

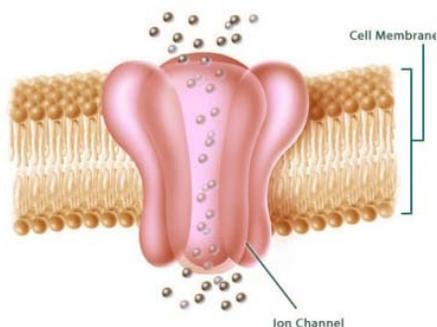
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Effect of membrane composition on temperature activation of TRPV1

Master of Science Thesis

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THESIS FOR THE DEGREE OF MASTER OF SCIENCE

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Abstract

The cholesterol content of the cell membrane shows an important role for physical properties of the membrane and also affects the functions of some ion channels. This thesis focuses on how the cholesterol content of the cell membrane effects the temperature activation of the TRPV1 ion channel. TRPV1 is a temperature activated ion channel, which acts as a heat pain sensor in peripheral nervous system and also plays an important role in inflammatory pain sensing (heat hyperalgesia).

Cholesterol content of the cell membrane was manipulated by using methylated β -cyclodextrins (either cholesterol depletion or enrichment). Whole cell patch clamping was used to record the current response of the ion channels. The experiments were carried out in a microfluidic chip which can create a patterned laminar flow for cell perfusion, and the chip was prefabricated with a thin gold layer onto the glass on the back side of the chip, so that the gold electrode can controllably heat the solution flowing out of the chip channel by applying certain voltage feedback controlled by a thermo-probe integrated system.

Results showed that untreated cells were activated at about 42°C which confirmed the results from other studies, whereas the activation temperatures of cholesterol depleted and enriched cells were both significantly increased. This suggests that the effect of cholesterol on temperature activation of TRPV1 ion channel either might be direct or indirect, i.e. that cholesterol changed the membrane physical properties which affected the ion channel or the activation temperature shift was due to direct cholesterol-protein interaction.

Key words: ion channels, TRPV1, cholesterol, temperature activation, cyclodextrins, thermo-control, patch clamp

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1. Introduction

The cell membrane isolates the intracellular environment from the extracellular milieu [1]. The phospholipid bilayer is an effective barrier for the movement of the charged particles, but this electrical insulation is not perfect: there are ion channels, transporters and there is also some leakage [1]. Ion channels can open under different stimuli to allow certain ions passing through the plasma membrane, and this profile of the ion channels gives them diverse functions: for the cells, ion channels are important sensor for both intra- and extra- cellular stimuli, and also related with cell proliferation, the control of salt and water balance [2] and even cell apoptosis [3]. For multicellular organisms, ion channels govern the electrical signaling in nerves, muscles and synapses [4], and are related with hormonal secretion, learning, memory and the regulation of blood pressure as well [2].

The transient receptor potential vanilloid 1 (TRPV1) ion channel is one member of the ion channels acting as temperature sensors. It is predominantly expressed in small diameter dorsal root ganglia (DRG) and trigeminal ganglia (TG) neurons [5]. The TRPV1 ion channel is a heat pain sensor activated approximately at 42°C or 43°C [6-7], and it plays an important role in inflammatory pain sensing (heat hyperalgesia) [8]. It is one of the most interesting ion channels in pharmaceutical targeting [9]. But the exact mechanism of temperature activation of TRPV1 is not known. This study may provide more information about the activation profile of TRPV1 to understand the temperature activation mechanism.

Cholesterol, one of the major components of the cell membrane, shows an important role on physical properties of the membrane [10] and also affects the functions of some ion channels [11]. Cholesterol content of the cell membrane can be manipulated with cyclic oligosaccharides called Cyclodextrins (CDs) [12]. The special cyclic structure and amphiphilic property enable cyclodextrins to either remove cholesterol from the cell membrane or enrich the membrane with cholesterol [13].

This study focuses on how the cholesterol content of the cell membrane can affect the temperature sensing properties of TRPV1 ion channel. A microfluidic chip, which can create a patterned laminar flow, is used, and a thin gold layer is fabricated onto the glass on the back side of the chip. The fabricated microfluidic chip combined with a temperature probe composes a thermo-control system, where the local temperature can be measured and controlled. Whole cell patch clamp recording is carried out in the thermo-control system to study the temperature activation

profile of the cells with different membrane cholesterol content (untreated, depleted and enriched). Results show significant increases of activation temperature both in the cholesterol depleted group and the cholesterol enriched group.

2. Theory

2.1 Electrical properties of the cell membrane

All living cells are enveloped by a plasma membrane that acts as a barrier between the intracellular and the extracellular environment [1]. The main constituents of cell membranes are phospholipids, which contain both hydrophobic and hydrophilic (polarized) residues. The amphiphilic property of phospholipid determines the bilayer structure of membrane by composing hydrophobic environment between two layers with hydrophobic residues and with polar group encountering the aqueous phase [1, 14]. The cell membrane also contains proteins, which may be integrated with the lipid bilayer or may simply be associated with cell membrane. The phospholipid bilayer forms a particularly effective barrier to charged molecules [1, 14]. So the membrane and the intracellular and extracellular media, from an electrophysical perspective, form a capacitor [1]. A capacitor can store charge, and the charges on either side of the plasma membrane are not in balance; the inside of all cells at rest is more negatively charged than the outside, and the difference causes an electrical potential over the membrane. The extracellular medium is the reference for the potentials in a cell system, so resting membrane potentials are negative[1]. Concentration differences (higher K^+ and lower Na^+ and Ca^{2+} in the cell) of ions between intra- and extra-cellular milieu result in concentration gradients for each ion across the membrane. Concentration gradients induce the diffusion of particles from higher to lower concentration. Thermodynamically, diffusion is a spontaneous process because it decreases the order in a system (increases entropy). This implies that diffusion releases energy. Walther Hermann Nernst (1864-1941) quantified this energy as

$$\Delta G = -RT \ln \frac{[ion]_o}{[ion]_i} \quad (1)$$

where ΔG is the Gibbs energy released by diffusion, R is the universal gas constant ($8.314 \text{ Jmol}^{-1}\text{K}^{-1}$), T is the temperature in Kelvin and $[ion]_o$ and $[ion]_i$ are the extracellular and intracellular concentration of the ion considered, respectively [1]. So if the cell membrane is permeable to potassium ions, K^+ ions will flow out of the cell and the outflow of positively charged ions will

increase the negative charge of the cell. Increased negative charge will start to attract the K^+ ions back into the cell. The electrical energy of this can be quantified as

$$\Delta G = -EzF \quad (2)$$

where E is the electrical potential across the membrane unit, z is the oxidation state of the ion under consideration and F is the Faraday constant ($9.65 \times 10^4 \text{ C mol}^{-1}$). Increased negative charge due to the diffusion of the potassium from the cell will increase the electrical attraction energy to K^+ . When the attraction energy is oppositely equal to the diffusion energy, there is no net movement of ions and the two energies are in equilibrium

$$-RT \ln \frac{[ion]_o}{[ion]_i} = -EzF \quad (3)$$

This relation can be rearranged to describe the equilibrium potential of the ion considered, and this is the all-important Nernst equation

$$E = \frac{RT}{zF} \ln \frac{[ion]_o}{[ion]_i} \quad (4)$$

where E is the equilibrium potential for the ion under consideration. Equilibrium potentials of all the ions, together with their relative permeabilities contribute to the cell membrane potential, and that is typically -50 to -80 mV at resting state [1].

The phospholipid bilayer is an effective barrier for the charged particles, thus the cell membrane is an insulator between two conductors (intra and extracellular watery salt solutions which are very conductive to ions). But this electrical insulation is not perfect: there are ion channels, transporters and there is also some leakage. So the resistance of the cell membrane to the movement of ions across is finite. The flow of ions through the cell membrane, which is current, is determined by

driving force and membrane resistance. The driving force is the difference between the equilibrium potential and the membrane potential E_m . So the bigger the driving force, the greater the net flow of ions. Thus, current is proportional to the driving force. The current is limited by the resistance of the membrane. In the other words, the current is inversely proportional to the resistance. This can be stated as

$$I_{leak} = \frac{E_m - E_{rm}}{R_{leak}} \quad (5)$$

When the ion channels are activated, I_{leak} is the current through the channels, $E_m - E_{rm}$ is the deviation from the resting membrane potential, R_{leak} is the channel resistance for the ions passing through [1].

2.2 Ion channels

Ion channels are pore forming proteins in the plasma membrane, that open and close upon different stimuli (Figure 1), and ion channels are crucial part of the cell membrane [15]. Most animal cells usually have relatively lower intracellular sodium concentration, while the potassium

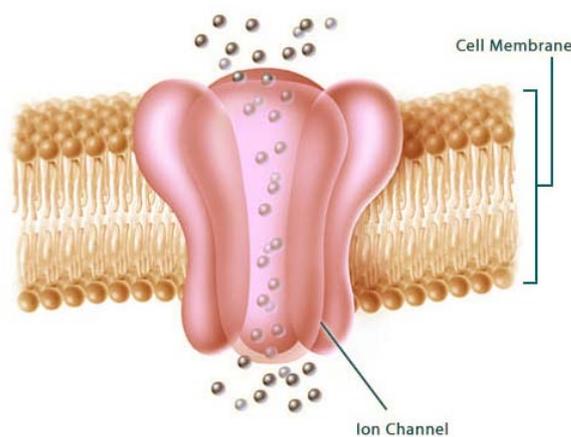


Figure1. Basic structure of an ion channel.

concentration is higher than in the extracellular milieu. The plasma membrane is an active barrier to separate the cell contents from the outside, so that ionic concentrations difference can be maintained between intra and extra cellular environment. This leads to an ionic concentration gradient across the plasma membrane for each ion, and also causes an electrical potential difference between the cytoplasm and the external medium. When the ion channels open, ions will flow down the concentration gradients in or out of the cell. This activity of ion channels will change the ionic concentration level difference, and also change the electrical potential. This membrane potential and the electrochemical gradients are substantially used by cells in their signaling and control system [15].

Ion channels are found in the membranes of all animal, plant and bacterial cells and play important roles in such diverse processes as nerve and muscle excitation, hormonal secretion, learning and memory, cell proliferation, sensory transduction, the control of salt and water balance and the regulation of blood pressure [2]. Ion channels also participate directly in cell apoptosis [3]. Considering their immense physiological functions and importance, it is not surprising that a considerable number of human and animal diseases are related with dysfunction of ion channels. Such diseases have been called 'channelopathies' [16]. Examples of such are long QT syndrome, Brugada syndrome, Episodic ataxia (EA), Spinocerebellar ataxia type 6 (SCA6), Myotonia, Malignant hyperthermia, Autosomal dominant deafness (DFNA2), and Cystic fibrosis (CF)[17].

Ion channel can be classified by its selectivity to the ions:

- K^+ channels
- Na^+ Channels
- Ca^{2+} Channels
- Cl^- Channels
- Non selective cation channels

Besides non selective cation channels, other channels specifically allow certain ions to pass through

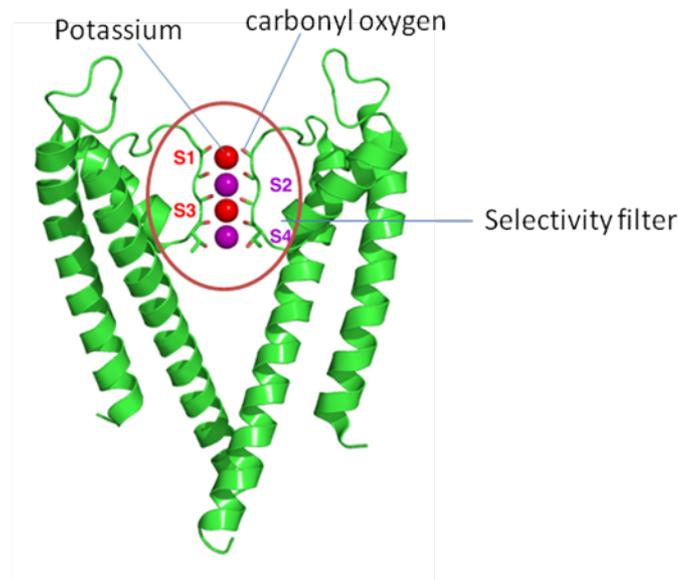


Figure 2. Crystallographic structure of the bacterial KcsA potassium channel (adapted from [18]).

the pore of the channel. For ion selectivity of the ion channels, Roderick MacKinnon used X-ray crystallography to study potassium ion channels and discovered how potassium ions pass through these channels and why (smaller) sodium ions do not [18]. As it is shown in the Figure 2, there is a selectivity filter structure in the extracellular part of the ion channel, which plays a decisive role for ion selectivity. Potassium ion should be dehydrated when it moves from bulk water into the low dielectric membrane environment [18]. Protein oxygen atoms surrounding each binding site in the selectivity filter have the same arrangement as water molecules of hydrated potassium ions. In other words, the selectivity filter mimics the water molecules of hydration surrounding a potassium ion. Therefore the energetic cost of dehydration is compensated in the selectivity filter and potassium ions are able to diffuse from water into selective filter. But passage of sodium ions would be energetically unfavorable, since a sodium ion is much smaller than a potassium ion, the selectivity filter cannot compensate the cost of dehydration of a Na^+ ion [18].

