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TITLE:

Recording Electrical Currents Across the Plasma Membrane of Mammalian Sperm Cells

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SUMMARY:

This protocol describes how to perform electrical recordings from mammalian sperm cells in a whole-cell configuration, with the goal of directly recording ion channel activity. The method has been instrumental in describing the electrophysiological profiles of several sperm ion channels and helped to reveal their molecular identity and regulation.

ABSTRACT:

Recording of the electrical activity from one of the smallest cells of a mammalian organism- a sperm cell- has been a challenging task for electrophysiologists for many decades. The method known as “spermatozoan patch clamp” was introduced in 2006. It has enabled the direct recording of ion channel activity in whole-cell and cell-attached configurations and has been instrumental in describing sperm cell physiology and the molecular identity of various calcium, potassium, sodium, chloride, and proton ion channels. However, recording from single spermatozoa requires advanced skills and training in electrophysiology. This detailed protocol summarizes the step-by-step procedure and highlights several ‘tricks-of-the-trade’ in order to make it available to anyone who wishes to explore the fascinating physiology of the sperm cell.

Specifically, the protocol describes recording from human and murine sperm cells but can be adapted to essentially any mammalian sperm cell of any species. The protocol covers important details of the application of this technique, such as isolation of sperm cells, selection of reagents and equipment, immobilization of the highly motile cells, formation of the tight (Gigaohm) seal between a recording electrode and the plasma membrane of the sperm cells, transition into the whole-spermatozoan mode (also known as break-in), and exemplary recordings of the sperm cell calcium ion channel, CatSper, from six mammalian species. The advantages and limitations of the sperm patch clamp method, as well as the most critical steps, are discussed.

INTRODUCTION

Similar to the traditional patch clamp invented by Erwin Neher and Bert Sakmann[1], sperm cell patch clamp enables interrogation of individual ion channel activity, as well as recording from the activity of the entire ion channel population within the single cell[2,3]. The method allows for the identification of a specific ion channel type under degrees of decoupling from enzymatic intracellular processes. This method is crucial for the determination of ion channel activity based on its electrophysiological and pharmacological fingerprints, and hence, provides a reliable identification strategy. The downside of the method is its inability to detect non-electrogenic transporters. Additionally, basic electrophysiological training is helpful to understand the nuances of the protocol. To master the patch clamp technique and apply it to mammalian spermatozoa, we recommend studying basic patch clamp literature[4,5]. In this paper we provide a detailed step-by-step procedure and highlight unique practices that make this technique easy to understand and available to anyone who wishes to practice sperm cell electrophysiology.

Ion homeostasis is an essential physiological function of sperm cells that heavily relies on ion channels and ion transporters to maintain physiologically important ion gradients, vary intracellular calcium, and change transmembrane voltage. Ion channels and ion transporters regulate essential sperm cell functions such as motility, navigation in the female reproductive tract, spermatozoan maturation, and in marine organisms, chemotaxis toward the egg[6-12]. Sperm motility is a gradually acquired process. Sperm cells are mainly quiescent during their maturation in the testis and during their consequent passage through epididymis. Their motility is restrained by an acidic epididymal environment that leads to an internal acidification of the sperm cell. This impairs the function of the axoneme since it is unable to function below pH 6.0[13,14]. However, upon exposure to the seminal fluids or a more alkaline environment, sperm intracellular ion concentrations and cytoplasmic pH undergo major changes and the spermatozoon becomes motile[15-17]. The movement of the sperm flagellum is powered by ATP hydrolysis that supports sliding of axonemal microtubules[18] and this process is highly pH-dependent[14]. Additionally, flagellar movement is also controlled by an elevation of intraflagellar calcium and cAMP[13,19-24]. These factors i.e., sperm intracellular calcium concentration $[Ca^{2+}]_i$,pH, ATP and cAMP are the main regulatory mechanisms allowing for motility changes and their concentrations are tightly regulated by the sperm ion channels and transporters.

Sperm cells are unique in that they express a number of proteins that cannot be found anywhere else in the body. Notable examples are sperm ion channels, such as the potassium channel, Slo3[25-29] and the *Cat*ionic channel of *Sper*m, CatSper[2,30-32]. The latter is the principal calcium channel of mammalian spermatozoa[31], and is regulated by intracellular alkalization[2,30-34]. CatSper is also regulated by species-specific cues[7,35] and is organized in quadrilateral longitudinal nanodomains along the sperm flagellum[36-38]. In primates, CatSper is activated by both flagellar alkalinity and progesterone[3,39-41], while in murine sperm, CatSper is activated solely by flagellar alkalinity[2,39]. Another specific feature of this channel is its multisubunit organization: CatSper is a complex of at least 10 different subunits[31,32,34,37,38,42-47]. Such sophisticated structure and specifics of its regulation hindered recombinant expression of CatSper in any known heterologous expression system, and hence physiological characterization of CatSper has been restricted to its native system of expression- the sperm cell. While molecular characterization of CatSper protein was achieved in seminal paper in 2000 by D. Ren et. al.[31], the ultimate proof that CatSper is a *bona fide* ion channel was possible only after the introduction of sperm patch clamp method in 2006[2]. Since then this technique allowed for precise characterization of many ion conducting pathways in sperm cells[9,28,37,39,40,44,46,48-54].

The classical and the most straightforward method to study ion channel characteristics- the patch clamp technique- was believed to be inapplicable to sperm cells due to their motility and specific morphology (**Figure 1A**). Specifically, the miniscule volume of the sperm cytoplasm and sperm plasma membrane's tight attachment to the rigid intracellular structures such as sperm fibrous sheath and the nucleus were the main challenges[55]. These two structural features result in a slim, arrow-shaped cell that is designed to penetrate through highly viscous environments such as egg's protective vestments, without significant deformation or damage to the plasma membrane.

The first step of the patch clamp method is an establishment of the tight seal between a recording pipette (a glass micropipette) and the cell plasma membrane. In order to achieve this, one has to pull enough plasma membrane inside the recording pipette for a mechanically stable gigaseal to form between the plasma membrane and glass. The plasma membrane must be flexible and not rigid (**Figure 1B**). As mentioned above, the entire surface of sperm plasma membrane is rather tightly adhered, except for the region known as cytoplasmic droplet (**Figure 1A** and **Figure 2**). Hence, the rigid nature of sperm plasma membrane was considered to be a main obstacle in obtaining the tight seal or 'gigaseal', so named because $>10^9$ ohms are required for good recordings. However, the introduction of the sperm patch clamp technique in 2006[2] removed this barrier and this method could be successfully applied to sperm cells of several mammalian species[2,41,51,56]. This breakthrough has been achieved by focusing on the cytoplasmic droplet (CD)[2,8], a tiny structure found along the midpiece of the sperm (**Figure 1A** and **Figure 2**) is simply the remnant of the elongated spermatid- a sperm cell precursor from which the head and the tail develop. Functionally, it may help adapt to changes in extracellular osmolarity during ejaculation. The important feature is that the plasma membrane within the CD is flexible enough to be drawn into the pipette to form a gigaohm seal. Thus, the sperm CD is the best part on the sperm surface through which one can achieve a successful gigaseal formation and transition to a whole-cell mode which ultimately electrically couples the sperm cell to a patch-clamp amplifier[2,8]. It is worth noting, that previous publications reported successful gigaseal formation at the sperm head, which enables recording in the cell-attached configuration[54,57-59]. However, the recordings in whole-cell configuration have so far only been reported by performing gigaseal formation at the CD region. This whole-cell mode allows the electrical access to the entire volume of the sperm cells, and therefore, allows detection of ion channel activities located on the sperm flagellum, as well as on the sperm head. For only a few years since its development, the sperm patch clamp technique has resulted in tremendous progress in our understanding of the sperm ion channels and is so far one of the most robust techniques to directly investigate the functionality of the sperm ion channels[9,28,37,39,40,44,46,48-53] (**Figure 1**).

Sperm patch clamp varies in some details from the classical patch clamp technique as outlined below. First, most of the sperm plasma membrane is tightly attached to the rigid intracellular structure and hence, spermatozoa have almost no "spare" plasma membrane to be drawn into the pipette. The only region that is flexible is CD's membrane that resembles plasma membrane of many somatic cells, and therefore, can be easily drawn into the pipette. To form a gigaohm seal with the CD, negative pressure is created by light suction at the top of the pipette to draw a small portion of the sperm plasma membrane into the tip of the micropipette (**Figure 1B**). This portion of the membrane forms a Ω-shaped invagination into the tip of the pipette and establishes a tight seal with its internal walls.

Second, the cytoplasmic droplet in human and mouse spermatozoa is between 1 and 2 µm (**Figure 1** and **Figure 2**). Hence, the application of the patch-clamp technique to such a small object requires high-resolution optics. Most sperm patch-clamp rigs are equipped with an inverted microscope with a differential interference contrast (DIC) or Nomarski optical components (**Figure 2** and **Figure 3**). Using a microscope equipped with DIC optics for sperm patch clamp is highly recommended over more conventional phase-contrast optics, since the spatial information seen in DIC helps achieve superior precision in positioning a patch pipette onto the tiny CD. We also suggest using a 60x water immersion objective or similar lens, with numerical aperture of 1.2. This objective has a long working distance (0.28 mm), which allows observation of free-swimming sperm cells in solution (**Figure 2**). The objective also has an adjustment collar to adjust to the thickness of the cover slip (variable from 0.13 to 0.21 mm). This combination of the long working distance and adjustment collar enables observation through two 0.13 mm cover slips; one cover slip serves as the glass bottom of the recording chamber, and the 5 mm coverslip with deposited sperm cells is placed on top. As discussed below, depositing sperm cells on easily exchangeable round 5 mm coverslips, rather than on the bottom of the recording chamber directly, is a convenient way to upload fresh sperm cells into the recording chamber.

Third, the sperm patch clamp rig must be equipped with a low noise patch-clamp amplifier and a digitizer to record tiny (picoampere range) electrical currents and miniscule changes in membrane potential. This equipment must ensure the lowest amplifier noise. The absence of vibration is an essential part of a successful patch clamp recording. Sperm patch clamping requires a drift-free precise micromanipulator that can be attached to the inverted microscope with a micromanipulator platform to ensure better stability than an independent micromanipulator stand (**Figure 3A**). To test the setup, one should not see any movement of the pipette tip (under 60x magnification) even when a person jumps up and down on the floor near the vibration-isolation table.

PROTOCOL:

All experiments were performed in accordance with NIH guidelines for animal research and approved by UC Berkeley Animal Care and Use Committee (AUP 2015-07-7742), with every effort made to minimize animal suffering. All described methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and IACUC committee. All experimental procedures utilizing human derived samples were approved by the Committee on Human Research at the University of California, Berkeley, IRB protocol number 2013-06-5395.

1. Making glass micropipettes for whole-cell sperm patch-clamp recording.

NOTE: The small size of cytoplasmic droplet requires glass micropipettes with fine tips.

1.1. For micropipette fabrication, start with borosilicate glass capillaries with an outer diameter 1.5 mm, inner diameter 0.86 mm and an internal filament and pull the tip on micropipette puller (**Figure 3B**).

1.2. For shaping and polishing of the pipette tip, position a hot wire in close proximity of the tip for a short moment. Perform this process of fire-polishing under 100x magnification using a pipette polisher (**Figure 3C,D**).

1.3. Ensure that the inner diameter of the pipette tip is ~2 µm before fire polishing and is reduced to ~0.5 µm after proper polishing.

NOTE: Only freshly pulled pipettes made on the day of recording are recommended for use. This avoids the accumulation of dust particles on the pipette tip.

2. Setting up the rig

2.1. Assembly of the recording chamber

2.1.1. Use a low-volume diamond-shaped perfusion chamber with laminar flow and relatively fast solution exchange rates (**Figure 4**). Connect the chamber via a manifold to a gravity-fed perfusion system (**Figure 5**).

2.1.2. To perfuse a solution through the recording chamber, assemble a simple self-made gravity-fed perfusion system with a polytetrafluoroethylene manifold that connect the perfusion chamber to eight separate perfusion lines.

NOTE: The tubing should not leach plasticizers into the solutions, since such chemicals can significantly impact the normal function of sperm cells[60] and alter ion channel activity[61,62]. A small diameter polytetrafluoroethylene tubing is highly recommended as the main perfusion tubing.

2.1.3. As polytetrafluoroethylene tubing is rather stiff, make flexible connections with high purity silicon tubing fit over the polytetrafluoroethylene tubing. In addition, use syringes (e.g., Luer Lock syringe- type) that lack lubricant, since mineral oil or other lubricant additives can interfere with ion channel recording (**Figure 5**).

2.2. Assembling an agar bridge

2.2.1. To avoid solid-liquid junction potential changes during recordings, keep the environment around the reference electrode stable by using an agar bridge (**Figure 4**).

2.2.2. To make an agar bridge, make an L-shaped glass capillary by bending it under small Bunsen burner fire and let it cool down.

2.2.3. Make a solution of 1% agarose in 1 M KCl and heat it in a microwave until the agarose melts and the solution becomes transparent. Carefully fill L-shaped glass capillary with the above solution to avoid air bubbles and let it cool to room temperature. The agar bridges can be stored in 1 M KCl for ~2 months at +4 ˚C.

