope, the cell's and pipette's positions can be observed. In addition, micromanipulators are used to position the tip of the micropipette precisely onto the cell membrane. [29, 43]

To control the configuration during the experiment, it is often emphasised to use a camera. This brings with it advantages as it allows to [29]:

- watch the visual signal simultaneously with the electrical signal.
- avoid the risk of mechanical interferences because of accidental contact with the microscope.
- record the patch clamping with cells for teaching and training or for the analysis of experiments.

The bath in which the preparation is immersed is usually a superfused petri dish (10 cm^2 or smaller) which on one side has a supply for the superfusate and one for the drain on the other side. A wire for the ground bath electrode is also immersed into the bath. [29]

The stable superfusion system consists of a set of containers and a drainage which are mounted onto a tripod stand, as shown in figure 5.18. The distinct containers that are filled with different solutions can be individually selected. The connection between the bath inlet and the containers are done with a tubing whose flow rate can be adjusted. It is also possible to install a temperature control device to preheat the solution before it enters the bath. Lastly, a drainage with an adjustable flowrate to match the inflow is required. [29]

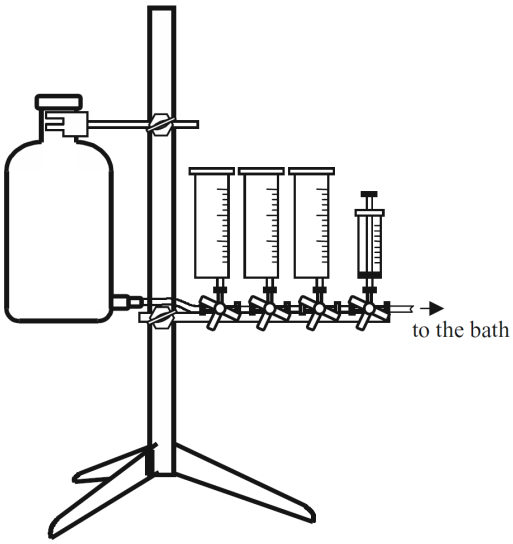

Figure 5.18.: A versatile superfusion system [29]

For the measurements, one normally needs two electrodes: the bath electrode and that inside the pipette. Here, they are a conductive solid that makes electrical contact with the fluid. The bath electrode is made of a silver wire or pellet that is immersed into the bathing solution and functions as ground electrode. [29]
For the measurements, a micro pipette made of glass is used which is filled with a salt solution. On the pipette tip there is a very small opening (approx. μm in size) to enclose the cell membrane. Inside the glass pipette is a second electrode coated with AgCl and connected to a highly sensitive amplifier that records the ions' movement through the ion channels. Also, the micropipette is connected to a small pressure system which is used to build the seal and a micromanipulator. [29]

With the conventional patch clamp technique, it is possible to directly measure the gating, permeability, selectivity and voltage sensing of ionic channels at the molecular level. Conventional patch clamping also has disadvantages such as very low throughput which makes it unsuitable for the research of cellular communication in neuronal networks. Another point is that the intracellular solution cannot be exchanged conveniently. To accomplish suction, exchange the solution and perform recordings under a microscope, a highly skilled and experienced operator is necessary. [44, 11]

5.3.4. Planar patch clamp technique

Due to the aforementioned disadvantages, the conventional patch clamp technique is widely used in research work in a laboratory. However, scientists were able to further develop and improve it: this recently developed technology is called planar patch clamp technique or chip-based patch clamping. [44, 11]

The advantages of this method are the higher throughput, the better control of the pipette (here a chip is used) and the simpler control of the bath solution. Another benefit is the lower level of complexity and time consumption. In comparison to conventional patch clamp techniques, the planar technique has a lower quality of record and lower flexibility. However, there are also disadvantages such as the risk that it can also attract cells that do not have a stable condition. Moreover, it can only make use of cell-attached and whole-cell configurations. [11, 44]

For the measurements in this thesis, the planar patch clamp technique from Nanion was used. This is an automated system that offers improved throughput with high quality and informative data. In figure 5.19, the schema of the planar patch clamp technique is illustrated. [39]

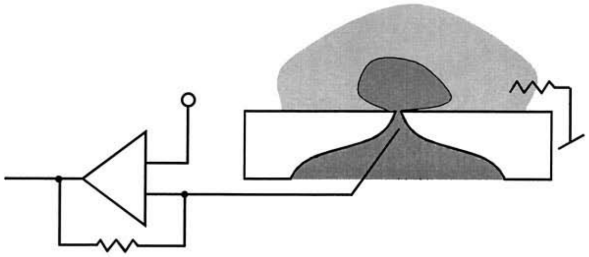

Figure 5.19.: Schema of a planar patch clamp technique [10]

The main difference to a conventional system is the chip where the suspension of cells is located, as shown in figure 5.20. Afterwards, suction is applied and a single cell is attracted and located on the aperture. It also differs in the setup: in the conventional technique, it is the pipette that is moved towards the cell, while in the planar technique, the cell is moved to the aperture. To build the whole-cell mode clamping, sealing and rupturing are reached by applying a suction pulse at the back of the pipette in the conventional technique. In the planar patch clamping, the cell solution is placed on the chip with an external and internal solution and suction can be controlled by a software. The latter is also used to adjust the clamped voltage and time interval. The setup of the planar technique is not as sensitive to interferences as that of conventional patch clamping. [39, 45]

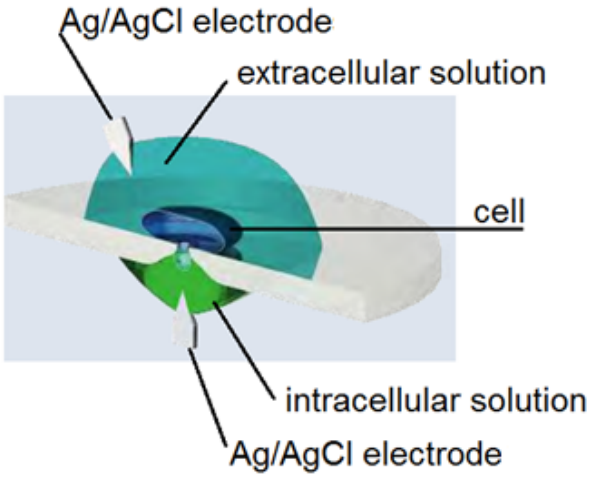

Figure 5.20.: Schema of the solution in an NPC-1 Chip [39]

5.3.5. Measurement setup of the planar patch clamp technique

The planar patch clamp system of Nanion consists of various devices that were also used for the present measurements. These devices are described in the following:

Port-a-Patch recording station
The Port-a-Patch recording station is a single channel patch clamp device which is connected to an external amplifier. This automated system is able to capture the cell and seal and achieve whole-cell configuration. It consists of the housing for the head stage, the chip holder, the Faraday lid and the external and internal electrodes. Figure 5.21 shows such a Port-a-Patch recording station. [39]

Port-a-Patch SuctionControl
SuctionControl is necessary to apply pressure between $-300\ mbar$ to $300\ mbar$ relative to the atmosphere of the Port-a-Patch recording station. The SuctionControl's nozzle is used for the tubing that is connected with the chip holder on the recording station and used to apply suction. Figure 5.22 shows the connection between the SuctionControl and the recording station. For the present measurement, the SuctionControl Pro (SN 2000530) was used which is an advanced SuctionControl unit. With this, it is possible to control and monitor the pressure which are applied to the cell via the amplifier. [39]

Figure 5.21.: Port-a-Patch recording station with the Faraday lid [39]

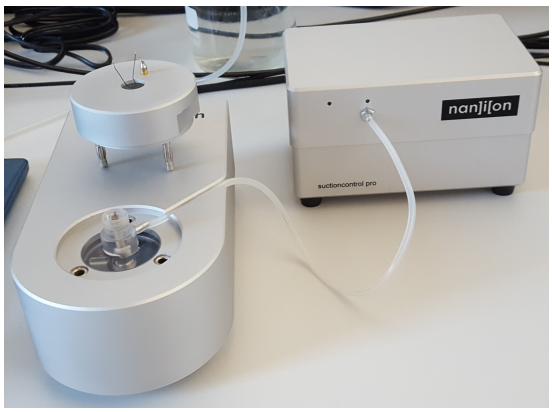

Figure 5.22.: On the right the Port-a-Patch SuctionControl Pro connected by a tube with the recording station

External Perfusion Panel, Laminar Flow Chamber
The Perfusion Panel (SN 4000295, see figure 5.23) is used in combination with the Laminar Flow Chamber and the Temperature Control. It is connected to the computer to regulate the perfusion through the chamber with the PatchControl software. The panel can also be handled manually via buttons. [39]
The Faraday lid of the Port-a-Patch recording station is replaced by the Laminar Flow Chamber, see figure 5.24. The chamber is easy to handle and connected via tubes with the Perfusions Panel. It is necessary for exchanging the solution inside the chamber. [39]

Temperature Control, multi channel system
The Temperature Control is used in combination with the Laminar Flow Chamber, see figure 5.25.
There are two heating elements: one (5.25 (1)) is built into the Laminar Flow Chamber to heat the whole chamber and the second heat elements (5.25 (2)), so called cannula, that heat the solution through the chamber. [39]
The multi channel system (TC 02) is needed (figure 5.26) to adjust the desired temperature inside the chamber. There are two possibilities to change the temperature of the cannula heating elements and the temperature control unit: either by using the buttons on the multi channel system or with the TCX-Control software.
To control the real temperature inside the chamber of the cell solution, a thermometer (Voltcraft K101 Digital Thermometer) is used because the real temperature often deviates from the adjusted one. [39]

Figure 5.23.: Perfusion panel [39]

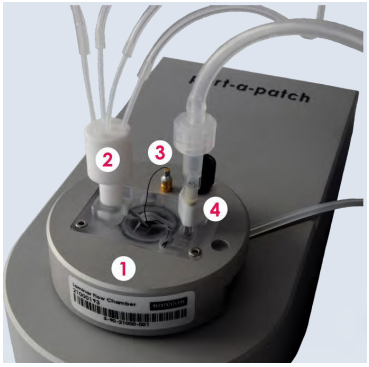

Figure 5.24.: Laminar Flow Chamber; Faraday lid with the chamber (1), including the nozzles (2), the external ground electrode (3), and the waste removal connector (4) [39]

NPC-1 chip
An essential part of the planar patch clamp technique is the NPC-1 chip which is the only disposable part that must be changed after every measurement. Figure 5.27 shows such a chip. The chip is a twist cap to exchange it fast and easily after each measurement from the chip holder. The borosilicate glass chip with the aperture is glued on this NPC-1 chip; for the present measurements, a chip with a chip resistance of $3-5\ M\Omega$ was used. [39]

Amplifier
For the measurement, the HEKA amplifier patch clamp EPC 10 USB was used, as shown in figure 5.29.

