**Response to review**

**Editorial comments:**  
\*Protocol text:

1) The protocol has about 3.25 pages highlighted, and much of the highlighting is not in complete sentences (for instance, 3.4.4 has only "Bouin's solution fixative" highlighted). Please review the highlighted sections of your protocol and ensure complete action statements are highlighted.

\*\*OK these sections have been corrected. I thought we just needed headings for the sections.

For instance, please review section 3.4.2 of your protocol and clarify if all steps will be videotaped. The number of steps that are most important to film should be less than 3 pages of text (~2.75 pages); i.e. which steps should be visualized to best supplement the written section of the protocol. The shorter length requirement is to allow our scriptwriter 0.25 pages to make transitions and references to the omitted text. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

\*\*OK we have limited the highlighted portion of the protocol to 2.75 pages.

2) As mentioned previously, please remove embedded figures and upload each figure file separately. To denote figure placement throughout the manuscript use brackets [Place figure 1 here].

\*\*OK

3) The manuscript will benefit from copyediting, as there are a number of errors throughout. Please thoroughly review the manuscript and edit any errors that you may find.

\*\*OK . The abbreviations of PD and others will be corrected. The fragmented sentences were due to our misunderstanding of listing topics/sections and not statements for the step wise fashion of the methodology. These steps were also made by the JoVE editor in breaking the parts to steps. We will double check for typos etc... Sorry about the oversight.

**Reviewers' comments:**

**Reviewer #1:**   
Concerns:  
1. Introduction: The significance of proprioception and the type of information processed by these neurons should be addressed in more depth rather than simply saying that an animal needs to know where its limbs are and that monitoring force is important. The relationship between chordotonal organs and their individual sensory neurons could also be addressed in more detail (roughly how many, why the need for multiple, etc.).

Response: We agree with the reviewer. We just tried keeping the text as short as possible. The following text has been added to address this point:

“…these receptors convey information about joint position, direction, speed, muscle tension and muscle-length. The crab leg has six joints and each joint has one or two proprioceptive chordotonal organs (Alexandrowicz, 1972) . Typically a chordotonal organ has 60-100 or more sensory neurons embedded within an elastic strand to monitor movement or changes in the strand. This information allows the animal to receive a fine degree of sensory information related to each joint. In addition, the tension receptors provide information on the force being generated to hold various joint positions or to move a joint. The various threshold of activation for the sensory receptors provide a range fractionation of movements. The input is then centrally processed for all the joints for the animal.”

2. Results: How will the data be analyzed and quantified? For example, the data shown in Fig. 10 - spike counting could determine (significant) differences between movement and static. In Fig. 12 - spike sorting would allow to identify individual neurons, etc.   
Why is this not part of the experiment/protocol?

Response: Good points. We do have students do just this type of analysis. For the JoVE report we just referenced to other studies that have conducted such analysis.

4.2) Further analysis of the spikes can be readily approached by sorting the relative amplitudes. This is an approach to demonstrate different populations of sensory neurons being recruited for positions or types of movements (Cooper, 2008). In sorting of amplitudes a frequency of occurrences in the various size ranges can also be graphically represented for analysis.

3. Discussion: Reads like an introduction. Maybe adding a sentence or two how these measurements provide insights that generalize beyond the arthropods? Proprioceptors play a major role in all organisms (as briefly mentioned in the introduction); basic principles are common among most organisms and results obtained from crabs will help understand these basic mechanisms. Elaborate on this.

Response: Yes we agree. The reviewer might not be aware of the space limitations we were restricted to with this large multi preparation and experimental design protocol. We are delighted to expand on these points. The following text has been added to the discussion:

“Proprioception and tension reception of skeletal muscles are sensory modalities that enable coordinated behaviors and responses to external and internal environment for all animals, not just crustaceans. The capacity to record from single neurons or scale up to entire sensory nerve bundles provides a wide range of physiological inquiry to convey the basic concepts of sensory reception. These relatively simple crustacean preparations allow one to address many fundamental aspects of proprioception and tension monitoring and to potentially address central integration of inputs that are relatively inaccessible in mammalian preparations.

