**To: Phillip Steindel ,Ph.D**

**Science Editor JoVE**

**MS#: ADr. Williamson-932**

**Authors: Slézia et al.**

**Dear Dr. Phillip Steindel 10. December 2018**

**Enclosed is the revised manuscript "Electrophoretic delivery of GABA into epileptic focus prevents epileptic seizures in mice" which we are submitting for consideration to JoVE as an invited manuscript.**

**Please find our responses to the editorial comments and reviewer remarks and questions below.**

**Sincerely yours,**

**Adam Williamson PhD, Aix Marseille Université , INS, UMR\_S 1106, Marseille, France**

**and**

**George G. Malliaras, PhD, University of Cambridge, Cambridge CB3 0FA , Cambridge, UK**

**Editorial comments:**

The authors thank for all the observations, corrections and suggestions of the editors.

*Find our responses to the editor and reviewer comments in italic*.

Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*We carefully checked the manuscript to minimize the number of spelling and grammar issues.*

2. Please print and sign the attached Author License Agreement - UK. Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account. As some authors are affiliated with UK institutions, can you please check whether open access is required by your funding agencies?

*We attached the signed Author License Agreement – UK.*

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

*We asked explicit copyright permission for the reused materials and are waiting for the response. We linked the editorial policy and appropriately cited figures in the text.*

4. Please revise lines 54-58 and 60-61 to avoid previously published text.

*We revised lines 54-58 and 60-61 and changed the text:*

*“Epileptic seizures occur when there is an imbalance within excitatory and inhibitory circuits either throughout the brain - generalized epilepsy - or in a localized part of the brain - focal epilepsy - , such that neurons discharge in an abnormal fashion 10-12. Antiepileptic drugs can act in two different ways in seizure-prevention: either decrease excitation or enhance inhibition. Specifically, they can either modify electrical activity of neuronal cells by affecting ion channels in the cell membrane 14 or act on chemical transmission between neurons by affecting either the inhibitory neurotransmitter GABA or the excitatory glutamate in the synapses 15,16”*

5. Figure 6: Please describe the different panels of the figure in the figure legend.

*We described the different panels of Figure 6.*

6. Please provide an email address for each author.

*We provided email addresses for each author.*

7. Please define all abbreviations before use.

*We defined all abbreviations we used.*

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), 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 and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Vaseline, Hamilton, SuperFrost, VECTASHIELD, etc.

*We used generic terms instead of commercial language in all cases and added (see table of materials) into the text.*

9. Please add more details 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. 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. See examples below.

*We added more details in protocol steps. In some cases, we added references which specify how to perform the action.*

10. 1.2: Please mention how proper anesthetization is confirmed.

*We added details to confirm that:*

*“Check the level of anesthesia by observing the respiratory rate, whisking and by checking response to pain. Note: When the breathing becomes regular, no whisking can be observed and the animal does not react to tail pinch, the anesthesia is deep enough to continue.”*

11. 2.6, 2.8: What is the diameter of the hole?

*We have added this information to the text:*

*“Drill an approximately 500 micrometer(µm) diameter hole on the skull for craniotomy, then remove gently the dura mater for duratomy with a fine forceps.”*

12. 5.1: Please provide advice on the exact volume of 4AP to draw.

*We added the recommended volume of 4AP in the text:*

*“Redraw 500 nl to 1 µl of 50 mM 4AP with the help of an automated injection pump”*

13. 7.1: At what temperature is the perfusion done?

*We suggested the temperature for the perfusion:*

*“Perfuse the mouse transcardially first with saline, then with 150 ml of ice-cold fixative solution containing 4% PFA in 0.1 M phosphate buffer (PB).”*

14. 7.2: Please specify all surgical equipment used. Please specify the region of interest where a tissue block is cut and describe how.

*We added the names of surgical equipment and specified the region of interest:*

*“Remove the brain, then cut a tissue block with the help of a brain matrix (see list of materials) from the region of interest (from the Bregma point AP: -1 mm till AP: -3 mm).”*

15. 7.5: Please describe the histological protocol or provide a relevant reference.

*We provided a relevant reference for the histological protocol used.*

16. 9.2: Please specify the maximal and minimal excitation and detection parameters used.

