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METHODS & TECHNIQUES

A simple device to immobilize protists for electrophysiology and microinjection

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ABSTRACT

We present a simple device to mechanically immobilize motile cells such as ciliates. It can be used in particular for intracellular electrophysiology and microinjection. A transparent filter with holes smaller than the specimen is stretched over an outlet. A flow is induced by either a peristaltic pump or a depressurized tank, mechanically entraining cells to the bottom, where they are immobilized against the filter. The cells start swimming again as soon as the flow is stopped. We demonstrate the device by recording action potentials in *Paramecium* and injecting a fluorescent dye into the cytosol.

KEY WORDS: *Paramecium*, Ciliate, Motility

INTRODUCTION

Paramecium is a well-studied ciliate that swims in freshwater and feeds on smaller microorganisms (Görtz, 1988; Wichterman, 1986). Its motility is electrically controlled by calcium-based action potentials: various types of sensory stimulation (mechanical, thermal, chemical) can depolarize the cell and trigger an action potential, and the entry of calcium leads to the reversal of ciliary beating, making the cell swim backwards (Eckert, 1972). Sensory stimuli may also hyperpolarize the cell, which leads to an increase in swimming speed. This makes *Paramecium* an interesting model organism for the study of sensorimotor mechanisms (Kung and Saimi, 1982; Hinrichsen and Schultz, 1988; Machemer, 2001) – some authors called it a 'swimming neuron' (Kung and Saimi, 1985).

Paramecium can swim at speeds exceeding 10 times its body length per second. Thus, a key requirement to experimentally manipulate *Paramecium* is to immobilize it without damaging it. Classically, intracellular electrophysiology in ciliates such as *Paramecium* and *Tetrahymena* was performed with the hanging droplet method (Hennessey and Kuruvilla, 1999; Naitoh and Eckert, 1972). A specimen is picked with as little fluid as possible and placed hanging below a coverslip. When water evaporates, the cell is captured by surface tension. A bent pipette is then gently but swiftly raised into the cell, effectively pinning the cell to the coverslip. The cell is then quickly covered by the bath before it dries out completely. The later use of inverted microscopes allowed the droplet to be placed on top of the coverslip, with no need for bending the pipette (Houten, 1979; Valentine and Van Houten, 2016). In either case, this technique requires substantial dexterity. An additional difficulty is that this technique provides no electrical signal during the impalement procedure because the reference electrode is not immersed. Thus, successful impalement relies on visual inspection, and can only be checked electrically after the chamber is flooded.

A less common strategy is to catch the swimming organism with a suction pipette (Grønlien et al., 2013; Jonsson and Sand, 1987), which can be challenging with fast specimens. For microinjection, the standard method consists in covering the specimen with oil, removing fluid with a needle until the cell is immobilized, then performing the microinjection and releasing the cell (Beisson et al., 2010).

Here, we present a simple device to mechanically immobilize swimming cells while providing an electrical signal. A transparent filter with holes smaller than the cells is placed at the bottom of the device, immersed in the bath. Fluid is then removed from the bottom using a peristaltic pump or a depressurized reservoir. In a few seconds, cells are immobilized against the filter. A pipette can then be inserted into the cell. If the pipette is filled with a conducting solution, successful impalement is indicated by a drop in measured potential. We demonstrate the use of the device by recording action potentials in *Paramecium tetraurelia* using two electrodes, and microinjecting Alexa Fluor into the cytosol. The immobilization device was also successfully tested on *Paramecium caudatum* and *Tetrahymena thermophila*.

MATERIALS AND METHODS

Ciliate culture and manipulation

Cultures of *Paramecium tetraurelia* (obtained from Éric Meyer, Institut de Biologie de l'Ecole Normale Supérieure) were maintained by reinjecting each week 1 ml of culture inoculated with *Klebsiella pneumoniae* into 5 ml of wheat grass powder (WGP) buffer supplemented with 1 µl of beta-sitosterol (4 mg ml^{-1}). Cultures were kept at room temperature. Prior to each experiment, the culture was filtered through a LCH Pure SN30 non-woven sterile swab, and cells were washed and concentrated in clean buffer (the extracellular solution used for electrophysiology; see below) using negative gravitaxis (Naitoh and Eckert, 1972). Paramecia tend to accumulate at the top of any aqueous solution, so placing a droplet of culture (typically 600 µl) in a narrow-necked volumetric flask enabled recovery of a concentrated population at the top of the flask.