Ion channels also can be classified by the different stimuli that activate the channels. As:

- Voltage gated channels
- Ligand gated channels
- Physiological stimulus (mechanical, temperature)

Voltage gated ion channels have a structural motif called a voltage sensor consisting of charged amino acids (Figure 3). The voltage sensors undergo conformational changes when charged amino

acid side chains respond to voltage and consequently affect the opening of the ion channel pore

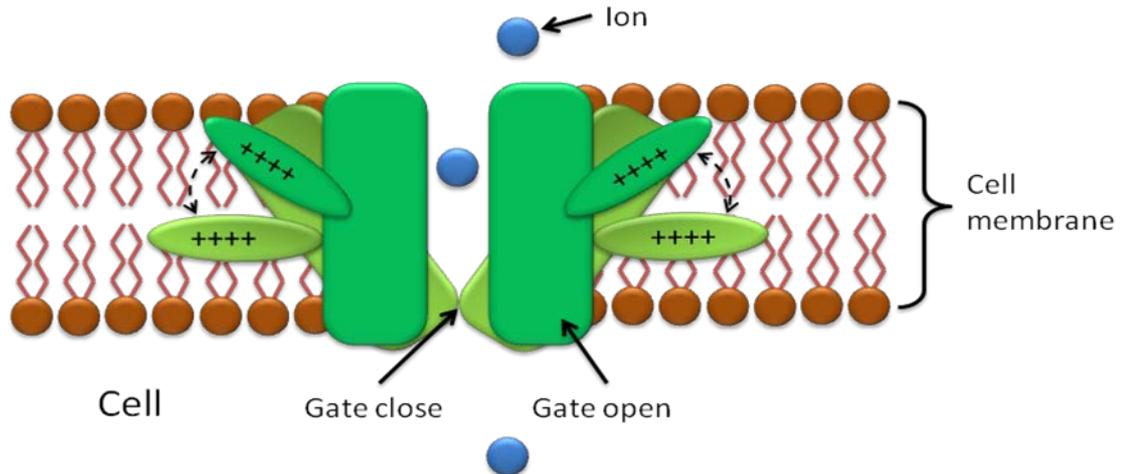


Figure 3. Voltage gated ion channel (adapted from [19]).

[19-20]. Ligand gated ion channels have specific binding sites for signaling molecules (Figure 4). Ligand binding to the channel protein results in a conformational change of the protein, and this leads to the pore opening [21]. Many ion channels also respond to other stimulus, such as mechanical change or temperature. Mechanosensitive ion channels are channels activated or inactivated by mechanical forces [22], and have been thought to be the primary molecular biosensors in such diverse physiological processes as touch, hearing, proprioception, or embryogenesis, as well as turgor control in plant cells and osmoregulation in bacteria [23]. Ion channels can be activated by one stimulating factor or by several different factors [24]. Temperature sensing ion channels were the target channels in this project.

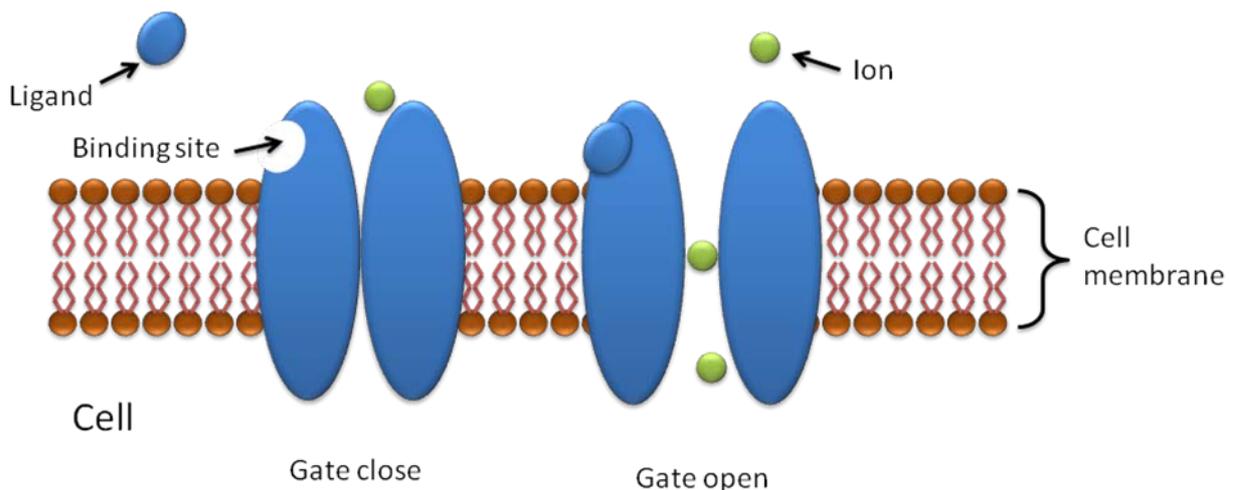


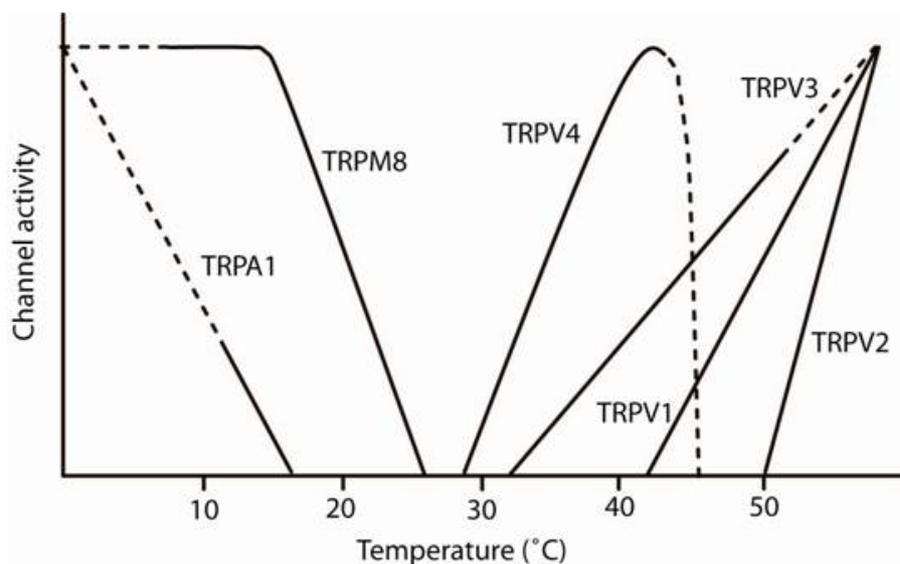
Figure 4. Ligand gated ion channel (adapted from [21]).

2.3 The Transient Receptor Potential family

Transient receptor potential (TRP) ion channels are the guards of our sensory systems, responding to touch, temperature, pain, osmolarity, pheromones, taste and other stimuli [25], so they are important sensory apparatus both for the cells and the multicellular organisms [26].

The TRP super family is divided into seven subfamilies, including TRPC, TRPV, TRPM, TRPN, TRPA, TRPP and TRPML [27], all of which have six putative transmembrane domains [26]. Some TRPCs may be store-operated channels, whereas others are activated by production of diacylglycerol or regulated through an exocytotic mechanism [26]. Many members of the TRPV subfamily function in sensory physiology and respond to heat, osmolarity changes, odorants, and mechanical stimuli [24]. The TRPM family functions as tumor suppressors and cold sensors [28]. The TRPN and TRPA include proteins with many ankyrin repeats. TRPN proteins function in mechanotransduction [26], whereas TRPA1 is activated by noxious cold and is also required for the auditory response [29]. TRPP and TRPML are distantly related to the other TRPs [26].

Temperature activated TRPs ion channels, which can be dubbed as thermo TRPs, have the distinctive feature that they can be activated alone by temperature [30]. Thermo TRPs detect



Graph 1. Ion channels related with temperature sensing (adapted from [32]).

almost the entire range of temperature sensed by most mammals [31-32]. These “temperature receptors” are showed in Graph 1. Ion channels TRPA1 and TRPM8 are cool sensors activated by cooling, while TRPV1, TRPV2, TRPV3, TRPV4 are heat sensors activated by heating [32]. The TRPV1 ion channel was the target channel in this project.

2.4 TRPV1

The transient receptor potential vanilloid 1 (TRPV1) ion channel was found in the peripheral nervous system (PNS), brain, spinal cord, skin, tongue and bladder [32], and it is predominantly expressed in small diameter dorsal root ganglia (DRG) and trigeminal ganglia (TG) neurons [5]. TRPV1 was first successfully cloned by David Julius and colleagues in 1997 [33]. The TRPV1 ion channel is a heat pain sensor activated approximately at 42°C or 43°C [6-7]. It plays an important role in inflammatory pain sensing (heat hyperalgesia) by different mechanisms; for example, bradykinin and nerve growth factor (NGF) enhance TRPV1 sensitiveness to heat and protons via phosphatidylinositol diphosphate (PIP₂) hydrolysis through phospholipase PLC γ [8]. So pharmaceutical blocking of TRPV1 presents a new strategy to release the pain by silencing the pain sensor, instead of stopping the propagation of the pain as most of the traditional pain-killers do [34]. The TRPV1 ion channel not only responds to heat, it can be activated by protons, voltage and ligands as well [24]. Capsaicin, the main vanilloid compound in hot pepper (or chili), is a strong activator of TRPV1. That is the reason why hot pepper can give a sense of heat pain [33].

As the putative configuration of the TRPs family, the TRPV1 ion channel is a tetrameric protein (Figure 5A) [35]. Each protein monomer is composed of 6 transmembrane domains with large N terminal and C terminal (Figure 5) [36]. The transmembrane domain of TRPV1 ion channel has qualitatively similar structure and topology as a mammalian voltage-dependent potassium ion (K⁺) channel (Kv1.2) [35], which is showed in Figure 7. Transmembrane domains S5 and S6 with pore helix between them (Figure 6) compose the ion conduction pore (Figure 7) [37].

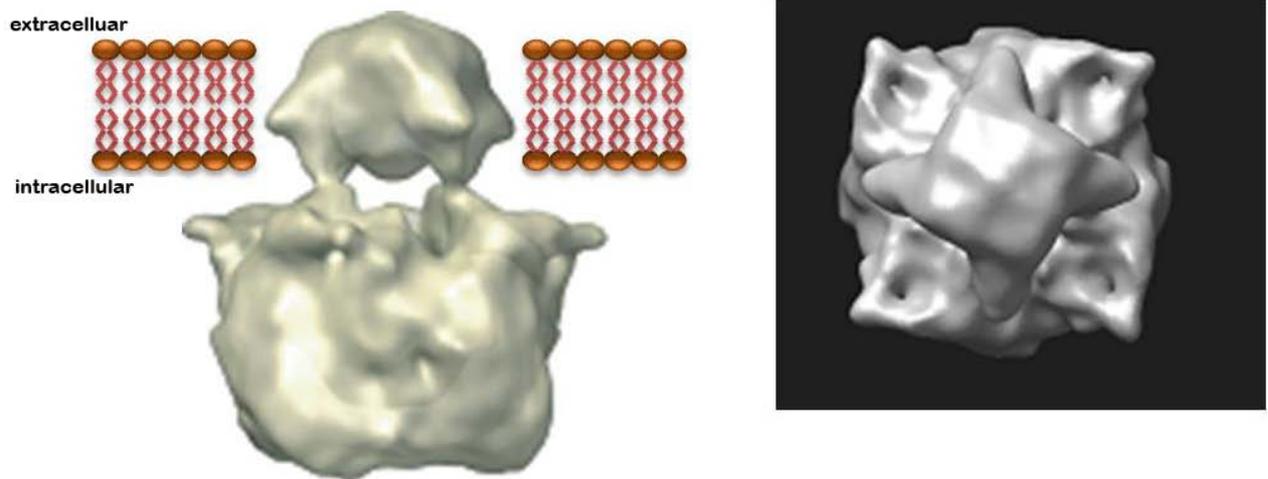


Figure 5. Topology of the TRPV1 ion channel.

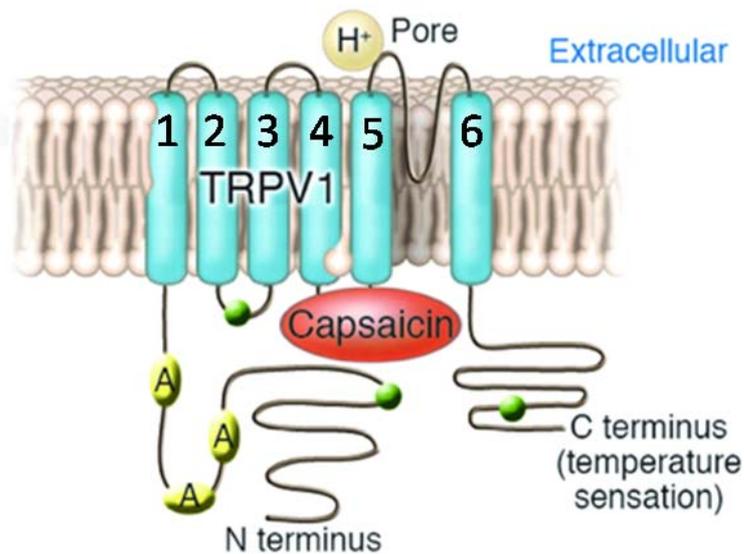


Figure 6. Topology of the TRPV1 ion channel unit.

In one recent study, C terminal swapping between TRPM8 (cool sensor) and TRPV1 (heat sensor) showed switched temperature phenotype, suggesting that the C terminal of TRPV1 might determine the directionality of the temperature response [38]. But the exact mechanism of temperature sensing has not been determined yet. There exist three possible mechanisms about temperature sensing of thermo-TRPs [39].

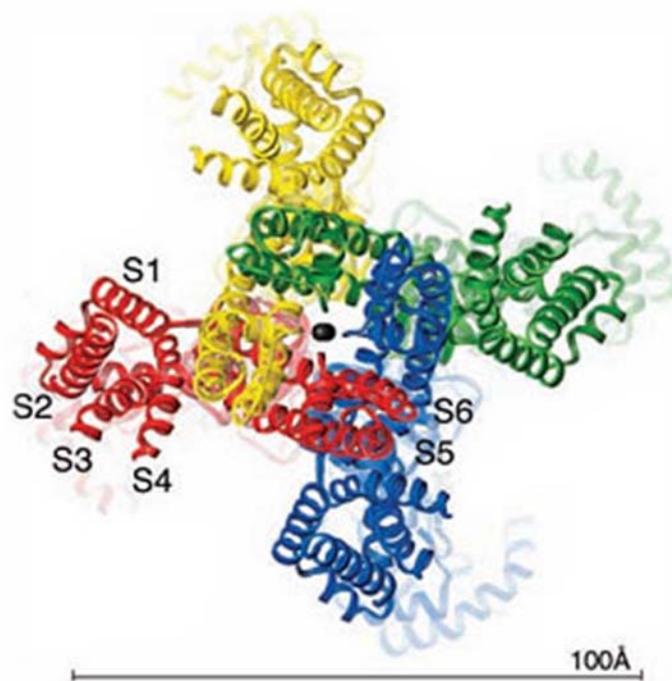


Figure 7. Stereo view of a ribbon representation viewed from the extra-Cellular side of the mammalian voltage-dependent potassium ion (K^+) channel (Kv1.2). Four subunits are colored uniquely (adapted from [37]).

