A Novel Single Compartment In Vitro Model for Electrophysiological Research Using the Perfluorocarbon FC-770

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Summary

Electrophysiological studies of whole organ systems *in vitro* often require measurement of nerve activity and/or stimulation of the organ *via* the associated nerves. Currently two-compartment setups are used for such studies. These setups are complicated and require two fluids in two separate compartments and stretching the nerve across one chamber to the other, which may damage the nerves. We aimed at developing a simple single compartment setup by testing the electrophysiological properties of FC-770 (a perfluorocarbon) for *in vitro* recording of bladder afferent nerve activity and electrical stimulation of the bladder. Perflurocarbons are especially suitable for such a setup because of their high oxygen carrying capacity and insulating properties. In male Wistar rats, afferent nerve activity was recorded from postganglionic branches of the pelvic nerve *in vitro*, *in situ* and *in vivo*. The bladder was stimulated electrically *via* the efferent nerves. Organ viability was monitored by recording spontaneous contractions of the bladder. Additionally, histological examinations were done to test the effect of FC-770 on the bladder tissue. Afferent nerve activity was successfully recorded in a total of 11 rats. The bladders were stimulated electrically and high amplitude contractions were evoked. Histological examinations and monitoring of spontaneous contractions showed that FC-770 maintained organ viability and did not cause damage to the tissue. We have shown that FC-770 enables a simple, one compartment *in vitro* alternative for the generally used two compartment setups for whole organ electrophysiological studies.

Key words

Electrophysiology • Bladder • *In vitro* • Single compartment setup

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Introduction

In electrophysiology, isolated whole organ systems are often studied for a better understanding of their function. In experiments requiring stimulation of the organs *via* efferent nerve fibers and/or recording of the afferent nerve activity from the organ, an *in vitro* setup is often preferred over an *in vivo* setup because: i) it offers greater control over the extracellular environment and therefore enables a more accurate measurement of the response of the organ to various stimuli (e.g. drugs, mechanical and/or electrical stimulation), without interference of the central nervous system or other functions such as circulation and breathing (Hutcheson *et al.* 2004), ii) experimental variation is less leading to better reproducibility, iii) it does not require complex surgical procedures or extensive monitoring (Namasivayam *et al.* 1998). Additionally, *in vitro* experimentation is a great step forward towards reduction of experimental animal usage (Russell and Burch 1959).

Two compartment *in vitro* setups have frequently been reported in the literature for the aforementioned research (Daly *et al.* 2014, Namasivayam *et al.* 1998, Rong and Burnstock 2004, Valderrama-Gonzalez *et al.* 2010, Yu and de Groat 2010). In a typical two compartment setup, the first compartment (filled with an oxygenated Krebs solution), containing the organ under investigation, serves the purpose of maintaining organ viability. The second compartment acts as a nerve recording/stimulating chamber and is filled with paraffin oil to provide electrical insulation. The nerve, still

attached to the organ, is pulled into the recording chamber through a small hole or gate between the two chambers. The opening is then sealed with silicon grease to prevent leakage (Namasivayam *et al.* 1998, Valderrama-Gonzalez *et al.* 2010). Apart from the implicit requirement of a relatively large length of nerve, guiding the nerve from the viability chamber to the recording chamber is a delicate procedure which can easily damage the nerve (e.g. by overstretching). Another popular technique used for electrophysiological studies on a nerve-organ system involves suction electrodes (Daly *et al.* 2014, Rong and Burnstock 2004). In this setup the nerve is sucked into a micropipette filled with a conductive fluid. This setup has some drawbacks. Firstly, the diameter of the tip of the micropipette has to be adjusted to the size of the nerves. Secondly, the micropipette tip is made of thin, fragile glass which can break easily during handling and may damage the nerves. Thirdly, only monopolar recordings can be made through the fluid filled suction electrodes, which have a low signal to noise ratio as compared to bipolar differential electrodes (Issa *et al.* 2009). Lastly, high frequency signal components cannot be measured as accurately as with bipolar electrodes (Issa *et al.* 2009).