*We specified the maximal and the minimal excitation and detection parameters in the text:*

*“Choose the optimal excitation and emission (exc/ems, nm) filter sets for the dyes as follows: DAPI 358/461, DiI 551/569, fluorescein (see list of materials) 488 490/525. Since staining varies section by section, for each section a proper range for minimal and maximal excitation and detection needs to be determined, where the least dense and most dense regions both show emission.”*

*“Choose the least dense region and set laser intensity and detection level values to high levels, then verify at most dense regions whether these values cause oversaturation of detected emission. If yes, lower values and re-check with least dense region. Iterate these steps until arriving to highest possible detection at low staining levels and proper, non-oversaturated levels at highly stained areas. Repeat the process for all dyes. “*

17. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

*We have combined the steps where it was possible*.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.

*We set single-line spaces in the required parts of the text.*

19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

*We highlighted the required amount of pages in the Protocol.*

20. 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 imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

*We highlighted whole sentences. We did not highlighted steps describing anesthetization and euthanasia.*

21. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

*We included all relevant details that are required to perform the step in the highlighting.*

22. Please number the figures in the sequence in which you refer to them in the manuscript text. For figures showing the experimental set-up, please reference them in the Protocol.

*We numbered the figures in sequence in the referred text.*

23. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

*We added modifications, troubleshootings and limitation of technique sentences.*

24. References: Please do not abbreviate journal titles.

*We changed abbreviations to full titles.*

25. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

*We sorted materials in alphabetical order.*

**Reviewers' comments:**

*We would like to thank the reviewers for careful and thorough reading of this manuscript and for*

*the thoughtful comments and constructive suggestions, which help to improve the quality of this*

*manuscript. Our response follows the reviewer’s comments.*  
  
**Reviewer #1:**

*The authors thank for all the observations, corrections and suggestions of Reviewer #1.*

Manuscript Summary:  
Slezia, Proctor, and co-workers present a very useful protocol for implementing their electrophoretic substance delivery tool for epilepsy research. Such drug delivery technologies are often proposed for in vivo application and new therapies, but are not always shown to be actually effective. In this regard, this JoVE submission is a very useful contribution to the field. Furthermore, most demonstrations of such bioelectronic tools do not include the protocol details as presented here. This would like be a great resource for all people working in the field.  
  
I had difficulty in deciding between minor and major revision, but chose to go with major based on the points below.  
  
Major Concerns:  
First, the manuscript needs a significant review for the quality of the English and the flow of the writing. While the focus of the manuscript is "visual", the text should still be of the highest possible quality.

We carefully checked the manuscript regarding spelling and grammar issues and improved the quality of English language.  
  
Second, the µFIP and the electrophoretic delivery concept are not sufficiently introduced. For a reader who doesn't already understand the mechanism, it's not at all clear why applying a voltage would cause GABA delivery. Likewise, the mention of "source and target" in step 6.3 are unclear without more introduction. Also, there is little or no mention of the placement of the µFIP relative to the epileptic focus and how this placement could effect the results (similar to how larger-scale 4AP treatment made the µFIP unable to turn off SLEs). Is there passive delivery of GABA when the µFIP is "off"? Does this need to be controlled for? And why is the µFIP better than, for example, an intrathecal pump? How is the µFIP made (or purchased)? I suggest that the authors revise their introductory text to address the µFIP in more detail.

*We added a paraghraph into the introductory text regarding µFIP to explain all these points.*

*“**The recently invented µFIP probes work by using an applied electric field to push charged drugs stored in a microfluidic channel across an ion exchange membrane (IEM) and out to the surrounding tissue. The IEM selectively transports only one type of ion (cation or anion) and thus works to limit both passive diffusion in the “off” state and transport of oppositely charged species from the surrounding tissue into the device. The electric field is created on demand by applying a small voltage (< 1 V) between the source electrode which is internal to the* *microfluidic channel and a target electrode which is external to the device (in this case the head screw on the animal model). The rate of drug delivery is proportional to the applied voltage and the measured current between the source and target electrodes. The precise tunability of drug delivery is one of the primary advantages of the µFIP. Another critical advantage compared to fluidic or pressure-based drug delivery systems is that in the µFIP there is only negligible pressure increase at the drug delivery outlet as drugs are delivered across the IEM without their carrier solution.*

*“There is a small amount of passive delivery of GABA when the µFIP is "off", but this was not found to effect seizure like activity. The µFIP were custom made following conventional microfabrication methods that we reported previously.*

*We added these sentences regarding µFIP placement and epileptic focus:*

*“The area of influence of the μFIP probe was estimated with a radius of approximately 550 μm from the outlet. It was proven that SLEs could only be affected if the 4AP injection was localized to this area. If the seizure focus or foci were outside of this region, the blockade of SLEs was not possible.”*

Minor Concerns:  
Line 48: "it needs to be set". The "it" is not clear here.

