18th July 2021

Dear Editor,

On behalf of the author team, let me thank you and the reviewers for providing valuable feedback for our article. Please find our comments and corrective actions towards all comments and recommendations of you and the reviewers on the following pages of this letter. The questions / recommendations of the reviewers are underlined, while our answers are in plain text. We also enclose the modified manuscript with highlighted changes (by using the Track changes function of Microsoft Word) and modified figures + the Materials and equipment list.

Thank you for considering our manuscript for publication in the Journal of Visualized Experiments.

Yours sincerely,

Marek Novák (also on behalf of the co-authors).

Editorial Changes

Editor's comment: 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Our reply: The manuscript has been thoroughly read and mistakes caused by bad spelling or grammar were corrected.

Editor's comment: 2. Please provide an institutional email address for each author.

Our reply: This was added to the manuscript.

Editor's comment: 3. Please define the abbreviations before use (ISFET, PCB, FEB, etc.)

Our reply: This was edited, thank you for pointing it out.

Editor's comment: 4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (TM), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

Our reply: We checked the manuscript and all commercial information should be strictly in the Table of Materials.

Editor's comment: 5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Our reply: Our method does not involve living animals. The stomach and esophagus were purchased from a local butchery as their standard product. This procedure is in accordance with Czech laws and we prefer it because of the "3R" principle (Replacement, Reduction and Refinement). We find it unethical to use a dedicated experimental animal only for the purpose of organ harvesting when another legal method which saves the animal is available.

Editor's comment: 6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Our reply: Changes to some steps (e.g. 2.5. and 3.6.s) were made to improve cohesion and provide more detailed information about the steps. We omitted the step 4.3. from the protocol as it is not directly related to the procedure, it only checks whether the step 4.2. was done correctly.

Editor's comment: 7. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

Our reply: The manuscript was corrected.

Editor's comment: 8. For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 μL, 7 cm2

Our reply: The manuscript was corrected.

Editor's comment: 9. Being a video-based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

b) Please mention how animals are anesthetized and how proper anesthetization is confirmed.

c) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

d) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

e) Discuss maintenance of sterile conditions during survival surgery.

f) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

g) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

h) Please do not highlight any steps describing euthanasia.

Our reply: This is not applicable – the method does not involve living animal experiments.

Editor's comment: 10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in the imperative tense.

Our reply: We checked the steps and are of an impression that the protocol is following these instructions. We are, however, open to any recommendations from you side.

Editor's comment: 11. Please ensure that the Discussion explicitly covers the following with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Our reply: The section was written to discuss all these topics. We are welcome to any further changes if these will be required.

Editor's comment: 12. Please consider combining some figures together to form a multipaneled single figure. Ensure to label the figures to make them more informative.

Our reply: We agree, multiple figures were combined.

Editor's comment: 13. Please sort the Table of Materials in alphabetical order.

Our reply: This was done – we created several "categories" of the material (according to the specific step they are connected to) and sorted them in alphabetical order. If requested, we can sort everything as a single table but we suggest keeping several sub-tables for clarity.

Reviewer 1

Reviewers's summary: This manuscript describes the fabrication, bench, and ex vivo testing of an implantable wireless pH sensor and passive receiver. It is interesting and would have value for others working in the field of biomedical engineering. In general, the protocol steps are well written and clear, but there are some steps that are very superficially described, and some essential background information is not given. I was also surprised at the lack of schematics or description of the electronic design - this would probably be more valuable than the clinical background for a methods paper. Materials are well described, but the equipment used should be more detailed. The representative results were reasonable, and it was good to see both in vitro and ex vivo data reported. Calculation of sensor error was performed using an unusual method - taking only the standard deviation of the absolute errors. This will give a measure of dispersion of error, but not its magnitude. This should be changed to the mean error (with SD quoted alongside). In the discussion, it would be useful to describe in more detail how other receivers could be used to collect data from the device. This would make the paper more generalisable and useful, as the passive receiver described is quite specific to this application. The code accompanying the paper is well commented, and it would be easy to understand and adapt.

Our reply: According to your summary, some of the steps which were described only briefly were expanded and the use of various instruments was described in more detail or the procedure was cited (e.g. step 2.8 and 2.9). The mean error was added alongside the standard deviation of absolute error to make the statistical analysis of the results clearer. Other types of receivers and specific settings which will lead to successful data reception are now discussed in the manuscript. The schematic diagram was embedded as a Supplementary material and the design of the circuit is now described in the Introduction.

Reviewers's comment: Abstract: Currently written in the style of "reporting research", should be adapted to make clear it is describing a method. GERD should be defined.

Our reply: The abstract was modified to better fit the nature of the article.