2.2.4. To use an agar bridge, fill an Ag/AgCl pellet reference electrode with 1 M KCl and insert the agar bridge carefully to avoid solidified agarose popping out of the glass capillary.

2.2.5. Next, insert the gold pin (2 mm) of the amplifier headstage wire into the reference electrode 2 mm (gold) slot and insert the end of the L-shaped agar bridge in the perfusion chamber as shown in **Figure 4**.

2.3. Solutions and recipes

NOTE: All reagents and chemicals must be of the highest purity. All prepared solutions are filtered through a sterile 0.22 µm PES filter and stored at 4 ˚C for up to one month. It should be noted that the exact adjustment of osmolarities is a prerequisite for successful patch clamping.

2.3.1. Prepare human tubular fluid (HTF) solution as described in **Table 1**. Adjust the pH to 7.4 with NaOH. Osmolarity should be 280 ± 5 mOsm, measured using a vapor pressure osmometer.

2.3.2. Prepare high saline (HS) “bath” or “extracellular” solution as given in **Table 2**. Adjust the pH to 7.4 with NaOH. Osmolarity should be 320 ± 5 mOsm.

2.3.3. Prepare cesium methanesulfonate “bath” or “extracellular” solution as described in **Table 3**. Adjust the pH to 7.4 with CsOH and the osmolarity to 320 ± 5 mOsm.

2.3.4. To prepare Cesium methanesulfonate ($CsMeSO_3$) intracellular “pipette” solution follow **Table 4** and adjust pH to 7.4 with CsOH. Osmolarity should be up to 10 mOsm higher than the HS solution.

3. Isolation and purification of mammalian spermatozoa

NOTE: Euthanize C57BL/6 male mice aged 3-6 months by inhalation of CO_2 followed by cervical dislocation. After cervical dislocation, perform tissue collection (cauda or corpus epididymis) from mice immediately.

3.1. Isolation of epididymal mouse spermatozoa

NOTE: Euthanize C57BL/6 male mice aged 3-6 months by inhalation of CO_2 followed by cervical dislocation. After cervical dislocation, perform tissue collection (cauda or corpus epididymis) from mice immediately.

3.1.1. Open the lower abdominal area of the mouse with scissors and extract both epididymides.

3.1.2. Place them in a 35 mm cell culture dish filled with HS solution (high saline solution, see **Table 2**). Ensure that an aliquot of the HS solution is prewarmed to room temperature before contact with the sperm cells.

3.1.3. Transfer the epididymides into a new cell culture dish containing HS solution and the remove thoroughly all residual fat.

3.1.4. Separate the epididymides into caput, corpus and cauda using a #15 scalpel blade (**Figure 6**).

3.1.5. Transfer the corpus (or cauda if needed) of each epididymis into a new cell culture dish containing HS solution. Make multiple incisions in the isolated part of the epididymis using a pointed #11 scalpel blade.

3.1.6. Transfer the parts of the epididymides with multiple incisions into a 1.5 mL microcentrifuge tube containing 1.5 mL HS solution.

3.1.7. Briefly shake sperm cells from the epididymis into the solution using super-fine Dumont type 5a forceps. Discard epididymides and leave the tube at room temperature for 10 min.

3.1.8. Wait till the solid matter (non-sperm cells) sediments to the bottom of the tube, and then transfer the supernatant into another 1.5 mL microcentrifuge tube.

3.1.9. Store the sperm mixture at room temperature with access to air for up to 2 h, during which the experiments can be performed. Isolated sperm lose their performance 3 h after isolation.

3.2. Capacitation of mouse spermatozoa

NOTE: All epididymal spermatozoa are suitable for patch-clamp, but only spermatozoa from the cauda epididymis are mature enough to become fertilization competent. Since caudal spermatozoa can undergo capacitation, it is suggested to perform experiments, particularly sperm capacitation, using caudal sperm cells.

3.2.1. Perform sperm capacitation on caudal or ejaculated spermatozoa (see step 3.1.4).

3.2.2. Take caudae isolated as described in step 3.1.4. and apply multiple incisions as described in the step 3.1.5. Transfer the caudae to a 10 mm cell culture dish with 2 mL of capacitation solution.

3.2.3. Incubate the caudal tissue in this capacitation solution initially for 10 min at 37 °C and 5% CO_2, after which discard the tissue and incubate the released sperm cells for another 50-80 min.

NOTE: In vitro capacitation of mouse caudal spermatozoa is achieved by 60-90 min incubation. Capacitated sperm cells are visually identified based on their excessive asymmetric bending of their flagella known as hyperactivation. This type of motility allows spermatozoa to swim in nonlinear manner.

3.3. Collection and purification of human sperm

NOTE: In this case, fresh semen samples were obtained from a total of 21 healthy volunteers aged 21-38 and spermatozoa purified by the room temperature swim-up technique as described in details in[48,63]. Briefly, the procedure is as follows:

3.3.1. Leave the human ejaculate samples at room temperature for 60 min to allow for liquefaction before being purified (longer durations may negatively affect sperm quality).

3.3.2. Preheat about 7 mL of HTF solution to 37 °C in a 50 mL centrifuge tube. Visually assess the volume of the ejaculate–if the volume is greater than 1 mL, prepare multiple tubes of HTF solution.

3.3.3. Carefully underlay 1 mL of liquefied ejaculate to the bottom of each tube without lifting strands of semen to the surface of the buffer. The semen-to-HTF-interface should be as clear-cut as possible. Avoid air bubbles.

3.3.4. Place the tubes in a 37 °C incubator for 1 h in a stand that holds them at a 45° angle for maximal surface area. If the incubator is a CO_2 incubator, close the cap tightly to prevent acidification of the buffer (CO_2 is not required for this procedure). During the incubation, motile sperm will gradually swim towards the surface of the HTF buffer.

3.3.5. After 1 h collect the uppermost 1 mL fraction from all samples in a 15 mL centrifuge tube, thus combining the highest quality, highly motile sperm for patch clamping. Do not attempt to remove all the supernatant–leave several mL of HTF remaining above the seminal plasma to avoid contamination. The purified cell suspension can be stored at room temperature for several hours.

NOTE: A normal ejaculate should contain at least 15 x 10^6 sperm cells per mL[64], but sperm counts vary widely between samples. The obstacle of a low cell count may be overcome by letting the purified sperm suspension sit for 30-60 min at room temperature before patch clamping. Under this condition, the cells will gradually accumulate at the bottom of the centrifuge tube and form a visible cell cloud from which you can pipette cells into the recording chamber (see below).

3.4. Capacitation of human spermatozoa

NOTE: In-vitro capacitation of human spermatozoa can be accomplished by 4 h incubation in capacitation solution: 20% fetal bovine serum, 25 mM $NaHCO_3$ in HTF or HS buffers[48,51]. Capacitated sperm cells can be visually identified based on their vigorous motility and excessive asymmetric bending of the flagella that allows spermatozoa to swim in nonlinear manner.

3.4.1. Prepare a 2x capacitation solution and mix 1:1 with sperm suspension.

3.4.2. Perform incubation at 37 ˚C and 5% CO_2 for a minimum of 4 h. To ensure a high percentage of capacitated cells, incubation time should be no shorter than 4 h.

4. Preparing coating solution (only needed for human sperm patch-clamp)

NOTE: An essential step is to lift the attached spermatozoon from the coverslip before break-in. This step is only necessary for human sperm cells and requires coating of the glass coverslip to create a less adhesive glass surface. Coverslip coating reduces sperm cells probability to stick to the coverslip and allows human spermatozoa to be lifted from the glass coverslip after successful gigaseal formation.

4.1. Dilute 200 µL of the ejaculate in 5 mL of HS solution in 15 mL centrifuge tube.

4.2. Spin down at 300 x *g* for 5 min and remove supernatant.

4.3. Resuspend the pellet in 1 mL of HS solution and transfer to a microcentrifuge tube.

4.4. Sonicate for 10 min at 25 ˚C using water-filled sonication bath.

4.5. Spin down at 10,000 x *g* for 5 min.

4.6. Transfer the supernatant to a fresh microcentrifuge tube and use it for coating (instructions below).

5. Recording ion conductance from the whole sperm plasma membrane.

5.1. Attaching sperm to the coverslip.

NOTE: Before the patch-clamp recording, spermatozoa are plated onto 5 mm round cover slips.

5.1.1. If working with human cells, briefly dip the coverslips into the prepared coating solution using sharp forceps.

5.1.2. Place four cover slips into a single well of a 4-well plate and cover with 300 µL of HS solution. Prevent the cover slips from floating by taping them to the bottom of the 4-well plate, pushing them with the plastic tip of the pipettor.

5.1.3. Gently resuspend the concentrated suspensions of human (step 3.3.5) or mouse spermatozoa (step 3.1.9) from the storage tube (20-50 µL of concentrated sperm) in 300 µL of HS solution above the coverslip.

5.1.4. Ensure that spermatozoa accumulate at the bottom of the well where their heads will attach to the coverslip and their flagella will vigorously beat in the solution just above the cover slip. It is important to allow spermatozoa to sediment onto the coverslip for about 10 min at room temperature before recording.

NOTE: If human spermatozoa attach poorly, go back to the step 5.1.1 and dilute the prepared coating solution with HS solution (1:20 to up to 1:2). This step must be adjusted to sperm samples from different human donors based on the variable sperm motility that is common in human sperm preparations.

5.2. Placing cover slip

5.2.1. Take one coverslip with slightly adhered cells from the well and place into a diamond-shaped well of the recording chamber containing HS solution (**Figure 4**).

5.2.2. Turn on the perfusion system (**Figure 5**) filled with HS solution to rinse the chamber and remove debris and excess detached spermatozoa from the chamber. This will increase the probability that the recording pipette stays clean when it reaches the cells. This step is helpful when working with non-capacitated cells that tend to stick to the coverslip stronger.

NOTE: If recording from the capacitated sperm, one must avoid turning on the perfusion immediately after a fresh cover slip is placed into the chamber. Capacitated spermatozoa have vigorous motility and only loosely attach to the coverslip. Therefore, to ensure that the largest number of capacitated sperm are available, it is important to wait ~ 10 min and let capacitated spermatozoa adhere to the coverslip before turning on perfusion. Once the appropriate capacitated cell is chosen and a gigaseal is formed, the perfusion can be turned back on.

5.3. Selection of a sperm cell for patch-clamp recording.

5.3.1. Find a suitable sperm cell with a cytoplasmic droplet using 600x magnification (see below). This is done using 60x water immersion lens as described in the methods with 10x magnification eyepiece. In sperm cells isolated from corpus epididymis, the CD is usually located close to the center of the midpiece. In caudal cells, the CD can usually be found close to the annulus (**Figure 2C**). For human sperm, the CD is located in the neck region (**Figure 2A,B).**

NOTE: Additional pull-out 1.6x magnification option of the microscope can be used to closely examine sperm morphology (magnification 960x).

5.3.2. Ensure that the cytoplasmic droplet is oval and have a slightly elongated (**Figure 2G**), spindle-like shape. CDs that appear very round and enlarged are often fragile and unsuitable for patching (**Figure 2D,F**).

5.3.3. Select spermatozoon that is motile with the head attached to the coverslip, such that the sperm cell is partly fixed, but the CD and the rest of the flagellum continues to move with flagellar beating. Ensure that the head of the sperm is loosely attached to the coverslip so that it rotates a little bit as the flagellum moves from side to side.

NOTE: The loose attachment is important since after formation of the gigaohm seal and before the transition into the whole-cell mode, the spermatozoon must be lifted from the coverslip into the solution.

6.4. Forming a gigaseal

NOTE: To record monovalent CatSper currents in the whole-cell mode, pipettes are filled with Cs-methanesulfonate (also referred as pipette or intracellular) solution (**Table 4**) – this yields a pipette resistance of 11-17 MΩ (**Figure 7A**), as automatically calculated by the amplifier upon applying a voltage step from 0 mV to 10 mV. Pipettes can also be filled with other solutions depending on the application. Pipettes can also be filled with other solutions depending on the application. It is recommended that solutions are made with mobile anions such as methanesulfonate, Cl^-, aspartate or similar ions to reduce pipette resistance. The osmolarity of the pipette solution should be up to 10 mOsm higher than the bath solution. Slightly higher tonicity of the pipette solution helps to keep the access resistance into the sperm cells as low as possible during patch-clamp recording.

6.4.1. After visual selection of a sperm cell with a proper morphology (**Figure 2G**), fill the micropipette with a pipette solution (**Table 4**) and secure it into the pipette holder.

6.4.2. In order to keep the pipette tip clean from debris, apply positive pressure to the pipette using U-tube shaped assembly to ensure that the pipette solution flows out of the tip after it is immersed into the extracellular solution, also referred below as “bath” solution (see **Table 2**).

NOTE: If conditions permit, the formation of the seal under constant perfusion is highly advised, as this condition ensures the cleanest pipette tip.

6.4.3. Lower the pipette down and immerse its tip into the bath solution (**Table 2**). At this time, pipette resistance can be measured (**Figure 7A**).