Procedure (general suggestions):

4. Individual sections would probably benefit from a brief summary of the general objective of each part appearing beforehand.  
Response: I am not sure this would fit the JoVE format. This format is a bit different with 1.2.3 procedures. I am going to refer to the JoVE Science Editor here as I am not sure of the format for including this approach.

5. Methodological considerations relevant to both procedures are sometimes omitted from one of the procedures. They should either appear in both or be discussed beforehand.  
 Response: I am not sure exactly all the considerations in the procedures the reviewer is addressing. Now I think the major points are addressed for the joint receptor part and the tension receptor parts.

6. A brief discussion of mechanisms, such as how the various stains work, or the logic or importance of certain steps, would be useful.

Response: The general uses of the stains are now described.

“Methylene blue provides some contrast of tissues (muscles and nerves) and can be visualized with visible light. More selective neuronal staining with a brief exposure to 4-di-2-ASP provides a definitive outline of the neurons but requires fluorescence imaging. Filling of the neurons for CoCl2 does not require fluorescence imaging and the targeted neurons will remain stained after fixation for long periods of time. However this staining process is more labor and time intensive, limitations that make it difficult to complete this procedure in a single instructional laboratory setting.”

7. Are there any health risks or hazards involved in the preparation or stains? If yes, they should be mentioned.

Response: I am going to refer to the JoVE Science Editor here for how much content needs to be listed here. All the chemicals are well labeled on the bottles for their potential hazards such as the CoCl2.  
Spelling:

8. There are typos in the manuscript.

Response: Very sorry for the typo oversights. The manuscript was carefully examined in the revised version.

a) "Detailed electrophysiological studies began in the 1930's and still being carried out today" - are still being carried out today

Response: corrected  
b) "Very little is known in the segmental connections of proprioceptors" - about the segmental connections

Response: corrected  
c) "around the Southern and Southeastern boarder of the United States" - border not boarder  
Response: corrected  
Specific comments:  
  
9. Starting with the Introduction, "PD" is never defined for the PD chordotonal organ (I am assuming it means: Propodite-Dactylopodite).  
Response: corrected

10. 1.1.) Was saline added to the first preparation? When?  
Response: corrected. Yes. See step 1.1.4

11. 1.1.1.) It appears that both C. sapidus and C. magister were used. It's not obvious from the text which animal was used for which protocol.

Response: Either crab species can be used. We state at the end of the Introduction

“Use of the blue crab (C. sapidus) in this procedure is for the reasons that they are readily accessible all around the Southern and Southeastern border of the United States. In addition this species likely serves as a representative of the chordotonal and tension nerve arrangements found in most crabs.”However we show some stained preparations of *C. magister* for comparative purposes.

The text states:

“4.5) 4-Di-2-ASP staining of a PD preparation from a different crab (*Cancer magister*) (**Figure 16**). If one has a high enough magnification the sensory endings can be seen inside of the supportive scolopales 12,13,19 (**Figures 17**)*.”*

12. 1.1.3.2) "condyle" should be explained.  
Response: corrected

13. Figure 3: An arrow pointing to the nerve would be helpful.  
Response: corrected

14. 2.1.2) I found this part confusing. Maybe rephrase.

Response: corrected

This section now reads,

“2.1.2) In order to stimulate the opener muscle the stretcher motor nerve in the carpopodite region is isolated and stimulated with a suction electrode. The proximal part of the leg is removed by transecting the meropodite with scissors.

15. 1.2.6) How are suction electrodes made?

Response: corrected

This section now reads,

“1.2.6) Position the micromanipulator so the attached suction electrode assembly will have easy access to the saline bath and preparation. The suction electrode is constructed as shown in an on line video (Baierlein et al. 2011).”

16. Figure 5: Maybe consider using a diagram here rather than pictures. If pictures are kept, an arrow pointing to the nerve (branching from the main nerve) would be helpful.

Response: corrected. Arrows now shown in figures.

2.1.3.2) Find the nerve branching from the main nerve bundle to the stretcher muscle (Figure 5E, at arrow) and this can be cut close to the muscle and pulled into a suction electrode to stimulate (Figure 5F&G, arrow depicts the branch).