Reviewers's comment: Introduction: How does neurostimulation work? What is the principle-of-operation of an ISFET, and why would it be chosen as a pH sensor for implantable devices? Which animal model have you chosen, and why is it a good model?

Our reply: The introduction section was expanded with the citations of current literature in terms of neurostimulation treatment. The principle of operation of an ISFET sensor as well as its suitability for implantable devices was mentioned. The chosen animal model represents the anatomy of a human body in terms of size and structure. This was also mentioned in the Introduction.

Reviewers's comment: 1.3 Define FEP

Our reply: Added to the manuscript.

Reviewers's comment: 1.8 How does black epoxy enable later inspection? Surely transparent epoxy would be better?

Our reply: A thorough inspection of the PCB is done before encapsulation. The reason for inspection is to ensure that no metallic component or PCB is exposed to the environment, and everything is covered with epoxy. Thus, black or colored opaque epoxy provides a better way to inspect possible voids in the epoxy layer. We acknowledge the fact that the explanation was not clear enough so we decided to provide a more detailed explanation within the step.

Reviewers's comment: 2 + 3: Schematic diagrams should be included for the circuits

Our reply: A schematic diagram for the electronics part (microcontroller + RF transmitter) was added as a Supplementary file (as it is not strictly required to reproduce the device as described in the procedure). As for the zero-bias Schottky receiver, the PCB was drawn directly without the schematic diagram. We think that due to a simple design (matching network, 2 diodes, and output capacitor), the circuit can be very easily decoded by a reader with experience in electronics circuit design.

Reviewers's comment: 2.3 Include orientation of polarised components (e.g. the microcontroller) on the PCB for figure 3.

Our reply: The pin 1 markings (previously grey) were changed to red color and the figure title was changed to make the orientation more clear. The crystal is not a polarised component.

Reviewers's comment: 2.4 Describe what equipment used to heat PCB, and timings.

Our reply: The hot air gun model was added to the Materials section. The temperature profile which was used was added to the manuscript.

Reviewers's comment: 2.9 State which programmer used to programme microcontroller

Our reply: Microchip PICkit 3 – it was added to the Material and equipment list.

Reviewers's comment: 2.10 What material is used for the antenna?

Our reply: SWG38 copper wire with transparent enamel coating – the type was added to the Material and equipment list.

Reviewers's comment: 2.14 Make clear why a 24h delay is introduced. Can initial testing of the completed device be performed before encapsulation to check function?

Our reply: The 24-hour delay is introduced for the epoxy to thoroughly cure (which takes at least several hours according to the epoxy datasheet) and for comfortable reproduction of the procedure. In our case, the lab for manufacturing the electronics is located far from the experimental endoscopic lab (around 180 km). Thus, the delay is needed for transportation of the finished pH sensors from one site to the other. Usually, the sensors were manufactured a day before the experimental implantation and one of the team members traveled with them in the evening before implantation. The delay can be altered in the source code very easily by modifying the delay. A short paragraph discussing this was added to the Discussion.

Thank you for recommending providing a procedure to check the sensor before encapsulation. We created another firmware without the 24-hour delay and altered the manuscript accordingly.

Reviewers's comment: 2.16 How is the epoxy applied?

Our reply: The same way as the pH sensor – syringe with a needle – the protocol was altered to provide this information to the reader.

Reviewers's comment: 2.18 Reference Fig 11

Our reply: Thanks for spotting the mistake – added to the manuscript.

Reviewers's comment: 3.2 Are both Fig 12 + 13 necessary? Would one be enough?

Our reply: The figures describe two different setups – one for matching and another for use as a receiver. Originally, we tried to make one picture (not multi paneled), however, there was too much text packed in a very small area which affected the conciseness of the figure. For this reason, we provided a multipanel figure (now Fig. 11) instead of two separate figures.

Reviewers's comment: 3.5 State which vector network analyser is used, and necessary specifications

Our reply: Added to the Materials and equipment list.

Reviewers's comment: 3.6 Explain how the values of the matching components can be calculated

Our reply: A freeware software from Iowa Hills software (http://www.iowahills.com/9SmithChartPage.html) was recommended in the protocol. In practice, the impedance matching components are rarely calculated by hand.

Reviewers's comment: 4.1 Change to Fig 16/17

Our reply: Fixed, thank you.

Reviewers's comment: 4.2 State the source of the pH buffers

Our reply: The buffers were prepared on-site according to standard laboratory procedures and subsequently verified with a laboratory pH meter. The type of the buffers was added to the step.

Reviewers's comment: 4.4 I can only see three pulses, not four? Amend Fig 16/17 screen capture to show more clearly which time is being measured.

