Editorial comments:  
• Your manuscript has been modified by your editor, please maintain the current formatting throughout the manuscript. **Please use the updated manuscript located in your Editorial Manager account (under “File Inventory”) for all subsequent revisions**. The updated manuscript is also attached.  
  
• 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. done  
  
• JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information. We applied the “JOVE” citation style using Mendeley. This was intended to have been applied on the original submission, and should fix the citation issues.   
  
• Please ensure that references are numbered in the manuscript in chronological order, that is, the first reference that appears in the manuscript should be numbered 1. done  
  
• Please provide the reference of Elias and Kriegstein, 2007, in the reference section. done  
  
•Scattered grammar/spelling issues should be corrected: all done  
-Step 1.1: "Remove the agar from [the] autoclave immediately..."  
-Step 4.1: This section is about slicing the brain, but the first step begins with "place a brain slice on top of the overturned dish..." We assume this is a typo since the brain has not yet been sliced.  
-Step 4.2: "Obtain sections from different planes from different orientation."  
-Step 4.8: Should be "vibratome" rather than "vibratone."  
-Step 7.4: Some grammatical articles (a, the) are missing in this step.  
  
•The abstracts indicate that this protocol is intended to demonstrate both slicing and calcium imaging, but the highlighted material covers only the slicing and mounting of slices. The highlighted material is not yet at the length limit, so the authors could include at least several steps on calcium imaging.  
More material was intended to be highlighted, and should now be highlighted.   
  
**Reviewers' comments:**  
**Reviewer #1:**  
*Manuscript Summary:*  
Neuzil and colleagues present a comprehensive manual on conducting calcium imaging in slice cultures, combined with the optical identification of neuronal subpopulations. Clearly, slices cultures represent a vital bridge between classical acute slice preparations and in vivo approaches. As pointed out by the authors, acute slice preparations require the addition potassium in the solution which might locally alter excitability, which is not needed in cultured slice. For optical recording of neuronal activity, here in the context of propagating waves, the authors introduce calcium imaging methods. The manuscript is overall well-written; the figure nicely illustrates the procedures. I would strongly support publication/production of their manuscript, at it would represent an excellent contribution to JoVE. However, there a quite a few key aspect that will need to be addressed by the authors, as otherwise the manuscript might mislead the readers concerning the concepts of calcium imaging  
1. Title: "Measuring calcium activity.." The term "calcium activity" makes no sense. Either a neuron can be active (neuronal activity), or the calcium levels can be monitored as a surrogate for neuronal activity, so this term needs to be replaced by either "calcium imaging" or "measure calcium dynamics", a term used by the authors (line 254)

The title has been adjusted to use the term “calcium dynamics”

2. Abstract: Following this logic, the authors need to be much more precise in explaining the origin of the signal which they are recording, this is vital; otherwise this will mislead the reader. Line 39 "…propagating waves of electric activity that induce calcium transients…" That is wrong. Not the propagating wave is "inducing" a calcium transient, but individual action potentials lead to an opening of voltage-gated calcium channels, thereby increasing intracellular calcium levels. So this needs to be rephrased, e.g. by stating "propagating waves of electric activity are mirrored by calcium transients…"

The introduction has been rewritten to clarify the purpose of calcium imaging and the relationship between calcium signals and electrical signaling. We continue to use the word “induce” in the abstract as we find it appropriate and efficient to state the relationship this way, though the sentence in question has been reworded for clarity. A large source of calcium released during wave activity is from internal stores in response to initial calcium entry through ligand and voltage gated ion channels that occurs with action potential firing.

3. Introduction, line 61, as stated above, as calcium imaging represents a key technique described here, the authors need to explain how intracellular calcium is mirroring action potential firing. The sentence "These waves induce rises in cellular calcium.." is not precise enough.

This has been clarified

4. Line 190 "Touch the curve to the membrane.." I am not sure I do understand this sentence, please re-phrase

“The curve” has been replaced by “meniscus”

5. 8.4 Measure calcium dynamics: The authors need to better explain the requirements of temporal resolution. They need to provide an estimate on acceptable sampling frequencies for the underlying activity patterns, e.g. "for resolving a calcium wave with the speed of ?? a minimum sampling frequency of ? is advisable"

These details were added

6. Representative Results: What do the authors mean by "calcium record"? The temporal profile of the calcium trace? This is a highly unusual nomenclature.