To overcome the complications of two compartment setups, we developed a single compartment set up using a perfluorocarbon (PF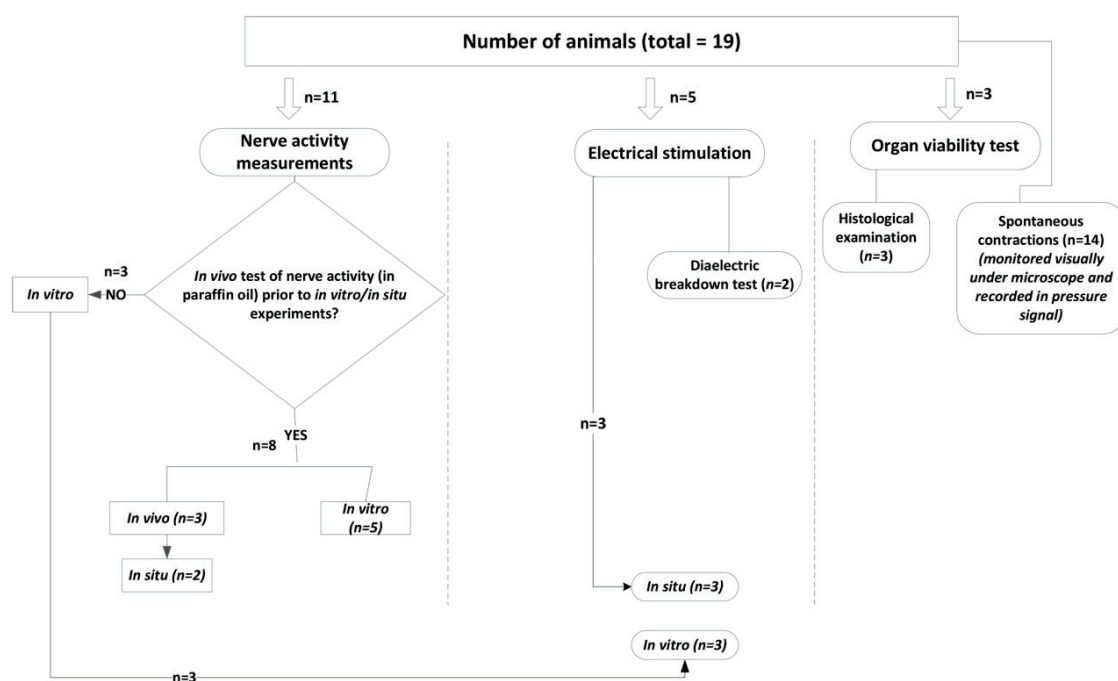C), FC-770 (Choudhary *et al.* 2013). PFCs are basically organofluorine compounds produced by replacing all hydrogen atoms of hydrocarbons with fluorine. They do not interact with biological tissue due to the high stability and inertness of carbon-fluoride bonds (Clark and Gollan 1966) and have a high capacity for dissolving respiratory gases at atmospheric pressure. Additionally, PFCs are highly insulating because of their high dielectric strength (>40 kV, 0.1" gap, k=1.9) (Datasheet/FC-770 2007).

The aim of the current study was to provide a proof-of-concept that FC-770 can be used as a new method for recording bladder afferent nerve activity and electrical stimulation of the bladder through efferent nerve fibers in a single compartment *in vitro* setup as well as for *in vivo* and *in situ* electrophysiology.

Methods

Experimental procedures

All laboratory and experimental procedures were conducted in accordance with Erasmus MC guidelines. Male Wistar rats were anesthetized with urethane (1 g/kg) intraperitoneally for the experiments. A total of 19 rats (animal weight = 390±40 g, 8-9 weeks old) were used in this study. Figure 1 shows the distribution of animals used for different (combinations of) experiments. The electrophysiological suitability and functionality of FC-770 was tested in *in vivo*, *in situ* and *in vitro* conditions.