We also tested the device on *Paramecium caudatum*, a larger ciliate, obtained from Sciento (UK), and on *Tetrahymena thermophila*, obtained from Emanuel Fronhofer (Institut des Sciences de l'Evolution, Université de Montpellier). Both ciliates were cultured as described above.

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Device fabrication and assembly

The device was engineered to provide immobilization of ciliates by suction on the filter. It was fabricated with a combination of laser-cutting and micro-milling techniques. The device consists of two thin Plexiglas plates (lower plate thickness ∼2.7 mm, upper plate thickness ∼1.3 mm) that are tightly screwed together to sandwich a filter (Fig. 1A). For our experiments, we have used in particular transparent engineered Whatman Cyclopore polycarbonate membranes (diameter 25 mm, pore diameter 12 µm for *Paramecium* spp. and 5 µm for *T. thermophila*). Note that before assembly, the filter is first wet with water to ensure good adhesion with the device. The upper plate was laser-cut with a circular and centred hole (diameter 5 mm) in order to form a pool-like structure in which ciliates can swim freely, once apposed against the lower plate. A mesa-like structure (diameter 4 mm, height 100 µm) was micro-milled in the centre of the lower plate using a three-axis commercial desktop CNC Mini-Mill machine (Minitech Machinery Corp.) as shown in Fig. 1B,C. The purpose of this mesa is to allow stretching of the filter just like a thin membrane is stretched on a drum. On the mesa structure, microfluidic channels (width 300 µm, depth 100 µm) were then micro-milled with the cross-like geometry shown in Fig. 1A,B. Finally, a small through-hole (diameter 300 µm) was drilled in the centre of the mesa, and eventually enlarged (diameter 600 µm) on 2 mm from the lower side of the plate. This allowed insertion of a small metallic tube (tubing connector SC23/8, Phymep) that acts as a fluid outlet to which external tubing can be easily connected, with which suction can be applied (Fig. 1C). The geometry of the microfluidic pattern was chosen to prevent any local bending of the filter while the mesa structure avoids larger height fluctuations, upon suction. Both plates were drilled with through-holes (diameter 2.2 mm) so screws (2 mm in diameter) combined with bolts could be used to assemble both parts of the device (see assembly diagram in Fig. S1).

Principle of the immobilization process

Ciliates are immobilized by simply sucking the liquid bath through the filter. As shown in the upper part of Fig. 1D, without suction, ciliates swim freely. But, as soon as suction is switched on (Fig. 1D, lower), the resulting hydrodynamic flux in the bath immobilizes the ciliates against the filter.

Pumping methods

The bathing liquid is pumped using either a peristaltic pump or a depression tank. It is reinjected into the pool to maintain a constant volume of the bath. In the case of a peristaltic pump, the tube of a Gilson Minipulse 3 pump is first filled with the medium (see extracellular solution composition in 'Electrophysiology', below). When using a depression tank, the device outlet is connected to a sealed glass jar with two entrances, one for the tube from the device and another used to depressurize it. To apply a controlled pressure, we use a microfluidic flow controller (OB1 Mk3, Elveflow). However, a simple syringe can also be used to lower the pressure in the jar. Volume of the bath is maintained by being supplemented with a gravity-driven perfusion system at a flow rate of 5 ml min^{-1}, while the excess solution is drained from the top by a peristaltic pump. In this way, it is possible to use the immobilization device together with the perfusion system, for example to exchange solutions while the ciliate is immobilized, as shown on Fig. S2.

Electrophysiology

For all experiments, we used a controlled extracellular solution consisting of 1 mmol l^{-1} $CaCl_2$, 4 mmol l^{-1} KCl and 1 mmol l^{-1} Tris-HCl, pH 7.2. Microelectrodes of ∼50 MΩ resistance were pulled with a single step from standard wall borosilicate capillary glass with filament (o.d. 1 mm, i.d. 0.5 mm, Harvard Apparatus) using a micropipette puller (P-1000, Sutter Instrument). They were filled with a 1 mol l^{-1} KCl solution using a MicroFil non-metallic syringe needle (MF 34G-5, World Precision Instruments).