- 1) Changes in temperature could lead to ligand production of the cell, and the ligand binds to the ion channel to activate it.
- 2) The channel protein may undergo temperature-dependent structural rearrangements leading to channel opening.
- 3) Thermo TRPs may be able to sense changes in membrane tension due to temperature dependent lipid bilayer rearrangement.

According to the 3rd mechanism, cell membrane structure or in the other words, cell membrane composition may affect the temperature sensing properties of TRPV1. Several researches on Chinese Hamster Ovary (CHO) cells, which were the cells also used in this project, reported that membrane fatty acid composition and membrane cholesterol content can affect the cell membrane fluidity and rigidity [10, 40]. Especially the cholesterol content of the cell membrane showed significant impact on the ion channels properties. For example, TRPM8 ion channel became more sensitive to high temperature after cholesterol depletion from the cell membrane [41], whereas the cation conductance in TRPC3-overexpressing cells were induced by cholesterol enrichment [42]. Depletion of cholesterol also inhibited the opening properties of the TRPV1 ion channel activated by various agonists [43]. Significant increase of activation temperature was also observed in cholesterol enriched cells, while the cholesterol depletion was found to have little effect on the heat response of TRPV1 ion channel [44].

2.5 The role of cholesterol in the cell membrane

Cholesterol is one of the important compounds of the cell, and is widely related with the cells physical and biological functions. The structure of cholesterol (Figure 8) consists of four fused rings. In addition to the steroid ring, cholesterol possesses a 3β -hydroxyl group and a hydrophobic tail

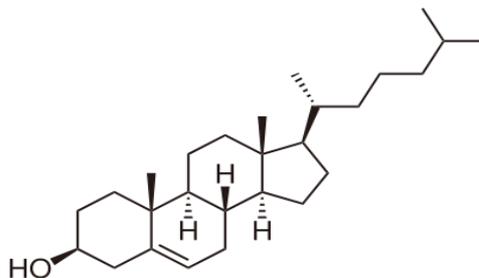


Figure 8. Structure of cholesterol.

[10]. While most of the molecule is hydrophobic, the 3β -hydroxyl is polar, and this structure gives the molecule an amphipathic character. So cholesterol can insert in a phospholipid bilayer, with its hydrophobic steroid ring oriented parallel to the hydrocarbon chains of the phospholipids and the polar hydroxyl group encountering the aqueous solvent [10]. This molecule is highly insoluble in water, but it is accommodated in membrane readily [10]. Cholesterol has a “specific ordering” effect on the cell membrane and cholesterol may be unique in such special effect. Cholesterol also modulates the functions of some membrane proteins and this is more likely by direct cholesterol-protein interactions [10-11].

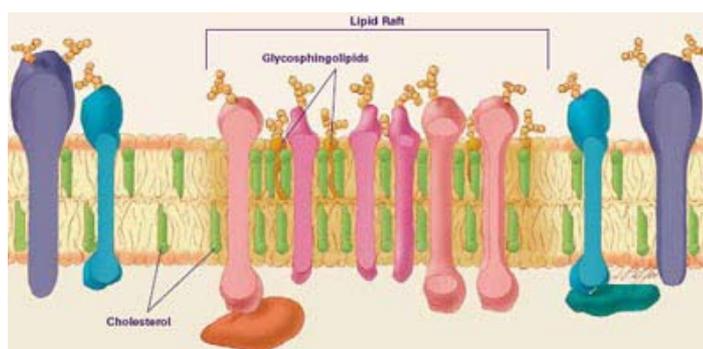


Figure 9. Lipid raft structure in the cell membrane.

There exists a debatable membrane structure called lipid raft. Lipid rafts (Figure 9) are commonly defined as cholesterol and sphingolipid-enriched membrane microdomains that function as platforms that concentrate and segregate proteins within the plane of the bilayer [45-46]. These specialized membrane microdomains are the organizing centers for the assembly of signaling molecules [47], influencing membrane fluidity and membrane protein trafficking, and regulating

neurotransmission and receptor trafficking [45]. Lipid rafts are more ordered and tightly packed than the surrounding bilayer, but float freely within phospholipid bilayer or also cluster with other rafts to form more ordered and larger platforms [48].

The “special ordering” effect, cholesterol-protein interaction and lipid raft structure indicate the crucial role of cholesterol on the cell membrane biophysical properties.

2.6 Cyclodextrins and cholesterol

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of 6-8 glucopyranoside units forming two rings with the wider rim displaying the 2- and 3-OH groups and the narrower rim displaying the 6-OH group on its flexible arm (Figure 10) [12]. The external faces of the cyclodextrin molecule are

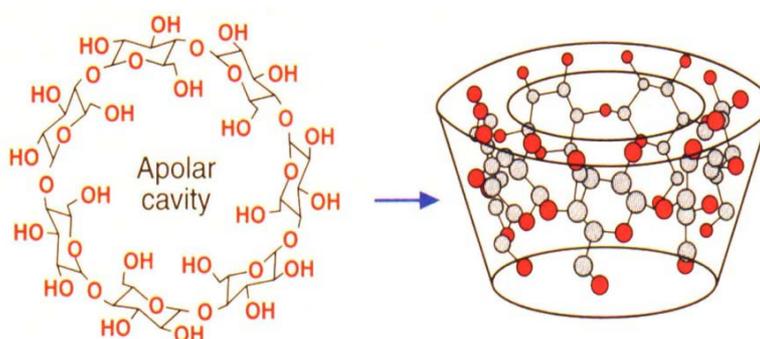


Figure 10. Topological structure of cyclodextrins.

hydrophilic, whereas the internal cavity is lined with C(3)H and C(5)H hydrogens and ether-like oxygens that provide an hydrophobic environment [12]. This internal cavity has the ability to encapsulate hydrophobic compounds while the exterior is sufficiently hydrophilic to impart cyclodextrins (or their complexes) aqueous solubility. Due to these properties, cyclodextrins are used for hydrophobic drug delivery in pharmacy [13, 49]. There are three different cyclodextrins which are named α -cyclodextrins (α -CDs), β -cyclodextrins (β -CD) and γ -cyclodextrins (γ -CD) with 6, 7 and 8 units respectively, and these have different cavity sizes (Figure 11). Different sizes are able to capsule different molecules and β -cyclodextrins are used for cholesterol [12].

Chemically modified β -cyclodextrins are widely used in cholesterol manipulation of cell membrane,

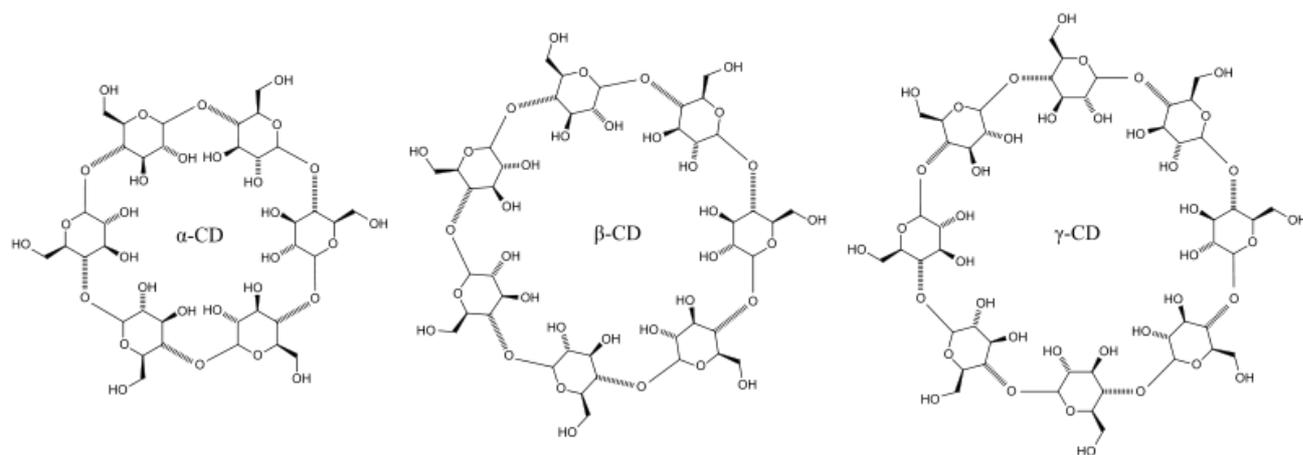


Figure 11. Cyclodextrins with different glucopyranoside units.

such as methylated β -cyclodextrins (M β CD). M β CD is efficient at attracting and removing the cholesterol from the cell (Figure 12A), and the M β CD-cholesterol complex is also efficient at enriching the cell membrane with cholesterol (Figure 10B) [12]. When the cell membrane is exposed to M β CD, cholesterol molecules in the cell membrane can be attracted to the cyclodextrins' hydrophobic cavity and removed from the membrane (cholesterol depletion). In the opposite manner, M β CD-cholesterol complexes (cyclodextrins with cholesterol in the cavity) can also release and give the cholesterol to the cell membrane (cholesterol enrichment, Figure 12B) [12].

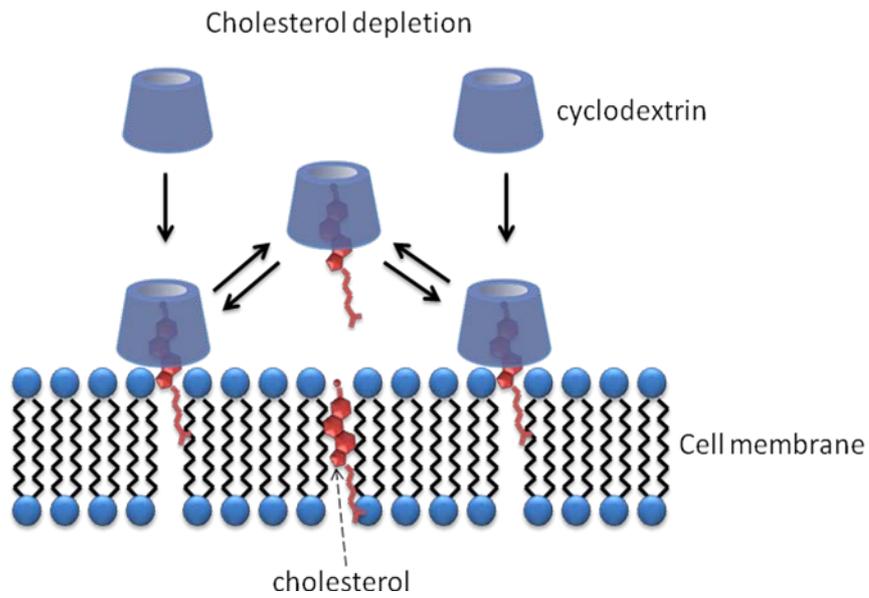


Figure 12A. Cell membrane cholesterol content depletion with M β CD. Adapted from [12].

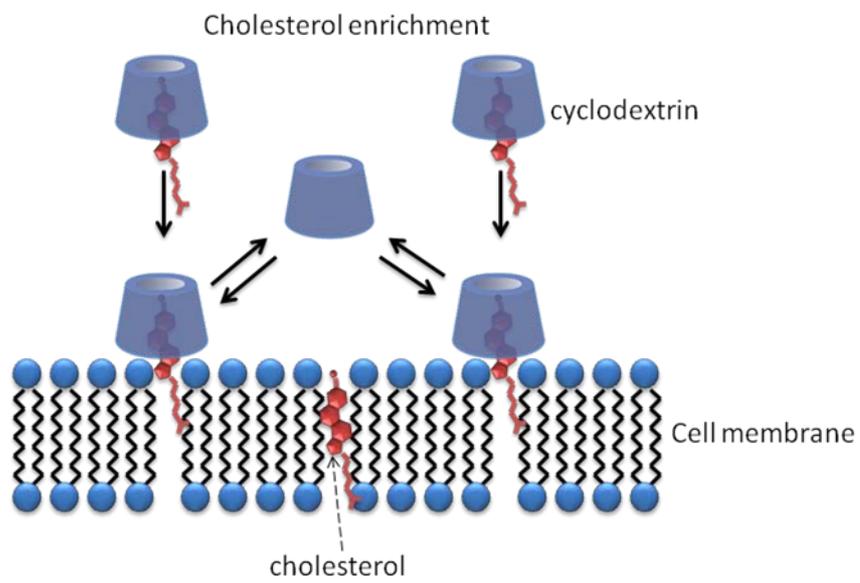


Figure 12B. Cell membrane cholesterol content enrichment with M β CD. Adapted from [12].

2.7 Amplex Red Cholesterol Assay

The Amplex Red Cholesterol Assay Kit was used to measure the cholesterol content of the cell membrane. The basic principle of the assay is showed in Figure 13. Cholesterol is oxidized by cholesterol oxidase to yield H_2O_2 and the corresponding ketone product. The H_2O_2 is then detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a highly sensitive and stable probe for H_2O_2 . In the presence of horseradish peroxidase (HRP), Amplex Red reagent reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin. The Amplex Red cholesterol assay can detect cholesterol at a concentration of 200 nM (80ng/mL) or lower and can accurately measure the cholesterol content in the equivalent of 0.01 μ L of human serum (product information of Amplex[®] Red Cholesterol Assay Kit (A12216)).