*We changed the text: “drug treatment needs to be set”.*

Line 67: what do the authors mean, specifically, by "connections"?

*We changed “conncetions” to “ neuronal network connections”*

Line 122: this is the first mention of PEDOT:PSS (and GOPS). These crucial materials may need some introduction?

*We have added an introduction and the full name of the materials as well.*

*“PEDOT:PSS is a conjugated polymer with a volumetric capacitance that is known to be biocompatible.*

*GOPS is a cross linker that is mixed with PEDOT:PSS to increase stability in aqueous media.”*

Line 210 and following: the use of colons is really confusing here.

*We rewrote and restructured this section to avoid confusion.*

Line 225: "among". Do the authors mean "between"?

*We changed “among” to “between”.*

Figure 3: "Ctx" has a spell-check squiggle underneath it.

*We corrected it.*

Figure 6: the scale-bar color (dark purple) is very hard to make out, particularly in the microscope image.

*We changed the color of the scale bar in Figure 6.*  
  
  
**Reviewer #2:**

*The authors thank for all the observations, corrections and suggestions of Reviewer #2.*

In their manuscript, Slezia and colleagues are reporting on their protocol about the simultaneous microinjection, electrophoretic injection and extracellular electrical recording of neuronal activity in rodent brains. They demonstrate it by inducing and terminating seizure-like activity in the hippocampus of intact, anesthetized mice.  
  
Although automated drug-injection is a routine approach nowadays even in clinical settings, the precise, non-pressure mediated microvolume deposition of pharmaceuticals/chemicals to a given brain regions is not properly established. Microfluidic channels designed in microfabricated electrodes gives a good option to maintain an optimal size/function balance, and the iontophoretic control of substance release allows for the precise, millisecond timing of the intervention.  
  
Conclusively, the demonstration and a step-by-step hands-on tutorial on the use of such a methodology is very timely and will be beneficial for a very broad audience in the field of neuroscience.  
  
Although I like the presented work a lot, given that the target audience will be most likely those people working in neuroscience already, I think the protocol in its current form needs some shift in its focus. My opinion is that the community would profit from this description way much more if it would focus on the details of how to use such a uFIP electrode, as this is not an obvious or standard skill that every neuroscientist would gain during their training. So I suggest to fine-tune the protocol in a way, that in addition to (or instead of) detailing the specifics of the anesthesia, surgery and histology, the emphasis would be put on how to get/handle/fill/use such a uFIP electrode.  
I think that if the authors can comply with this request, the result is going to be an extremely useful video-manual on how to perform micro-iontophoresis in rodents.  
My specific comments in order of their appearance are:  
  
Long Abstract:  
-Please spell out 4-AP at its first appearance.

*We corrected this.*

-How does the GABA induced overinhibition comply with the possible seizure mechanism based on overinhibition induced rebound bursts?

*We added the sentence:*

*“GABA concentration was kept in physiological range by the precise control of GABA delivery to reach antiepileptic effect in seizure focus but not to cause overinhibition induced rebound bursts.”*

Introduction:  
-„only neurosurgery can attenuate the occurrence of seizures"  
This is only true in the case of seizures with a constant single seizure generator locus, which is at a resecable part of the brain.

*We modified the text:*

*“Worse yet, in 30 % of the cases patients are resistant to medication, and in case of constant single seizure generator locus, only neurosurgery can attenuate the occurrence of seizures.”*

-„For some drugs, the mode of action is unknown": Please give examples, references.

*We gave a reference.*

-Please mention in the Introduction that drugs have a continuous effect and cannot adapt to the prevalence dynamics of the seizures. An optimal treatment would not touch the brain interictally but would act immediately when a seizure starts developing.