6.4.4. In order to clearly visualize the cell, position the tip of the pipette above the CD and the opening of the tip is aligned diagonally (approximately 45˚) toward the CD.

6.4.5. Quickly lower the tip of the pipette toward the CD to be in the same focal plane, within a few µm apart from the CD.

6.4.6. As soon as the tip of the pipette touches the CD, apply negative pressure to the pipette using "mouth-piece" to move part of the droplet into the tip and form a gigaohm seal - also known in electrophysiology as a "suction event". Usually, after initial contact with the pipette tip, the negative pressure in the pipette can be maintained up to a minute to gradually achieve a gigaohm seal.

6.4.7. After successful formation of the gigaohm seal ($>$ 4-20 GΩ), lift the spermatozoon from the coverslip (**Figure 7B**).

NOTE: This step is not required for rodent sperm cells due to their length, however, partial detachment of the sperm flagellum is beneficial to alleviate potential pipette drift. Sperm detachment is an essential step, since the CD is small (1~3 µm); if the cell is left attached to the coverslip, even a slight drift of the pipette during the experiment can disrupt the CD. After the spermatozoon is lifted, its flagellum normally continues to beat, while the cell is attached only to a micropipette.

6.5. Break-in and transition into the whole-cell mode.

6.5.1. Compensate stray capacitance transients using amplifier's compensatory mode before transitioning to the whole-cell mode (**Figure 7B**). To initially observe capacitance transients, hold the membrane potential at 0 mV and 10 mV pulses are applied using the "Membrane Test" tool (**Figure 7B**).

6.5.2. Perform a break-in and transition into the whole-cell mode by applying short (1 ms) gradually increasing (430-650 mV, ~50 mV increment) voltage pulses combined with a very light suction (**Figure 7C**).

NOTE: To apply the break-in pulses, use previously programmed **break-in** protocol in patch clamp software. The break-in voltage pulses are applied to the spermatozoon using the high voltage command input (rear-switched) on the back of the amplifier.

6.5.3. After application of each break-in voltage pulse, launch the **Membrane Test** tool to check whether larger capacitance transients appear.

NOTE: The presence of large capacitance transients (**Figure 7C**) indicates that the break-in has occurred, and the entire capacitance of the cell is being measured (~1 pF for human sperm and ~2.5 pF or more for murine sperm).

6.5.4. Fit the large capacitance transients using **Membrane Test** Tool to determine the capacitance of the whole cell as well as its access resistance.

NOTE: The access resistance should be as low as possible for efficient perfusion of the interior of the sperm cell with the micropipette solution, as well as for reliable voltage clamp of the sperm plasma membrane. Access resistance is usually 25-30 MΩ and 50-70 MΩ for Cs^+/K^+- and NMDG-based intracellular solutions, respectively.

6.5.5. After successful break-in, proceed with the planned whole cell patch-clamp experiments, such as applying various bath solutions (**Figure 8**) containing different compounds or measuring channel activities using voltage-step (**Figure 8**, **Figure 9**) or voltage-ramp protocols. If the break-in was not successful, return to step 6.4.1. by choosing fresh micropipette and select another appropriate sperm cell.

REPRESENTATIVE RESULTS:

Sperm patch clamp method allows for direct recording of the CatSper channel.

As mentioned above CatSper recordings were performed by establishing a high resistance (gigaohm) seal between the patch pipette and mammalian spermatozoon at its cytoplasmic droplet. Upon breaking in and transitioning into a whole-cell mode, the full electrical access to the entire body of the sperm cells and its interiors, including sperm head and flagellum is obtained[2,8,39,51]. This condition ultimately allows recording from any active ion channel located on sperm plasma membrane. Bath nominally divalent-free (DVF) solution containing either cesium or sodium as the main permeant ions is preferable for recording of the monovalent CatSper currents [2,8,39,51]. While CatSper channel conducts divalent ions such as Ca^{2+} and Ba^{2+}, they are moving through CatSper pore at a much slower rate, resulting in barely detectable conductances of few picoamperes (~10-20 pA)[2,8,39,51]. Hence, measuring monovalent and, therefore, larger currents, through the CatSper channel is a more convenient way to assess the current (**Figure 8**). It is important to note that CatSper is also permeable to potassium; therefore, the CatSper channel must be blocked, or CatSper-deficient sperm cells used in situations where one wants to study only sperm cell potassium channels[2,3,8,28,65]. By varying ion composition of the pipette and bath solution, one can selectively exclude certain ion channels, while creating conditions for selective recording from only specific ion channel type(s). For example, addition of Cs^+ into the pipette solution results in blocking ion permeability through sperm potassium channel.

The CatSper channel is regulated differently among mammalian species.

Sperm cells of different species are diverse in their morphology and internal regulatory pathways[66]. It is no surprise that their ion channels are also regulated uniquely in ways that reflect the specialized microenvironments of the male and female reproductive tracts. The sperm patch-clamp method has been successfully applied to six mammalian species: murine[2], rat[56], human[39,51], bovine, boar, and macaque[41] as shown on Figure 9. For these experiments sperm cells from adult male rhesus macaques [*Macaca mulatta*] were obtained from the California National Primate Research Center in compliance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) under approved animal protocols by University of California, Davis as described in[41]; and all studies were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Bull and boar semen were obtained as by-products exempt from specific IACUC approval from UCD Department of Animal Science facilities and all animals were maintained in AAALAC-approved facilities. Bull and boar semen can also be obtained from commercial sources.

Primate (*Rhesus macaque*) and human spermatozoa showed similar CatSper channel properties and regulation. Interestingly, progesterone activation of CatSper seems to be unique for primate spermatozoa (**Figure 9** and [41]), as boar, bull, and rodent sperm did not display any progesterone stimulated alteration of their CatSper currents. In bull and boar spermatozoa, even basal CatSper channel activity was below detectable limits (**Figure 9**), suggesting that in these species, calcium influx and consequent hyperactivation is driven by other channels/transporters, or that a different natural stimulator is needed for activation of their CatSper channels. It must be stated, that with all sperm species mentioned here, including bull and boar sperm cells, the full electrical access to the interior of the sperm cells was obtained and cells were recorded in the whole-cell mode, as was evident by the appearance of the large capacitance artifacts upon breaking-in (**Figure 7**). This condition permits easy recording of the functional CatSper channel, and its absence from boar and bovine spermatozoa indicate that either this channel is blocked by yet unknown endogenous inhibitor present in sperm cells of these species or requires specific modulator to be activated. However, these are preliminary experiments and additional experiments will be required for boar and bull sperm cells to ensure functional importance of CatSper channel in these species. This broad spectrum of sperm ion channel diversity among species could be related to the sperm to egg size ratio, the relation between sperm size and the egg protective vestments, or serve as a barrier to fertilization by other species[66].

FIGURE AND TABLE LEGENDS:

Figure 1: Mammalian sperm morphological diversity. (**A**) *Bottom panel:* schematic representation of a spermatozoon; cellular compartments are labeled. *Top panels:* DIC images of spermatozoa from different species in clockwise: rat (Rn; *Rattus norvegicus*); mouse (Mm; *Mus musculus*); bull (Bt; *Bos taurus*); boar (Sd; *sus scrofa domesticus*); human (Hs; *Homo sapiens*), and rhesus macaque (Mmu; *Macaca mulatta*). The scale bar applies to all DIC images. Inserts indicate cytoplasmic droplets. (**B**) Patching of the mammalian sperm cells. To achieve successful seal formation between the pipette tip and the plasma membrane, part of the plasma membrane is gently sucked into the pipette tip. Transfer to whole-cell mode is performed by rupturing the plasma membrane between the tip and the cell (this figure has been reproduced from[8]). Right panel: human sperm cells attached to a recording micropipette. (**C**) Schematic representation of human spermatozoon and some of the flagellar ion channels studied in human sperm cells with the patch clamp method, as well as the ions they conduct. CatSper- calcium ion channel[39,51]; Hv1-

proton channel[51,56,67]; Slo3/Slo1- potassium channels[50,53,65,68]; TRPV4- transient receptor potential cation channel vanilloid type 4[48].

Figure 2: Sperm size and variable morphology of cytoplasmic droplets. The DIC images of intact live sperm cells. (**A**) Comparison of the size of a human sperm cell (bottom) and two CHO cells (upper). (**B**) Intact human (*Homo sapiens*) spermatozoon (bottom) and a headless sperm cell (flagellum, upper). Cytoplasmic droplets are indicated by yellow arrowheads; this figure has been reproduced from[8]. (**C**) Intact murine (*Mus musculus*) spermatozoon with the normal shaped cytoplasmic droplets (CD) indicated by the yellow arrowhead. (**D-G**) Epididymal murine sperm cells have cytoplasmic droplets of different sizes and shapes; only (**C**) and (**G**) are suitable for patch-clamp. (**D**) CD is microscopic and one-sided; (**E**) CD is missing; (**F**) CD has particles inside that may clog the recording pipette; (**G**) CD is smooth, uniform, and not swollen. Forming a gigaseal with this type CD will likely result in a successful recording.

Figure 3: Sperm patch clamp rig components. (**A**) Typical sperm electrophysiology rig with essential components: (1) inverted microscope; (2) low noise digitizer; (3) amplifier; (4) low drift micromanipulator that is coupled to the inverted microscope with a micromanipulator platform; (5) PC computer; (6) a vibration-damping air table; (7) Faraday cage to shield the setup from ambient electrical interference. It is essential that all electrically powered components of the rig, including computer keyboard and mouse, produce low or no electrical (50Hz or 60Hz) noise and that all components of the rig are properly grounded. (**B**) Micropipette puller used for recording pipette fabrication. (**C**) (1) MicroForge used for pipette fire-polishing; (2) Borosilicate glass capillaries with outer diameter 1.5 mm, inner diameter 0.86 mm and an internal filament; (3) pipette collection box. (**D**) Stages of successful pipette fire-polishing: (a) Unpolished pipette with the inner diameter of 2 mm; (b) Fire-polished pipette with an inner diameter of 0.5 mm; (c) Over-polished sealed pipette not suitable for recording.

Figure 4: Components of the recording chamber system and its assembly. (**A**) Essential components of the recording chamber system: (1) microscope stage adapter for series 20 platforms with (2) two stage holding clamps; (3-4) PM series magnetic heated platform with (3) magnetic clamps to hold the perfusion chamber; (5) perfusion chamber; (6) agar bridge; (7) magnetic clamp, reference electrode with 2 mm jack to Ag/AgCl pellet; (8) magnetic holder (MAG-1) for the suction line; (9) suction tube; (10) suction tube O-holder. (**B**) Assembled recording chamber system with indicated components from (A).

Figure 5: Components of the perfusion system. (**A**) Assembled perfusion line and (**B**) its essential components: (1) 20 mL and 3 mL syringes; (2) stopcock with Luer connections; 4-way; male lock; (3) female luer hose barb adapter, 1/16”; (4) Polytetrafluoroethylene (PTFE) perfusion tubing (Microbore PTFE Tubing, 0.022” ID × 0.042” OD); (5) Polytetrafluoroethylene 8-position perfusion manifold; (6) Silicone connector tubing (platinum-cured silicone tubing, 1/32” ID × 3/32” OD); (7) Manifold connector tubing (PTFE Tubing, 1/32” ID × 1/16” OD).

Figure 6: Male mouse dissection. (**A**) Murine male reproductive organs; both testis and epididymis are shown. (**B**) The epididymides are transferred into a 35 mm cell culture dish

containing HS solution and the residual fat and vas deferens removed. (**C**) Each epididymis is then divided into caput, corpus and cauda using a #15 scalpel blade.

Figure 7: Gigaseal formation and break-in with the murine sperm cell. The interface of the "Membrane Test" tool of the commercial patch clamp software. Three stages of sperm patch clamping: (**A**) recorded pipette is immersed in a bath HS solution producing a pipette resistance of 14.8 MΩ; (**B**) A gigaseal is formed (resistance is 4.7 GΩ), capacitance transients are compensated, and the spermatozoon is lifted from the coverslip; (**C**) Transition into whole-cell mode. Break-in and transition into the whole-cell mode is performed by applying short (1 ms) gradually increasing (430-650 mV, ~50 mV increment) voltage pulses combined with a light suction, as shown on the left. Break-in has occurred as evident from the appearance of the large capacitance transients that reflect the entire capacitance of the cell (~2.93 pF for this sperm cell).

Figure 8: Murine CatSper recording from wild type (WT) cauda epididymal, capacitated and CatSper knockout spermatozoa. To record monovalent CatSper activity, a ramp protocol is applied every 5 s and CatSper currents elicited by voltage ramps from a holding potential of 0 mV[39,51]. Voltage ramps (-80 mV to 80 mV; 850 ms) are applied in HS and nominally divalent-free solution (DVF). Data were sampled at 2-5 kHz and filtered at 1 kHz. Baseline currents recorded in HS solution, which produces no CatSper current due to inhibition by high extracellular magnesium[39,51]. Baseline currents are useful to estimate the leak conductance (non-ion channel pathways). Representative, Cs^+ whole-cell CatSper currents densities (pA/pF; blue) recorded from caudal WT murine sperm cells (noncapacitated; left and capacitated; middle) and CatSper-deficient caudal murine sperm cells (right). Currents were elicited by voltage ramps from a holding potential of 0 mV and ramps were applied from -80 mV to 80 mV in HS and nominally divalent-free solution. Baseline currents (black) recorded in HS solution. To obtain current densities, CatSper current amplitudes were normalized to cell capacitance (pA/pF).