Fig. 1. Schematic overview of experiments on all animals used. All experiments were done in FC-770 except the *in vivo* test of nerve activity (shown in the diamond box).

In vivo experiments

In anesthetized rats, bladder filling (volumetric flow rate = 0.11 ml/min) and measurement of pressure were done by inserting a 23G needle at the top of the bladder. The other end of the needle was attached to a disposable pressure transducer (BD DTX Plus™) and an infusion pump using a 2 way connector. Pressure was measured using a Statham SP1400 blood pressure monitor. The pressure transducer was calibrated using a column of water before the start of each experiment. Postganglionic bladder nerves, presumably branches of the pelvic nerve, were mounted on a bipolar electrode consisting of two thin (diameter 0.1 mm) platinum-iridium hook shaped wires separated by a distance of 0.5-1 mm. The bladder was repeatedly filled with saline up to a certain volume (~1 ml) or until voiding occurred. The nerve activity was recorded and amplified by a DISA 15C01 EMG amplifier (gain: 100-200,000) and band-pass filtered with a Krohn-Hite 3944 filter (Bessel, 4th order, 200-2000 Hz). Bladder pressure and nerve activity were sampled at 25 Hz and 25 kHz respectively and 30-900 s episodes were stored using a custom written Labview® program. The recorded signals were processed with a custom written MATLAB® program.

In situ experiments

For *in situ* experiments, the rats were euthanized after the *in vivo* measurements by injecting an overdose of KCl in the heart, while keeping the original setup intact. Care was taken not to disturb the nerve-electrode configuration. The bladder was repeatedly filled with saline and the corresponding pressure-nerve activity was recorded as described in the preceding section.

In vitro experiments

After euthanizing the rats, the bladder along with prostate, urethra, attached nerves and surrounding tissue were removed and placed in a pre-warmed (30 °C) Krebs solution. After careful removal of the surrounding tissue, the preparation was transferred to a preheated (~30 °C) FC-770 (3M™ Fluorinert™ Electronic Liquid) filled single compartment setup. Some tissue surrounding the bladder was not removed and was used to fixate the preparation to the bottom of the compartment, to avoid floating of bladder. The whole setup was placed on a heated platform which maintained the temperature of the fluid around 30 °C. The FC-770 was perfused continuously with carbogen throughout the experiment.

Bladder filling and nerve activity recording were performed in the same way as described in the *in vivo* experiments section. After filling the bladder with up to ~1 ml of saline, a mechanical stimulus was applied by pushing a cotton swab on the bladder for a period of ~4-5 s until there was a discernible increase in the nerve activity. The bladder pressure and nerve activity were stored, as described in the *in vivo* experiments section.

Three types of experiments were done

Nerve activity measurements

In 8 rats, nerve activity was tested *in vivo* prior to the *in vitro* and *in situ* experiments. Rats in which nerve activity was recorded successfully in at least 25 % of the *in vivo* measurements were selected for further *in vitro*/*in situ* experiments. In 3 rats nerve activity was measured *in vitro*, without prior *in vivo* experiments. To quantify the nerve activity, first the baseline nerve activity was determined by taking the average of 30 s of the resting nerve activity at the beginning of filling when the bladder was empty and pressure was low. Next, the nerve signal was divided into windows of 1 s and the peaks (local maxima) exceeding the baseline nerve activity were counted with a custom written Matlab® program. A measurement was considered successful when there was a good correlation between nerve activity and bladder pressure i.e. the number of peaks in the nerve activity was maximal during the maximum pressure. We also determined the effect of FC-770 on the linear dependence of nerve activity on bladder pressure. A linear regression model was fitted to the afferent activity-pressure data in MATLAB® to calculate slope and offset.