*We added:*

*“.Thus, drug treatments have a continuous effect on the patients and cannot adapt to the prevalence dynamics of the seizures.”*

*“ An optimal treatment would not touch the brain interictally but would act immediately when a seizure starts developing.”*

-"…It is difficult to determine if these reorganizations are adaptive responses or whether they are causally related to epileptogenesis or seizure genesis and propagation": This is the exact problem with the primarily generalized seizures: there is no definite seizure onset (epileptogenic) zone. Please discuss that to what extent is the proposed method superior to e.g. DBS which is also only suitable for seizures with a single generator focus (not talking about e.g. thalamic stimulation)

*We added:*

*“Since GABA is an endogenous substrate, it leaves intrinsic neuronal properties unchanged in physiological concentrations. Local application of low levels of GABA will only affect cells naturally responsive to inhibition, and will only cause similar effects to physiological inhibition, contrary to DBS, which has unspecific actions by stimulating all cells of the neuronal network in its environment, causing a mixed response involving both excitation and inhibition. In* *conclusion, the proposed method provides a more specific approach to seizure control than deep brain stimulation (DBS). “*

-Please add a short introduction on the uFIP probes, by mentioning why are they a superior choice compared to standard pressurized drug injection system, and cite references on their manufacturing, handling, etc.

*We added a short introduction:*

*The recently invented µFIP probes work by using an applied electric field to push charged drugs stored in a microfluidic channel across an ion exchange membrane (IEM) and out to the surrounding tissue. The IEM selectively transports only one type of ion (cation or anion) and thus works to limit both passive diffusion in the “off” state and transport of oppositely charged species from the surrounding tissue into the device. The electric field is created on demand by applying a small voltage (< 1 V) between the source electrode which is internal to the microfluidic channel and a target electrode which is external to the device (in this case the head screw on the animal model). The rate of drug delivery is proportional to the applied voltage and the measured current between the source and target electrodes. The precise tunability of drug delivery is one of the primary advantages of the µFIP. Another critical advantage compared to fluidic or pressure-based drug delivery systems is that in the µFIP there is only negligible pressure increase at the drug delivery outlet as drugs are delivered across the IEM without their carrier solution.*

*“There is a small amount of passive delivery of GABA when the µFIP is "off", but this was not found to effect seizure like activity. The µFIP were custom made following conventional microfabrication methods that we reported previously.”*

Protocol:  
-1.2.1: "Place it gently into the rectum": How deep?

*We have added the depth as required: “(1-2 cm deep)”*

-1.2.2: What concentrations:

*The concentration is in the text:*

*“Inject intraperitoneally a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg body weight, respectively) to anesthetize the animal.”*

-2.6: Give stereotaxic coordinates

*The stereotaxic coordinates are given in the text:*

*“With the help of the stereotaxic frame, measure the stereotaxic coordinates for the desired brain region.; For example, our region of interest was the hippocampus, anteroposterior (AP) -1.8 mm and mediolateral (ML) 1.8 mm from the Bregma point on the basis of the brain atlas for mice. Note: These are coordinates for the right hemisphere.”*

-2.8.: Mention that the craniotomy must be filled with a droplet of saline solution to prevent drying. Please also state that the saline droplet must be regularly refilled.

*We added the sentence:*

*“Note: the craniotomy must be filled with a droplet of saline solution to prevent drying, and then regularly refilled during the experiment”.*

-3.3: State that the probe must be lowered with axial movements relative to the shank, otherwise it brakes.

*We added the sentence:*

*“Note: µFIP is very flexible, it may benefit from the support of a small and clean paint brush to keep it straight until it reaches the brain surfaces. After that step, µFIP can be lowered gently with axial movements.”*

-4 If these uFIPs are not commercially available refer to a protocol how to prepare them.

*The µFIP were custom made following conventional microfabrication methods that we reported previously. These points are discussed in more detail in the referenced work.*

-4.1. How to perform filling?

*The uFIP probes are filled with drug solution using standard fluidic tubing and connectors (seen in Fig 6b).*

-5. Might worth to introduce how to attach a glass micropipette to the Hamilton Syringe, as this is a delicate step which needs some good manuality and tweaking

*We added the sentences:*

*“Change the metal needle of the syringe (see materials). Remove the needle holding metal part, place and fix the micropipette, then replace the needle holding element.”*

-6. Better to use the word insertion instead of implantation.