Incubator
The cell suspension should be kept at temperatures in the area of 37 $°C$, which is why an incubator(Midi 40 CO_2 incubator) was used.

Biosafety Cabinet
The Biosafety Cabinet (BSC-700II-I) is used to exchange some of the cell suspensions in sample vessels so that the remaining cell suspension can be stored in an incubator for later measurements.

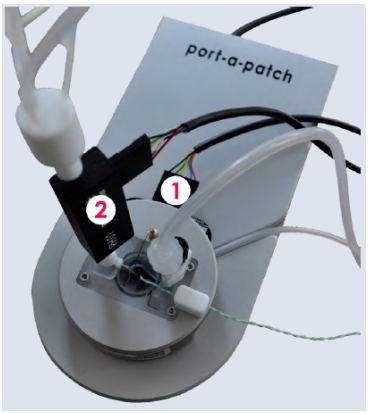

Figure 5.25.: Temperature Control
(1) to keep the whole chamber at a constant temperature
(2) cannula heating system [39]

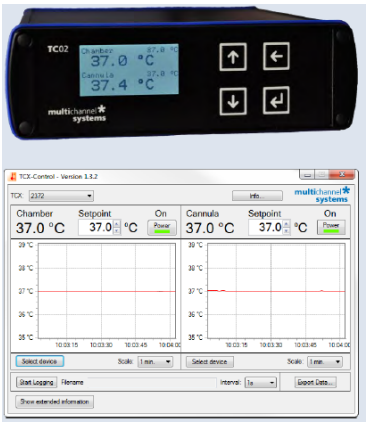

Figure 5.26.: Multi Channel System, below the software on the computer [39]

Figure 5.27.: NPC-1 Chip [39]

Figure 5.28.: Pipette internal solution at the bottom of the chip [39]

Figure 5.29.: HEKA Amplifier EPC 10 USB

Electrode

The external electrode is placed in the socket of the Faraday lid and connected to the ground (see figure 5.24 (3)). The internal electrode is attached to the chip holder of the Port-a-Patch recording station, thereby it is directly connected to the head stage. The two electrodes are made of silver and coated with AgCl. [39]

Extracellular or external solution
The composition of the extracellular solution is as follows:

- 160 mM NaCl
- 4.5 mM KCl
- 1 mM $MgCl_2$
- 2 mM $CaCl_2$
- 5 mM D-Glucose monohydrate
- 10 mM HEPES/NaOH, pH 7.4

Intracellular or internal solution
The composition of the intracellular solution is as follows:

- 10 mM NaCl
- 75 mM KCl
- 70 mM K-Fluorid
- 2 mM $MgCl_2$
- 10 mM EGTA
- 10 mM HEPES/KOH, pH 7.2

Seal enhancer solution
The composition of the seal enhancer solution is as follows:

- 80 mM NaCl
- 3 mM KCl
- 35 mM $CaCl_2$
- 10 mM $MgCl_2$
- 10 mM HEPES/NaOH, pH 7.4

PatchControl software and PatchMaster software
In the PatchControl software, default parameter files are set that define the whole execution of the measurement. The parameter file (suction protocol) regulates the suction which is applied during the measurement and can be loaded, modified and saved. The file is displayed in the parameter window. In general, the parameter file is divided into 8 steps that are described in more detail in the next sub-chapter *Execution*. [39]
The electrophysiological software PatchMaster starts automatically if PatchControl is opened. In this software, the voltage and time intervals for the measurement are defined along with numerous further factors that can be adjusted. [39]

Experimental settings

In fact, there exist important standard cellular and measuring parameters that are described in this sub-chapter.

Cell capacitance *C_slow*
The slow capacitance in the PatchControl software represents the capacitance of the cell membrane C_m during the whole-cell mode.

Glass capacitance C_fast
The PatchControl software C_$fast$ indicates the glass capacitance.

Capacitive transients can occur if the holding potential changes to the distinct voltage and produces artefacts because the R_*access* and R_*pipette* are in series with the membrane capacitance (C_*slow*), as can be seen in figure 5.15 equivalent circuit for the whole-cell mode. To avoid such artefacts, most amplifiers can generate RC responses and subtract them from the output signal. This RC circuit compensation of C_$fast$ and C_*slow* can be carried out before each measurement and sets an amplitude and time constant which results in a large, quick transient. [29]

Series resistance *R_s*
The series resistance R_*s* represents the sum of the access resistance R_*access* and the pipette resistance R_*pipette*. It is recorded after the capacitive transient cancellation [29]. In chapter 6, the *R_s* is denoted as *R_inside*.

Seal resistance *R_memb*
In the PatchControl software, R_*memb* is defined as the seal resistance between the cell membrane and the aperture of the chip which indicates the resolution. The seal resistance should be in the range of GΩ because the higher the R_*memb*, the better the resolution, and the weaker the noise [29]. In chapter 6, the *R_memb* is denoted as *R_outside*.

The criterion for an acceptable measurement, i.e. one avoiding high leakages, is that the seal resistance (R_*memb*) should be approximately four times higher than the series resistance R_*s*, whereby the R_*s* should not be greater than 50 $M\Omega$.

Another point is the sampling frequency that is 20 KHz. This entails roughly 260 000 data values for each measurement, whereby every measuring has 13 different voltage steps (sweeps).

Execution

As soon as the setup is prepared, the devices switched on, the settings adjusted and the files loaded in the PatchControl software to prepare the solution, the measurement can be executed.
In the beginning, the NPC-1 chip needs to be filled with 5 μl intracellular solution on the bottom, as shown in figure 5.28. The chip has to be screwed tightly on the chip holder to make contact with the internal electrode.
The Faraday lid (Laminar Flow Chamber) has to be placed on the Port-a-Patch recording station and the external electrode should be positioned inside the chamber on top of the chip. One must pipette 5 μl of the external solution onto the chip so that the electrode is immersed in the liquid. [39, 45]

The PatchControl protocol can be started by clicking the play button in the experiment window, as shown in figure 5.30.
The first step in the parameter file is *Wait for contact*, in which the electrical contact between the external and internal solution is checked. A defined pressure is applied to the aperture, which does not mix the external and internal solution, and the chip resistance (that should be between $3 - 5$ $M\Omega$) is read from the electrophysiological software. If the resistance is below the threshold, an electrical contact is achieved and the system proceeds to the next step. In the Oscilloscope window, a square pulse should be displayed that indicates the electrical contact.
The next step in the parameter file is the *Adjust VpOffset*, which, as the name already indicates, adjusts the VpOffset. This can be done automatically or manually, depending on the amplifier.
If the VpOffset is suitable, the Experiment window *Add Cells!* flashes green, as can be seen in figure 5.31. Before the cell suspension is pipetted into the middle of the external solution droplet, the cell composition should be shaken to ensure that the cells do not cluster at the bottom of the vial. Press the button *Add Cells!* and the software adjusts the offset again while suction is applied to the aperture. The next step *Wait for cells* appears and the 5 μl cell suspension should be added. [39, 45]

Figure 5.30.: Push the play button in the experiment window to start the measurement [39]

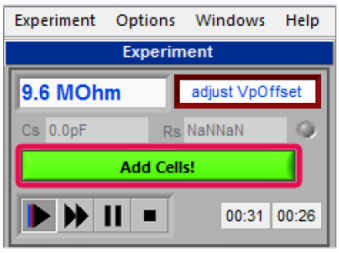

Figure 5.31.: Push *Add Cells!* in the experiment window [39]

Automatic suction is applied that attracts a cell to the patch clamp aperture, which results in an increase of the chip resistance.
If a cell is captured and the adjusted threshold for the chip resistance is reached, the next step is *Wait for seal*.
To enhance the connection between the chip and the cell, a seal enhancer should be added. If the resistance threshold is reached, the software goes on to the next step *Improve Seal* 1 and afterwards automatically proceeds to step *Improve Seal* 2.
If the threshold is reached, the next step is *Wait for whole cell*. In this step, more suction pulses are applied to reach the thresholds for C_*slow* and R_*s*, which are adjusted in the parameter file. In the Oscilloscope window, the whole-cell mode is indicated by the series resistance that increase when the membrane patch breaks.
The last step of PatchControl is *maintain whole cell*, in which the system tries to hold the adjusted holding pressure and potential.
With the help of the perfusion system, the seal solution should be exchanged with the external solution. Before starting the test protocol to measure the currents at different potentials, one should compensate the fast (C_$fast$) and the slow (C_*slow*) capacitance (by pressing the *Auto* button) [39].

The settings of the present test protocol are described in detail in chapter 5.3.6. Figure 5.32 shows the whole setup for this measurement of the planar patch clamp technique from Nanion.