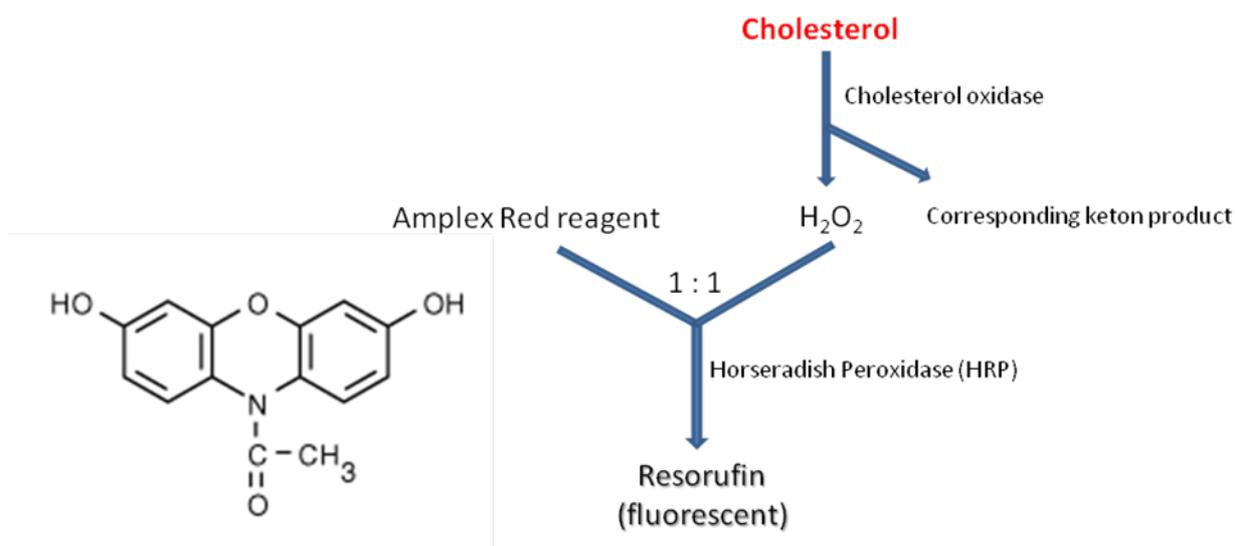


Figure 13. Basic principle of Amplex Red Cholesterol Assay.

2.8 The patch clamping technique

2.8.1 Patch clamp

Ions leaving or entering the cell changes the membrane potential, and this is often the physiological effect of the ion channel activity, so recording the changes of the membrane potential is an important indicator of the ion channel activities [1]. However, membrane potential changes also affect the ion channel function since: 1, changing the membrane potential causes the driving force the ion flow through the channels to change; 2, many ion channels are voltage gated (voltage based activation); 3, some ion channels have the ion flow restricted at certain membrane potential [1]. Therefore, it is often desirable to control the membrane potential and record the membrane current directly. This is called voltage clamp. In voltage clamp, the measured potential is compared with the holding potential (set by experimenter), and any deviation of the recorded potential from the holding potential is instantly corrected by compensatory current injection controlled by an electrical feedback system. This current is then an accurate representation of the ionic current over the membrane under investigation [1]. Most patch clamp experiments are voltage clamp experiments. If a fixed amount of current is injected instead of clamping the voltage, then this is called current clamp [1].

2.8.2 Patch clamp configuration

Figure 14 shows the procedure and the graph observed in an oscilloscope when a test pulse is applied between the two electrodes; one which is positioned in a pipette and one which is positioned in the solution surrounding the cell. The pipette touches the cell membrane and a slight suction is applied to make a tight seal between the pipette tip and the cell membrane. Seal resistance is often required to be bigger than $1G\Omega$ [1] for reducing the effect of leaking current from the seal. A seal with resistance of more than $1G\Omega$ is called a gigaseal. Some unfavorable consequences may happen under the suction. Seal break: loss of the seal between the pipette tip and the cell membrane showing low seal resistance or high leakage current. Patch break: cell membrane is ruptured before getting a gigaseal. When the gigaseal is obtained, bigger suction applied can rupture the cell membrane in the pipette tip (possibility of breaking the seal still exists) and the patched cell is ready for measurement. This measurement is called as whole cell

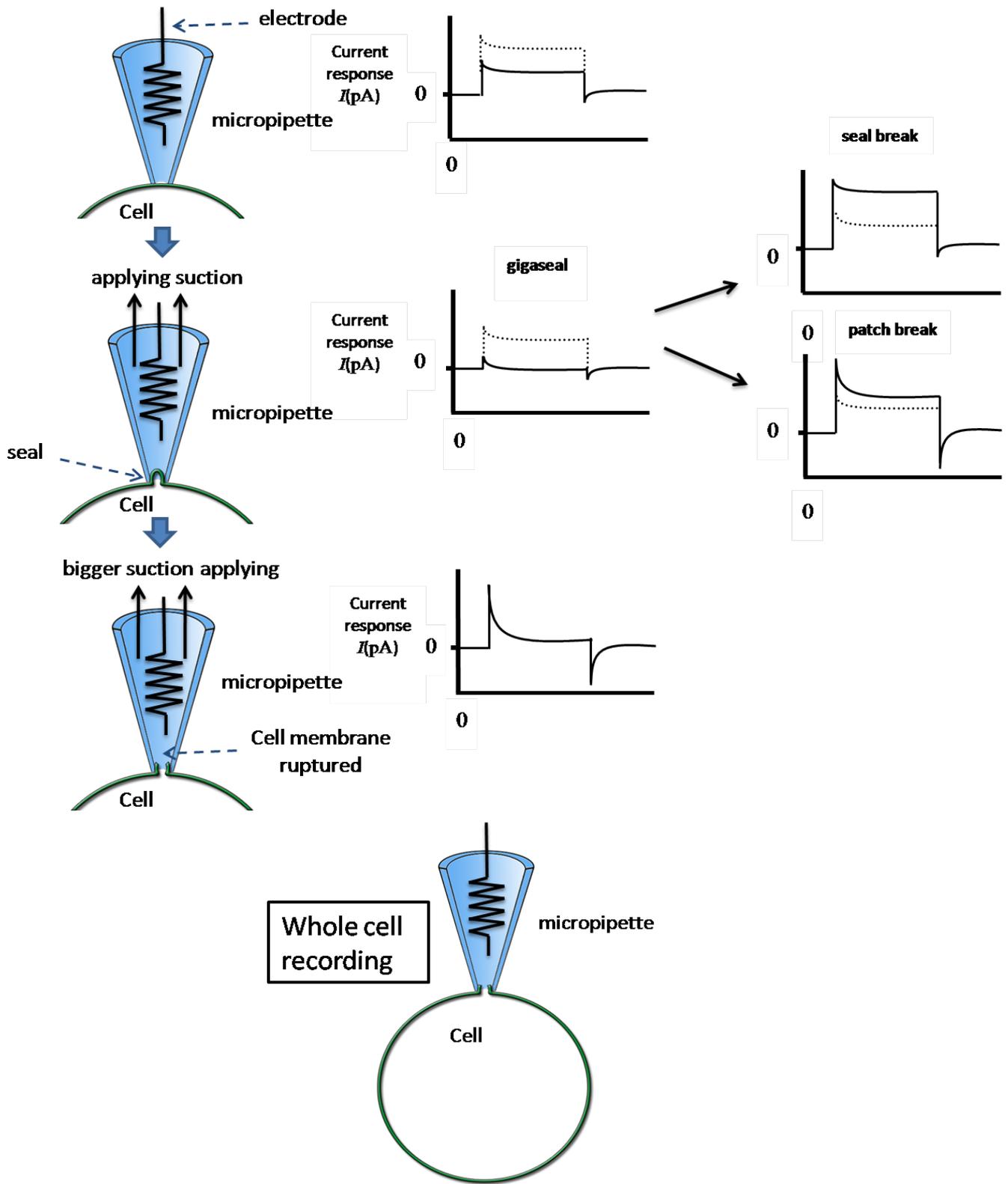


Figure 14. Schematic diagram of cell patching: whole cell recording.

recording, since it records the electrophysiological properties of the whole cell membrane. In addition to whole cell recording, there are two other configurations that can measure single channel activities. As is shown in Figure 15, when the whole cell configuration is obtained, the pipette is gently pulled away from the cell. The membrane around the pipette will break and reseal to form a new patch, which is then an outside out configuration. Inside out configuration is more difficult to establish. When the gigaseal is obtained, pulling away the pipette can break the membrane around the pipette, but subsequently resealing will make a vesicle which is not desirable. Out-ward facing membrane can be ruptured by air exposing, which can be done by briefly lifting the pipette tip out of the bathing solution. Outside out and inside out patching

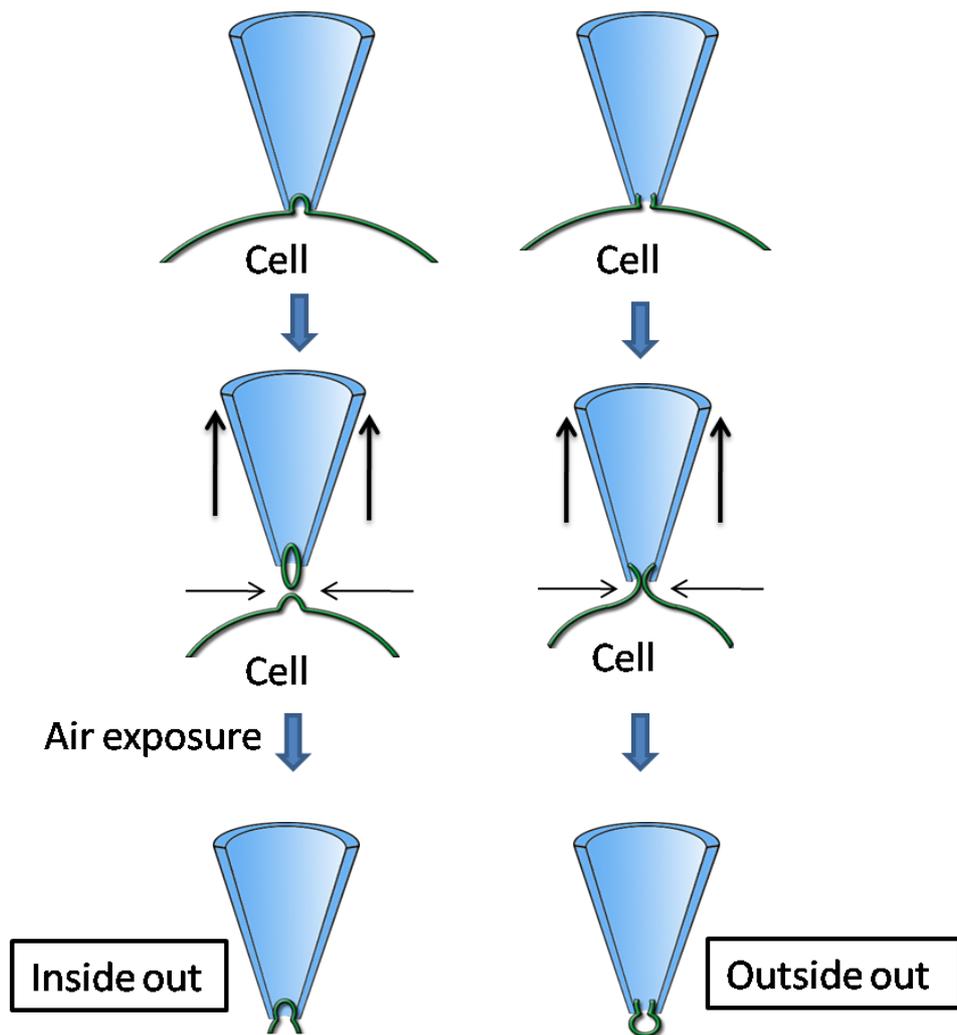


Figure 15. Schematic diagram of cell patching: inside out and outside in configuration.

configuration provides an access to measure single channel activity by exposing extracellular and intracellular site of the single channel, respectively, to different stimuli.

2.8.3 Whole cell recording

The whole cell configuration, showed in Figure 14, is obtained by breaking the cell membrane under the pipette so that the electrode in the pipette has direct electrical contact with the cytoplasm [1]. The equivalent circuit for the cell configuration is showed in Figure 16. The series circuit consists of the pipette resistance $R_{pipette}$, the access resistance R_{access} and the membrane resistance R_m . The sum of R_{access} and $R_{pipette}$ is sometimes referred to as series resistance. The membrane resistance is the largest current limiting resistor, so this configuration allows the observation of current through R_m . Because this current is the sum of currents through all activated single ion channels in the membrane, it is named whole-cell current or macro-current [1].

The membrane capacitance imparts a delay on potential change of the membrane: any membrane potential change must first overcome a change in stored charge (either reduces it or increases it), so the membrane capacitance is a limiting factor in action potential propagation speed [1]. Similarly in whole-cell recording, the membrane capacitance affects the voltage clamp time characteristics, and any change in holding potential will be delayed [1]. There are some methods to quantify and minimize the delaying effects. The quality criteria for whole cell recording are up to the experimenter, but a typical criteria could look like this [1]:

1. *The seal resistance (R_{leak}) must be better than $1\text{ G}\Omega$ (gigaseal) . Seal resistance should be as high as possible to minimize short-circuiting of the membrane current which is caused by the leakage of the seal.*
2. *The series resistance must be lower than $20\text{M}\Omega$ and stay that way throughout the recording.* Voltage clamp of the cell membrane is adversely affected by the series resistance.
3. *Cell capacitance and resistance must be stable.* Membrane and seal leaking will increases the response current.