“record” refers to a record of the calcium indicator fluorescence over time

7. 296 "Calcium events" again, the authors need to be precise. How is a calcium event defined? I guess they refer to a transient exceeding a threshold, or significant events in the calcium recordings.

This is indeed what we are referring to. A calcium event is defined in the “representative results” section.

8. Line 354 "…brain slices that usually reflect activity patterns that are present in-vivo" I would strongly argue against this statement. There might be processes that can be recapitulated or modelled in slices, but where is the evidence that slices "usually" reflect the in vivo situation?

This statement has been removed.

9. Line 361,362, maybe this is a typo, but this sentence makes no sense:"..bath application of calcium indicators is less effective in adult brain slices, and thus GECIS are not preferable.." Exactly the opposite is true; GECIS ARE preferably due to the rightfully mentioned shortcomings of the bath-applied acute indicators.

This was indeed a typo. Thank you for catching this

10. Figure 1 The authors did not normalize the calcium traces, just stating A.U. This is not state of the art; usually normalization is conducted, leading to df/f measures. The authors should at least describe these standard procedures of normalization.

We find that normalization procedures in the field are highly variable, and different experiments call for different normalization techniques. The important idea we want to illustrate is that amplitude is not usually a meaningful measurement in our data. In the representative results section we mention several aspects of data analysis to take into consideration. The figure legend here has been modified to clarify that amplitude measurements should not be compared between cells.   
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
N/A  
  
*Additional Comments to Authors:*  
N/A  
  
  
**Reviewer #2:**  
*Manuscript Summary:*  
The manuscript by Neuzil et al describes a method for brain slice preparation that allows measurement of calcium activity. They are utilizing organotypic slice cultures to increase cell health. The authors provide details of the experimental setup. Many of these details have been published previously as stated in the introduction.  
  
*Major Concerns:*  
-I believe that there's no reason to have any particular concern about the procedures described in this manuscript. However, the goal of this paper and the technique they want to emphasize should be more focused. From one end the title state that the purpose is to measure calcium activity from interneurons, which are genetically labelled, but when looking on the protocol, they only mention the GCamp6 mouse, who is used to detect calcium signals.

The title has been modified for clarity. Section 9, “Identify Cell Types”, depicts how to identify genetically labeled cell types. We do not use GCamp animals for these experiments, and have removed the small reference to GCamp. The basis of our system is widefield epifluorescence imaging of calcium dynamics paired with spinning disk confocal imaging of RFP expression in genetically targeted cell types. We have clarified the title and short abstract to hopefully better represent this idea.

-The authors mention that slices have advantage over cultures, as they preserve the network settings. Although true, they also should mention that the network settings and physiological properties on neurons are altered following the addition of culture media that contain growth factors and penicillin as per the described method (step 1.5).

The use of culture media has now been addressed in the introduction. We do not use growth factors  
  
*Minor Concerns:*  
-A big part of the introduction and method sections is devoted for the slicing method and the advantages of slices over cultures, however there is no adequate reference to the disadvantages of using culture media (especially antibiotics) on physiological properties of cells. Furthermore, recent advances in slice preparations that extend slices viability significantly should be discussed (Buskila et al., 2014).

We now cite previous studies where we have shown that ion channel properties and morphological features of neurons develop normally in culture.

-In the short abstract - does the goal is to measure individual neurons? Or calcium activity in individual neurons?

We reworded the short abstract as we agree that the initial wording was unclear.

-There is no mention of labelling interneurons, or using mice with labelled interneurons in the protocol. I guess step 9.1.1 is a general step to identify neurons, but should be amended to include interneurons.

We have reworded the entire article to be less centered on interneurons, although our example figure shows interneurons labeled. These techniques could be applied to a wide variety of neuronal subtypes. We have labeled interneurons by utilizing a Cre-dependent, RFP-expressing, transgenic mouse paired with a Cre-expressing transgenic mouse where Cre is under control of an interneuron-specific promoter region. The use of a different promoter to drive Cre could label a different subpopulation of neurons.

-The authors should mention the software used for calcium detection in the protocol.

This has been added to the materials section of the submission

-The paper would benefit from additional proof reading. For example-  
Line 362 in the discussion: " genetically encoded calcium indicator dyes" should be "genetically encoded calcium indicators" and the conclusion from this sentence should be that genetically encoded calcium indicators are more preferable in adult slices rather than not preferable.

done  
  
*Additional Comments to Authors:*  
N/A