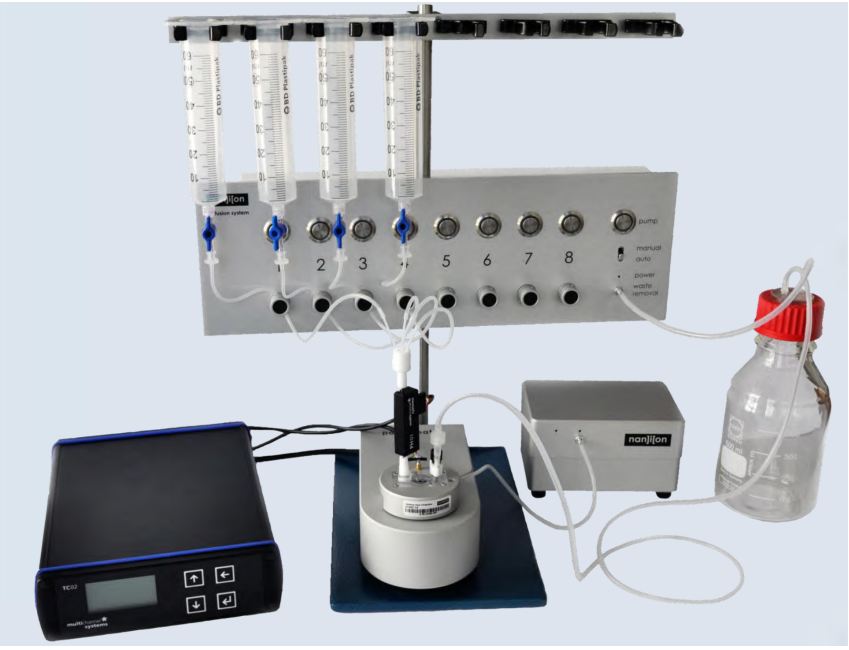

Figure 5.32.: Measurement setup for the planar patch clamping with temperature control [39]

5.3.6. Test protocol

For the present measurements, a previously defined test protocol was used which was created and saved in the PatchMaster software.
The test protocol should include the measurement of the ion current at different voltage steps over a defined time.
In figure 5.33, the structures are represented in detail and defined as follows:

- 0 ms to 100 ms: -80 mV is clamped
- 100 ms to 900 ms: voltages between -60 mV to 60 mV in steps of 10 mV are clamped
- 900 ms to 1 s: -80 mV is clamped

The result of an A549 cell measurement with this test protocol is displayed in figure 5.34. The x-axis shows the time plotted and the y-axis the measured current. The current differences at the first and last 100 ms are not striking because -80 mV is the holding potential of the cell. In contrast, there are current differences between the time sequences of 100 ms and 900 ms as the cell seeks to retain its holding potential and sends ions through the ion channels of the cell membrane – which can be measured in the current flow. The results of the measurement 3E2_4 are represented in figure 5.34, whereby 3E2_4 indicates that this is the third measurement on this day with the second cell and the fourth measurement cycle of said cell.

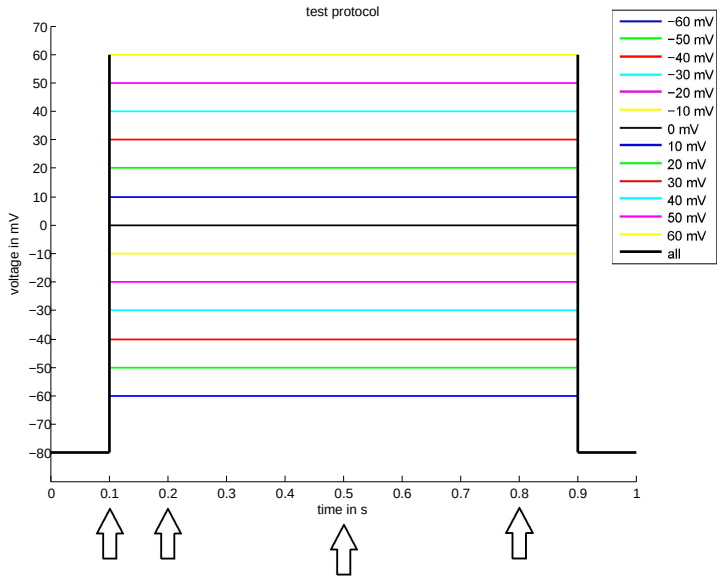

Figure 5.33.: Test protocol, with time points (labelled by arrows) for the current voltage curves

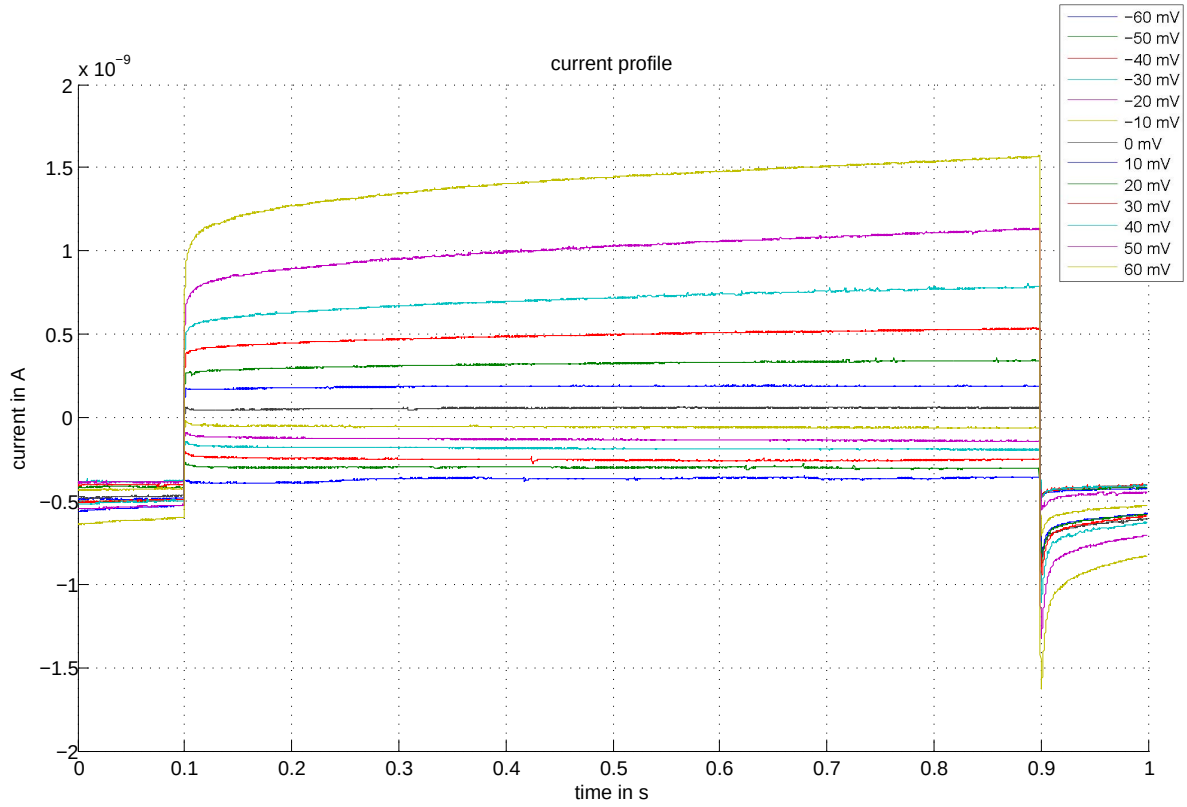

Figure 5.34.: Current profile of an A549 measurement (3E2_4)

For the evaluation of the voltage-activated channel conductance at different temperatures, current voltage curves were plotted.
These I/U curves (represented in chapter 6.2) are constructed with datasets of the patch clamp system and generated with the help of the software MATLAB.
It is necessary to define points in time where the current voltage curves are plotted. Thus, there are four slots: 100 ms, 200 ms, 500 ms and 800 ms, which are marked by arrows, as shown in figure 5.33. The x-axis shows the voltage values plotted, which corresponds to the test protocol, while the y-axis shows the measured current at particular voltages and time values.
The special time at 100 ms, on which the voltage is changed from the holding potential to the distinct voltages, needs to be explained in more detail. Due to the voltage change, capacitive transients caused by the RC circuits occur and produce artefacts [29]. However, this capacitive transients occur shortly before 100 ms are reached, which is demonstrated in figure 5.35 that shows the zoomed out A549 measurement (3E2_4). Therefore, at 100 ms, the artefacts of the RC circuit are already over and the distinct voltages are clamped and can be used for the analysis.

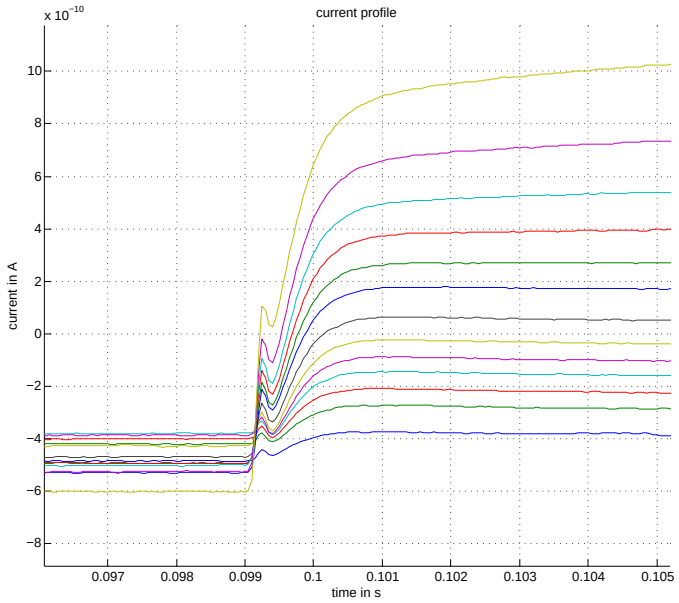

Figure 5.35.: Current profile of an A549 measurement (3E2_4) zoomed to view the capacitive transients shortly before 100 *ms* are reached

6. Results

The results for this master thesis are divided into two parts: a general conclusion of the patch clamp technique and, second, the results of the measurements and the analysis.