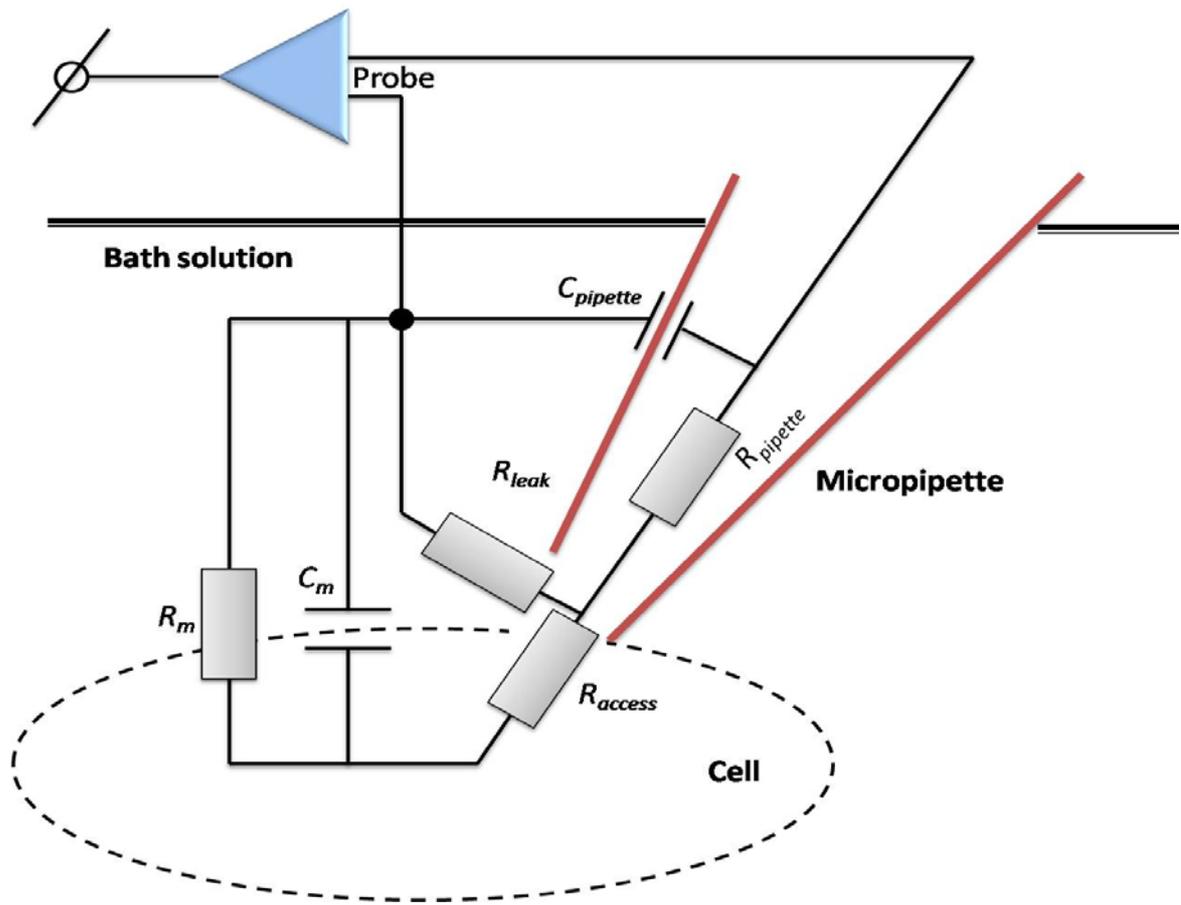
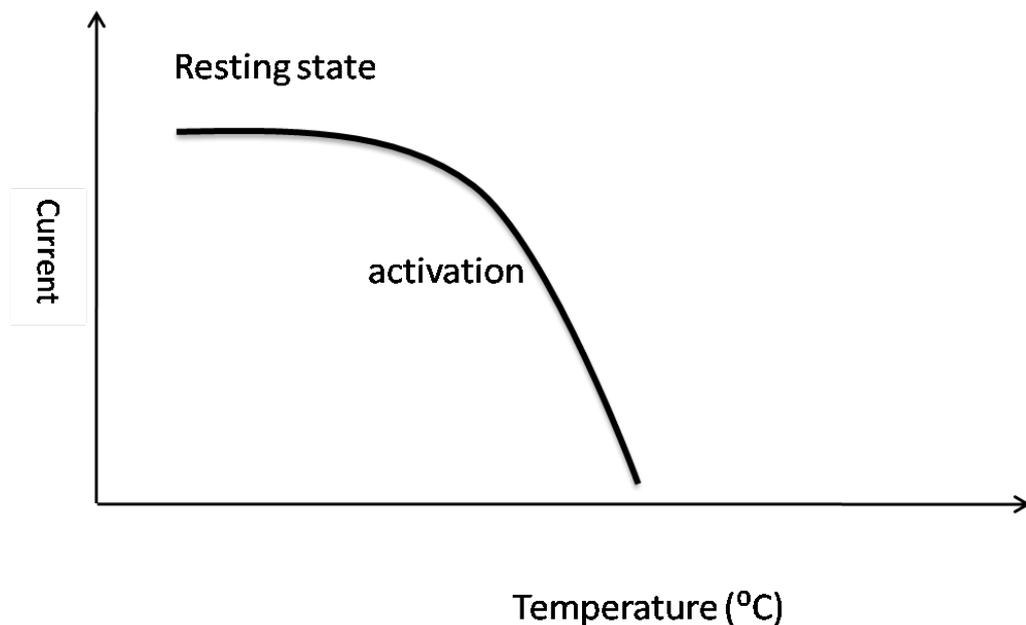


Figure 16. Equivalent circuit for the whole-cell recording configuration.

3. Aim

The aim of this project was to check the activation temperature (temperature threshold) for TRPV1 at different cell membrane cholesterol contents. When the ion channel is activated, there is a flow of ions across the membrane, which is a current. Since cholesterol has important effect on both cell membrane properties and ion channel activities, we decided to manipulate the cholesterol content of the cell membrane, i.e. either enrich or deplete it. By exposing the manipulated cells to gradually increased temperature and measure the current responses of the cells, we could observe how the cholesterol content of the cell membrane can affect the activation properties of the ion channels. In other words, the aim of this project was using this current response to check the activation temperature (temperature threshold) for TRPV1 at different cell membrane cholesterol contents. Expected activation curve is showed in Graph 2.



Graph 2. Temperature activation curve of the TRPV1 ion channel.

4. Materials and methods

4.1 Cell culturing

Cells were cultured in DMEM/F12 (PAA) with 10% fetal bovine serum (PAA) + ZeocinTM (350 μ l/ml, Invitrogen) + Blastidicin (5 μ l/ml, Sigma) at 37°C with 5% CO₂. Expression of TRPV1 was induced overnight with doxycyclin (1 μ g/ml, Sigma) before the experiment. (Functions of the antibiotics are given in the appendix I).

4.2 Cyclodextrin solutions

M β CD solution: 0.033g M β CD was weighed up and dissolved in 10g of DMEM/F12, with an end M β CD concentration of 2.5mM.

M β CD+cholesterol solution: 0.066g M β CD was weighed up and dissolved in 20g of DMEM/F12, with an end M β CD concentration of 2.5mM. 38.6 μ l cholesterol solution (with concentration 50mg/ml) was taken and dried with N₂. 20ml prepared M β CD solution was added to the dried cholesterol. Then, the mixture was sonicated for ~3min and put on a shaking table overnight at 37°C. Overnight shaken M β CD: cholesterol solution was filtered through a hydrophilic filter with pore size 0.45 μ m.

4.3 Microfluidic chip

The microfluidic chip (Figure 17) used in this project consists of a PDMS mold plasma-bonded to a glass slide. The chip contains 16 sample wells individually addressing a tightly packed channel system that emanates into an open bath with the size of 35 × 20 × 4 mm ($w \times l \times h$). At the outlet, the 50- μ m-wide and 57- μ m-high channels are closely spaced and separated by 22- μ m-thick walls [50]. When an air pressure is applied to the chip, solutions in the 16 wells flow out of the channels to the container. At an in-channel mean velocity of 3 mm·s⁻¹, suitable for applications involving cells, the microfluidic chip can create a patterned laminar flow at the channel outlets accessible from the open bath [50]. Scanning and positioning of micropipettes, electrodes, or patch clamped cells are possible in the system, and the probes can be exposed to a sequence of solutions precisely and with fast switching times in tens of milliseconds [50].

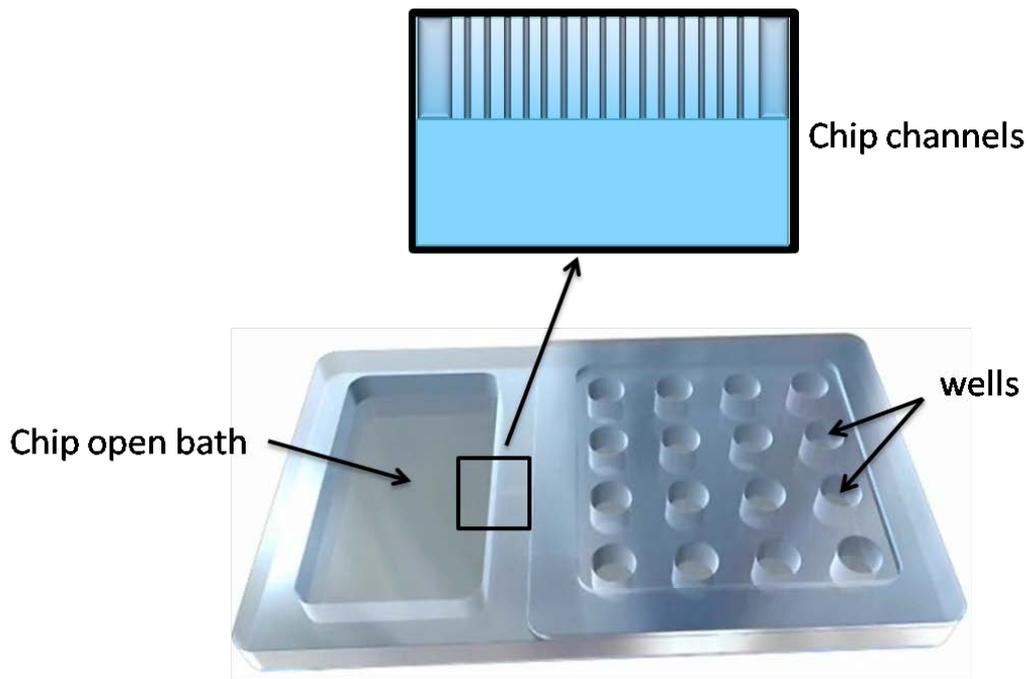


Figure 17. Microfluidic chip (Collectricon Dynaflo[®]Pro II).

4.4 Cholesterol content manipulation

Induced cells were washed 3 times with pre-warmed DMEM/F12, and then they were incubated in M β CD solution at 37 °C for 10 minutes to deplete the cholesterol content of the cell membrane. After 10 minutes, cells were washed with extracellular buffer (ECB) with pH 7.4 (detailed composition of the buffer is given in appendix II) 3 times, and incubated with extracellular buffer at 37 °C for about 30 minutes. The extracellular buffer was removed and the cells were detached by incubation at 37 °C with 750 μ l preheated accutase (PAA) for 5 minutes (note: cells can NOT be incubated more than 5 minutes, otherwise Accutase could start degrading membrane proteins). 750 μ l ECB was put into the dish to dilute the accutase after digestion, and a glass pipette with a heat smoothed tip was used to detach and disperse the cells by flushing them up and down about 5 times (note: should avoid bobbles flushing into the dish). A proper amount of cells were dropped in the microfluidic chip open bath filled with ECB at pH 7.4. The chip container had been pretreated with 2.5mg/ml BSA for 3-5 minutes to prevent cell adhesion to the bath surface. All channels of the chip had been filled with ECB pH 7.4. The same process was used for cholesterol enrichment, but the cells were incubated with M β CD + cholesterol solution for 1.5h instead of with M β CD for 10 minutes. The Amplex Red Cholesterol Assay Kit (Invitrogen) was used for cholesterol content determination.

4.5 Patch clamping

Whole cell patch clamping was used in all experiments, and all data was recorded with HEKA EPC10 (HEKA Instruments Inc., Bellmore, USA) patch-clamp amplifier. Micropipettes with resistance 2-10M Ω were filled with intracellular buffer (ICB pH 7.2, exact content is given in appendix II). The whole cell recording configuration was obtained in the chip open bath, and the cells were clamped at -60mV. Current signals were recorded at a sampling frequency of 5 kHz and low pass filtered with frequency of 1 kHz.

4.6 Thermo control system

The microfluidic chip was used for on-chip fabrication with a 3.5nm Cr adhesion layer and a 7.5nm Au layer evaporated onto the glass on the back side of the chip [51] (Details of the fabrication process is given in appendix III). The coils under the chip channels can generate resistant heat by

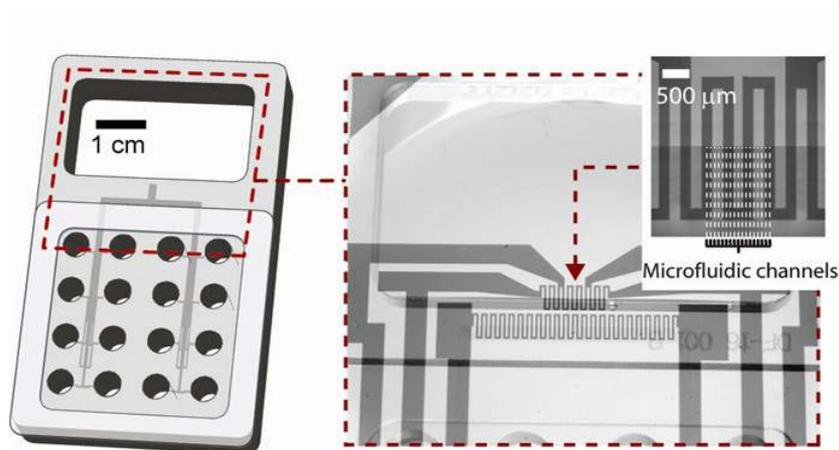


Figure 18. Configuration of the temperature controlled microfluidic chip (adapted from [48]).

employing voltage (Figure 18). The channel outlets are located in the middle of this heating zone, so the outlet solution from the channels can be heated by the coils.

As it is showed in Figure 19, a thermocouple was used as thermo-probe. Thermocouples work on the basis of the thermoelectric effect, i.e. that a junction of two different metals produces a voltage related to a temperature change, and they have accuracy within 0.5% [52]. The thermo probe and patched cell were closely located in front of the channels. The amount of voltage applied to the heating coil was feedback controlled by the local temperature signal from thermocouple. So the local temperature can be controlled by preset program (Cadinterface, programmed in house) and the uniformity of the temperature across the channel outlets is within ± 0.2 °C

[51].