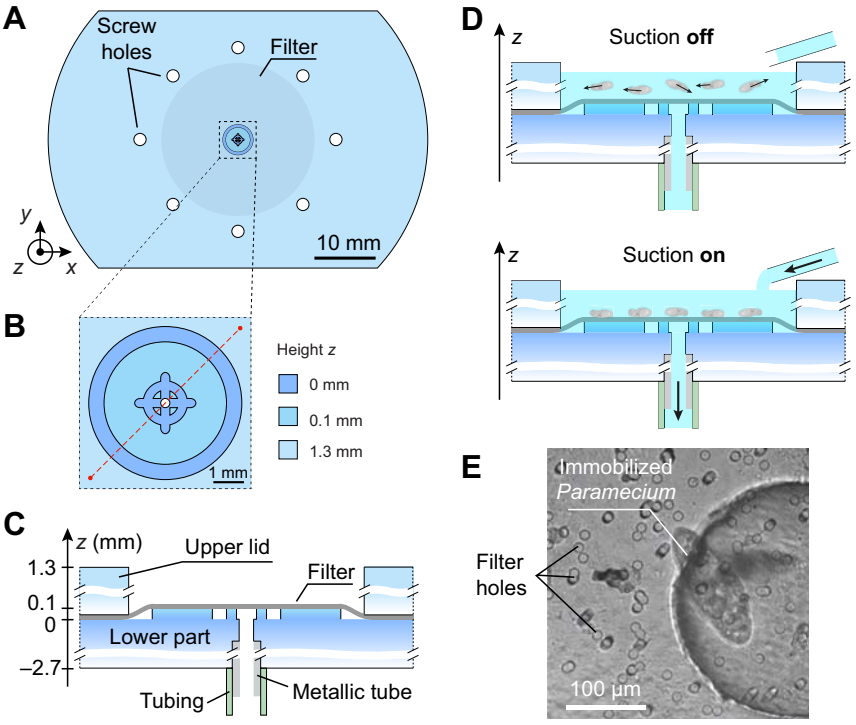

Fig. 1. Diagram of the immobilization device. (A) Top view of the device. The filter (shaded grey disk) is sandwiched between an upper lid and a lower part all made of Plexiglas, tightened together with 8 screws. (B) Close-up of the centred microfluidic mesa-like structure. The *z*=0 height origin is arbitrarily taken at the base of the mesa-like structure. (C) Lateral view along the red dashed line in B. (D) Principle of the immobilization process. Without suction (upper panel), ciliates in the centred pool swim freely. Once suction is switched on (lower panel), ciliates are immobilized against the filter. Their bathing liquid is pumped using either a peristaltic pump or a depression tank. The liquid is reinjected (when using the pump) or supplemented (when using the depression tank) in the pool to maintain the level of the bath constant. (E) Bright-field image of a single *Paramecium* immobilized with the current device (top view). The round object is a microfluidic channel below the filter, the left end of the cross in B.

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Custom Python programs (https://github.com/romainbrette/clampy) were used to control the analog–digital acquisition board (USB-6343, National Instruments) connected to the amplifier (Axoclamp 2B, Axon Instruments) operating at a sampling frequency of 40 kHz. After cell immobilization, the microelectrode is lowered into the cell until the measured potential drops by about 20 mV. The procedure is repeated with a second electrode. The pump or depression is then stopped, and the cell is maintained against the coverslip by the electrodes. Square current pulses of 500 pA amplitude and 100 ms duration are then injected to tune the amplifier's capacitance neutralization circuit. Fig. 2C shows a typical voltage response when 100 ms long current pulses of varying amplitude, from −4 nA to 4 nA, are injected through one electrode and recorded with the other.

Microscopy

We imaged ciliates using an upright LNScope microscope (Luigs & Newmann) with two objectives, a 20× air objective (SLMPLN Plan Achromat, Olympus) used to locate cells, and a 40× water immersion objective (LUMPLFLN, Olympus) with DIC contrast enhancement for electrophysiology and microinjection. For visualization and recording, we used a high-speed and high-sensitivity CCD camera (Lumenera Infinity 3-6UR, 2752×2192 pixels^2, 8 or 14 bit depth, 27 frames s^{-1} at full resolution). For fluorescence measurements, the setup was illuminated with a CoolLED pE-300 ultra combined with a Cy3 filter.