6.1. Conclusion of the patch clamp technique

This section explains the outcome of the patch clamp reading, the pre-processing steps and the major problems during the actual planar patch clamp measurements.

Current profile
The output of a whole-cell patch clamp measurement is the current profile which shows the summation of currents of the ion channels at specific voltages. Figure 5.34 illustrates such a current profile of an A549 cell. It can also be seen in the current profile (see figure 5.34) that with each voltage step, the activity of the current running through the ion channel increases. Ion currents that are evoked by positive voltage changes (here 10 mV to 60 mV) can be divided into an instantaneous activating and a slow activating conductance. The major role of the conductance carries the instantaneous current. [37]

The detailed information at the adjusted and measured temperatures, $R_{outside}$ (equivalent with R_{memb}), R_{inside} (equivalent with R_{series}) and the respective measurement are listed in table 6.1. There, $R_{outside}$ represents the seal resistance between the attached cell and the chip, while R_{inside} shows the interior resistance of the measurement (cf. chapter 5.3.2. $R_{outside}$ is defined as R_{leak} and R_{inside} as $R_{pipette} + R_{access}$). The last column of the table below shows the respective measures.

Adjusted temperature	**Measured temperature**	$R_{outside}$	R_{inside}	**Measurement**
$°C$	$°C$	Ω	Ω	
28	27	200 M	40 M	3E2_4
36	38.3	700 M	33.9 M	3E5_7

Table 6.1.: Measurement 3E2_4 and 3E5_7 with adjusted and measured temperatures and measured $R_{outside}$ and R_{inside}

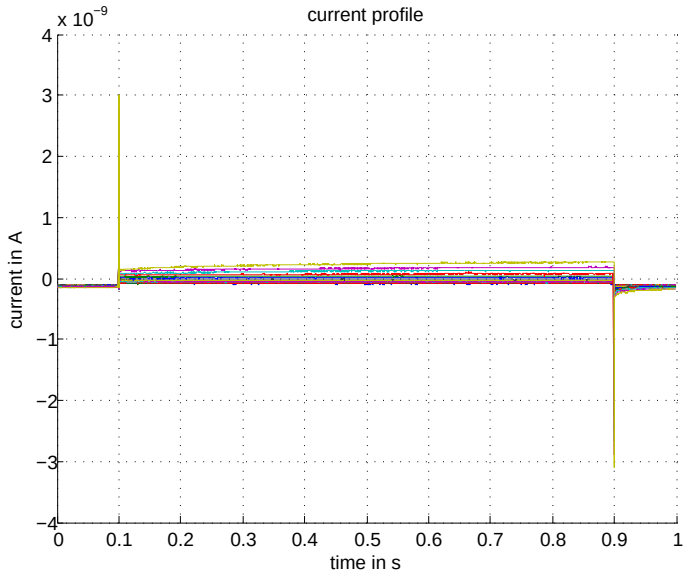

Figure 6.1.: Measurement 3E5_7

Method for preprocessing

The measurements' results to a certain extent include noise disorders.
For example, figure 6.1 shows measurement 3E5_7, (for more information, see table 6.1). The original current profile is depicted as very small due to the peak in positive and negative direction at 100 ms and 900 ms. This peak are by reason of not good compensation of the capacitance before the measurement started. If this current profile is zoomed out, the signal looks as expected but a lot of noise is being illustrated, as can be seen in figure 6.2.
For further processing steps, the profile can be filtered in different ways.
The moving average filter is the common and easiest digital filter regarding the understanding and use in the digital signal processing [46]. This type of filter is often used to smooth the signal and reduce random noise. In detail, it allows to discover essential patterns and filtering unimportant signals such as noise from the measurement [47]. The moving average filter averages the input points in accordance with the sample rate to produce each point in the output signal [46, 47]. The equation looks as follows:

$$y[i] = \frac{1}{M} \sum_{j=0}^{M-1} x[i+j] \tag{6.1}$$

Here, x[ ] is the input signal, y[ ] is the output signal and M is the sample rate indicating the number of points in average. Figure 6.3 shows a moving average filter with a sample rate of 80 being applied to the signal with the help of MATLAB. This sample rate was chosen by the author of this thesis and can be variably adjusted for different uses.

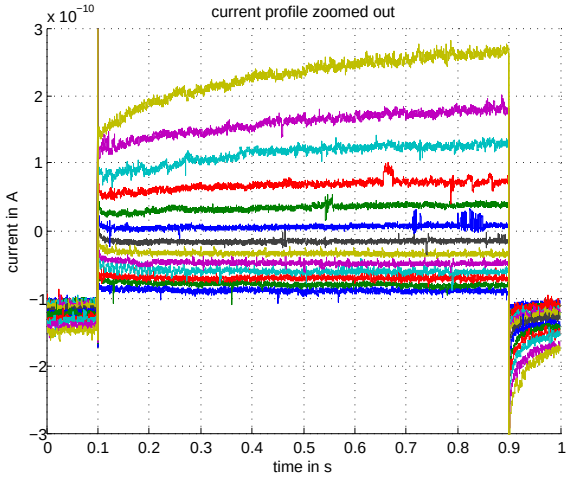

Figure 6.2.: Plot 3E5_7 zoomed

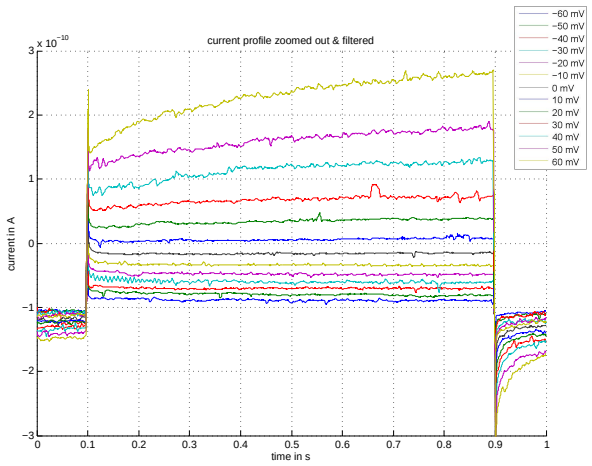

Figure 6.3.: Plot 3E5_7 zoomed and filtered

Main problems during the measurement

During the application of the patch clamp technique, it is important to build a good seal between the cell membrane and the chip. In many of the measurements, the seal was not good which made the connectivity between the membrane and the chip lose its tightness and let ion current pass through this link. If the seal is in a giga ohm range, the connectivity is good and the system measures the sum of the cell's ion current as the whole-cell mode is applied.

During the measurements, it often happened that the system was unable to create a connection between the external and internal electrode. The problem was that the AgCl-layer from the electrode was detached after a few measurements and had to be coated for 20 minutes in an AgCl solution.

Another rather big problem of the patch clamp system is that the chamber in which the cell suspension is located did not reach the adjusted temperature. With the help of an additional thermometer, the real temperature inside the chamber was measured. It seemed that the Temperature Control merely heated the Port-a-Patch recording system to high temperatures but not the solution inside the chamber. When the perfusion was started, the temperature in the chamber rose better to the adjusted value because of the cannula heating system. The adjusted and measured temperature more or less differs at all performed measurements, which can be seen in the tables of the individual execution. However, high temperatures show more distinctions between adjusted and measured temperatures than low temperatures because the system is not able to bring the temperature to higher adjusted values.

Furthermore, the measurements are more physiological only if external solutions run through the system. The problem with the present measurements was that if the chamber was perfused with the external solution, the seal got lost.

6.2. Results of the current voltage curve

In the next sub-chapters, the different current voltage curves of the realised measurement are represented. It should be noted that all results are single measurements.

6.2.1. A549 - different cells

Figures 6.4, 6.5, 6.6 and 6.7 show the current voltage curves at four time points. In table 6.2, more detailed information with regard to these figures can be found.

Adjusted temperature	**Measured temperature**	$R_{outside}$	R_{inside}	**Measurement**
$°C$	$°C$	Ω	Ω	
28	27	200 M	40 M	3E2_4
42	33	400 M	36.1 M	3E4_1
36	37.3	700 M	33.9 M	3E5_9
45	40	430 M	36.1 M	3E4_7
55	47	470 M	33.9 M	3E5_15

Table 6.2.: A549 measurement with different cells with adjusted and measured temperatures and measured $R_{outside}$ and R_{inside}

The striking feature of the above mentioned four figures is that the hypothermia curve at 27 $°C$ shows the maximal changes of the ion current characteristics. At all four points in time, the current at -60 mV is the lowest and the one at 60 mV is highest.
The first section of the curve at 33 $°C$ is in the same range as the body temperature and hyperthermia curve. However, there are more current changes in the last section of all measurements at the green curve.

In contrast, the hyperthermia curve at 47 $°C$ is similar to the elevated body temperature curve at 37.3 $°C$. Furthermore, the curve at 40 $°C$ shows the same properties as the curve at 47 $°C$ and elevated body temperature, only in the last voltage part of the early time stage (0.1 s) it shows a little more current than the other two. Also, only at 60 mV at 0.5 s and 0.8 s is the current value higher.

This is a remarkable result as it suggests that low temperatures have a greater influence on ion channels in A549 lung cancer cells at all times, compared to hyperthermia with higher temperatures. It should, however, be kept in mind that the results of different cells – only 33 $°C$ and 40 $°C$ are the same cells, as are 37.3 $°C$ and 47 $°C$.
Also, it should be considered that the seal between cell and chip is not strong in all measurements, especially not at 27 $°C$.