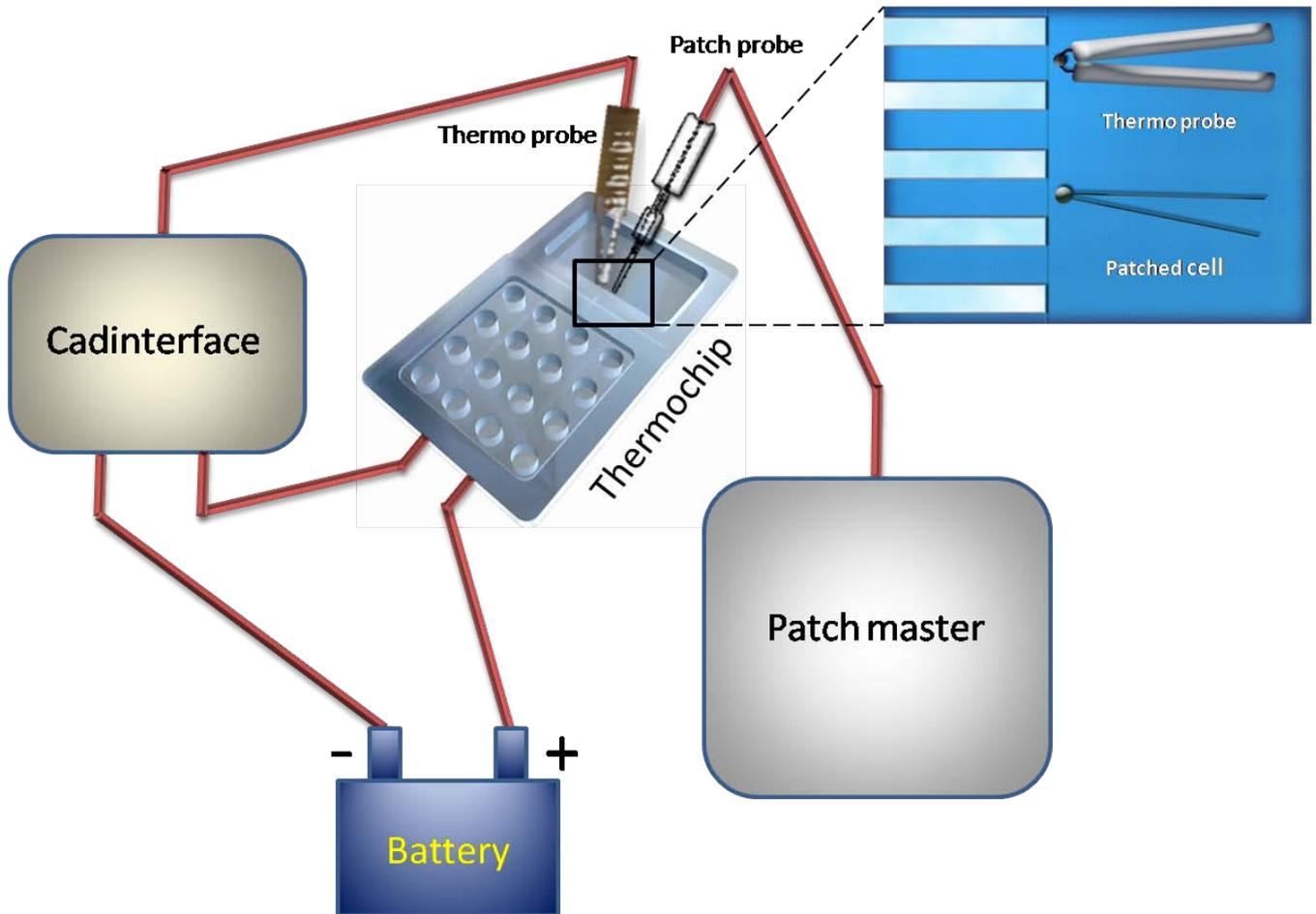


Figure 19. General configuration of thermo control system.

Figure 20 shows the general scheme of temperature controlled ion channel activation measurement in this project. As it shows, temperature was stepwise increased and the current across the cell membrane was measured. Each step was 1°C and it was held for 10s at each temperature point. The temperature was reduced back to the initial value when it had reached the highest measuring temperature.

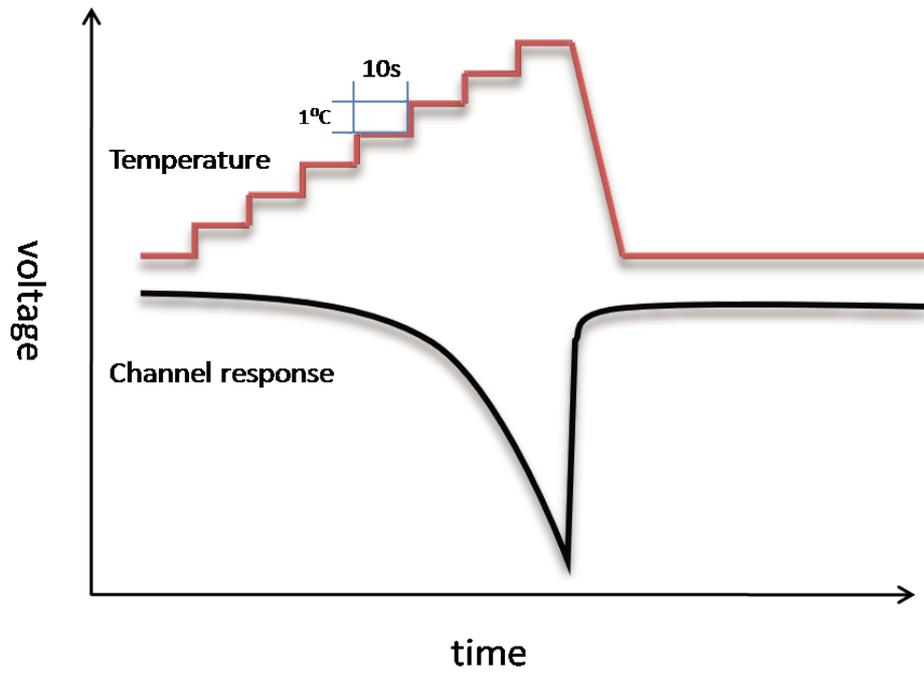
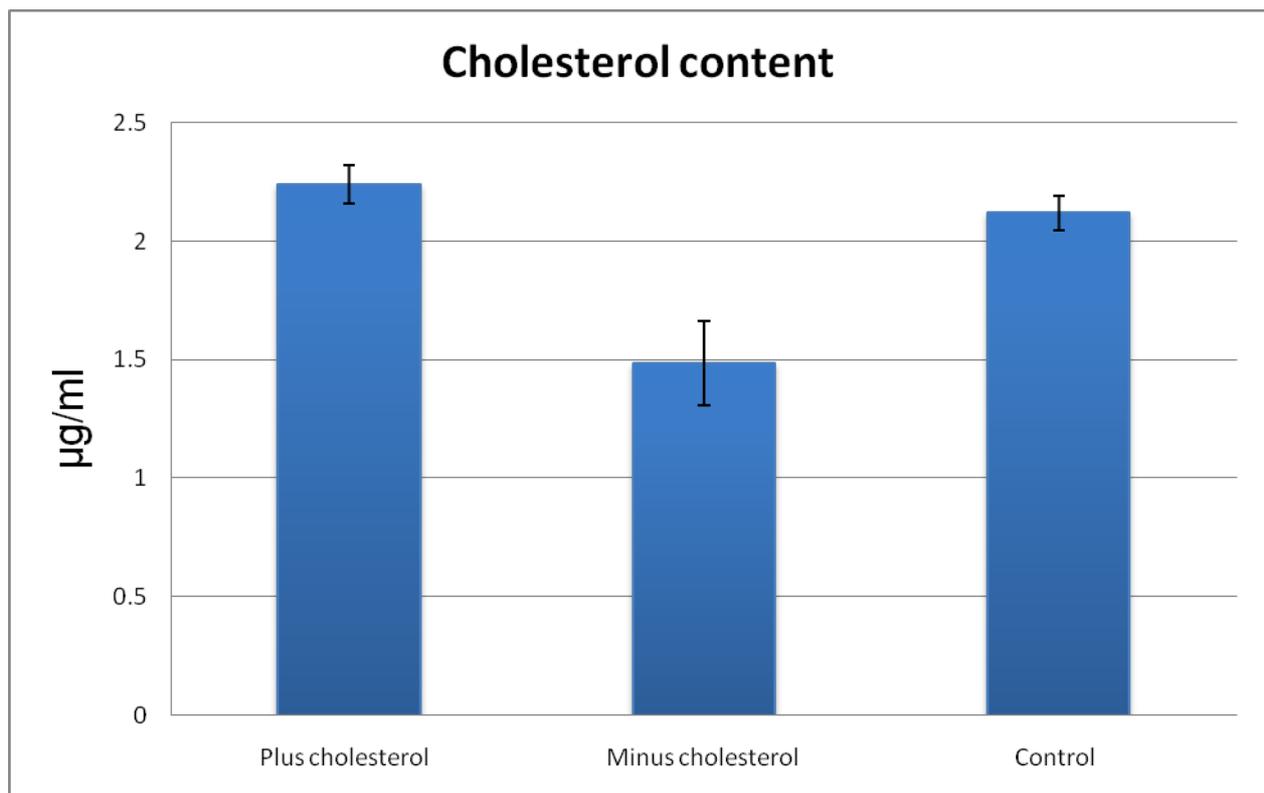


Figure 20. Temperature controlled ion channel activation measurement.

5. Results

5.1 Cholesterol content

Graph 3 shows the result from the cholesterol content manipulation with cyclodextrin. Compared with untreated cells (control), cholesterol content increased slightly in cholesterol enriched cells, while there was an obvious decrease obtained in cholesterol depleted cells.

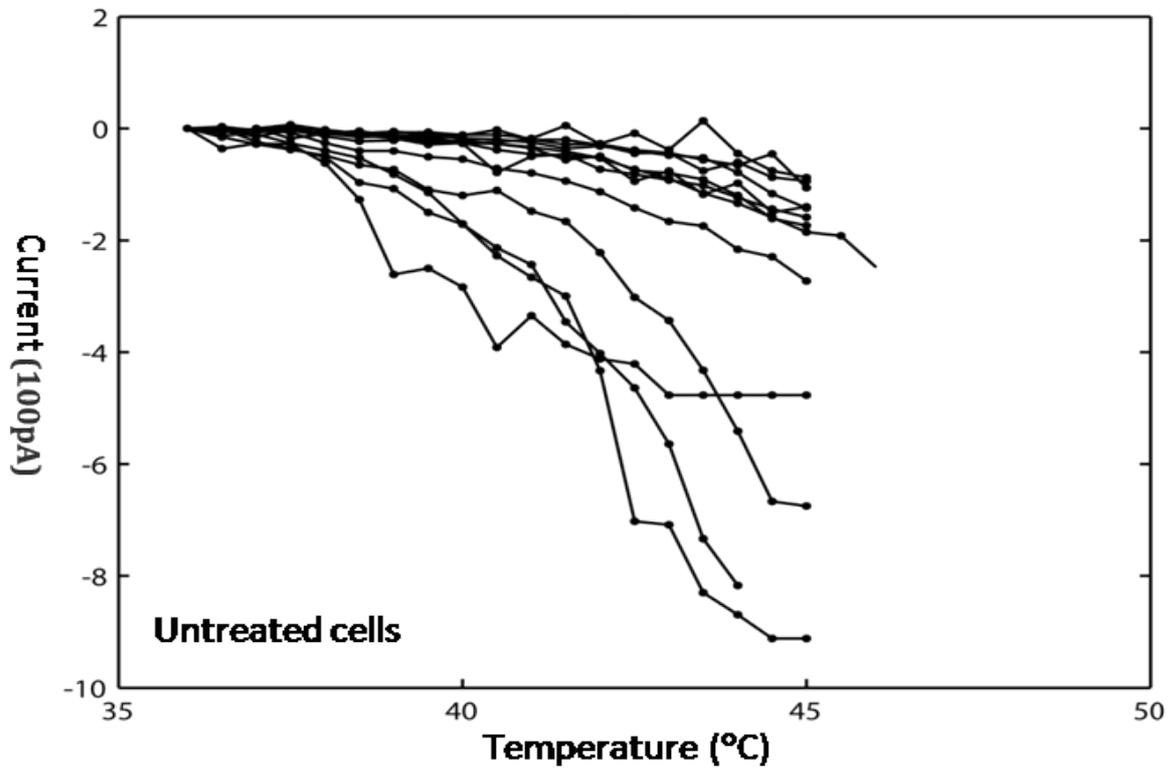


Graph 3. Cholesterol content of different manipulation with cyclodextrins and control, the unit is µg cholesterol per ml sample.