Figure 9: Progesterone regulation of CatSper in different mammalian species. (**A**) Representative CatSper current elicited from sperm cells of different species by a voltage ramp protocol as indicated. Species: human (Hs; *H. sapiens);* rhesus macaque (Mmu; *M. mulatta*), mouse (Mm; *M. musculus*), bull (Bt; *B. taurus*); rat (Rn; *R. norvegicus*); boar (Sd; *S. scrofa domesticus*). CatSper currents in the absence (blue) and presence (red) of 1 mM progesterone were recorded, as well as the basal currents in HS solution (black). (**B**) CatSper current amplitudes ($I_{CatSper}$, *pA*) and (C) Average current densities (pA/pF) recordings from sperm cells of different species as indicated; n indicates the number of individual sperm cells used. Data are Mean +/- S.E.M.

Figure 10: The difference in flagellar motility. Two representative examples of cytoplasmic droplets and flagellar motility. The overlaid images of the same rat (Rn) and human (Hs) sperm cells were taken at two different time points when they show the most distal flagellar deflection. Dotted rectangles indicate the region with cytoplasmic droplets and their corresponding spatial mobility.

Figure 11: U-tube assembly and its essential components. (**A**) Components of the U-tube: (1) 10 mL serological pipette; (2) Silicone tubing; (3) Silicone connector tubing; (4) 1 mL syringe; (5) Female luer barb adapter; (6) male Luer integral lock adapter 1/8”; (7) stopcock with Luer connections; 4-way; male Lock; (8) male Luer series barb adapter, 1/16”. (**B**) Fully assembled U-tube and (**C**) U-tube attached to the Faraday cage.

Figure 12: A schematic representation of the U-tube assembly. Left panel: A positive air pressure is provided by mouth to create a difference in liquid levels in the U-tube. The level of liquid in its right horn raises by 2 cm. After such level difference is created, the stopcock is turned to connect U-tube to the line leading to a recording pipette. Right panel: the higher liquid level in the right horn creates a positive pressure that constantly pushed pipette solution out of the pipette tip and keeps the tip clean of the debris.

Table 1: Human tubular fluid (HTF) solution

Table 2: High-Saline (HS) Solution

Table 3: $CsMeSO_3$ bath solution (Divalent Free bath solution: DVF)

Table 4: $CsMeSO_3$ pipette solution

DISCUSSION:

We describe a detailed protocol to perform electrophysiological recordings from sperm cells of various species. Given the physiological significance of ion channels and electrogenic transporters for spermatozoa, this technique is a powerful tool to study sperm cell physiology as well as defects that lead to male infertility. The experimenter might find the execution of this technique challenging at first, but with perseverance and endurance, success follows.

Mammalian spermatozoa are long (usually >50 µm), narrow, and highly motile. The basal beat frequency (BF) of mammalian spermatozoa varies greatly with values averaging from 4 Hz (mouse [69]), 7-15 Hz (boar [70,71]), 11 Hz (rat [72]), 11-20 Hz (bull [18]), 24 Hz (rhesus macaque [23]), and up to 25 Hz (human [3]). The cytoplasmic droplet (CD) is the entryway for recording from sperm cells. In rodent spermatozoa the CD is often distal but moves alongside the flagellum (**Figure 10**), creating an additional obstacle to recording. However, in human sperm cells the CD is more commonly located near the head. The key components of a successful sperm patch-clamp are therefore excellent optics to enable a clear, sharp view of CD and a highly precise micromanipulator system without drift or vibration. The initial high rate of failures is expected and is normal within the first several days of sperm patch clamp. We recommend routine practice involving numerous attempts per week. Achieving several recordings per day per week will establish a routine and improve motor skills.

Until recently, the identification and pharmacological characterization of sperm ion channels was hindered by an inability to study them directly. The field largely relied on immunocytochemistry studies, which often suffer from nonspecificity of antibodies and/or the lack of corresponding genetics models. To study calcium channels, the classical calcium imaging method has been widely used, which has its own advantages and limitations[73-77]. While calcium imaging is a relatively easy method that is applicable for medium-to-high throughput studies[78-81] and is less invasive, it requires relatively intact cells, and hence poses a hurdle to dissect the function of ion channels decoupled from intracellular signaling cascades or to distinguish them from calcium ion exchangers. Additionally, it is difficult to control membrane potential and therefore, harder to exclude contribution of the voltage-gated calcium channels. Among several advantages of calcium fluorometry is the use of calcium ratiometric dyes that allows precise measurement of the changes in calcium ions concentration. At the same time, one must be aware of that the sensitivity of these dyes can vary based on the changes of intracellular pH.

Below we describe the critical steps within the protocol, including troubleshooting steps of the method. It is essential to use only pure reagents for the preparations of the experimental solutions, as even a small contamination with undesirable ions (such as magnesium or heavy metals) can impair the detection of monovalent currents. Given small size of the sperm cells, one can expect a relatively low numbers of ion channels per cell. Hence, the net current ranges from few pA to several hundred pA. Therefore, the internal electrical noise of the rig must be minimal to ensure detection of small currents, and the use of drift-free equipment highly recommended. In order to distinguish a specific conductance from electrical noise and background leak, the recording apparatus and grounding system must be maximized. This is achieved by properly grounding the rig to avoid any electrical interference[82]. The use of a Faraday cage is highly recommended to protect from electrical interference produced by a variety of electrical devices, such as building lights and in-wall electrical wiring. It is essential that all electrically powered components of the rig, including the computer keyboard and mouse radiate little or no electrical (50 Hz or 60 Hz) noise and that all components of the rig are properly grounded. The electrical noise in the whole-cell configuration when all ion channels are closed should be < 0.5-1 pA.

Another important point is to monitor correct osmolarities of the working solutions. The composition of the intra- and extracellular solutions must be precisely determined and their osmolarities are measured correctly. The extracellular solution must be slightly hypotonic in comparison to the pipette solution as it leads to miniscule cell swelling and prevents the pipette being clogged by the sperm membrane. Note: if the pipette solution is too hypertonic and differs from bath solution more than 10 mOsm, excessive cell swelling, and seal rupture ensues. As a result, the cell will be fragile and the gigaseal lost within seconds after break-in. In our experience, inaccurate solution preparation is one of the most common mistakes that prevents successful patch-clamping.

Another potential obstacle to avoid is plasticizer/phthalate-containing plastic, as well as mineral oil lubricated syringes, which must be avoided. The tubing, syringes and all plastic equipment that encounters solutions, and hence sperm cells, should not leach plasticizers or other environmental toxins or oils, since such chemicals can significantly alter ion channel activity. We use small diameter Teflon tubing as the main perfusion line. Teflon (PTFE) has few leachable compounds but is rather stiff. Flexible connections are made of high purity silicon tubing that fits over the Teflon tubing. All syringes used for the perfusion system lack any lubricant, since the mineral oil or other lubricating additives can interfere with ion channel recording.

We cannot overstate the importance of using the right glass and pulling the correct micropipette shape. Hence, the optimal fabrication of glass micropipettes is a prerequisite for successful patching. We use glass micropipettes made only from borosilicate glass and containing a filament for better solution filling. The tip of the pipettes must be fire-polished to provide the ideal tight seal. Pipette tips that exceed 2 μm in diameters (and hence have a resistance of 10 MΩ or below) are generally not suitable for sperm cell patch-clamp.

Another important step is to ensure that the micropipette tip must be kept clean of any debris or air bubbles before seal formation. This is a difficult task given that the micropipette is loaded into a solution full of the motile cells. One factor that helps avoid accidental “bumping” of the pipette into free-swimming sperm cells, is to use a constant perfusion to wash away all nonadherent cells. Another tool is a home-made “U-tube” that allows one to switch between positive and negative pressure modes to keep the tip clean (**Figure 11** and **Figure 12**).

As sperm cells vary greatly in the shapes and sizes of their cytoplasmic droplets (CD), it is important to pick the droplet with suitable morphology. As shown on Figure 2, only CDs that are small (1-3 μm), smooth, uniform, and not overly swollen are suitable for patch-clamp. Tiny, one-sided; “bloated”, fully transparent CDs produce weak or no seals. CDs that have large soluble particles inside may clog the recording pipette. When testicular mouse spermatozoa enter the epididymis, their CDs are located in the neck region, close to the head. As they travel through the epididymis, their CDs move along the midpiece and eventually arrive at the connection between midpiece and principal piece (the annulus) when spermatozoa reach the cauda epididymis. Therefore, as mentioned above in sperm cells isolated from corpus epididymis, the CD is usually located close to the center of the midpiece. In caudal cells, the CD can usually be found close to the annulus (**Figure 2C**). For human sperm, the CD is located in the neck region (**Figure 2A,B**).

While this is not an issue for spermatozoa isolated from laboratory animals, significant variability exists between human donors. Variation in sperm quality within the same donor mainly affects the quality of the sperm plasma membrane and sometimes make seal formation relatively difficult. There is less variability in ion channel behavior and pharmacology, factors that probably correlate with individual genetics or physiology. One has to be persistent and assess samples from various donation during multiple days, as well as rely on multiple human donor participants. Working with human material requires extra patience, since donated sperm vary greatly in sperm quality within the same donor, depending on various environmental factors (stress, diet, health, etc.). We recommend assessing samples from various donation days to make a final decision on the donor status. While ejaculated purified spermatozoa are generally suitable for electrophysiology within hours (up to 12 hours after isolation for human sperm), epididymal murine sperm cells are suitable for patching only within a 2-hour window after isolation.

And last, but not the least, gigaseal formation differs among sperm cells. For murine/rodent sperm cells, gigaseal formation happens almost instantaneously, while several seconds (and sometimes up to a minute) are required to form a gigaseal with a human sperm. Often the initial suction results in an input resistance ranging from 200 MΩ to 800 MΩ. Switching holding potential to -60 mV and providing “Membrane Test” short pulses up to 10mV often helps rescue gigaseal formation (through voltage field induced movement of the membrane in the pipette).

The sperm cell patch clamp technique enables the detailed study of specific ion channels in their natural expression system. The success of the technique depends on proper equipment, high quality viable sperm cells, pure reagents, and basic electrophysiology skills, patience and persistence. The method opens new frontiers in sperm physiology by studying ion channel evolutionary diversity, mechanisms of their regulation, and alterations in their function as they move from the male to the female reproductive tract and are altered by exogenous conditions, such as pH and ligands.

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The authors have nothing to disclose.

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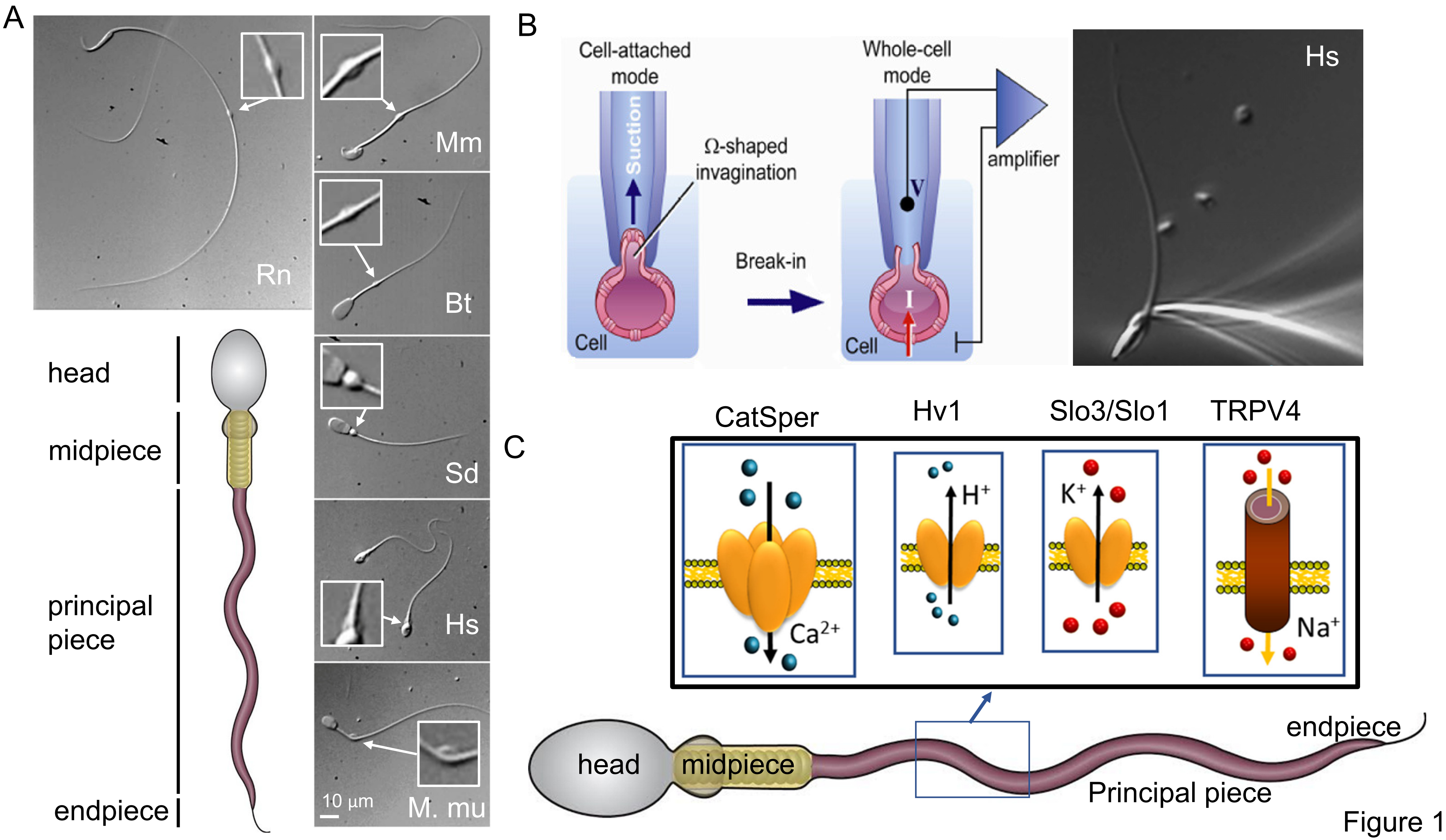