Microinjection

Glass microinjection pipettes were pulled to an outer diameter of 0.7–0.9 µm in one step using the same pipette puller as described above. The back of the pipette was connected to a microfluidic flow controller (Elveflow, OB1-Mk3) and controlled with ESI software (Elveflow Smart interface). The baseline pressure was set to 5 mbar (500 Pa), such that there was no net flow through the micropipette. Paramecia were injected with a solution containing 60 µmol l^{-1} Alexa-594 fluorophore dye and 20 mmol l^{-1} KCl, by applying a 100 ms long pulse at a pressure of 300 mbar (30 kPa).

RESULTS AND DISCUSSION

Immobilization

Paramecia (*P. tetraurelia*) swimming in a large drop of culture are placed over the device. A downward flow can then be induced by two means. In the first configuration, a peristaltic pump draws fluid from the bath and pours it back in at the top (see Movie 1). When the flow rate is greater than about 0.7 ml min^{-1}, paramecia are pulled down and immobilized against the filter, typically after a few seconds. Although paramecia cannot swim, their cilia still beat. When the pump is stopped, paramecia immediately swim away from the filter. Note that in practice, paramecia can be immobilized by one or several holes constraining them in vertical or horizontal positions. The hole diameter of the filter must thus be smaller than the cell size.

In an earlier version of the device, the filter moved in the vertical direction by about 30 µm when the pump was turned on, as the pump pulled on the filter. To solve this issue, the filter was put on a slightly raised platform (see Materials and Methods), so that it is stretched when the top cover is screwed over the bottom part of the device. No measurable movement was then observed when the pump was turned on.

As the peristaltic pump can introduce a periodic pulsation of the paramecia's vertical position, we also implemented a second configuration in which downward flow is induced by a negative pressure. In this configuration, the outlet is connected to a sealed reservoir. When the reservoir is depressurized at about −150 mbar (–15 kPa), paramecia are immobilized against the filter (see Movie 2). This pressure difference imposes a flow rate of about 0.7 ml min^{-1} into the reservoir, as in the first configuration. To maintain the liquid bath surface level in the pool, we use a gravity-based perfusion system that yields a flow rate of 5 ml min^{-1}, while the excess fluid is removed with a peristaltic pump. Perfusion can be used simultaneously with the depression; Movie 3 and Fig. S2 show a solution exchange while *Paramecium* is immobilized by depression.

We also tested the immobilization device on a larger ciliate species, *P. caudatum* (about 200 µm), and on a smaller one, *T. thermophila* (about 50 µm). Both could be immobilized with minor adjustments (see Movies 4 and 5): *P. caudatum* was immobilized with the peristaltic pump rotating at about twice the speed used for *P. tetraurelia* (corresponding to 1.4 ml min^{-1}); *T. thermophila* was immobilized with −85 mbar (−8500 Pa) depression and a filter with smaller holes (5 µm diameter).

The device can be used to observe various cellular phenomena. For example, Movie 6 shows the periodic beating of contractile vacuoles of *P. tetraurelia* (Tominaga et al., 1998), as well as cytoplasmic streaming (Sikora, 1981), while the cell is immobilized by depression for 2 min. Below, we demonstrate the use of the device for electrophysiology and microinjection.

Electrophysiology

After immobilization, a pipette can be lowered into the cell. Fig. 2A shows a *Paramecium* impaled with two sharp microelectrodes. Impalement is facilitated by the fact that, in contrast with the droplet technique, an electrical signal is available while the electrode is lowered. Indeed, entry of the microelectrode into the cytosol

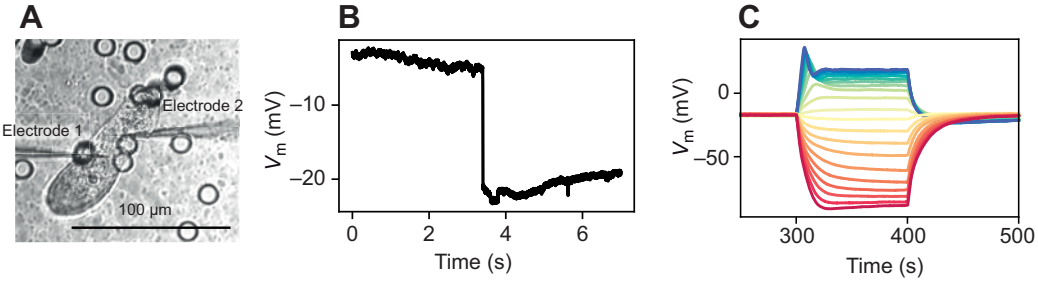

Fig. 2. Electrophysiology experiments on immobilized *Paramecium*. (A) Immobilized *Paramecium* impaled by two microelectrodes. (B) Membrane potential (V_m) measured while the electrode is impaled into the cell. Successful impalement is signalled by a sudden voltage drop. (C) Action potentials recorded in response to current pulses of −4 nA (red) to 4 nA (blue), in steps of 400 pA.