Our reply: Four pulses were used in the previous version of the firmware, the text was corrected. As for the amendment of Fig 16/17, the measured time is shown by on-screen cursors (two orange vertical dashed lines). Is it necessary to provide another visual clue for the reader?

Reviewers's comment: 5. Provide more detail on the ex vivo animal model - source, animal size/age, how dissection was performed.

Our reply: No living animal model was used, a pig stomach with the esophagus (weight of animal of around 40-50 kg) was purchased from a local pig farm as it is a consistently offered item. The animal from which the stomach and esophagus were obtained was not dedicated for the experiment. The clarification was added to the Materials and equipment list.

Reviewers's comment: 5.2 Should be Figs 18-20

Our reply: Fixed, thank you.

Reviewers's comment: 5.5 How is the sensor attached to the esophageal wall?

Our reply: The description of the sensor implantation was clarified.

Reviewers's comment: 6.2 Describe in more detail how solutions were injected into the esophagus. What volume? How did you ensure the previous solution was removed?

Our reply: This was clarified in the manuscript. A wash with 100 ml of DI water was introduced between injections. The volume of each solution was also added.

Reviewers's comment: Line 309: between the end of the second pulse and what?

Our reply: Fixed, thank you – between the end of the second pulse and the beginning of the third pulse.

Reviewers's comment: Line 316: report the error mean ± SD, not just the SD of the errors

Our reply: ± SD as well as the error mean was added.

Reviewers's comment: Line 328: Table 4 is not needed, a text description of the effect is sufficient

Our reply: We agree, the table was removed.

Reviewers's comment: Discussion: Describe in more detail how other receivers could be used to measure the output from the implanted sensor.

Our reply: The possible use of a spectrum analyzer or even a common heterodyne receiver was described in more detail, including the pros and cons.

Reviewer #2:

Reviewer's comment: * Specify the advantage of this battery-powered pH sensor over wirelessly powered battery-less implantable pH sensor.

Our reply: This topic was addressed in the discussion section.

Reviewer's comment:* The authors have shown the change in output due to mobile interference in 15 cm proximity. I suggest the authors show the effect of interference in further close proximity.

Our reply: After submitting the paper for peer review, we spotted a fault in the used equipment – the antenna which is connected to the passive receiver was faulty (short-circuit at the connector). The output voltage of the passive receiver seemed quite low but we attributed this to the small size of the transmitting antenna and its proximity to metallic parts (mainly batteries). We replaced the antenna, did the measurements again and we got a much stronger response from the sensor. As a result, we revised this part of the manuscript. It was shown that the GSM signal injects strong peaks to the signal but due to chosen ASK modulation scheme of the sensor, these can be easily filtered out by rejecting the high-frequency component of the signal with a passive filter.

Reviewer's comment:* The authors have specified a limit of 10 cm proximity for the receiver from the sensor. I suggest the author show the variance in the output and the incurred error with the change in the proximity of the receiver from the implanted sensor.

Our reply: The measurement on now Fig. 13 was re-done at 20 cm which is well beyond the range between the transmitter and receiver when using it as intended (communication between the pH sensor and an implantable neurostimulator, both in the proximity of a lower esophageal sphincter). During the GSM interference test, the distance was 10 cm. Rather than incurring errors to the transmitted data, the signal will be indistinguishable from the noise and surrounding RF transmitters.

As the antennas are close to each other during all experiments, wireless communication happens in a near-field. Thus, equations and models which are usually used (i.e. free space path loss) do not work and even small variances in the angular position of the antennas and construction may lead to drastically different outcomes. Our opinion is that showing two distinct data points (10 cm and 20 cm) gives sufficient information about the expected performance.

Reviewer's comment:* The authors have used an AG1 battery. I suggest the authors to mention what kind of modifications are required to incorporate other types of batteries.

Our reply: This was added to the discussion. Generally, silver oxide/alkaline/carbon-zinc batteries provide better performance and simplify the circuit than using primary lithium

batteries or Li-Ion batteries. Small primary lithium batteries have high internal resistance which would cause significant voltage drops, potentially leading to the brown-out of the microcontroller and RF transmitter during normal operation. Lithium-ion batteries, on the other hand, are incompatible with 3.3 V microcontrollers (the operating voltage of Li-Ion batteries is around 3.0 V to 4.2 V), adding complexity to the circuitry (requirement of a regulator or DC/DC step-down converter). Two primary 1.5 V button cells are the best commonly found type of battery based on the availability, operating voltage, and internal resistance.

Reviewer's comment:* I request the authors to specify whether the used commercial ISFET pH sensor offers higher sensitivity compared to other sensors available.