17. 2.2.9.1) How much (volume) of the neuromodulators should be applied?

Response: corrected.

2.2.9.1) Use a pipette and just drip 1 or 2 ml over the preparation.

18. 3.2.1) What is the stock solution?

Response: corrected.  
“3.2.1) Dilute one part methylene blue chloride stock solution (0.25%) with two parts of distilled water. “

19. 3.3.2) What volume is used?  
Response: corrected.

3.3.2) Use a 10 µM concentration of 4-Di-2-ASP solution and leave the preparation in the refrigerator for 15 minutes. Use just enough solution to cover the preparation.

20. Figure 13: Should be labeled A) and B). What is on the y-axis? What do the different colors mean? More explanation is needed.

Response: corrected.  
*Figure 13: The relative forces that are developed with the joint fully flexed and stimulated at the various frequencies. A, The relative forces are shown with each stimulation frequency. B, The traces in panel A are superimposed in different colors for ease in comparison.*

**Reviewer #2:**

*Manuscript Summary:*   
This paper presents a very thorough demonstration of a preparation which is outstanding for teaching purposes.   
Response: Thank you.

*Major Concerns:*

While the content of the paper is excellent, the writing …….. “requires editing.” (sic).

The fragmented sentences were due to our misunderstanding of listing topics/sections and not statements for the step wise fashion of the methodology. These steps were also made by the JoVE editor in breaking the parts to steps. We will double check for typos etc... Sorry about the oversight.

*Minor Concerns:*

P1 p5 line 2 adding a comma after proprioception makes the sentence easier to read  
P1 p 5 line 7 delete "of a chordotonal organ"  
p2 p1 line 3 a methodology "for recording from primary"...  
P2 p 1 line 4 "...(individually or as a group)...  
P2 p10 line 10 ...which neurons "carry" out...  
P2 p1 line 10 ...relate "this" to the location...  
P2 p1 line 16 ... "are" still being carried out...  
Fig 13 add scale bars and label traces  
Fig 14 add scale bars

Response: Corrections made and manuscript proof read by 3rd party.

It's probably a good idea to mention that blue crabs are rather aggressive, and very fast, particularly since they can actually draw blood if they get a good pinch in.

Response: Good idea. Thank you. The following caution has been added to the methods section:

**1.1) Dissection**

1.1.1) Hold the crab across the carapace from behind, and avoiding the claws, pinch the proximal part of the meropodite with forceps. The leg will autotomize. This avoids the animal from bleeding to death. Blue crabs are rather aggressive and very fast one needs to use caution when handling.

*Additional Comments to Authors:*  
N/A

**Reviewer #3:**

*Manuscript Summary:*   
This manuscript shows the recording and staining techniques for a sense organ in a crab. The paper describes is not one, but a suite of experimental procedures including dissection, extracellular recording, stimulation, and cellular staining. Tasks include:  
1. Isolating a small sensory organ and a small motor nerve;  
2. Recording from the sensory organ while the limb is being moved passively;  
3. Recording from the sensory organ while the limb is actively moving from neural stimulation;  
4. Recording muscle tension caused by varying neural stimulation;  
5. Two staining methods to visualize the sense organ. **NOTE: We used three staining methods.**

*Major Concerns:*  
This seems to be a challenging preparation for teaching proprioception. Sticking with decapod crustaceans, the abdominal muscle receptor organ can be recorded in crayfish (smaller, more readily available), is a simpler dissection, shows many of the same physiological properties (e.g., neurons responding to movement and position), and is well documented in widely distributed teaching materials (e.g., the Crawdad Project; http://www.crawdad.cornell.edu/). Given that, this article needs to make a stronger case for the benefits of using this particular preparation compared to others. Perhaps emphasizing the possible role of these sensory organs in active walking, swimming, and so one, might provide justification for studying these particular sensory structures.

Response: We agree that crayfish have some advantages but one can not record from joint receptors within the walking legs as well in crayfish preparations as compared to the crab legs. We do have a video recording of the methodology for recording from the crayfish MRO presented for educational purposes.