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is witnessed by a voltage drop (Fig. 2B; Movie 7). Once both microelectrodes are in place, the pumping flow is stopped. Fig. 2C shows action potentials recorded by one electrode in response to current steps injected through the other electrode. Several known aspects of *Paramecium* electrophysiology can be observed in this figure. Positive currents trigger action potentials with graded amplitude (not all-or-none), which are due to an inactivating calcium current shortly followed by the opening of delayed rectifier potassium channels (Eckert and Brehm, 1979). At the end of a positive pulse, the membrane hyperpolarizes below resting potential, due to a calcium-gated potassium current (Satow and Kung, 1980). When a strong negative pulse is injected, the membrane first hyperpolarizes, then slightly depolarizes. This is due to a mixture of inward hyperpolarization-activated currents, including a potassium inward rectifier current (Oertel et al., 1978) and two calcium currents (Nakaoka and Iwatsuki, 1992; Preston et al., 1992). The resting potential is known to vary substantially depending on the extracellular solution and culturing conditions. Naitoh and Eckert (1968) report −23 to −19 mV in *P. caudatum* with the same extracellular solution as the one we used; the resting potential was −17 mV in Fig. 2C.

If the pump is left running during the recording, the pulsation can be observed on the cell's membrane (Movie 8). As *Paramecium* and many ciliates are highly mechanosensitive (Machemer and Deitmer, 1985), it is therefore advisable to switch off the pump during the recording, or to avoid the pulsation by using the depression configuration. In the recording shown in Movie 8, the pulsation did not seem to impact the measured membrane potential, but we noticed transient hyperpolarizations synchronized with the pulsation in one other recording.

Microinjection

Microinjection in the cytosol of *Paramecium* has been used to investigate various cellular mechanisms: injection of EGTA, calcium and cyclic nucleotides to investigate ciliary regulation (Nakaoka and Machemer, 1990); transfer of cytoplasm from normal to mutant cells to understand the basis of mutant phenotypes (Haga et al., 1982); and injection of a calcium indicator to investigate trichocyst exocytosis (Klauke and Plattner, 1997). As a proof of principle, we performed microinjection of a fluorescent solution into the cytosol of immobilized *Paramecium*. As our specimens display green autofluorescence (Wyroba et al., 1981), we chose the red fluorophore Alexa Fluor-594 (Fig. 3; Movie 9). While the pump is still running, the fluorophore is injected by pressure (Fig. 3A) and then the pipette is removed. Fig. 3B shows the fluorescent *Paramecium* a few minutes after microinjection. Noticeably, it swims normally once immobilization is stopped and retains its fluorescent content.

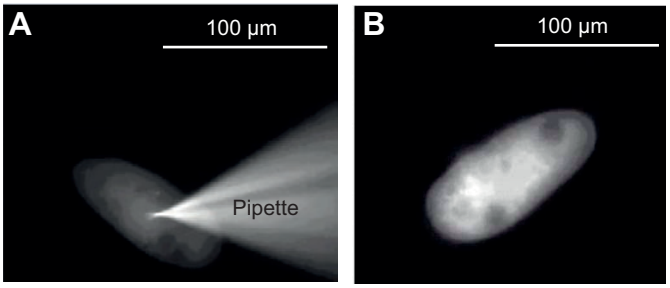

Fig. 3. Microinjection of a fluorescent probe into an immobilized *Paramecium*. (A) *Paramecium* impaled by a microinjection pipette that contains Alexa Fluor-594. (B) Snapshot of a freely swimming *Paramecium* a few minutes after injection.

