**Editorial comments:**  
  
1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor removed the trademark symbols and pictures from the Table of Materials and Equipment. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions.  
  
2) Prior to peer review, the protocol length is exactly at our 3 page limit. If, in response to peer review, additional details are added to the protocol, please use yellow highlighting to identify a total of 2.75 pages of protocol text (which includes headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification and remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.  
  
3) Prior to peer review, the length of the Short Abstract is exactly at our 50 word limit. If, in response to peer review comments, changes are made to the Short Abstract, please ensure that the final length does not exceed 50 words.  
  
4) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.  
  
5) Please disregard the comment below if all of your figures are original.  
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."   
  
**Reviewers' comments:**  
  
**Reviewer #1:**   
*Manuscript Summary:*   
Here Mingming et al. have described a novel unfolded hippocampus preparation that can be advantageous in studying transverse and longitudinal circuit's in-vitro. Here the authors have utilized microarray technique to describe the effectiveness of this novel hippocampal unfolding technique and have suggested its advantage over the traditional in-vitro hippocampal slice preparation.  
The technique of unfolded hippocampal preparation is physiologically important and makes the study of hippocampal neural circuit much easier. The only drawback as mentioned by the authors in discussion is the loss of the perforant pathway. I believe the preparation would be well perceived. By having a visual protocol for the method would certainly help researchers utilize it for their studies.  
In general the paper is well summarized. The representative data presented are clear and straightforward. However there are few points that I would like the authors to address to improve the manuscript -   
  
*Major Concerns:*  
1. The method mentioned is very valuable for studies in the hippocampus of animals of age 3 weak or below. Several researchers would experiment and utilize the unfolding protocol in older animals. What might be the changes one may require to use the protocol for older animals? Have the authors tried the unfolding protocol in adult/older animals? Mention your thoughts in discussion.

Answer to this question:

Thank you for pointing this issue out and the suggestion is very valuable. We have carried out the same surgery process to unfold adult mice hippocampus (more than one month old), and there is nothing different in the protocol for adults rat brains. We choose to use younger animal because the hippocampal neurons have a higher chance to survive in the *in-vitro* environment. We have included related statements in the Discussion.

2. It would be a good to mention in short (few lines) the fixative and section details used to obtain the crystal violet staining (Fig 2). Would hear perfusion of animal with tissue fixative pose difficulty in hippocampal unfolding?

Answer to this question:

We only processed the tissue after the dissection was performed. The hippocampus was first dissected and then underwent the post histology preparation procedure. The brief description in a few sentences has been updated in the caption for Figure 2.

*Minor Concerns:*

1. Having an array of microelectrodes is extremely valuable for network studies. Do the authors believe that the preparation can be utilized extensively for single or double patch clamp studies in younger and older animals?

Answer to this question:

We have not tried yet to carry out patch clamp in this preparation. It would be somewhat more difficult since the cell bodies are deeper and would require blind patch.

2. Expand as artificial cerebrospinal fluid (aCSF) in Protocol 1.1

Answer to this question: text has been changed in the manuscript.

3. Change recovering to recovery in Protocol 1.1

Answer to this question: text has been changed in the manuscript.

4. Protocol 2.4 - I think pre-prepared was meant to be written as "prepared".

Answer to this question: text has been changed in the manuscript.

5. Protocol 2.6 - Correct the sentence "Once the hippocampus is exposed from inside of the cortex, two or three drops of ice-cold sucrose aCSF is placed on the tissue and remove extra solution around it"

Answer to this question: text has been changed in the manuscript.

6. Protocol 2.9 - Correct aSCF as aCSF

Answer to this question: text has been changed in the manuscript.

7. 3.1 - Write "PGA" as pin grid array, also correct Figure 3B caption as PGA package.

Answer to this question: text has been changed in the manuscript.

3. For the representative results - "Since the electrodes have a height of 200 μm and the electrode tips are located just below the cell layer (Figure 2, 3C)". Rephrase the sentence or beforehand explain figure 2, followed by the description of 3C giving special importance to the white dots.

Answer to this question: text has been changed in the manuscript.

4. Figure caption 4B - correct white arrows as blue arrows or simple arrows.

Answer to this question: text has been changed in the manuscript.

*Additional Comments to Authors:*

In general the method is well summarized and the data presented are clear and straightforward. The mentioned technique would prove helpful for several labs.

**Reviewer #2:**

*Manuscript Summary:*

In their article, "Neural activity propagation in an unfolded hippocampal preparation with a penetrating micro-electrode array", Zhang et al describe using a micro-electrode array with high signal-to-noise ratio and penetration to record from CA1-CA3 regions of the hippocampus. They describe this technique on an unfolded intact hippocampus, a preparation that gives access to both longitudinal and transverse connections. The array, made of 64 spikes 200 μm tall, was able to successfully record from deep tissue.   
  
Overall the method appears to be an effective, elegant method of recording from deep hippocampus with a high signal to noise ratio. The experimental procedure as well as the rational and background of the technique is adequately explained. Seeing this technique could be very useful to other scientists in the field.   
  
*Major Concerns:*

None  
  
*Minor Concerns:*

Minor points –

1. The long introduction should clearly indicate what the reader should expect to see in this paper.

Answer to this question: text has been changed in the manuscript.

2. This technique is specific to CA1-CA3 region of the hippocampus. It will be helpful to know whether and how this technique can be applied for other preparations.