Our reply: The sensitivity of ISFET pH sensors (and pH sensors in general) usually follows the Nernst equation if the circuit is designed properly. In this system, the sensor exhibited almost ideal Nernst response of around -51.7 mV/pH (Nernst equation gives an ideal slope of -58 mV/pH for 25 °C). Thus, its sensitivity is almost on par with traditional glass electrodes and better than reported values for antimony pH sensors (-45 mV/pH [1]).

[1] https://www.annalsthoracicsurgery.org/article/S0003-4975(10)60791-6/pdf

Reviewer's comment:* Demonstration of removal of faulty soldering and redoing the soldering would help the readers.

Our reply: The assembly of the printed circuit board with miniature 0402 components and a small-pitch QFN package requires a certain level of skill and prior experience. All components are temperature and ESD sensitive, so we advise that the procedure of manufacturing of the ISFET pH sensor assembly and the electronics is performed only by a person with prior experience in the assembly of printed circuit boards. While a guide for de-soldering of the components and SMT rework could be helpful for some readers, creating a comprehensive and useful guide with visual demonstrations would most likely disrupt the cohesion of the article/method. However, if the request to provide a de-soldering guide persists, we will be glad to provide it to the article.

Reviewer's comment:* In 1.3, mention the model number for the open-short circuit tester.

Our reply: It was added to the Material and equipment list.

Reviewer's comment:* In 1.4, mention whether the soldering needs to be dried before cleansing. If so, how much time for drying.

Our reply: Sonication or chemical treatment of bare dies is generally not recommended as it can disrupt the rather delicate internal structure which, in the case of a pH sensor, is fully exposed to the environment. Thus, it is advisable to skip the cleaning process for the ISFET sensor. We have added this information to the protocol.

Reviewer's comment:* In 1.5, mention the ratio of flux remover and water mixture used and the optimum range of the ultrasound power.

Our reply: The concentration of the flux remover solution and optimum range of ultrasound power was added to the manuscript.

Reviewer's comment:* In 1.11, specify the temperature is used by the authors in this method.

Our reply: This was added to the manuscript.

Reviewer's comment:* In 2.9, I recommend the authors to show the steps in programming the PCB using a microcontroller.

Our reply: Another article published in JoVE by us which describes this procedure in detail for the used PICkit 3 programmer was cited in the protocol. If a full explanation in the article is more suitable, we can add it based on the editor's opinion.

Reviewer's comment:* In 2.17, specify a list of materials that can be used in the place of titanium and mention why titanium is used.

Our reply: Step 2.17 was amended according to this comment.

Reviewer's comment:* In 4.1, I guess the output figure number is 16 and not 14.

Our reply: Yes, this is a mistake on our side, it was pointed out by another reviewer, too. It was fixed, thank you.

Reviewer's comment:* In 4.3, specify the pH meter used for calibration in the beaker test.

Our reply: The pH meter model was listed in the Material and equipment list.

Reviewer #3:

Reviewer's comment: 1. The authors used several abbreviated terms. For example, GERD and ASK modulated wireless output. Please introduce the full term prior to the abbreviation.

Our reply: Fixed, thank you for pointing this out.

Reviewer's comment: 2. The authors could consider giving a brief explanation in introduction section about the zero-bias Schottky diode-based receiver and ASK modulation. It will help readers from different fields to understand the principles and why specific wireless modulation module are chosen as a technical background.

Our reply: The introduction was altered according to this recommendation, thank you very much for it. The receiver only detects received power (often abbreviated as "RSS" – received signal strength). Thus, ASK modulation is the only viable modulation scheme. To detect other types of modulations (namely FSK and PSK), an active receiver is required.

Reviewer's comment: 3. In the protocol section 1, the authors could used epoxy to seal the electronics part. I wonder about the biocompatibility and the acid tolerance of the specific type of the epoxy

Our reply: For the initial experiments, we used automotive-grade epoxy because it was available at the time and was tested to successfully bond to FR-4 substrate in past. Depending on the nature of the experiment which will be done by the researcher, suitable epoxy (cost vs. performance) can be chosen. As we plan to proceed with experiments combining this sensor with a neurostimulator in a living animal, we conducted experiments with different epoxies during the peer-review of the article. Following this testing, we switched to Loctite Hysol EA M-31 CL medical-grade ISO 10993 compliant epoxy. The bond of the epoxy to FR-4 substrate was shown to be sufficient for encapsulation. The Materials and equipment list was altered accordingly as well as the discussion.

To enhance the biocompatibility for long-term implantation, further coating with a biocompatible material (i.e. parylene or Teflon) can be done. A short mention of this was also included in the manuscript. However, we think that a detailed description of the design of a long-term implantable device from the point of biocompatibility is outside of the scope of the article, and present literature from the material science field documents this topic well.