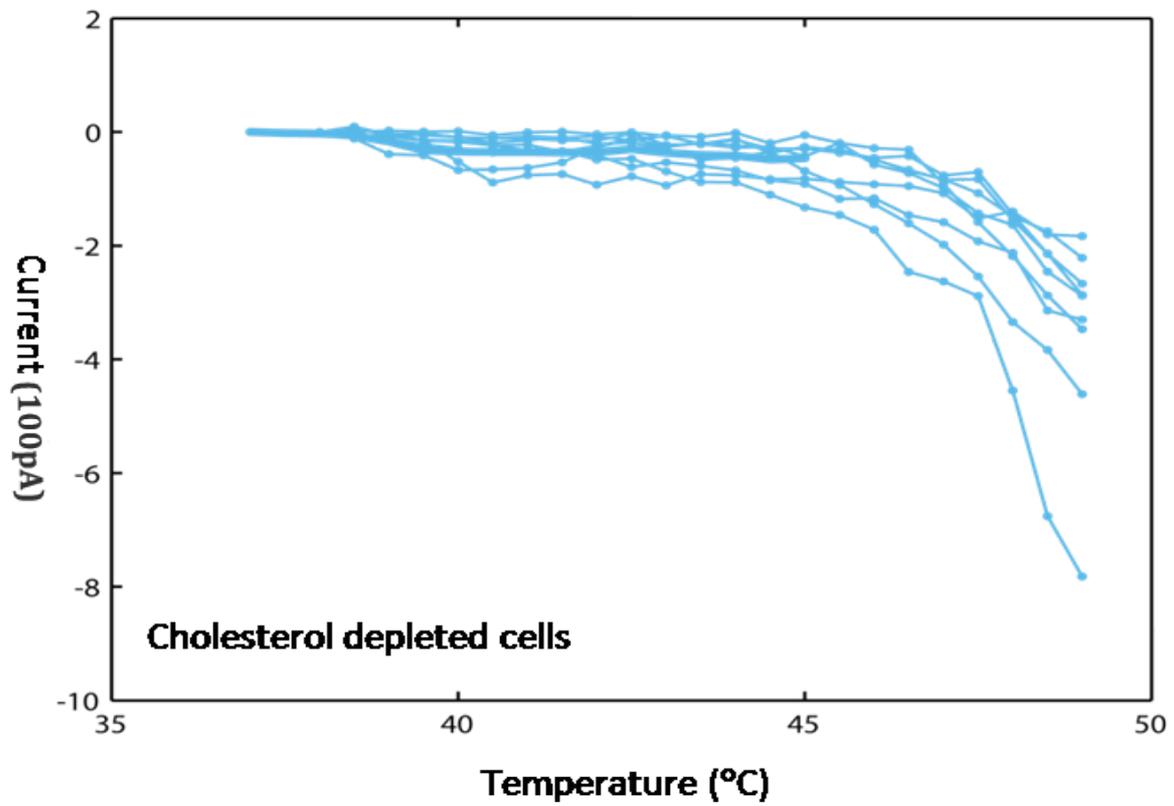
5.2 Ion channel response on different cholesterol content

The results show how the differently treated cells responded to the temperature. For the first group of untreated cells (Graph 4A), the obvious deviation of a few results from the main region was most likely due to not well manipulating lab process, since once these cells were incubated in accutase more than 5 minutes. The experimental temperature range for the first group of untreated cells was from 36°C to 45°C. Cholesterol depleted cells did not respond in this temperature range, but showed activation at higher temperature (Graph 4B). So the experimental

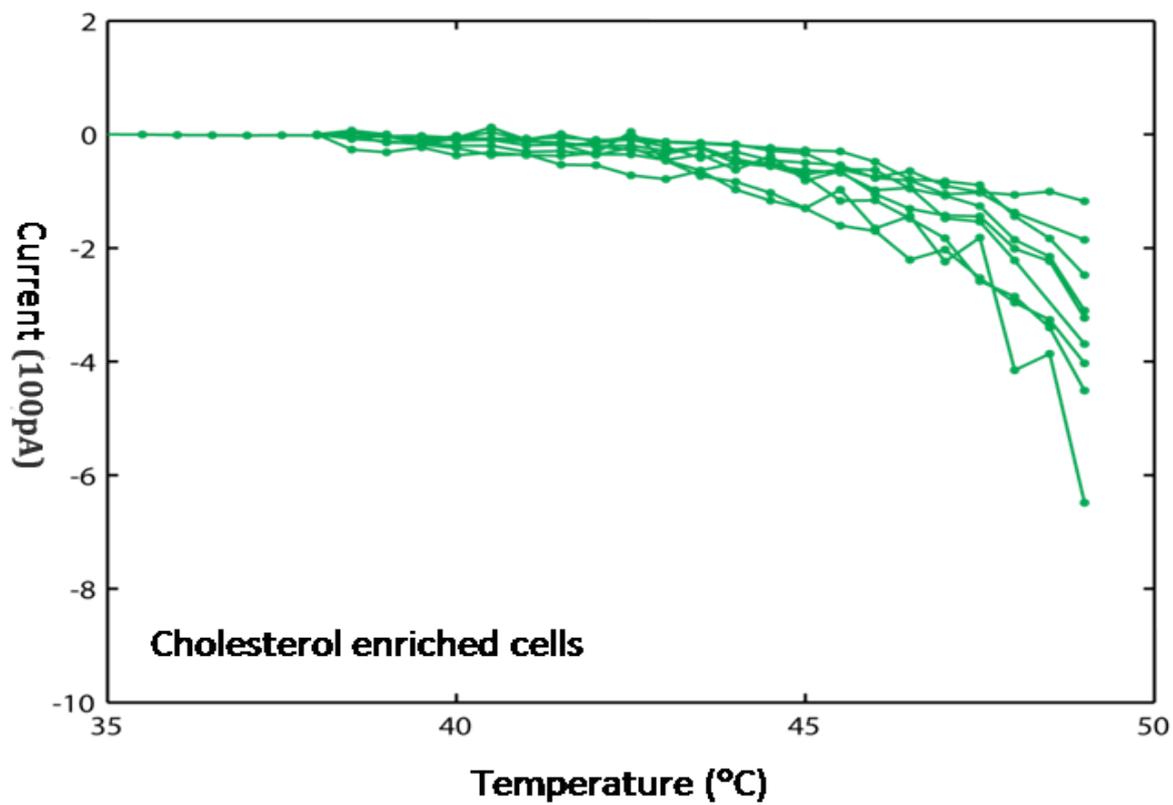
temperature range for cholesterol depleted cells was changed to 38°C to 49°C. The same temperature range was used for cholesterol enriched cells and similar responses were observed (Graph 4C). Since the temperature range for the first untreated cell group was only up to 45°C, and in this temperature range some of the responses showed almost the same tendency as both cholesterol depleted and enriched cells, it implied a possibility that untreated cells might have similar response as cholesterol depleted or enriched cells at higher temperature. So a new untreated cell group (Graph 4D) was done with an experimental temperature range of 36°C to 49°C, but, unfortunately, the responses were very low compared to both cholesterol depleted and enriched cells. So when the plots were put in same Graph (Graph 4E), the activation of the untreated cells were not obviously seen. This way of plotting was not practical on comparing temperature activation properties of the cells with different cholesterol contents. In addition, due to that the sizes of the cells and the density of TRPV1 in the cell membrane differ among the cells, the responses of each cell were different in the magnitude. So the result of each cell was normalized at the response which was obtained at the highest temperature; the first untreated cell group was normalized at 45°C, while the others were normalized at 49°C. Normalized data is showed in Graph 5. In both the old and the new untreated cell group, obvious increases of currents can be seen in the temperature range from 41°C to 43°C. It confirmed the expected temperature threshold of TRPV1 in untreated cells, which is 42°C to 43°C. Since the responses of the new untreated cells are all quite low, small difference of each response can result in significant variation, and this can be the reason of the substantial standard deviation. But the tendencies of the responses were similar. For the other cell groups, the activation temperature shifted to higher temperature, both when the membranes of the cells were depleted and enriched with cholesterol.



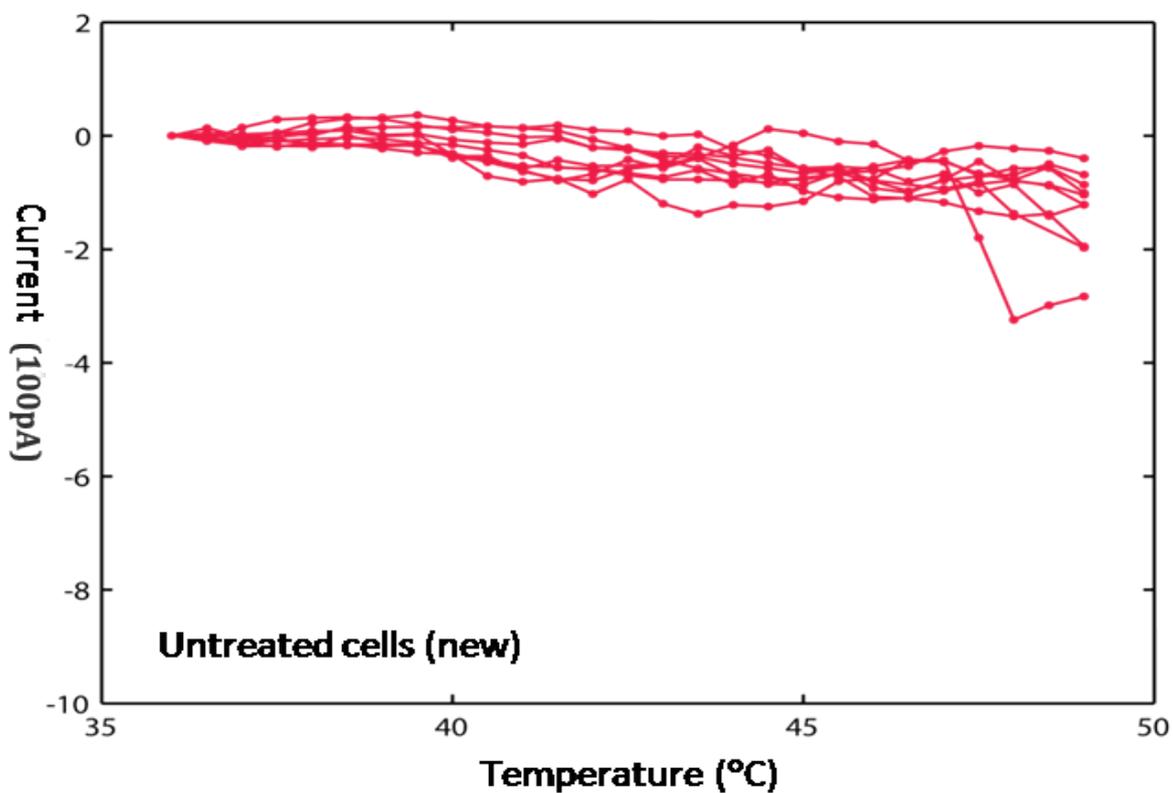
Graph 4A. Current responses of untreated cells.



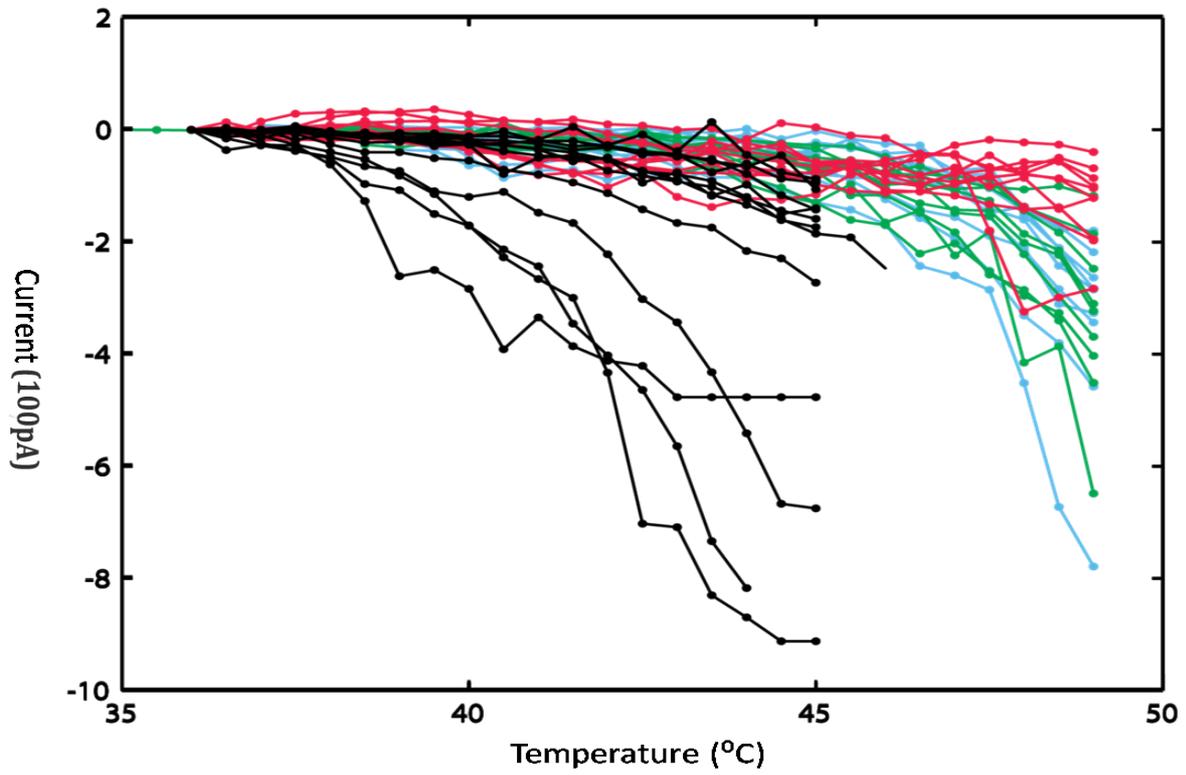
Graph 4B. Current response of cholesterol depleted cells.



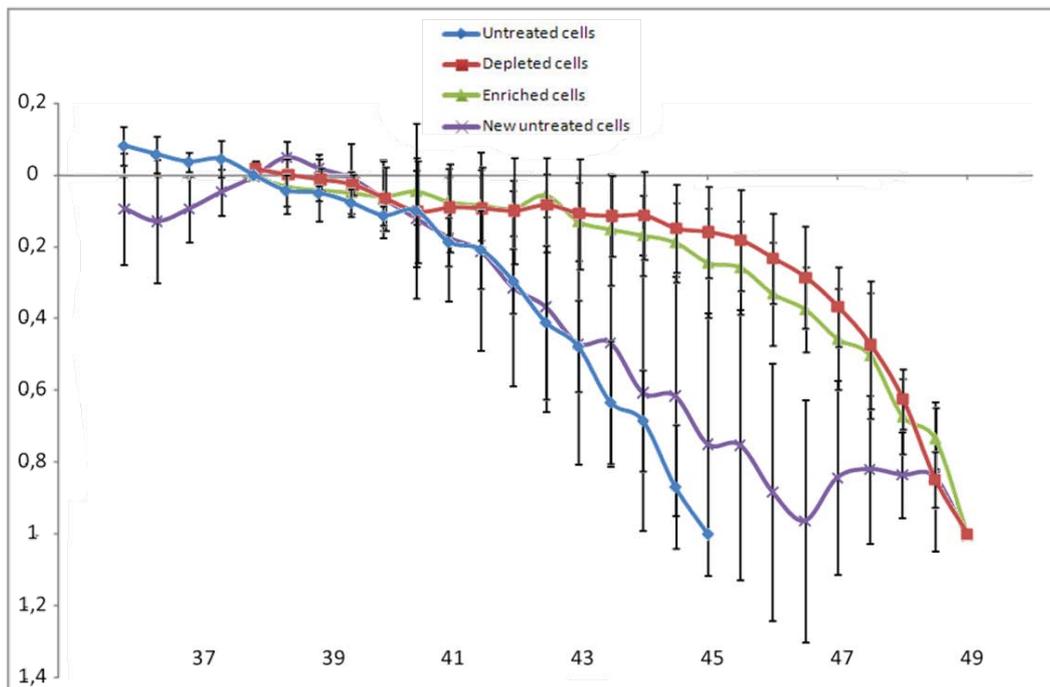
Graph 4C. Current response of cholesterol enriched cells.