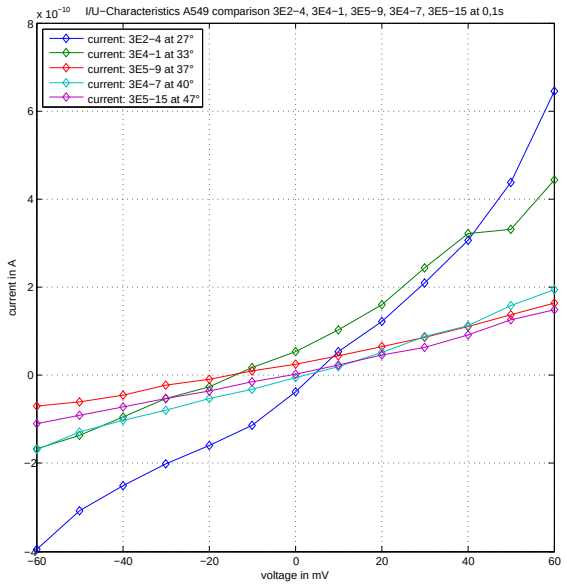

Figure 6.4.: A549 - different cells at 0.1 s

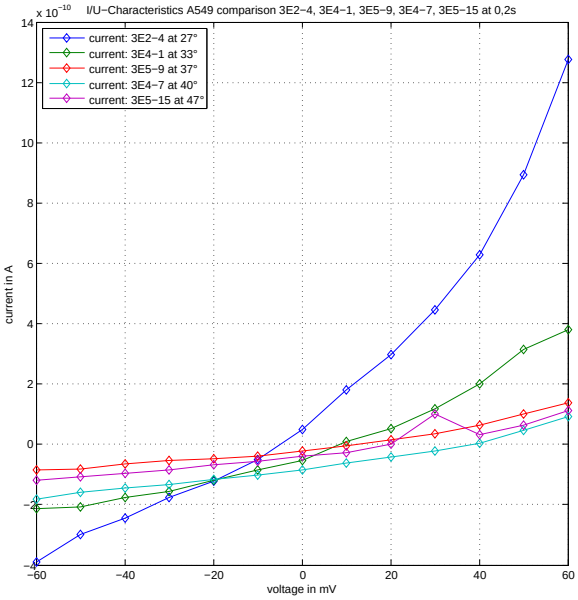

Figure 6.5.: A549 - different cells at 0.2 s

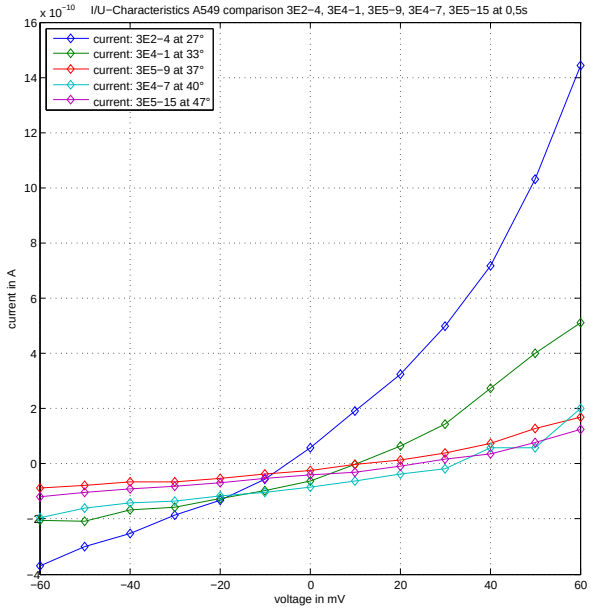

Figure 6.6.: A549 - different cells at 0.5 s

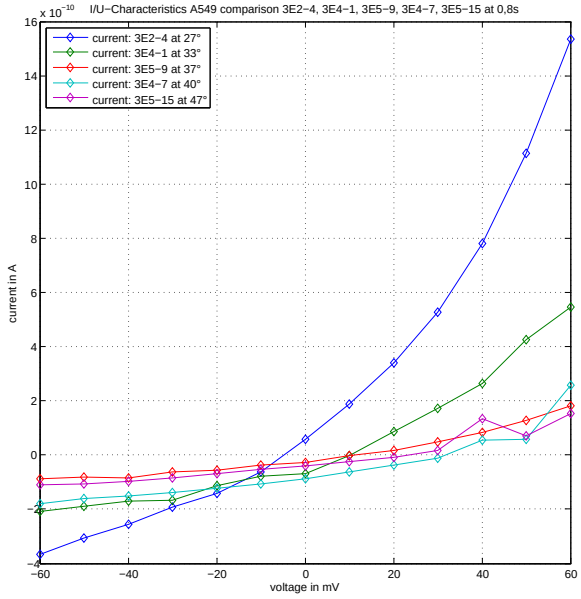

Figure 6.7.: A549 - different cells at 0.8 s

6.2.2. A549 - same cells

It was attempted to compare the same A549 cancer cells at different temperatures.

Due to the problems with the patch clamp system, it was immensely difficult to perform a measurement with a cell from hypothermia to hyperthermia. Mostly, the cell ruptured before it could be exposed to high temperatures, the measurements had to be terminated and a new one with another cell had to be started.
However, one measurement could be sufficiently executed in all temperature ranges, which is therefore represented in this subsection.

Figures 6.8, 6.9, 6.10 and 6.11 show the current voltage curves at different time points over the voltage pulse length. Table 6.3 again lists detailed information on the figures.

Adjusted temperature	**Measured temperature**	$R_{outside}$	R_{inside}	**Measurement**
$°C$	$°C$	Ω	Ω	
30	28.5	340 M	36.5 M	2E2_5
40	34	500 M	36.5 M	2E2_17
45	40	450 M	36.5 M	3E2_18

Table 6.3.: A549 measurement with the same cell with adjusted and measured temperatures and measured $R_{outside}$ and R_{inside}

In early stages at 0.1 s and 0.2 s, the measurement results were basically similar to the results of different A549 cells. The hypothermia at approximately 28.5 $°C$ shows the most current changes with regard to all times, which also applies to different cells. The difference is that the current change of 28.5 $°C$ is not as high as in measurement 3E2_4 at 27 $°C$. These results can be ascribed to different reasons, e.g. the bad seal or no perfusion with the external solution. It is necessary to execute more measurements to better control the outputs.

The difference between hypothermia and hyperthermia is not clear in the figures. Only at an early stage (0.1 s) does hypothermia show higher ion currents at higher voltage changes. At all other times, hypothermia shows almost as low ion currents as hyperthermia. The peak at the 40 $°C$ hyperthermia curve at 0.8 s in figure 6.11 may be caused by a measurement failure.

If considering the curves at 40 $°C$ and 34 $°C$ at 0.1 s and 0.2 s, this seems to repeat itself: only some parts of the curve differ, albeit only slightly. At 0.5 s and 0.8 s and in the last part with approx. 20 mV, the current is clearly different from the others. Thereby, the hyperthermia curve receives higher currents than the low body temperature curve.