A
Rn
Mm
Bt
Sd
Hs
M. mu
10 μm
head
midpiece
principal piece
endpiece
B
Cell-attached mode
Suction
Ω-shaped invagination
Cell
Break-in
Whole-cell mode
V
I
Cell
amplifier
Hs
C
CatSper
Hv1
Slo3/Slo1
TRPV4
Ca^{2+}
H^+
K^+
Na^+
head
midpiece
Principal piece
endpiece

Figure 1

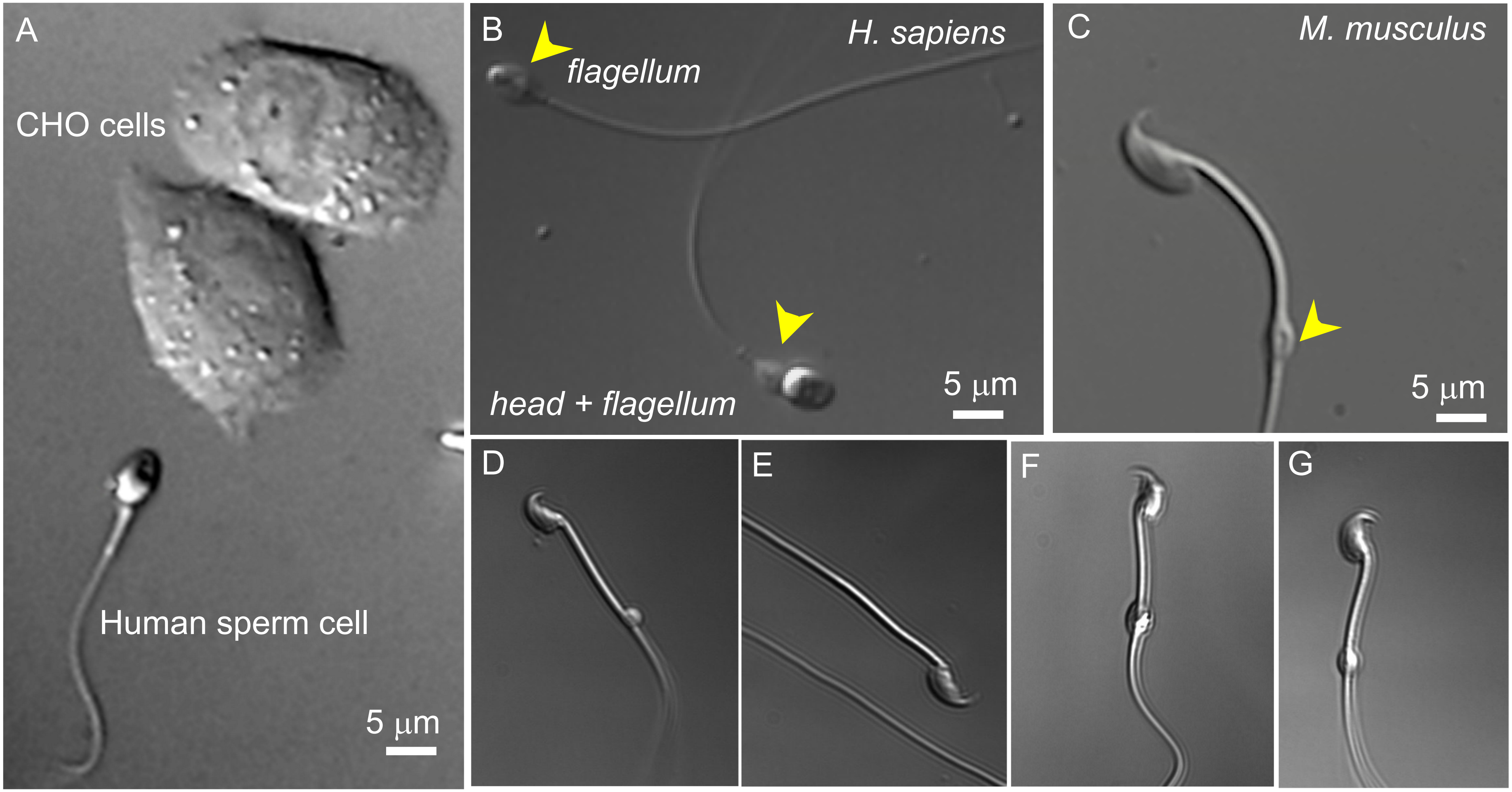

A
CHO cells
Human sperm cell
5 µm
B
H. sapiens
flagellum
head + flagellum
5 µm
C
M. musculus
5 µm
D
E
F
G

Figure 2

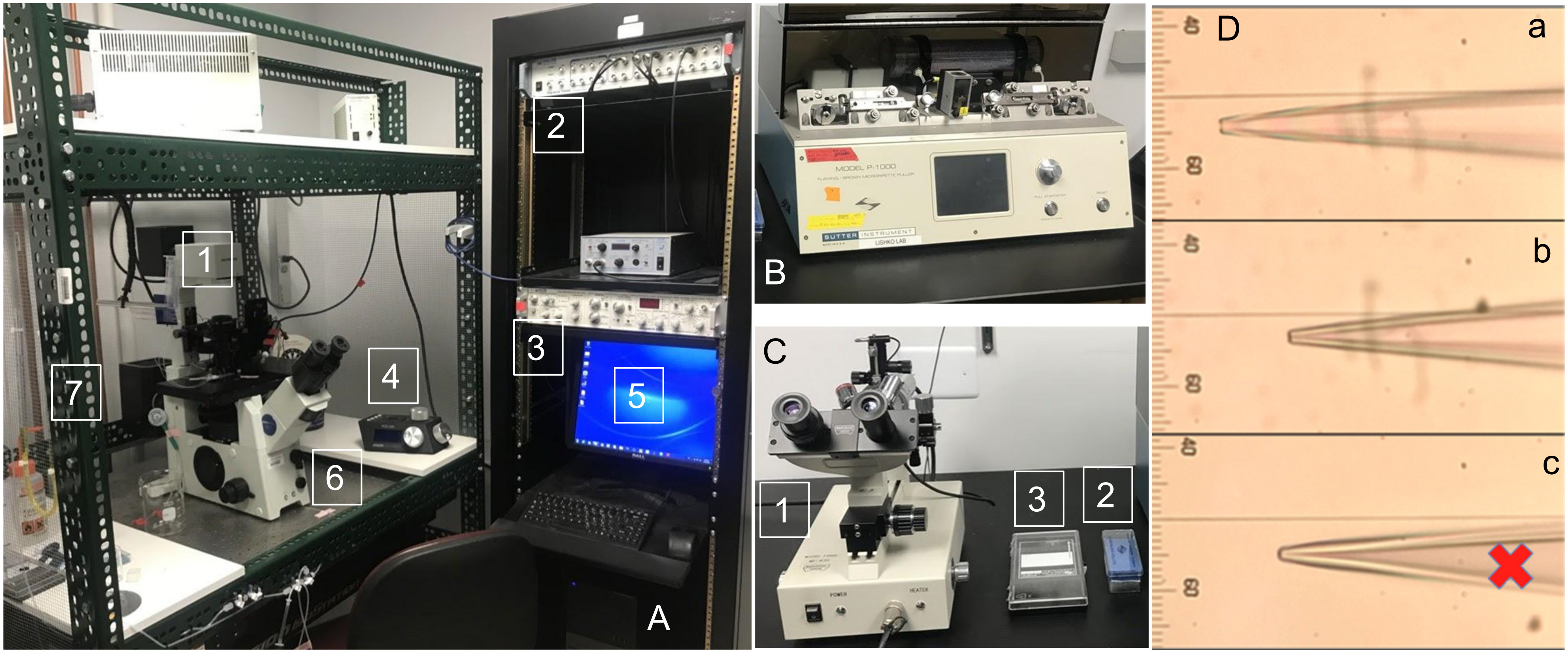

1
2
3
4
5
6
7
A
MODEL P-1000
SUTTER INSTRUMENT
LISHKO LAB
B
C
1
3
2
D
a
b
c

Figure 3

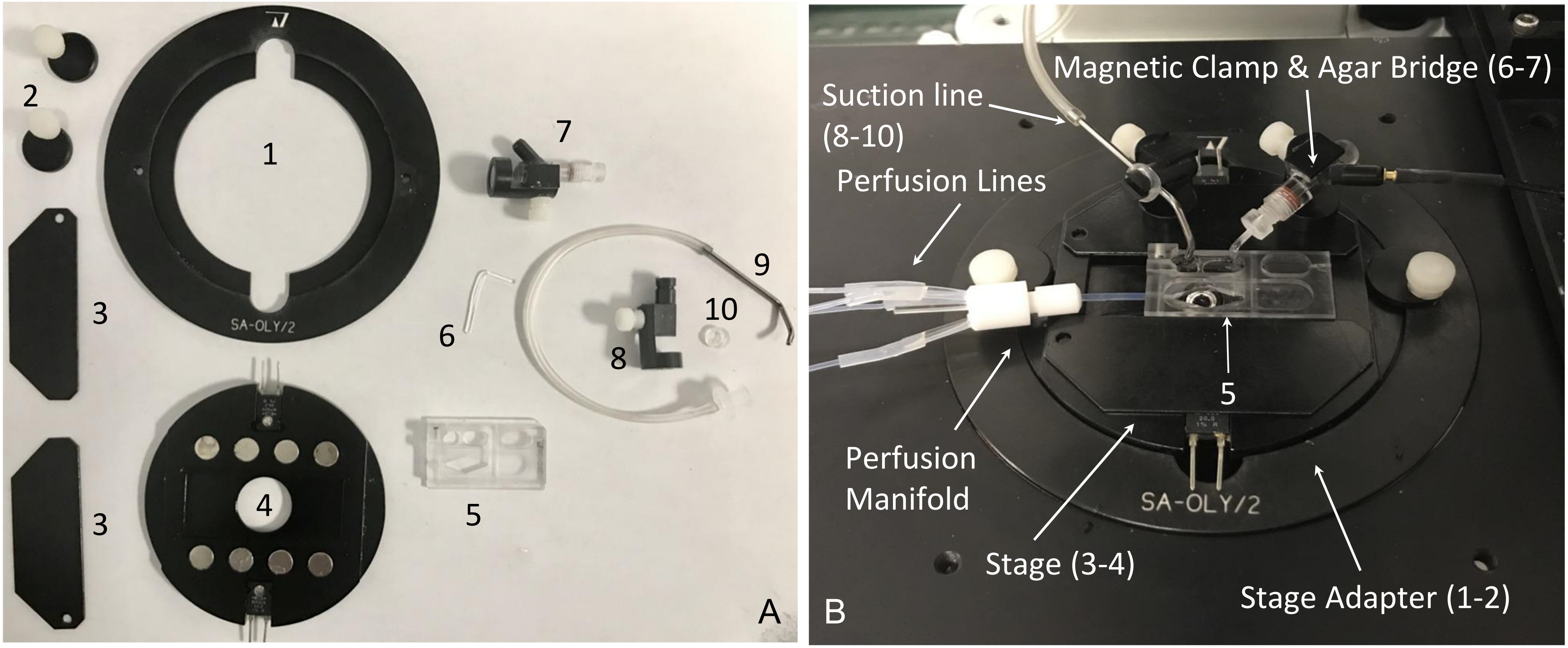

2
1
7
3
9
6
10
8
SA-OLY/2
3
4
5
A
Magnetic Clamp & Agar Bridge (6-7)
Suction line
(8-10)
Perfusion Lines
5
Perfusion
Manifold
SA-OLY/2
Stage (3-4)
Stage Adapter (1-2)
B