Electrical stimulation

Bladders were stimulated electrically *via* the pelvic nerve *in situ* and *in vitro* by rectangular pulses of width 400 µs, frequency 10 Hz and an amplitude (starting with 1 V) increasing in steps of 1 V until a bladder contraction in the form of a pressure rise of approximately 10 cm H_2O above the baseline was observed and then the stimulation was switched off. Bladders were stimulated for ~2 h, with rest periods of 10 min between successive stimulations of ~3-4 s. To verify that the bladders were stimulated *via* the nerves and not *via* the muscle, the electrical coupling between nerve and bladder was verified at the end of experiments by cutting the nerve and stimulating again.

Organ viability test

To test the viability of bladder and nerves in FC-770, the pressure during spontaneous contractions of the bladder in nerve activity and electrical stimulation experiments was recorded upon filling with saline. The spontaneous contractions were also observed under the microscope. The effect of FC-770 on bladder tissue was histologically compared to that of a physiological buffer (carbogenated and heated Krebs solution).
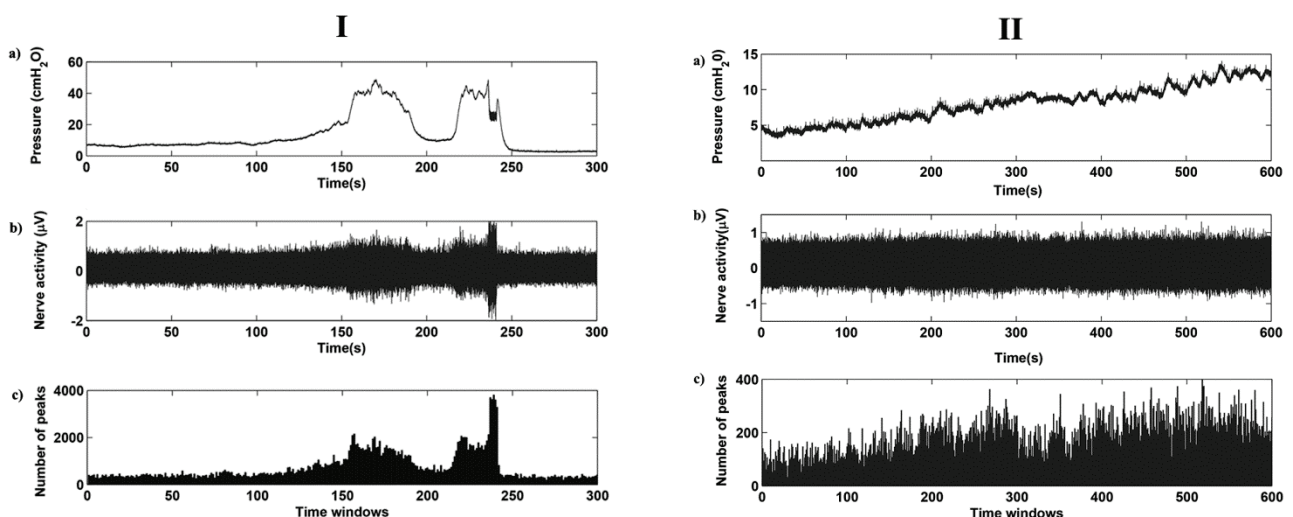
Statistical analysis