*We changed “implantation” to “insertion”.*

-7. Add step before: "Remove the inserted probes, overanesthesize the animal…"

*We added the requested step*:

*“ Gently remove the inserted probes and the headscrew, remove the animal from the stereotaxic equipment. After overanesthesizing the animal with a lethal dose of anaesthetics, the mouse is ready for the perfusion.”*

-7.1. Cite some reference for the transcardial perfusion (Is there any JoVE video for this?), as this is not obvious either, and requires a good skillset.

*We cited a reference of a JoVE video about transcardial perfusion.*  
Representative results:  
-Should test the injection of vehicle as a control, too, to show that it's not the injection current or volume excess causing the seizure suppression.

*We prepared Figure 7 as a vehicle control.*  
  
Discussion:  
-"mostly these are the patients where conventional drug treatments are inadequate": This seems counterintuitive to me, and I cannot find this statement in the cited reference. Please give a reasoning and support it with solid evidence from robust studies. This gets back to the same problem I mention in my remark regarding the long abstract.

*We thank the comment of the Reviewer. We removed the sentence from the discussion.*  
  
**Reviewer #3:**

The authors thank for all the observations, corrections and suggestions of Reviewer #3.

Andrea Slezia and co-authors present an electrophoretic GABA delivery system into epileptic focus that can prevent epileptic seizures in mouse. In the presented protocol, which acutely induces seizure in a localized spot in the hippocampus, the neural activity was recorded with incorporated electrodes and multichannel silicon probe and 4AP-induced seizure was successfully controlled in the epileptic focus by the microfluidic ion pump (μFIP) filled with GABA solution. Additionally, the authors show histological data to evaluate the implantation placement of three different implants (silicon probe, μFIP, and micropipette). In my opinion, as its characterizations were well demonstrated, this manuscript is sufficient to be published in Journal of Visualized Experiments after minor revision.  
  
1. In the manuscript Page 6, line 165 (6.3), although the authors stated that the μFIP is filled with 0.05 M GABA solution, there is no mention about the dosage of GABA release (GABA volume) to prevent epileptic seizure in mouse.

*We added the dosage of GABA in the text.*

*As we previously reported, we delivered 1 nmol of GABA in the seizure prevention experiments.*

*We added the sentence:*

*“Note: The delivered GABA concentration is around 1 nmol.”*

And I wonder that if GABA more than necessary is released in the epileptic focus, is there any side effect to their body and brain?

*We added the sentence:*

*“GABA concentration was kept in physiological range by the precise control of GABA delivery to reach antiepileptic effect in seizure focus but not to cause overinhibition induced rebound bursts.”*

2. The authors mentioned that two devices (μFIP; mediolateral, dorsoventral coordinate: 1200 μm and micropipette with Hamilton syringe; lateromedial, dorsoventral coordinate: 1500 μm) were implanted into specific hippocampus region. Then, there is a difference of 300 μm at the dorsoventral coordinate. What's the general distance between μFIP outlet and pipette tip? In my opinion, the release of GABA closer to the pipette tip is likely to affect the epilepsy focus more directly.

*As we previously reported, we estimate the distance between the μFIP outlet and pipette tip within a few hundred microns (< 300 μm).*

*In our experiments we tried to put the three devices as close as possible, considering this 300 μm distance of outlet from the μFIP tip. On the other hand, it was necessary to avoid any mechanical issues among the devices and their connectors during insertion. In addition, mechanical issues on the neural tissue regarding brain movements in the consequence of breathing, blood pressure changes during maintenance of anesthesia, etc. were also necessary to avoid. We chose the closest placing possible of our devices for the closest GABA release site relative to epileptic focus.*

And I recommend that fully-implanted mouse photographs are attached on figure set to easily understand.

*Unfortunately, we don’t have any publish-quality photos but we can take these photos during the the filming and can add later to the manuscript if it is possible.*

3. Please add the full name. e.g. PEDOT: PSS, GOPS and ACSF.

*We added the full name of the materials.*

4. Please check if it's a typing error;  
Page 4, line 113, '2.1' instead of '2.2'

*We corrected numbering.*

Page 7, line 213 (Fig. 1) and 219 (Fig. 2), 'reference # 31' instead of '#32'

*We changed references and in Fig1 and Fig2.*