Figure 4

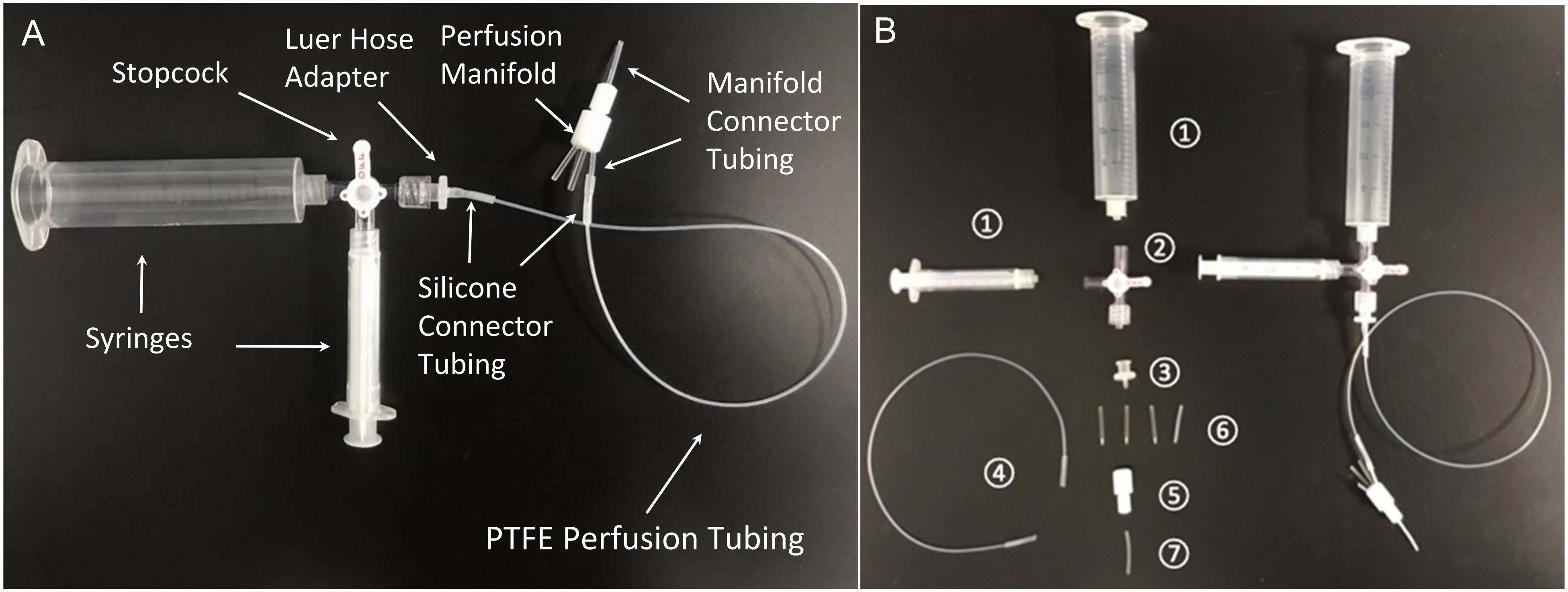

A
Stopcock
Luer Hose Adapter
Perfusion Manifold
Manifold Connector Tubing
Syringes
Silicone Connector Tubing
PTFE Perfusion Tubing
B
①
①
②
③
④
⑤
⑥
⑦

Figure 5

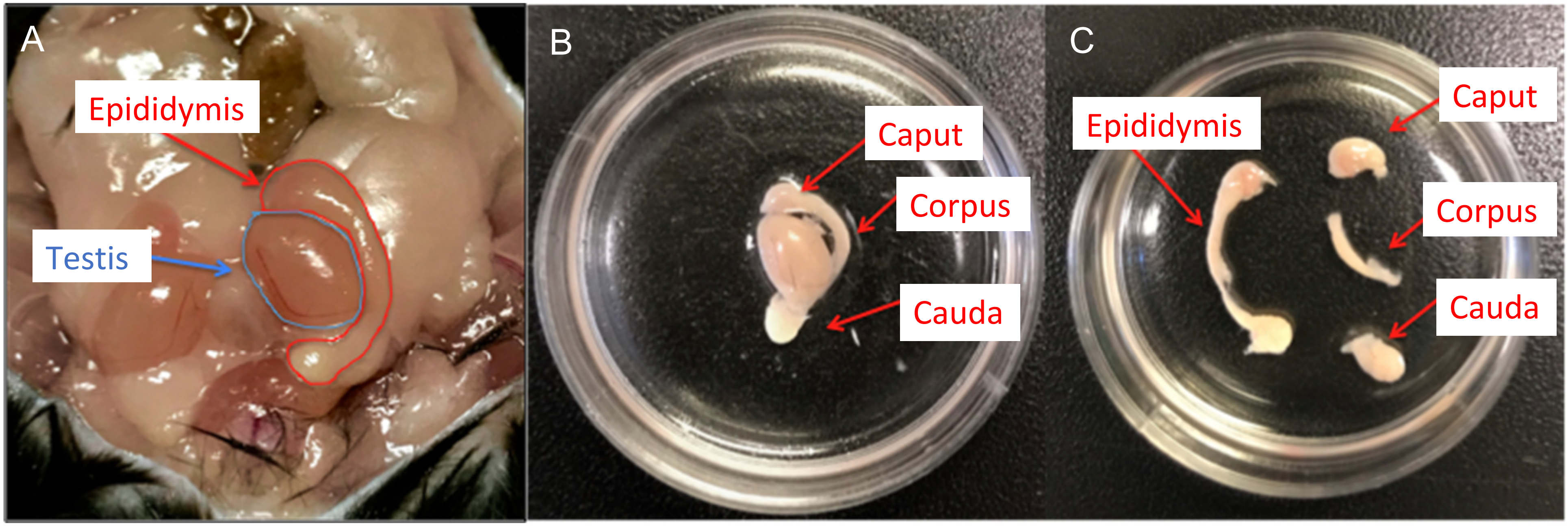
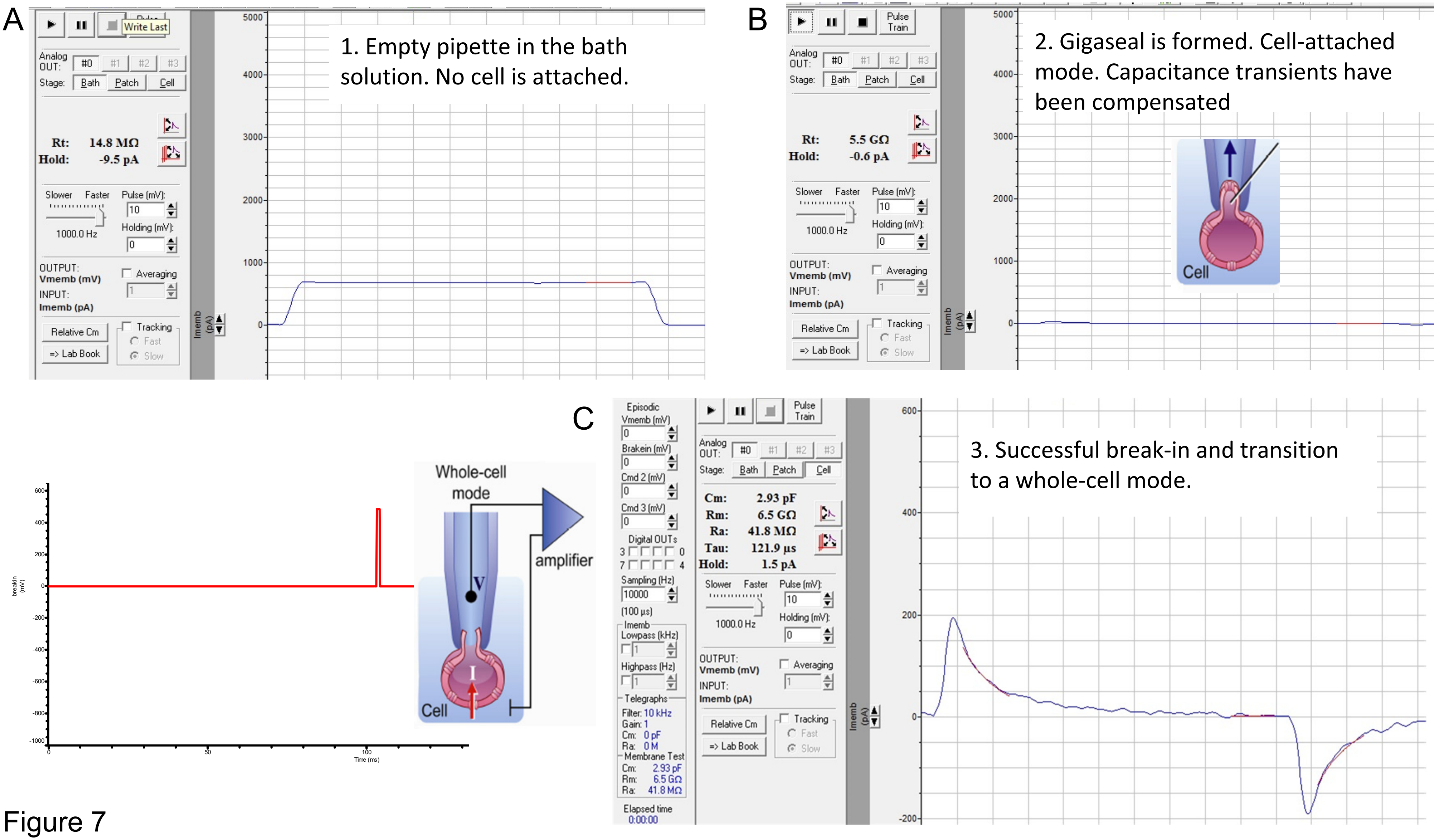

A
Epididymis
Testis
B
Caput
Corpus
Cauda
C
Epididymis
Caput
Corpus
Cauda

Figure 6



1. Empty pipette in the bath solution. No cell is attached.

Rt: 14.8 MΩ
Hold: -9.5 pA
1000.0 Hz
Pulse (mV): 10
Holding (mV): 0
OUTPUT: Vmemb (mV)
INPUT: Imemb (pA)
☐ Averaging 1

2. Gigaseal is formed. Cell-attached mode. Capacitance transients have been compensated

Rt: 5.5 GΩ
Hold: -0.6 pA
1000.0 Hz
Pulse (mV): 10
Holding (mV): 0
OUTPUT: Vmemb (mV)
INPUT: Imemb (pA)
☐ Averaging 1

3. Successful break-in and transition to a whole-cell mode.

Episodic
Vmemb (mV) 0
Brakein (mV) 0
Cmd 2 (mV) 0
Cmd 3 (mV) 0
Sampling (Hz) 10000 (100 µs)
Imemb
Lowpass (kHz) ☐ 1
Highpass (Hz) ☐ 1
Telegraphs
Filter: 10 kHz
Gain: 1
Cm: 0 pF
Ra: 0 M
Membrane Test
Cm: 2.93 pF
Rm: 6.5 GΩ
Ra: 41.8 MΩ
Elapsed time 0:00:00

Cm: 2.93 pF
Rm: 6.5 GΩ
Ra: 41.8 MΩ
Tau: 121.9 µs
Hold: 1.5 pA
1000.0 Hz
Pulse (mV): 10
Holding (mV): 0
OUTPUT: Vmemb (mV)
INPUT: Imemb (pA)
☐ Averaging 1

Figure 7

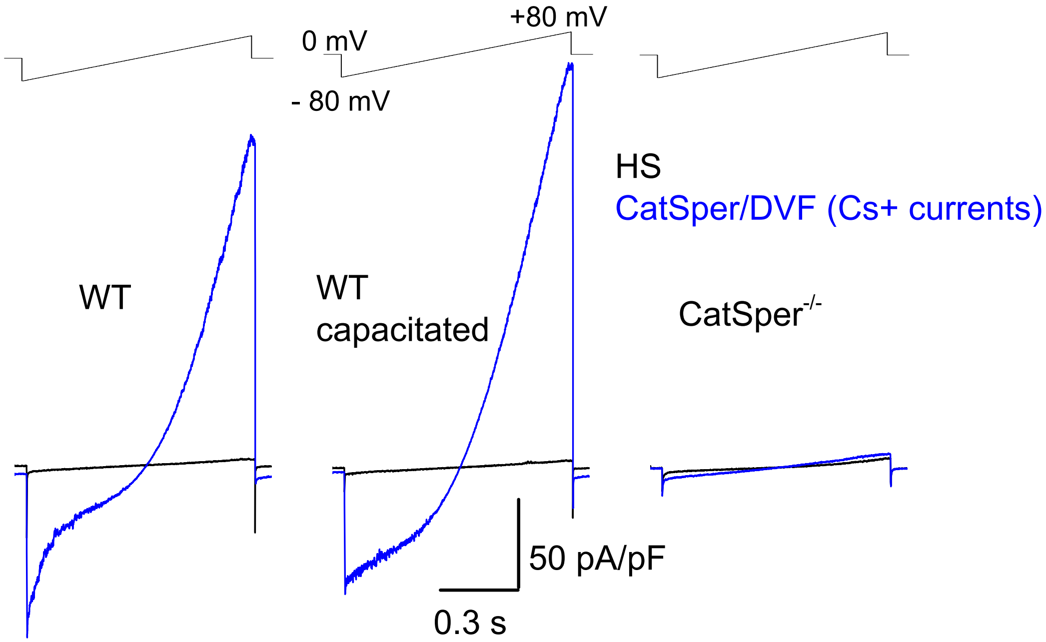

0 mV
+80 mV
- 80 mV
HS
CatSper/DVF (Cs+ currents)
WT
WT
capacitated
CatSper-/-
50 pA/pF
0.3 s