Answer to this question:

The unfolding technique is specifically designed for the hippocampus with a curved structure. However, the penetrating microelectrode array can be used for any flat tissue preparation *in-vitro*,such as cortical slices, hippocampus transverse slices and etc. The text has been updated in the discussion part.

3. The authors should provide references for alternate recording methods and describe how this method is superior.

Answer to this question:

We have modified the text and revised in the Discussion section.

This method with PMEA is obviously superior over signal channel recording before the array could monitor neural activity in a 2-D plane. In this manuscript, we have also provided the references for the comparison of PMEA recording to other recording methods. In the 2nd and 3rd paragraph of the Discussion section, we have compared the PMEA and regular MEA to show the improved signal noise ratio in the recording, especially for the recording in the unfolded hippocampus. The PMEA also have advantage over voltage sensitive dye (VSD) recording since it is free of toxicity and photo bleaching. The text has been rephrased in the Discussion part.

4. The authors point out that cleaning of electrodes is an issue since tissue stuck to them can cause erroneous recordings. They should suggest some ways of cleaning these electrodes.

Answer to this question: we have added the text in the Discussion part.

**Reviewer #3:**

*Manuscript Summary:*

This article describes how to dissect, prepare, and perform electrophysiological recordings from the unfolded hippocampus. The authors use a custom made penetrating micro-electrode array (PMEA) that provides a better signal-to-noise ratio than more widely used flat electrode arrays. Using this PMEA, they are able to show that electrical activity propagates from hippocampal area CA3 to CA1 as a diagonal traveling wave.  
  
*Major Concerns:*

I would suggest revising the title so as to not highlight "neural activity propagation" as that is not the main point of this article. For example, "Recording neural activity in the unfolded hippocampus with a penetrating micro-electrode array" would be a more appropriate title.

Answer to this question:

In principle the reviewer is correct. We would prefer to keep the current title because this preparation was designed to detect propagation on two direction in a flat array of cells.

It is not clear why the DG and subiculum are cut from the unfolded preparation? It does not appear that this was the case in the authors' previous Kibler et al 2012 J Neurosci Methods manuscript. If the criticism of other hippocampal preparations is that they have tissue missing, then why cut off two of the major structures of the hippocampus? The DG is a major input and the subiculum is a major output pathway. I imagine that there is a rationale, but it is not stated.

Answer to this question:

The reviewer is correct and the DG does not have to be removed in principle. In practice it is easier to remove it because it facilitates the unfolding of the tissue. We have modified the text.

While considering that this is a Methods article and the results aren't necessarily primary to the manuscript, the "finding" that neural activity moves from one area of the hippocampus to another is not exactly illuminating. It would be useful if the authors cited or discussed some of the greater utility of these techniques. For example, has the unfolded hippocampus preparation revealed anything unexpected or non-obvious about hippocampal circuitry?

Answer to this question:

We disagree with the reviewer on that point as it is the first time that propagation in both the longitudinal and transverse direction could be obtained simultaneously. This was clearly shown in a previous publication (Zhang, M. et al. J Neurosci, 2014) where propagation was shown to propagate diagonally across CA1 and CA3, could cross both layer as similar speed and suggested a new mechanism for propagation.

[**Editorial comment:** While addressing the above comment, please note that while we do not require in depth results for publication in JoVE, the results must accurately demonstrate the efficacy of the proposed method. Additionally, the results must substantiate all claims presented within the manuscript.]

Increased SNR is useful, but that on its own is may not yield anything beyond what is available with more widely used recording techniques and tissue preparations

Answer to this question:

Without unfolding it is not possible to image the propagation of the activity across the CA3-CA1 layers on a flat array.

*Minor Concerns:*

Page 2, line 64: I would not use the word "spikes" in your description of the electrodes due to it being synonymous for action potentials amongst electrophysiologists.

Answer to this question: we have changed the text in the Discussion part.

Page 2, line 76: The hippocampus is not generally considered a brain area for emotion as you state. Also, the references 1-3 are unusual choices to back up this statement about the hippocampus.

Answer to this question: we have changed the text and fixed the citations.

Page 3, line 90: "difficulty" should be "difficult"

Answer to this question: we have changed the text in the Discussion part.

Page 5, line 12: Please define "PGA package". Also, this is referred to as a "PKA package" in your Figure Legends (line 277). Which one is correct?

Answer to this question: we have changed the text and fixed the typo.

Page 5, line 196: Do the authors mean a glass vacuum tube or flask, rather than a "dust container" to collect solutions?

Answer to this question: we have changed the text and it is a vacuum tube connected flask.

Page 7, line 259: Figure Legend refers to "sulcus" but the figure says "sulculus" (small sulcus). Sulcus seems to be the more appropriate term, but either way, just be consistent within the manuscript.

Answer to this question: we have changed the text in the figure and it should be sulcus.

Page 7, line 275: "insertion" should be changed to "insert"

Answer to this question: we have changed the text.

Page 7, line 280: Please define "OCT".

Answer to this question: it is Optical Coherence Tomography, it is already added in the text.

Table of Materials:

Numbers 1, 3: "Custome" should be replaced by "custom"

Answer to this question: already changed.

Number 3: should read "recovery" chamber

Answer to this question: already changed.

Number 17: "completely" should be "complete"

 Answer to this question: already changed.