All data are presented as mean ± SD. Using the SPSS® statistical package (version 21.0, SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to test normality and Levene's test for homogeneity of variance. A one-way ANOVA test was used for normally distributed data and a Kruskal-Wallis test for non-normally distributed. A $p<0.05$ was considered significant.
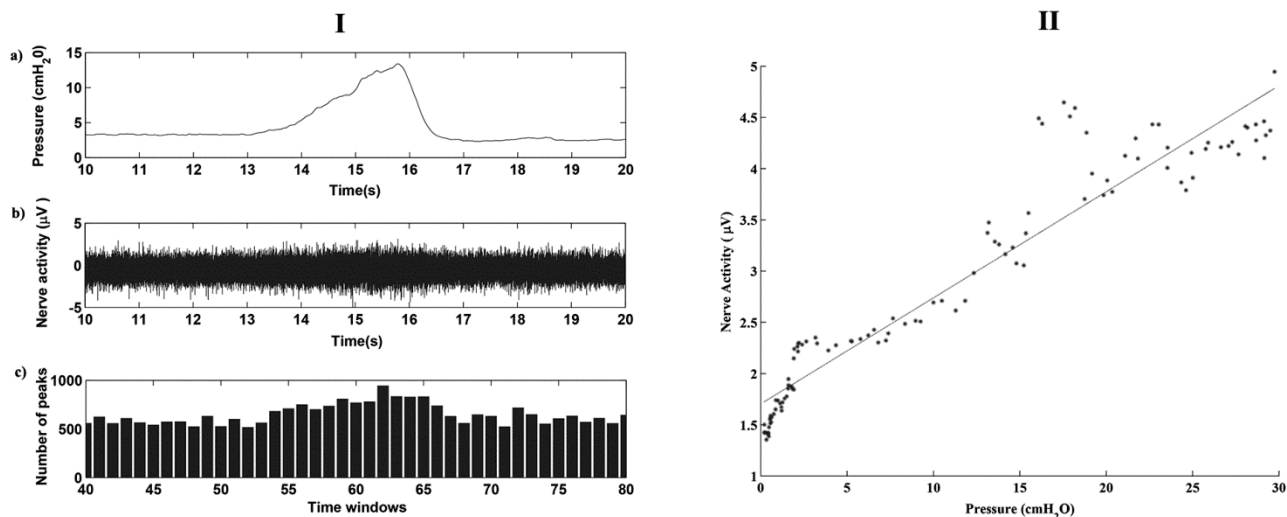
Results

Nerve activity measurements

Nerve activity measurements were done in a total of 11 rats. In 8 of these it was measured *in vivo* in paraffin oil and minimally 3 measurements *per* rat were obtained in which the number of peaks in the nerve ac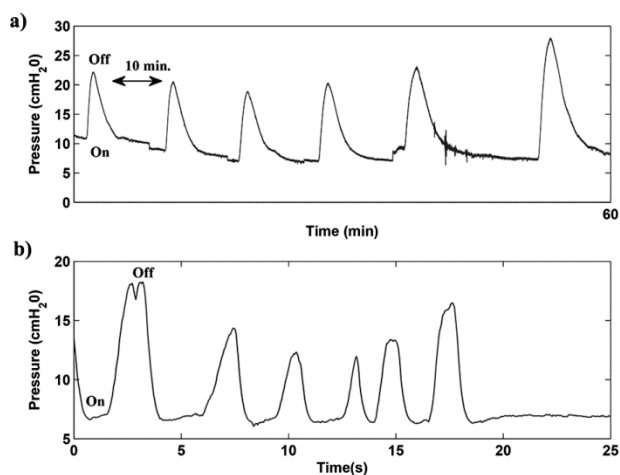tivity was maximal during the maximum pressure. The effect of FC-770 on the electrical conduction of the nerves was tested in 3 rats by comparing the amplitude of the nerve activity recordings *in vivo* (7 measurements) in paraffin oil with that of the nerve activity recordings *in vivo* in FC-770 (5 measurements). No significant difference (p=0.8, one-way ANOVA) in the number of peaks (175052±171168 vs. 156725±163342) was found. An example of nerve activity recorded *in vivo* in FC-770 is shown in Figure 2(I). The slope and intercept of nerve activity and pressure was compared between measurements done in paraffin oil and FC-770, where no significant difference (0.20±0.21 vs. 0.20±0.18, p>0.05, Kruskal-Wallis test) was found. Furthermore, in 2 of these 3 rats, afferent nerve activity was also measured *in situ*, i.e. after sacrificing the rats, where 3 episodes of nerve activity were recorded (Fig. 2(II)). After confirming the presence of nerve activity in the *in vivo* settings, nerve activity experiments were done in 5 bladder preparations in the single compartment *in vitro* setup. 6 successful measurements were done in 3 rats (Fig. 3(I)), whereas no nerve activity could be recorded in the other 2 rats. A linear polynomial fit of nerve activity as a function of bladder pressure is shown in Fig. 3(II). In 3 other rats, nerve activity was recorded *in vitro* without any prior *in vivo* nerve activity test. In 2 of those rats, 3 successful measurements of nerve activity were recorded *in vitro*.