Figure 8

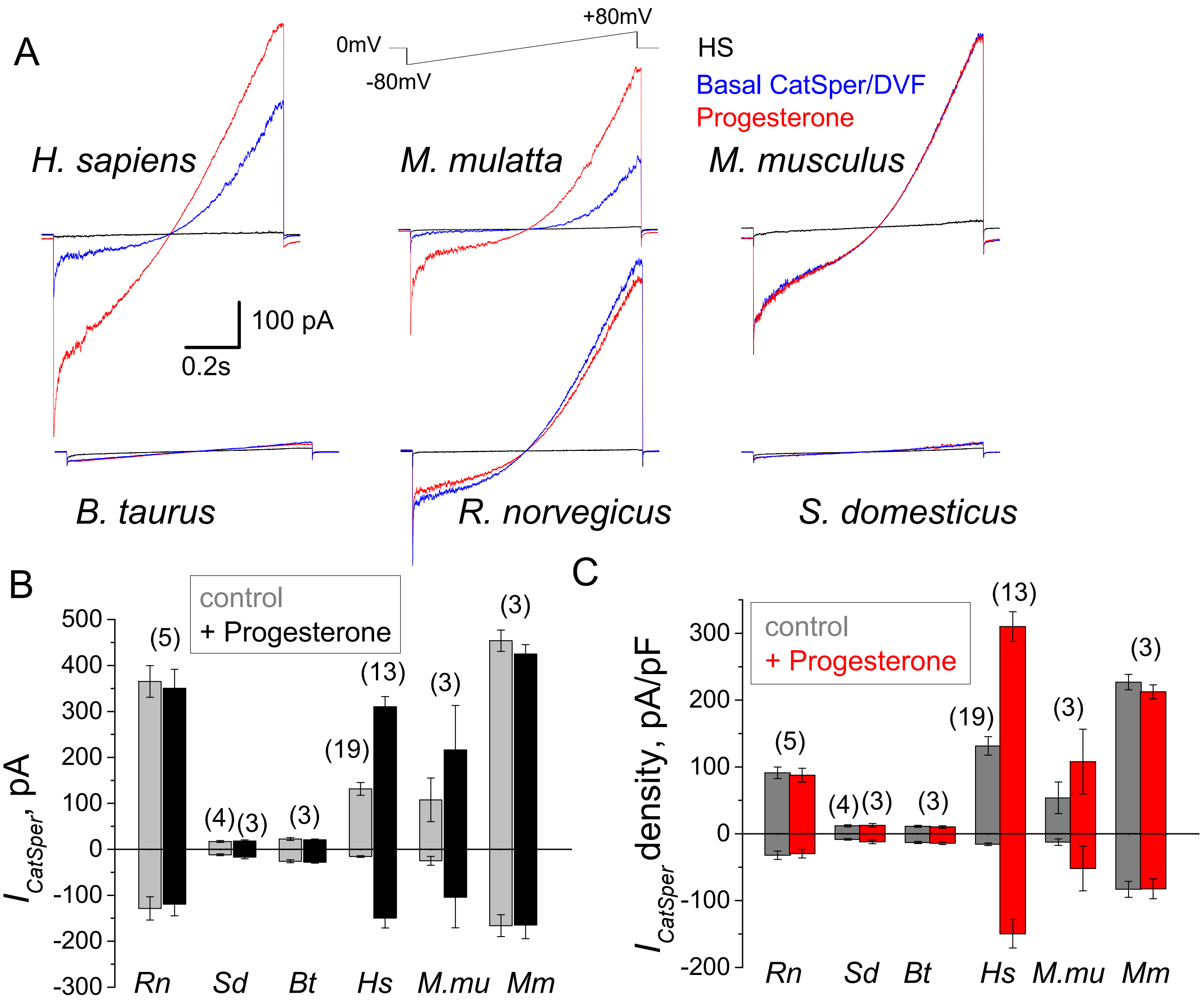

A
0mV
+80mV
-80mV
HS
Basal CatSper/DVF
Progesterone
H. sapiens
M. mulatta
M. musculus
100 pA
0.2s
B. taurus
R. norvegicus
S. domesticus
B
control
+ Progesterone
$I_{CatSper}$, pA
500
400
300
200
100
0
-100
-200
-300
(5)
(4)
(3)
(3)
(19)
(13)
(3)
(3)
Rn
Sd
Bt
Hs
M.mu
Mm
C
control
+ Progesterone
$I_{CatSper}$ density, pA/pF
300
200
100
0
-100
-200
(5)
(4)
(3)
(3)
(19)
(13)
(3)
(3)
Rn
Sd
Bt
Hs
M.mu
Mm

Figure 9

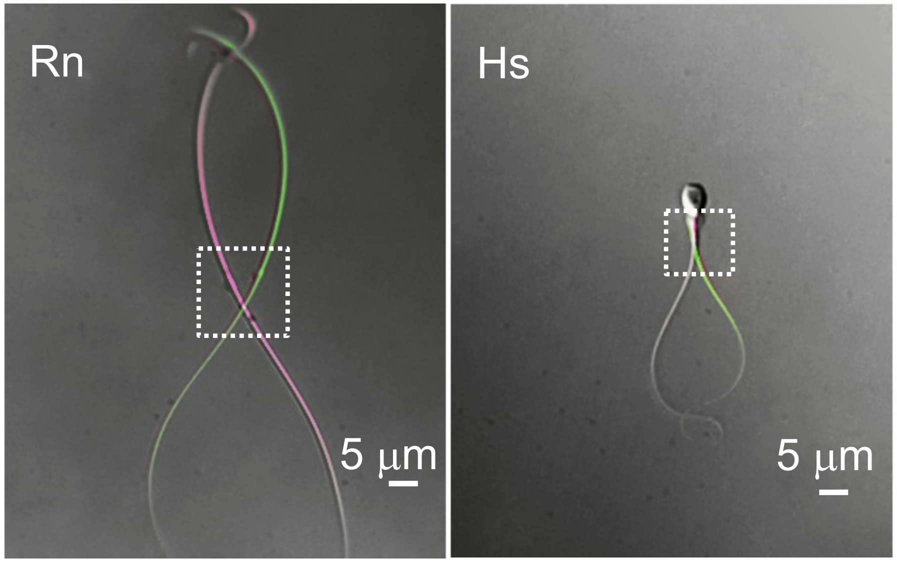


Figure 10

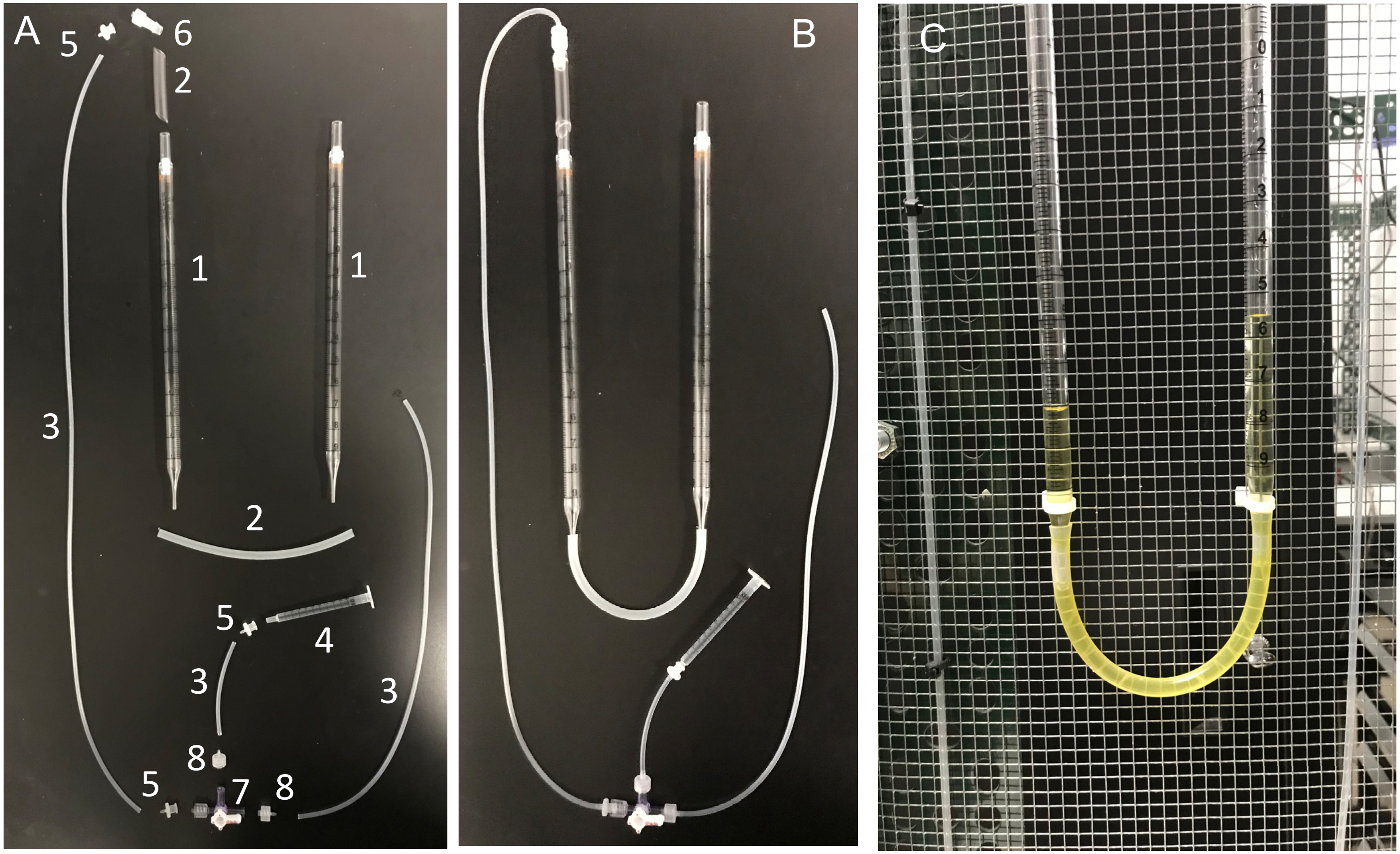


Figure 11

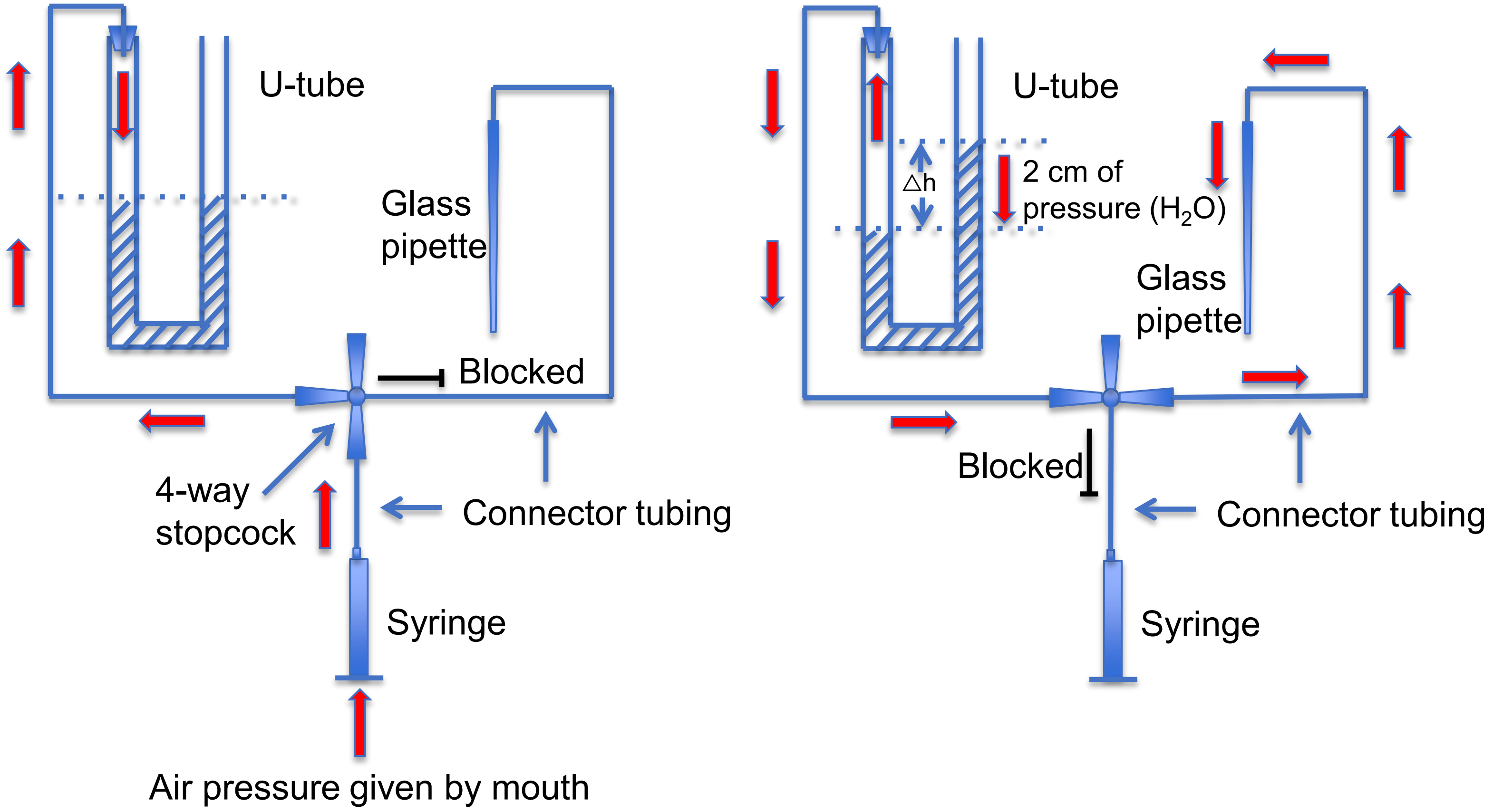

U-tube
Glass pipette
Blocked
4-way stopcock
Connector tubing
Syringe
Air pressure given by mouth
U-tube
△h
2 cm of pressure (H_2O)
Glass pipette
Blocked
Connector tubing
Syringe