Graph 4D. Current response of untreated cells (new).



Graph 4E. Current responses of all cell groups.



Graph 5. Normalized data of all cell groups, data from each group were normalized at the maximum temperature.

6. Discussion

Upon activation, ion channels undergo a conformational change to open the pore, and allow ions to pass through the channels. This conformational change of the ion channel protein will affect the lipid bilayer surrounding the protein. In other words, lipid bilayer also has a resistance to pore opening of the ion channels [53]. Since cholesterol can change the mechanical properties of the cell membrane, different cholesterol content give different resistance forces. The delayed response to heat of cholesterol enriched cells might be due to the increased resistance of the lipid bilayer to the channel opening [41]. But the activation temperature also increased in cholesterol depleted cells, where resistance should be smaller because of the decreased cholesterol content in the cell membrane. This implied more complicated mechanisms in cholesterol-ion channel interaction, possibly both direct and indirect. Lipid raft structure has significant importance for TRPV1 ion channel functions [43] and the sensitivity to capsaicin and protons [8], strongly suggesting that TRPV1 might be localized at lipid rafts. So if the lipid raft structure was disrupted by cholesterol depletion, it possibly affected the TRPV1 ion channel located in the lipid raft. In contrast to TRPM8 [41], TRPV1 might prefer a higher resistance force, which the lipid rafts can provide, to keep the protein structure in an optimal conformation. Disrupted lipid rafts might no longer have this optimizing capability, resulting in reduced temperature sensitivity of TRPV1 ion channels. Sequence analysis showed that another member of TRPs family, TRPC3, hosts seven potential cholesterol-binding motifs (cholesterol recognition amino acid consensus) [42]. This indicates a possibility of direct cholesterol-protein interaction, suggesting cholesterol is an important part of the protein structure and functioning. Cholesterol depletion might also remove the cholesterol bound to the protein, consequently leading to the delayed response to the temperature. Similar result for cholesterol enriched cells was observed in a study about heat activation of TRPV1 [44] (the temperature threshold increased to about 46°C). But cholesterol depleted cells were found to have little effect on heat activation (the temperature threshold was observed at 43-44°C). Since the treatment with M β CD was 2h instead of 10 min used in this project, and the membrane cholesterol content after treatment was not given, it is hard to say whether the cholesterol content of the cell membrane was significantly decreased after 2 hours of treatment.

The responded signals were quite noisy at higher temperature (mostly at above 45°C). This was most likely because of solid-to-liquid transition of the membrane due to temperature increase, and which resulted in change of ion permeability of lipid bilayer membrane [54]. So there is a possibility that an artifact exists, that cell membrane itself had some effect on the response at higher

temperature. Temperature dependent chemical or biological activities of other proteins on or in the cell membrane also contribute to this possibility.

7. Future work

There are some more studies that can be done to have more comprehensive understanding of the effect of cell membrane on temperature sensing profile of the TRPV1 ion channel.

- *Measure the response of uninduced cells, which do not express TRPV1, to the temperature.* Since the permeability of the cell membrane to the ions changes upon different temperature [54], measuring the response of uninduced cell to the temperature is necessary to rule out the artifact that the cell membrane itself had major effect on the responses to the temperature changes.
- *Measure the effect of other membrane compositional compounds on temperature sensing of TRPV1.* Fatty acid composition changes the membrane fluidity [40], so it may also effect the temperature activity of TRPV1.
- *Stepwise temperature control can be replaced by linear temperature control.* We used stepwise temperature increase, as 1°C for one step and kept the temperature for 10s at each temperature point, because we thought that the ion channel might not respond to the temperature immediately and it might be better to keep the temperature at each temperature point for a while well enough for the ion channel to respond, so that we would not miss the exact activation temperature. But the stepwise temperature control resulted in uneven spread of the data: quite much data at each temperature point but quite little between the points, which was not enough to indicate the precise responses of the ion channel at the temperatures between each temperature points. The experiments showed that the ion channel had instant response to the temperature change. So the stepwise temperature control can be replaced by linear control to get more data on temperatures between each temperature points in stepwise control.

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Appendix I

Antibiotics in cell culturing (user manual from Invitrogen™)

The T-REx™ System

The CHO cell line used in this project has been transfected with an inducible expression plasmid for our target gene TRPV1. The plasmid is specific for the mammalian expression system (T-REx™ System), in which expression of the gene of interest can be induced by tetracycline and repressed in the absence of tetracycline.

As it shows in the Figure A, the tetracycline (Tet) repressor forms a homodimer that binds with extremely high affinity to each TetO2 sequence in the promoter of the inducible expression vector, and represses transcription of TRPV1. When tetracycline is added it binds to each Tet repressor homodimer with high affinity, and then the Tet repressor-tetracycline complex dissociates from the Tet operator and allows expression of TRPV1. Doxycycline, which was used in the project, is similar to tetracycline in its mechanism, and it has a longer half-life than tetracycline (48 hours vs. 24 hours, respectively).

The cells are transfected with two different plasmids; one containing the TRPV1 gene and one containing the gene for the Tet repressor. The plasmid containing the TRPV1 gene also contains a Zeocin resistant gene, and the plasmid containing the Tet repressor gene contains a Blastidicin resistant gene. Hence, Blastidicin and Zeocin are effective tools for selecting cells that contain both plasmids. Blastidicin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells and Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces*. It shows strong toxicity against bacteria, fungi (including yeast), plants and mammalian cell line. Under the treatment with Blastidicin and Zeocin™, the cells without the plasmid are killed, while the cells with both plasmids are resistance to the antibiotics.

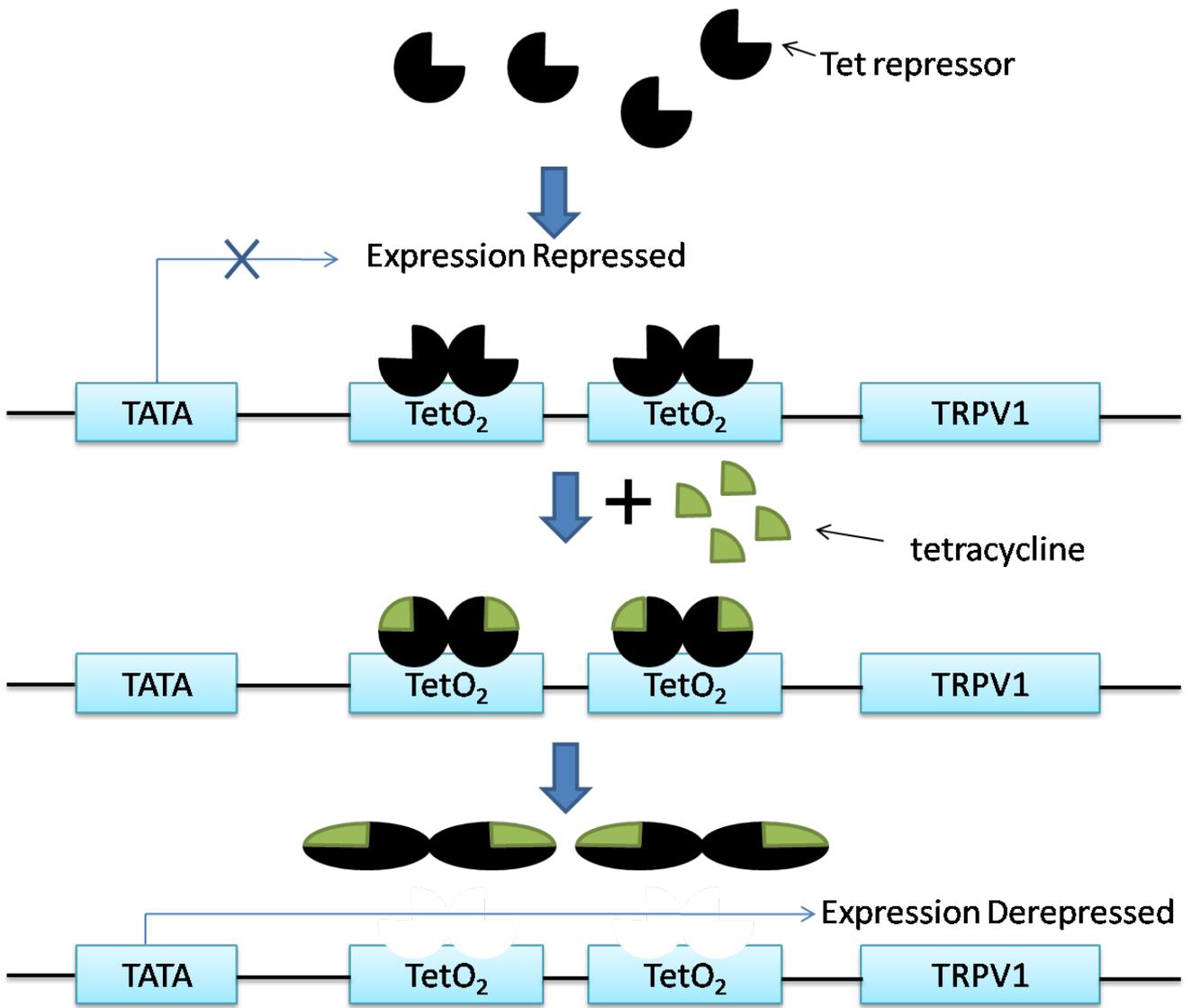


Figure A. Tetracycline-regulated inducible expression.

Appendix II

Content of the buffers

1. Extracellular buffer

Solutes	NaCl	KCl	CaCl ₂	MgCl ₂	HEPES	D-glucose
Concentration (mM)	140	5	1	1	10	10

pH is adjusted to 7.4 with NaOH

2. Intracellular buffer

Solutes	KCl	MgCl ₂	CaCl ₂	EGTA	HEPES
Concentration (mM)	120	2	1	11	10

pH is adjusted to 7.2 with KOH

Appendix III

Fabrication of the microfluidic chip

Thin metal film heating structures are deposited onto the glass under side of the chip by a standard photolithographic process [51], which is showed in Figure B below. ShipleyS1813 is spin-coated onto the glass underside of the chip (Figure B2). Subsequently, the chip is exposed to UV under the mask for 10s to outline the heating structure (Figure B3). The chip is the fixed in a metal enclosure, where only the rectangular glass surface is exposed to the developer (Figure B4). The elastic PDMS effectively seals the enclosure and avoids contamination of the channels and sample wells. Following development, the chip is blow-dried with air, plasma cleaned in an oxygen plasma for 15s (RIE batch top plasma chamber, 50 WRF power and 10s ccmO₂ at 250 mtorr pressure) and a 3.5nm Cr adhesion layer and a 7.5nm Au layer is evaporated onto the glass (Figure B5). Shipley 1165 resist remover is used for lift-off (Figure B6), and enclosing case is used again to avoid contamination of the channels and sample wells.

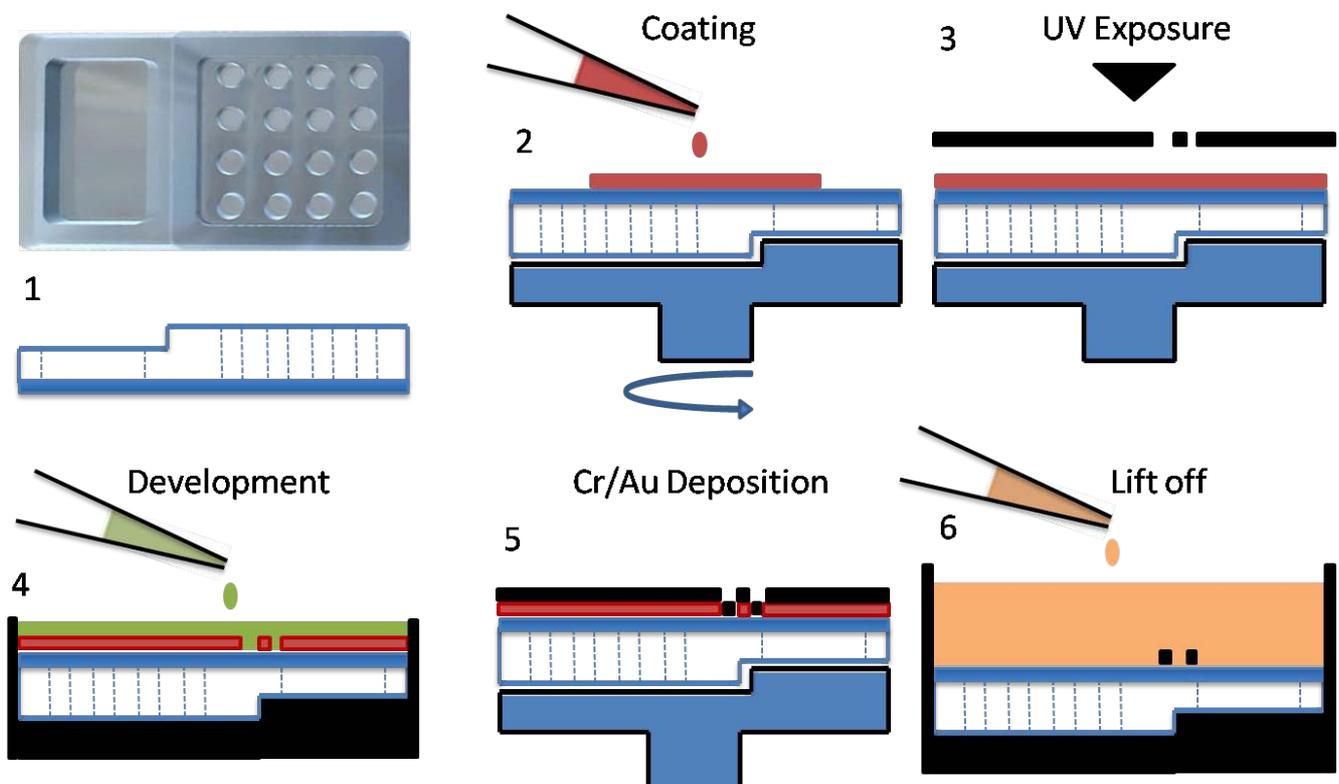


Figure B. Procedure of on chip fabrication (adapted from [51]).