Conclusions

The device presented here was designed to ease the manipulation of motile *Paramecium* for both intracellular electrophysiology and microinjection measurements. Traditional methods mostly rely on trapping *Paramecium* in microdroplets. Two typical configurations are used, either trapping a single *Paramecium* in an aqueous droplet immersed in oil (Beisson et al., 2010) or confining it in an evaporating water droplet (Naitoh and Eckert, 1972). In the latter case, the time window during which one can introduce a micropipette before *Paramecium* dies is very narrow, which makes the experiment difficult. The former case is not adapted to electrophysiology because the micropipette tip gets contaminated with oil. In contrast, our method is easy to implement, highly reproducible, inexpensive and does not alter *Paramecium*'s viability. In particular, the immobilization can be obtained with any device that imposes a fluid flow such as peristaltic pumps, pressure controllers or syringe pumps. An additional benefit is that an electrical signal is available during the entire procedure, allowing one to verify the proper insertion of microelectrodes. Finally, our device allows for a straightforward medium exchange and is thus appropriate for easy drug testing on *Paramecium*.

Our immobilization method is similar in principle to the suction pipette technique (Grønlien et al., 2013; Jonsson and Sand, 1987), but it has a few advantages. It does not require dexterity, in particular to catch fast specimens. It also does not necessitate the use of a micromanipulator. For electrophysiological recordings, it avoids the application of a constant pressure: once the cell is impaled, the fluid flow is stopped and the cell is immobilized between the coverslip and the microelectrode. In other words, the recording configuration is the same as the classical method and therefore we do not expect the device to interfere with these highly mechanosensitive cells (Machemer and Deitmer, 1985). Finally, the device allows simultaneous imaging of multiple specimens.

We have successfully tested this device on three ciliate species of different sizes, *T. thermophila*, *P. tetraurelia* and *P. caudatum*. The device should thus be useful to efficiently trap any other type of motile protist or microorganism provided that their typical size remains larger than the size of the filter holes. We have demonstrated its use for electrophysiology and microinjection in the cytosol. It could potentially also be used for microinjection of genetic material in the macronucleus (Beisson et al., 2010), although this remains to be tested specifically. In this work, cells were immobilized for a few minutes. The device may also allow imaging of live cells over long periods of time, such as the sexual cycle. In the future, this immobilization technique could be straightforwardly automated by controlling the pump or using solenoid valves, which could allow complete automation of an electrophysiological or microinjection experiment.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.K., I.E., N.E., A.M.P., L.P., R.B.; Methodology: A.K., I.E., N.E., A.M.P., L.P., R.B.; Software: A.K., R.B.; Validation: A.K., I.E., A.M.P., L.P., R.B.; Investigation: A.K., I.E., N.E., A.M.P., L.P., R.B.; Resources: A.M.P., L.P., R.B.; Writing

- original draft: A.K., A.M.P., L.P., R.B.; Writing - review & editing: A.K., N.E., A.M.P., L.P., R.B.; Visualization: A.M.P., L.P., R.B.; Supervision: A.M.P., L.P., R.B.; Project administration: A.M.P., L.P., R.B.; Funding acquisition: A.M.P., L.P., R.B.

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Supplementary information
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References

Beisson, J., Bétermier, M., Bré, M.-H., Cohen, J., Duharcourt, S., Duret, L., Kung, C., Malinsky, S., Meyer, E., Preer, J. R. et al. (2010). DNA microinjection into the macronucleus of paramecium. *Cold Spring Harb. Protoc.* **2010**, pdb.prot5364. doi:10.1101/pdb.prot5364

Kung, C. and Saimi, Y. (1982). The physiological basis of taxes in paramecium. *Annu. Rev. Physiol.* **44**, 519-534. doi:10.1146/annurev.ph.44.030182.002511

Eckert, R. (1972). Bioelectric control of ciliary activity. *Science* **176**, 473-481. doi:10.1126/science.176.4034.473

Eckert, R. and Brehm, P. (1979). Ionic mechanisms of excitation in Paramecium. *Annu. Rev. Biophys. Bioeng.* **8**, 353-383. doi:10.1146/annurev.bb.08.060179.002033

Görtz, H.-D (ed.). (1988). *Paramecium*. Berlin Heidelberg: Springer-Verlag.

Grønlien, H. K., Bruskeland, G. E., Jansen, A. K. and Sand, O. (2013). Electrophysiological properties of the microstome and macrostome morph of the polymorphic ciliate Tetrahymena vorax. *J. Eukaryot. Microbiol.* **60**, 57-69. doi:10.1111/jeu.12006

Haga, N., Forte, M., Saimi, Y. and Kung, C. (1982). Microinjection of cytoplasm as a test of complementation in Paramecium. *J. Cell Biol.* **92**, 559-564. doi:10.1083/jcb.92.2.559

Hennessey, T. M. and Kuruvilla, H. G. (1999). Chapter 17 Electrophysiology of Tetrahymena. In *Methods in Cell Biology* (ed. D. J. Asai and J. D. Forney), pp. 363-377. Academic Press.