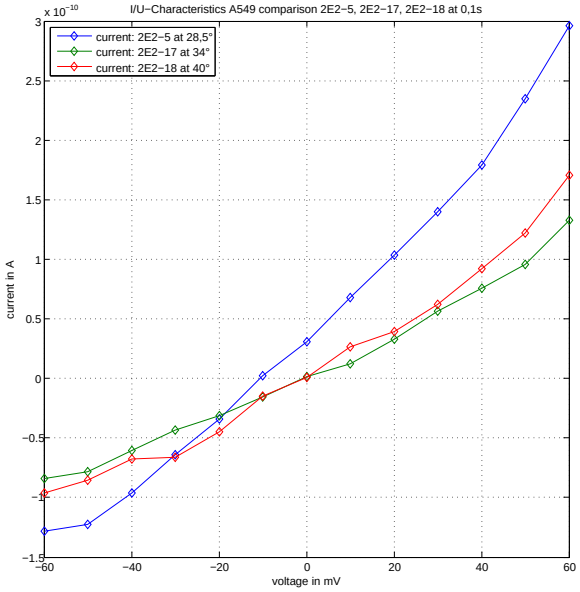

Figure 6.8.: A549 - same cell at 0.1 s

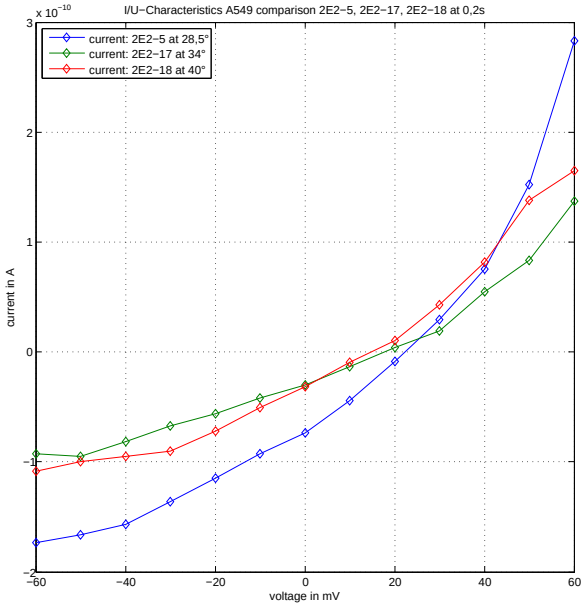

Figure 6.9.: A549 - same cell at 0.2 s

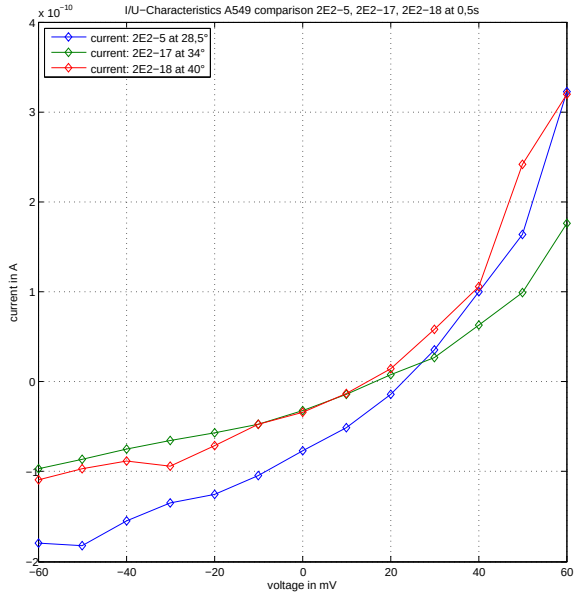

Figure 6.10.: A549 - same cell at 0.5 s

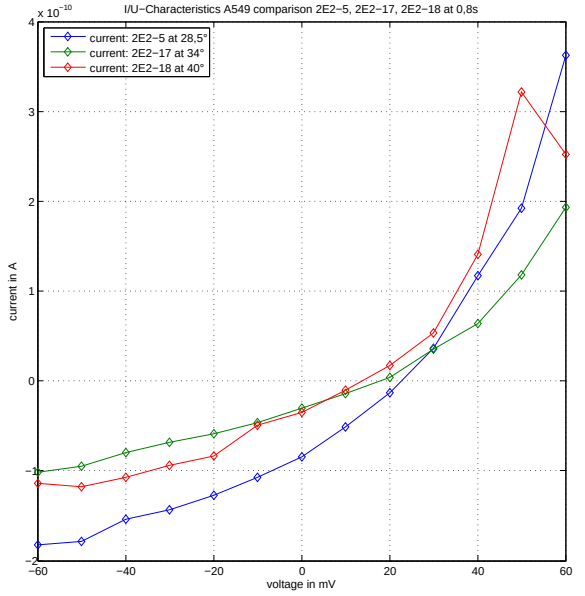

Figure 6.11.: A549 - same cell at 0.8 s

6.2.3. H1299

Figures 6.12, 6.13, 6.14 and 6.15 show the current voltage curves of H1299 cells at different points in time. In table 6.4, detailed information on the figures is represented.

It should be noted that these current curves were executed with different cells as it was not possible to execute measurements with the same cells from low to high temperature. However, the seals of the H1299 measurements are very tight in comparison to those of the A549 measurements. At the measurement E2_8, the cell was perfused through the whole measurement with seal solution, hence the good seal. At the measurements E2_14, E4_6 and E4_7 shortly before the experiment started, the seal solution runs through the chamber. Only at measurement E3_1, no perfusion was started.

Adjusted temperature	**Measured temperature**	$R_{outside}$	R_{inside}	**Measurement**
$°C$	$°C$	Ω	Ω	
25	27	1.3 G	37.4 M	E3_1
36	31.5	780 M	19.7 M	E4_6
40	34	1.1 G	19.7 M	E4_7
45	38	3 G	35.2 M	E2_8
55	44	1 G	35.2 M	E2_14

Table 6.4.: H1299 cells with adjusted and measured temperatures and measured $R_{outside}$ and R_{inside}

The hypothermia and hyperthermia results of the H1299 cell line are different from the A549 cell line.
H1299 cells react almost in the same way at both low and high temperatures except in the very early phase (0.1 s). At all times, apart from 0.1 s at -60 mV until approximately 10 mV, the current of low temperatures is lower than at higher temperatures and from 0 mV, all temperature curves are almost in the same area, excluding at 38 $°C$. The slightly elevated body temperature almost shows the same and not so high current changes.
The result at 0.1 s of the current voltage curves differs from the other times. In this phase, the curves' behaviour is different at various temperatures: hypothermia has the lowest current at 27 $°C$. This figure also shows that the highest ion current can be found at 31 $°C$ and 34 $°C$. Hyperthermia at 44 $°C$ is similar to a slightly elevated body temperature at 38 $°C$. It could also be observed that the low hypothermia curve (blue) at 0.1 s does not have as big current changes as the other times.

The main conclusion that can be drawn from the information above is that the early phase shows more current changes than the late phase of the current voltage curves. As stated before, it is also necessary here to execute more measurements to confirm the results.

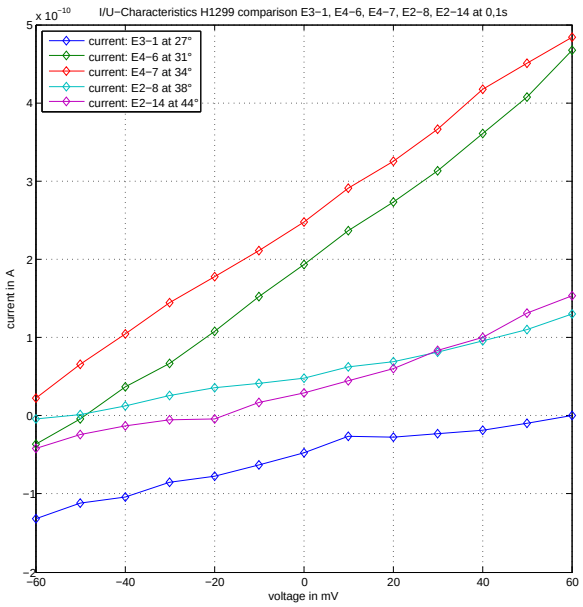

Figure 6.12.: H1299 cells at 0.1 s

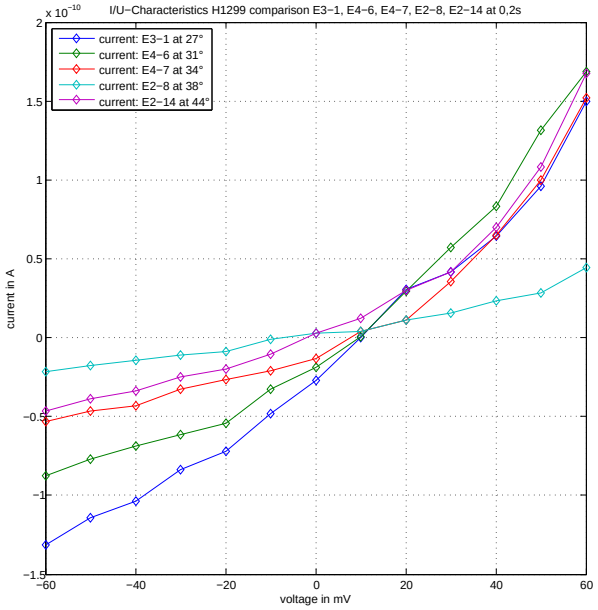

Figure 6.13.: H1299 cells at 0.2 s

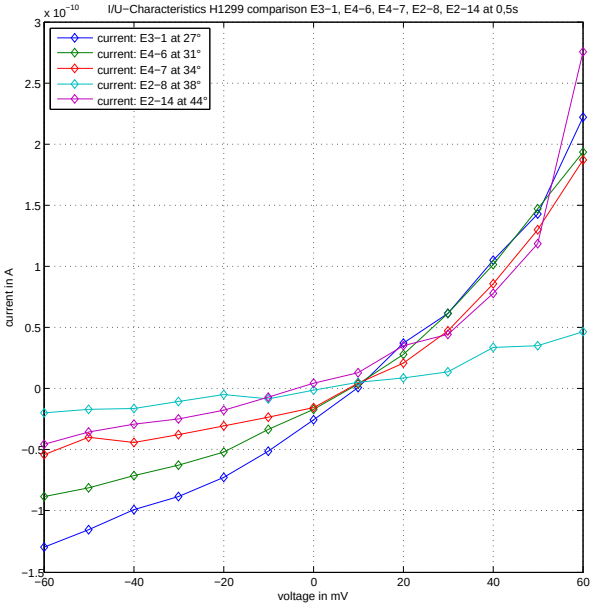

Figure 6.14.: H1299 cells at 0.5 s

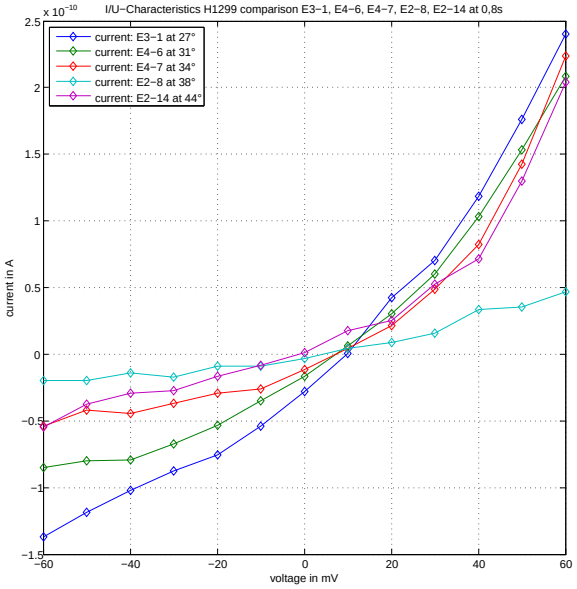

Figure 6.15.: H1299 cells at 0.8 s

6.2.4. Differentiation of A549 with H1299

In the following subsection, the difference between the various cell lines A549 and H1299 is represented.
Figures 6.16, 6.17, 6.18 and 6.19 illustrate the current voltage curves at different times and table 6.5 again further details the figures' information. In this measurement, the cells are not the same and the seal of the H1299 cells is tighter than that of the A549 cells.