Figure 12

Name of Material/Equipment	Company	Catalog Number
IX71 with DIC optics	Olympus Inc	IX71
UplanSApo 60x	Olympus Inc	
Vibration-damping air table	Newport Inc	
Axopatch™ 200B amplifier	Axon™ /Molecular Devices	
Axon Digidata analog to digital converter	Axon™ /Molecular Devices	1440 or 1550
Vapor pressure osmometer	Wescor	model 5600
MPC 385 micromanipulator	Sutter Instruments, Novato CA	MPC 385
Micropipette puller	Sutter Instruments, Novato CA	P1000
MicroForge	Narishige	MF-830
Faraday cage	Homemade	
5 mm glass Cover Slips	WPI	#502040
Perfusion chamber	Warner Instruments, Inc	RC-24E
Borosilicate glass capilary	Sutter Instruments, Novato CA	BF 150-85-7.5
Teflon manifold MP-8	Warner Instruments, Inc	64-0211
Nunc 4-well plate	Nunc	#179820
1 X HTF buffer	EmbryoMax	MR-070-D
SA-Oly/2 stage adapter	Warner Instruments, Inc	for series 20 platforms
Magnetic heated platform	Warner Instruments, Inc	PM-1 or similar series
MAG-1 magnetic clamp	Warner Instruments, Inc	#64-0358
Microelectrode holder with 2mm Ag/AgCl pellet	WPI	MEH3F4515
Stopcock with Luer connections; 4-way; male lock	Cole-Parmer, Inc	EW-30600–09
female luer hose barb adapter, 1/16”	Cole-Parmer, Inc	EW-45508–00
Polytetrafluoroethylene (PTFE) perfusion tubing	Cole-Parmer, Inc	EW-06417–21

Silicone connector tubing (platinum-cured silicone tubing, 1/32” ID × 3/32” OD)	Cole-Parmer, Inc	EW-95802–01
Manifold connector tubing (PTFE Tubing, 1/32” ID × 1/16” OD)	Cole-Parmer, Inc	EW-06407–41
male Luer series barb adapter, 1/16”	Cole-Parmer, Inc	45518–00
Male Luer integral lock adapter 1/8”	Cole-Parmer, Inc	45-503-04
Silicone connector tubing	Dow Silicone Corporation; MI	#508-008
Syringes	Fisher Scientific or VWR	Air-Tite, Norm-Ject Luer
NaCl	Sigma-Aldrich	S7653
KH2PO4	Sigma-Aldrich	60216
$MgSO_4$ x $7^{\cdot}H_2O$	Sigma-Aldrich	63140
$CaCl_2$ x $2^{\cdot}H_2O$	Sigma-Aldrich	21097
HEPES	Sigma-Aldrich	H7523-250G
Glucose	Sigma-Aldrich	G8270
Sodium lactate (60% w/w)	Sigma-Aldrich	L7900
Sodium pyruvate	Sigma-Aldrich	P2256
EDTA	Sigma-Aldrich	BCBG2421V
$CsMeSO_3$	Sigma-Aldrich	C1426
KCl	Fisher Scientific	P217
EGTA	Sigma-Aldrich	BCBF5871V
Tris-HCl	Quality Biological	315-006-721
NaOH	Sigma-Aldrich	221465
CsOH	Sigma-Aldrich	232041
Embryomax Human Tubal Fluid medium: (Embryomax-HTF)	Millipore	MR-070-D
Animals		
Male Wistar rats	Wistar	Harlan Laboratories (Livermore, CA)

Male C57BL/6 mice	C57BL/6 mice	Harlan Laboratories (Livermore, CA)

Comments/Description
Nikon TiU microscope can be used as well
water immersion objective
TMC airtables or similar can be used
Sutter I PA®/Double IPA ® Integrated patch clamp system is also an excellent amplifier
can be used as well
The Eppendorf micromanipulator TransferMan series can be also used
P97 can be used
Should be equiped with Nikon MPlan 100/0.80 ELWD 210/0 objective
to shield the setup from ambient electrical interference
outer diameter 1.5 mm, inner diameter 0.86 mm and an internal filament
Teflon 8-position perfusion manifold
capacitation solution
only for Olympus microscope
to hold RC-24E chamber
(Microbore PTFE Tubing, 0.022” ID × 0.042” OD)

1 mL, 3mL, and 20 mL

Cesium methanesulfonate

1 M solution of similar

capacitation medium for murine sperm cells

adult rats

3-6 month old

Table 1. Human tubular fluid (HTF) solution

Chemicals	Molar weight (g/mol)	mM
NaCl	58.44	97.8
KCl	75.56	5
KH_2PO_4	136.09	0.37
$MgSO_4 \times 7 \cdot H_2O$	246.48	0.2
$CaCl_2 \times 2 \cdot H_2O$	147.02	2
HEPES	238.3	20
Glucose	180.2	3
Sodium lactate (60% w/w)	112.06	20
Sodium pyruvate	110	0.4

Table 2. High-Saline (HS) Solution

Chemicals	Molar weight (g/mol)	mM
NaCl	58.44	135
KCl	75.56	5
$CaCl_2 \times 2 \cdot H_2O$	147.02	2
$MgSO_4 \times 7 \cdot H_2O$	246.48	1
HEPES	238.3	20
Glucose	180.2	5
Sodium lactate (60% w/w)	112.06	10
Sodium pyruvate	110	1

Table 3. $CsMeSO_3$ bath solution (Divalent Free bath solution: DVF)

Chemicals	Molar weight (g/mol)	mM
$CsMeSO_3$	228.0	140
HEPES	238.3	40
EDTA	292.24	1

Table 4. $CsMeSO_3$ pipette solution

Chemicals	Molar weight (g/mol)	mM
$CsMeSO_3$	228.0	130
HEPES	238.3	70
EDTA	292.24	2
EGTA	380.35	3
Tris-HCl	1 M solution	1

g for 1L
5.72 g
0.373 g
50.4 mg
49.3 mg
0.294 g
4.766 g
0.540 g
3 ml
44 mg

g for 1L
7.889 g
0.373 g
0.294 g
0.247 g
4.766 g
0.901 g
1.5 ml
0.110 g

g (for 500 ml)
15.960 g
4.766 g
0.146 g

mg (for 25 ml)
741 mg
417 mg
7.3 mg
47.5 mg
25 μl

UNIVERSITY OF CALIFORNIA, BERKELEY

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November 25, 2020

Dear *JoVEs* Editors,

We would like to thank all reviewers for the constructive and helpful criticism. We have extensively revised the manuscript by making it more structured, removed redundant parts and added missing references. As mentioned in our response below, we were not able to perform some of the additional experiments that were suggested due to the current COVID related situation that keeps our lab at low occupancy and prevented animal experimentation. Below is our point-to-point response (in italic) to the reviewers' suggestions.

Reviewers' comments:
Reviewer #1:.

Minor Concerns:
1) Line 235: you name HS solution: High Saline solution. Is this correct? The solution seems to be composed of a standard salt concentration.
A: corrected, thank you!

2) It is unclear how the U-tube itself affects positive and negative pressure (line 545).
A: correction and explanations added in Figure 11-12.

3) The legend of Figure 1 refers to the human sperm cells attached to the pipette being the left panel, when it is on the right (line 617).
A: corrected, thank you!

Reviewer #2:
Specific comments:
L97ff: The authors state in the abstract that the technique has also been instrumental to record chloride channels in sperm. Citation for the recording of Cl channels are missing in this list of citations. Please, either add a citation or edit the abstract.
A: we have removed all citations from the abstract, and included it in the line 108 in the introduction.

L 126: The authors state, that all attempts to form a gigaseal have been unsuccessful outside the CD. Nevertheless, there are papers describing recordings in the cell-attached configuration from the head of sperm cells (e.g. Gu Y, Kirkman-Brown JC, Korchev Y, Barratt CL, Publicover SJ. Multistate, 4-aminopyridine-sensitive ion channels in human spermatozoa. Dev Biol. 2004; 274:308-317.) and others. Please specify this.
A: we have now properly cited these papers

L149-140: Same comment also holds true for this.
A: see above

L173-175: It remains unclear for the reviewer, why only these two patch-clamp amplifier should work. Every

modern patch-clamp amplifier should be able to record these currents from sperm cells. Especially, since all these amplifiers are suited to record currents from single channels.
A: we have removed all vendor mentioning, but based on our experience we have used these two types successfully, and listed them in the methods.

L331ff: This procedure to prevent sperm cells from sticking to the glass seems to be quite complicated. In the field of motility analysis of sperm cells, which faces the same problem, usually HSA is used for this purpose.

A: we have not used HSA and therefore do not know whether this compound can alter sperm ion channel behavior by chelating lipids and/or cholesterol. We describe in details the method that has been working successfully in our hands. Besides, we apply coating solution made from the same donor sperm to the same sperm populations.

L396: Here the authors report a pipette resistance of 11-17 MΩ. In line 194 it is 11-13 MΩ. Please specify the value and keep it consistent throughout the protocol.
A: corrected, thank you!

L508: Here the authors say that the difference between pipette and bath solution should not exceed 10mOsm. In line 399 it is less than 15 mOsm. Please specify the value and keep it consistent throughout the protocol.
A: corrected, thank you!

L594: The study cited here as an example for high throughput Ca2+ imaging did not use Ca2+ fluorometry but motility and acrosome reaction as readout. There are much more and better suited studies that used medium- or high-throughput Ca2+ fluorometry in sperm cells. Moreover, the comparison between the two methods is not very precise. There is no doubt, that the direct study of ion channels via patch-clamp is best suited to characterize ion channels. Nevertheless, ionic imaging also has a lot of advantages, since it is much less invasive. Also the statement, that only change nd a proper calibration also allows to study Ca2+ concentrations in a quantitative manner.s in the Ca2+ concentration can be measured is incorrect. Using ratiometric dyes a proper calibration also allows to study Ca2+ concentrations in a quantitative manner.
A: Thank you for this statement. We have adjusted references and corrected this part.

Minor comments:
L153: 2μM has to be 2 μm
A: corrected

Table4: The amount of Tris-HCl has to be 25 μl.
A: corrected.

Reviewer #3:
Major Concerns:
My only criticism would be that it is not clear who the ms is aimed at. In parts it describes patch clamping from first principles and appears to be addressing readers with no background in electrophysiology, but the more technical parts may only really be understood by readers with some patching experience. It might be worth indicating some reading on basic patch clamp techniques and concepts, and suggesting that readers look into (and try) standard patching techniques first before tackling the particular problems of patching sperm. Similarly, it might be worth emphasising what is particular to sperm patching. For instance - sections 2.2 and 3 are not really specific to patching sperm.
A: corrected. We have added additional reading sources

Minor Concerns:
Lines 126-7 and lines 148-150 both state that seals cannot/have not been achieved outside of the CD. Though it is true that whole-cell patch has only been achieved via the CD, cell-attached seals on the sperm head have been reported a number of times and in several species. Alberto Darszon's group has done this on sea urchin (Guerrero et al, 1987), mouse (Espinosa et al, 1998), and human (Orta et al, 2012). Gorelick et al (2002) reported achieving seals on the head of sea urchin sperm using SICM (smart patch) and Jimenez-Gonzalez et al 2007 used cell-attached patches to map channel activity on the human sperm head.
A: corrected. We have added those citations.

Line 190 - might be worth explaining what 'fire polished' means
Line 444 - should this be step 5.2.4?
Line 506 - should read "The slightly hypotonic extracellular solution in comparison to the pipette solution"?

A: all above were corrected.

Sincerely,
Polina Lishko, Ph.D.

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