Hinrichsen, R. D. and Schultz, J. E. (1988). Paramecium: a model system for the study of excitable cells. *Trends Neurosci.* **11**, 27-32. doi:10.1016/0166-2236(88)90046-X

Houten, J. V. (1979). Membrane potential changes during chemokinesis in Paramecium. *Science* **204**, 1100-1103. doi:10.1126/science.572085

Jonsson, L. and Sand, O. (1987). Electrophysiological recordings from ciliates. In *Chronobiotechnology and Chronobiological Engineering, NATO ASI Series* (ed. L. E. Scheving, F. Halberg and C. F. Ehret), pp. 432-434. Dordrecht: Springer Netherlands.

Klauke, N. and Plattner, H. (1997). Imaging of Ca^{2+} transients induced in Paramecium cells by a polyamine secretagogue. *J. Cell Sci.* **110**, 975-983.

Kung, C. and Saimi, Y. (1985). Chapter 3 Ca2+ channels of paramecium: a multidisciplinary study. In *Current Topics in Membranes and Transport, Genes and Membranes: Transport Proteins and Receptors* (ed. F. Bronner and A. Kleinzeller), pp. 45-66. Academic Press.

Machemer, H. (2001). The swimming cell and its world: structures and mechanisms of orientation in protists. *Eur. J. Protistol.* **37**, 3-14. doi:10.1078/0932-4739-00816

Machemer, H. and Deitmer, J. W. (1985). Mechanoreception in ciliates. In *Progress in Sensory Physiology, Progress in Sensory Physiology* (ed. H. Autrum, D. Ottoson, E. R. Perl, R. F. Schmidt, H. Shimazu and W. D. Willis), pp. 81-118. Berlin, Heidelberg: Springer Berlin Heidelberg.

Naitoh, Y. and Eckert, R. (1968). Electrical properties of Paramecium caudatum: modification by bound and free cations. *Z. Für. Vgl. Physiol.* **61**, 427-452. doi:10.1007/BF00297875

Naitoh, Y. and Eckert, R. (1972). Electrophysiology of ciliate protozoa. *Exp. Physiol. Biochem.* **5**, 17-31.

Nakaoka, Y. and Iwatsuki, K. (1992). Hyperpolarization-activated inward current associated with the frequency increase in ciliary beating of Paramecium. *J. Comp. Physiol. A* **170**, 723-727. doi:10.1007/BF00198983

Nakaoka, Y. and Machemer, H. (1990). Effects of cyclic nucleotides and intracellular Ca on voltage-activated ciliary beating in Paramecium. *J. Comp. Physiol. A* **166**, 401-406. doi:10.1007/BF00204813

Oertel, D., Schein, S. J. and Kung, C. (1978). A potassium conductance activated by hyperpolarization in paramecium. *J. Membr. Biol.* **43**, 169-185. doi:10.1007/BF01933477

Preston, R. R., Saimi, Y. and Kung, C. (1992). Calcium current activated upon hyperpolarization of Paramecium tetraurelia. *J. Gen. Physiol.* **100**, 233-251. doi:10.1085/jgp.100.2.233

Satow, Y. and Kung, C. (1980). Ca-Induced K+-outward current in Paramecium tetraurelia. *J. Exp. Biol.* **88**, 293-304.

Sikora, J. (1981). Cytoplasmic streaming in Paramecium. *Protoplasma* **109**, 57-77. doi:10.1007/BF01287630

Tominaga, T., Allen, R. D. and Naitoh, Y. (1998). Electrophysiology of the in situ contractile vacuole complex of Paramecium reveals its membrane dynamics and electrogenic site during osmoregulatory activity. *J. Exp. Biol.* **201**, 451-460.

Valentine, M. S. and Van Houten, J. L. (2016). Methods for studying ciliary-mediated chemoresponse in paramecium. *Methods Mol. Biol. Clifton NJ* **1454**, 149-168. doi:10.1007/978-1-4939-3789-9_10

Wichterman, R. (1986). *The Biology of Paramecium*. Springer US.

Wyroba, E., Bottiroli, G. and Giordano, P. (1981). Autofluorescence of axenically cultivated Paramecium aurelia. *Acta Protozool.* **20**, 165-170.