Adjusted temperature	**Measured temperature**	$R_{outside}$	R_{inside}	**Measurement**
$°C$	$°C$	Ω	Ω	
30	28.5	340 M	36.5 M	A549 2E2_5
25	27	1.3 G	37.4 M	H1299 1E3_1
50	44.5	700 M	33.9 M	A549 3E5_12
55	44	1 G	35.2 M	H1299 1E2_14

Table 6.5.: H1299 and A549 cells with adjusted and measured temperatures and measured $R_{outside}$ and R_{inside}

At all points in time, the graphs show that A549 cells at low temperatures (in this case 28.5 $°C$) have the lowest current in the first part and the highest current in the last part of the measurements. Especially at 0.1 s, the ion current of the 28 $°C$ A549 cell is much higher in the last section than the one of the H1299 hypothermia cell. As shown in figure 6.12, H1299 cells demonstrate a clear increase of the current at low temperatures at 0.2 s, 0.5 s and 0.8 s, while at 0.1 s, it rises only slightly. In addition, it was observed that the A549 cell's current in hypothermia (at 0.2 s, 0.5 s and 0.8 s, and in the first part of the measurement) is clearly below the current of the hypothermia H1299 cell. In the last section of the figures, the current of the A549 cell rises above that of the H1299 cell. At 0.1 s, the current of the H1299 cell at the beginning is a little bit higher, while at -50 mV that of the A549 cell is lower; however, at all other voltage values, the current of the A549 cell is much higher.
When investigating hyperthermia – here at 44 $°C$ with A549 and H1299 cells – it can be seen that their currents rise in line with the voltage. Also, the current of H1299 cells is higher than that of the A549 cell. Only at 0.5 s at 50 mV, is the current of H1299 cells a little bit lower than that of the A549 cell.

The difference of A549 and H1299 cells at hypothermia and hyperthermia can be interpreted in such a way that at hyperthermia, the two cell lines work in the same way – with the only difference that the H1299 cell transports more ions through the channel which results in a higher current curve. At hypothermia, the two cell lines work almost in the same way at 0.2 s, 0.5 s and 0.8 s but again, the ion current of H1299 is higher at the first voltage steps. At 0.1 s, the A549 cells show more ion flowing through the channel than H1299 cells.

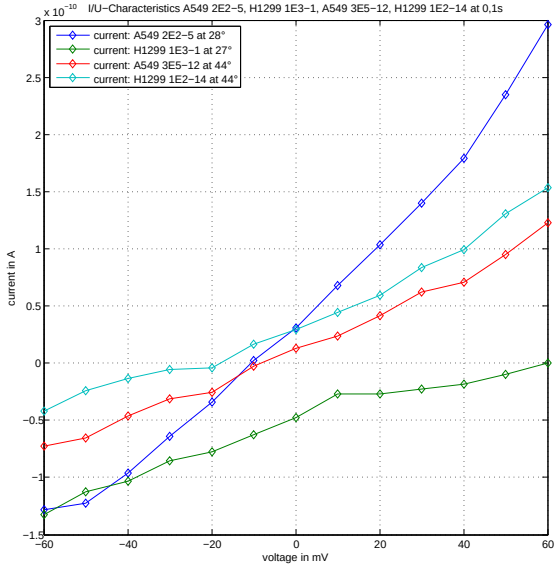

Figure 6.16.: Differentiation with H1299 and A549 cells at 0.1 s

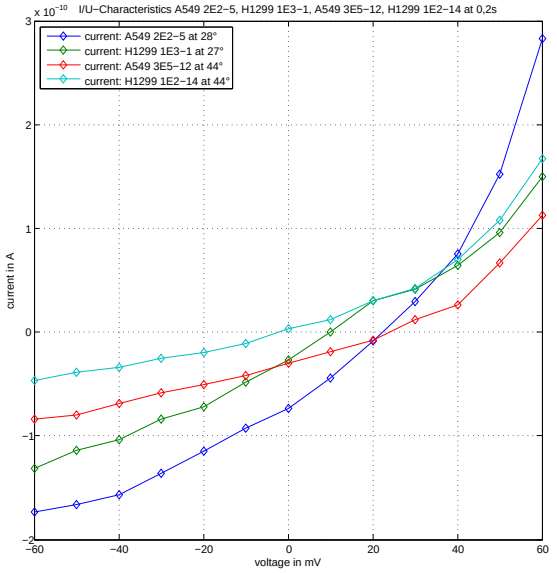

Figure 6.17.: Differentiation with H1299 and A549 cells at 0.2 s

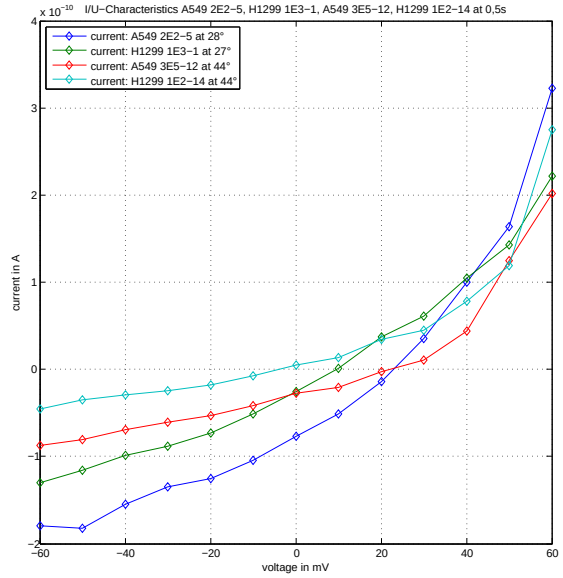

Figure 6.18.: Differentiation with H1299 and A549 cells at 0.5 s

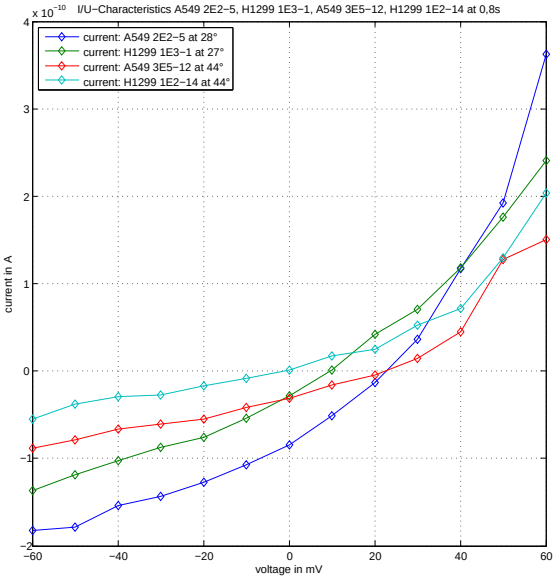

Figure 6.19.: Differentiation with H1299 and A549 cells at 0.8 s

7. Discussion and conclusion

The most important results of this master thesis need to be discussed in more detail, which is the purpose of the present chapter. Some of the aspects related to the measurements with the automated planar patch clamp method need to be revised before more measurements can be executed.

First of all, it should be discussed why four points in time were chosen for analysing the results. In fact, this allowed to determine more precisely what happened at a very early stage, after some time elapsed and one last time before the measurement was finished. Therefore, two measurement points at an early stage (0.1 s and 0.2 s), one at an intermediate stage (0.5 s) and one at a later stage (0.8 s) were chosen.
It becomes clear in the results, that there is little difference between the time points 0.2 s, 0.5 s and 0.8 s. However, the very early stage, especially with regard to H1299 cells, shows a different result: indeed, the current voltage curves at different temperatures show a different behaviour when compared to the other points in time.
It should be noted that it was at 0.1 s where the distinct voltages were clamped, which is why the voltage from the holding potential changes to the clamped voltage. The latter is reached at 0.1 s, which can be seen in figure 5.35. There is no capacitive leakage at this point in time and it can thus be concluded that the ion channels of H1299 cells in a very early stage react differently than that of A549 cells. The reason for this may be that there are different ion channels in H1299 and A549 cells addressed with the voltage changes. Thus, H1299 cells need more time to respond to the voltage changes while the ion channels in A549 cells react faster. If the other times are considered as well, it shows that the voltages are already clamped before, which is why the current voltage curves do not differ much.
Furthermore, the current profile of the A549 measurements evoked two characteristic current conductance at positive voltages. The first was an instantaneous activating of ion channels in the very early state, and the second a slow activating conductance, which indicates the slow current increase until the voltage changes to the holding potential. [37]

It should be stated that more measurements were in fact executed for this master thesis than are represented in chapter 6.2.
For the analysis, however, only those measurements were used that showed a reasonably good seal resistance and a current profile with a uniform curve at each voltage value. There is no fixed value for the seal resistance (R_memb), but it should be in an acceptable relation with the series resistance (R_s). This means that the R_memb should be approximately four times higher than the series resistance, whereby R_s represents the sum of the access resistance and the pipette resistance, and should not be higher than 50 $M\Omega$.

The current profile without the uniform curve indicates that the Port-a-Patch unit may have either captured a dust particle instead of a cell, or – if measurements had already been done – it may indicate that the cell was destroyed, no more measurement could be executed with this cell and new ones had to be started.
However, this gives rise to the problem that if a measurement from low hypothermia to high hyperthermia is required for the analysis, the behaviour of the new catched cell can differ from that of the destroyed cell.
The conventional patch clamp technique has some advantages because, first, the user himself attaches the pipette to the cell and, second, with the high quality microscope, they can assure that a cell and not a dust particle is attached. The planar patch clamp technique, in contrast, automatically captures the cell and the user cannot be sure whether it is indeed a cell or rather a dust particle.
The measurements show that the greatest differences between them can be found with hypothermia of A549 cells, as shown in chapters 6.2.1 and 6.2.2. The A549 measurement 3E2_4 (chapter 6.2.1) at 27 $°C$ shows large changes in the current up to the point of 1.53 nA in contrast to the measurement 2E2_5 (chapter 6.2.2) at 28.5 $°C$, where only currents in the same range of hyperthermia curves up to 0.362 nA are depicted. It should, however, be noted that the seals in both cases were not as tight as desired. The results show that more measurements are necessary to be able to prepare a more precise analysis.
Furthermore, the measurement results at higher temperatures differ from each other, even if the variations are not as high as those at low temperatures.
It should be noted here that these results were not expected – especially not that connected to hypothermia. Due to the information presented in the theoretical part of the thesis, it was expected that ion channels react to high rather than low temperatures.