Fig. 2. An *in vivo* and *in situ* pressure-nerve activity measurement with the abdominal cavity of the anesthetized rat filled with FC-770. **Ia)** The pressure development during the filling phase and a voiding contraction. A typical rat voiding contraction is marked by high frequency oscillations (HFO) of the urethral sphincter. **IIa)** The pressure development during bladder filling in an *in situ* preparation. In both **I** and **II, b)** shows the corresponding nerve activity. **c)** The number of peaks in nerve activity overcoming the baseline.

Fig. 3. An *in vitro* pressure-nerve activity measurement of a rat bladder-nerve preparation in FC-770. **I)** After filling the bladder up to a certain volume (approx. 1 ml), the bladder was slightly pushed with a cotton swab and then the stimulus was removed. **a)** Shows the recorded pressure during the mechanical activation. **b)** The corresponding nerve activity. **c)** The number of peaks in nerve activity overcoming the baseline nerve activity. **II)** A linear polynomial fit of nerve activity as a function of bladder pressure recorded during the filling phase of a voiding contraction in one rat.

Fig. 4. Electrical nerve stimulation of a rat bladder-nerve preparation *in situ* and *in vitro* in FC-770. The upper panel **(a)** shows the bladder pressure change during electrical stimulation *in situ*. In this rat the bladder was stimulated intermittently with 10 min rest periods between consecutive stimulations. The bladder was stimulated until a peak in pressure was observed, then the stimulation was switched off. The lower panel **(b)** shows evoked bladder contractions upon electrical stimulation *in vitro*. In this example multiple On/Off electrical stimulations were done in a short period of time.

Electrical stimulation

Electrical stimulation was performed in a total of 6 rats *in situ* and *in vitro*. In 3 of these rats nerve activity measurements had been done before. The pelvic nerve was stimulated until a rise of ~10 cm H_2O above the baseline in the bladder pressure was observed and then stimulation was switched off, which resulted in an immediate decline in pressure. In 3 rats, the bladder nerve was electrically stimulated during a period of 2 h *in situ* and 15 episodes of bladder contraction upon stimulation were recorded. Figure 4a shows an example of such an *in situ* electrical stimulation. In the other 3 rats, 8 episodes of high amplitude bladder contractions were recorded upon stimulation *in vitro*. An example of an *in vitro* electrical stimulation is shown in Figure 4b. In 2 of these 3 rats, the nerve was cut midway between the bladder and the stimulation site and no contractions could be evoked after that, verifying that the nerve was electrically stimulated, not the muscle.

Test of organ viability

Spontaneous bladder contractions

Bladder viability was tested by continuous filling of the bladder with saline. In 14 out of the 16 animals in which nerve activity and electrical stimulation experiments (*in vitro* and *in situ*) were done, spontaneous bladder contractions were observed for ~90-120 min after sacrificing the rats.

Histological examinations

Histological examination of tissue from 3 rats showed no evidence of autolysis and necrosis. Muscle bundles were found to be intact with no visible signs of edema.

Discussion

In the current study, we showed that a single compartment *in vitro* setup using FC-770 can be used as a new method for recording rat bladder afferent nerve activity and electrical stimulation of the bladder *via* efferent bladder nerve fibers.