A further important aspect is the seal between the cell membrane and the chip. In fact, its resistance should be in a $G\Omega$ range and not as low as in the present measurements. Therefore, it should be ensured – as mentioned above – that the seal resistance R_{memb} is expected to be four times higher than the series resistance R_s. It must also be considered that R_s should not be higher than 50 $M\Omega$. To be able to objectively analyse the measurements, it is also necessary that the seal resistance does not vary, which means that the relation between the seal resistance and the series resistance needs to be fulfilled – which was, unfortunately, another problem faced in the measurements for the thesis in hand.

The next factor refers to the perfusion of the planar patch clamp system. In the present case, however, there were some problems: normally, the seal enhancer should initially establish a high resistance seal between the cell membrane and the aperture of the chip with the external solution subsequently representing the extracellular environment, thus forming the solution with which the measurement can be executed.
However, the difficulties of the present measurements were that if the perfusion of the external solution was started, the seal would be lost, resulting in the loss of the entire cell and, further, the termination of the measurement.

Therefore, some of the measurements were carried out with the seal solution even though this affected the measurements' outcome. The H1299 measurement E2_8 was executed with the perfusion of the seal enhancer during the whole experiment – this explains why the seal resistance is very high compared to the other measurements. In the H1299 measurements E2_14, E4_6 and E4_7, the perfusion of the seal enhancer was started shortly before the measurements were executed to bring the temperature to the desired value. No A549 measurement used in the analysis was executed with a perfusion during or before the experiment.

Another aspect is the preparation of the cell suspension. In fact, the company Nanion attaches great importance to the procedures and conditions for the cell culture. It needs great expertise to prepare the cell culture in such a way that every cell is in a stable condition. When using the conventional patch clamp technique, it is not so important that all cells are in a stable state because the operators select a cell with the help of the microscope. When working with the automated planar patch clamp technique, all cells should be in a stable condition because it is the system that chooses the cell. [39]

In the present measurements, the cell suspensions from ZMF Graz were used, that did not require additional preparation. Thereby, attention was paid to storing the sample vessels with the entire cell suspension in an incubator at 37 $°C$ and exchanging some cell suspension for the measurement in smaller sample vessels. In addition, the cell suspension in the incubator was frequently shaken so that the cells did not cluster at the bottom of the sample vessels.
It should be noted that the sample vessels with some of the cell suspension were kept at room temperature during a few measurements instead of in the incubator throughout the entire measurement.

Another essential aspect that emerged when comparing the results was that it is necessary to execute the measurements under completely equal conditions.
This means that the same cells should primarily be used throughout the whole measurement, i.e. from low to high hyperthermia. For this to work, the problems with the patch clamp system need to be solved to enable testing with the same cells in higher temperatures. Thus, the planar patch clamp system needs to be improved, particularly with regard to the seals stability and the temperature control.

On 14.11.2016, measurements of the A549 cells were also carried out with the planar patch clamp system but there arose even larger problems with this system.
First of all, the desired seal between the aperture of the chip and the cell membrane, which should be four times higher than the series resistance could not be reached at any of the measurements. The highest value could be reached at the beginning of this measurement day and measured 500 $M\Omega$ for the seal resistance and 35.6 $M\Omega$ for the series resistance at room temperature.

In the course of the day, the seal resistance worsened and, in the end, seals in the range of only 100 $M\Omega$ remained, which was too little to obtain results for the analysis. It was curious that the series resistance R_s was very high with approx. 100 $M\Omega$ in comparison to earlier measurements. The reason for this could be e.g. that the cell could not be ruptured or, instead of a cell, something else was captured to the aperture.
The next problem this day was the temperature: this should be measured with one cell from low to high hyperthermia. However, this was impossible because the cell died before the temperature could be elevated. For example, one measurement was executed with 39 $°C$ but at there, the series resistance was 24.4 $M\Omega$ and the seal resistance was only 71 $M\Omega$, which was too little.
It was also attempted to perfuse the cell with extracellular solution instead of seal enhancer to try and obtain a result, which could be used for the analysis. However, if the extracellular solution was used, at every measurement cycle in which the temperature increases to a higher value, the seal resistance decreases. After each measurement cycle, the seal resistance became worse until the cell died. Only one measurement could be used for the analysis: it had a seal resistance of 450 $M\Omega$, a series resistance of 35.6 $M\Omega$ and shows currents at 0.8 s up to 0.72 nA at room temperature (approximately 21 $°C$). This is similar to the A549 measurements conducted at the first measurement series.
It should be noted that these cells of the second measurement series had a passage number of 6 and the cells of the first measurement series one of 50, which is indeed problematic when comparing the two cells. Because of all these problems, no data analysis was possible for the first and second measurement series with A549 cells.

Finally, it should be noted that the measurements with the planar patch clamp technique were single measurements, which makes them slightly less significant. For a statistical analysis, more investigations would be necessary to confirm the findings.
All of the aforementioned aspects need to be clarified and solved before executing further experiments with this system. It is difficult to propose a solution because there is not only one problem. Certainly, when executing measurements with temperature control, it needs revisions of the laminar flow chamber so that the cell suspension can be heated. Also, it is necessary to pay more attention to the cell suspension and make sure that the cell preparation is in good condition. Working with cells in a stable state might already greatly improve the measurement results.
Afterwards, additional experiments on lung cancer cells in hyperthermia and hypothermia can – and should be – performed because this seems important with regard to the research of further therapy methods to treat cancer.

In order to discuss confirmed results, it should be referred to the article *Fever-Range Hyperthermia vs. Hypothermia* [48] that examines the effects of fever-range hyperthermia and mild hypothermia on different human cancer cells by concentrating on cell viability and proliferation. It investigates the following cells: A549 and H1299 lung carcinoma, MCF7 breast adenocarcinoma, U87MG and T98G glioblastoma, DU145 and PC3 prostate carcinoma and MRC5 normal fetal lung fibroblast cell lines.
The results of the viability/proliferation experiments illustrate that MRC5 fibroblasts were extremely sensitive to hyperthermia and mostly resistant to hypothermia. T98G and A549 were thermo-tolerant (at temperatures of approx. 40 $°C$), and the remaining were thermo-sensitive to a different extent. Importantly, a universal effect was that hypothermia at 34 $°C$ reduced the viability/proliferation in all cell lines. The article concluded that the existence of thermo-tolerant and thermo-sensitive cancer cell lines can indeed be confirmed and that mild hypothermia had a negative effect on cancer cell proliferation. [48]

In comparison to the present measurement, which investigated the ion flow through the ion channel at different temperatures, it can be stated that A549 cells are also more or less thermo-tolerant. However, due to the fact that the hypothermia effect on A549 cells is not as clear as desired, more measurements are required for a precise statement. Also, the H1299 cells of the present tests indicated that they were almost equally sensitive to low and high temperatures.

Summing up, it can be stated that the patch clamp method is a highly useful technique to investigate the ion current characteristics at different temperatures. For a statistical analysis, it needs more experiments with similar conditions, especially if measurements are carried out at different temperatures.
Concluding, it remains without doubt that hyperthermia is a method with huge potential when it comes to the therapy of cancer. Thus, this field definitely deserves more research in order to discover more possibilities for using high temperatures for therapeutical purposes in oncology.
Besides hyperthermia – due to the astonishing and unexpected results at low temperatures – hypothermia may also have a significant effect on the electrophysiological behaviour of cancer cells and it would doubtlessly deserve to be investigated in more detail so as to be able to study and possibly even discover new methods for treating cancer.

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A. Glossar

apoptosis	genetically programmed cell death [49]
asphyxia	respiratory arrest, i.e. suffocation due to oxygen depletion of the blood [49]
atherosclerosis	degenerative disease of the arteries by reason of thickening of arterial walls caused by deposit of fatty materials [25]
cardiac arrhythmia	irregular heartbeat [49]
cerebral edema	accumulation of fluid in the brain leads to the swelling of the brain [25]
chronic obstructive pulmonary disease (COPD)	chronic, progressive lung disease which is characterised by poor airflow in the lungs and especially impeded breathing during exhalation [25]
cytokine	intracellular protein signalling molecules involved in cell regulation and proliferation [25]
cytology	study of cells, including their origin, structure, physiology, pathology etc. [25]
free radicals	a highly chemically active groups of atoms that are able to freely exist under certain conditions for a very short time. Composite with at least one unpaired electron which makes them highly reactive [25]
glioblastoma	malignant tumour located in the cerebrum [49]
hematogenous	originating from the blood [49]
hypoxic-ischemic encephalopathy	disorders or diseases of the brain due to severely reduced oxygen intake [25]
ischemia	lack of blood supply of some organs [49]
lymphomas	inflammation of the lymph nodes [49]
necrosis	cell injury which leads to early cell death in tissue [49]
pleomorphic	two or more forms, e.g. of an organism during lifetime or cells of a certain type [25]

point mutations	alterations at one point in the DNA sequence [2]
proliferation	amplification of tissue due to increase of cells [49]
thrombin	important enzyme for blood coagulation [49]
thrombocytopenia	an abnormally low level of thrombocytes in the bloodstream [25]

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