One of the essential requirements for recording nerve activity in an *in vitro* setup is the insulation of electrodes, which is primarily the reason why two-compartment setups are used. We have shown that FC-770 provides the necessary electrical insulation in a single compartment setup by successfully recording afferent nerve activity in our *in vitro* setup. Additionally, no significant difference was found between the nerve activity recorded *in vivo* in paraffin oil and FC-770, which implies that FC-770 affect the amplitude of nerve activity no more than paraffin oil does.

We also tested the functionality of FC-770 in an *in situ* model, in which afferent nerve activity recording and electrical stimulation of the bladder were measured successfully. The *in situ* model enables a quick and easy switch from *in vivo* to *in situ* without changing the setup, hence reducing the damage to bladder and nerves. The *in situ* set up also enables the study of the rat micturition cycle without interference from the central nervous system in a whole animal model. The spontaneous contractions recorded in our *in situ* experiments have also been reported in other animal models in isolated bladders (Drake *et al.* 2003). The origin of these non-voiding contractions has been shown to be autonomous in nature and their frequency and amplitude largely depends on bladder volume (Clavica *et al.* 2015, Streng *et al.* 2006). Therefore, the recorded *in situ* nerve activity is assumed to be afferent. However, the autonomous spontaneous contractions in *in vivo* studies, have been associated with pelvic efferent nerve discharge (Choudhary *et al.* 2015, Satchell 1991), indicating a regulation by the central nervous system. A similar mechanism might exist *in situ* leading to an efferent component in the recorded measurements.

Electrical stimulation of the bladder *via* efferent nerve fibers was favorably tested *in vitro* and *in situ* in FC-770. In both cases high amplitude bladder contractions were evoked upon stimulation. In 3 rats, bladder contractions were evoked up to 2 h after sacrifice. Due to the high oxygen carrying capacity (approx. 25 times that of blood), FC-770 provides good organ viability, which was confirmed by the spontaneous contractions of the bladder throughout the experiments. The viability of the bladder nerves was ascertained by evoking bladder contractions upon electrical stimulation of these nerves. In our study, histological examinations of bladders kept in FC-770 showed no signs of cell death, muscle damage or any disruption in the anatomy of the tissue, which strongly suggests that FC-770 maintained tissue viability without damaging it.

FC-770 is immiscible with aqueous and hydrophobic solutions, which facilitates the removal of blood or any other unwanted liquid from the chamber. A limitation caused by this property of FC-770 is that it makes extravesicular administration of drugs difficult, which can be overcome by preparation of emulsions and nutrient solutions of PFCs (Haiss *et al.* 2009). Additionally, most of the drug administration tests done on bladders can also be done *via* intravesicular administration (Fowler 2000, Pandita and Andersson 2002) or through arterial perfusion (Sadananda *et al.* 2011). Another minor limitation of FC-770 is that due to its high density (Datasheet/FC-770 2007), the bladder floats on the surface of the fluid. This requires fixation of the bladder to the bottom of the chamber (Kuroda *et al.* 1990).

Our proposed single compartment model can be applied to a broad field of electrophysiology in various other animal models in which two compartment models are generally used. For example, in a study of afferent nerve fibers in ureters (Cervero and Sann 1989), a two chamber setup – an oxygenating chamber and paraffin oil filled recording chamber was used. In another study, for control of a prosthetic hand, an *in vitro* peripheral nerve preparation in a two chamber grease-gap bath setup was used for stimulation and recording (Valderrama-Gonzalez *et al.* 2010). A similar two compartment design was also used in the study of esophagus and stomach afferents (Page and Blackshaw 1998). The setup design described in preceding examples requires difficult preparation and is vulnerable to failures. These limitations could be overcome with our proposed one compartment setup.

Conclusions

We have shown that FC-770 enables a simple, one compartment *in vitro* alternative for the existing two compartment models used in various whole organ electrophysiological studies. The results of this study provide sufficient indications that PFC could be a valuable tool for electrophysiological studies. Further

studies are warranted on a larger group of animals in order to definitively prove the value of this setup in a broader field of electrophysiology.

Conflict of Interest

There is no conflict of interest.

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