Leksrisawat, B., Cooper, A. S., Gilberts, A. B., Cooper, R. L. Muscle Receptor Organs in the Crayfish Abdomen: A Student Laboratory Exercise in Proprioception . J. Vis. Exp. (45), e2323, doi:10.3791/2323 (2010).

Good point on justifying the crab leg for relating to understanding sensory organs in active walking and swimming etc…

This text was added to the Introduction:

“The muscle receptor organ in the abdomen of the crayfish is also a well documented preparation (e.g., the Crawdad Project; <http://www.crawdad.cornell.edu/>) for teaching purposes of proprioception with only two neurons per abdominal hemi-segment (Leksrisawat et al., 2010); however the chordotonal organ in the joints of walking legs in crabs allows one to emphasize recruitment of neurons in range fractionation as well as sensory processing as part of locomotion.”

"3.3.2) Use a 10 <mu>M concentration?" How much dye should be used per volume of saline?

Response:

3.3.2) Use a 10 µM concentration of 4-Di-2-ASP solution and leave the preparation in the refrigerator for 15 minutes. Use just enough solution to cover the preparation.

*Minor Concerns:*

There is some specialized terminology that may be unfamiliar to novices. E.g., PD organ is not defined (though can probably be figured out from context); condyle.

Response: Corrections made.

“In this demonstration we use the Propodite-Dactylopodite (PD) chordotonal organ.”

“1.3.2) Using the same technique cut a smaller window on the pigmentless (medial) side of the propodite, but leaving the condyle (the socket joint or hinge between segments) attachment intact.”

"3.4.1.2) ? If any CoCl2 spills into the saline bath the entire preparation will stain black, and the preparation should be discarded." If students are aware of the spill, a quick and complete change of saline can prevent everything from staining.

Response: Corrections made. Good point.

“3.4.1.2) Make a petroleum jelly well to hold the CoCl2. If any CoCl2 spills into the saline bath the entire preparation will stain black, and the preparation should be discarded. **NOTE: that may work with crayfish but doesn't work with crab preps.**

"3.4.2.2) ? Good metal tools are not to be used to handle the preparation after this step (you should use specific tools for this)." This may be cryptic to new readers; what sort of tools? I assume the authors mean using forceps that have already been exposed to fixatives, etc.

Response: Corrections made. Good point.

“3.4.2.2) Transfer the isolated preparation to a small glass Petri dish containing about 10 ml of crab saline. The neurons are washed and the following steps are done in situ. Good metal tools are not to be used to handle the preparation after this step (you should use specific tools that are not to be used at a later time for physiology). “

"3.4.3.2) Make certain that the development solution you have poured into the sink drain is followed by running tap water for a few minutes." A waste bottle might be preferred by some safety officers rather than the sink.  
Response: Corrections made. Good point.  
“3.4.3.2) Make certain that the development solution you have poured into the sink drain is followed by running tap water for a few minutes or in a waste bottle with a lid.”

"3.4.4.2) ? With time the filled cells will become more apparent because the surrounding tissue will become clearer." Worth noting that the stained neurons will also fade unless intensified.

Response: Corrections made. Good point.

“3.4.4.2) After about 10-15 minutes in two changes in 100 % alcohol, clear the tissue by replacing alcohol with 100% methyl salicylate. The preparation will stay in this solution permanently for repeated viewing. With time the filled cells will be come more apparent because the surrounding tissue will become clearer. Intensification methods can be used to help prevent fading over time (Delaney and Gelperin, 1990).”

The introductory review is somewhat cursory. "Detailed electrophysiological studies [are] still being carried out today for research and for teaching purposes.5,6,7" has no references past the 1950s, which does not support the claim that this prep is actively being used. More recent references might include:  
  
Bévengut M, Simmers AJ, Clarac F. 1983. Central neuronal projections and neuromuscular organization of the basal region of the shore crab leg. The Journal of Comparative Neurology 221(2): 185-198. http://dx.doi.org/10.1002/cne.902210207  
  
El Manira A, Cattaert D, Clarac F. 1991. Monosynaptic connections mediate resistance reflex in crayfish (*Procambarus clarkii*) legs. Journal of Comparative Physiology A 168(3): 337-349. http://dx.doi.org/10.1007/BF00198353  
  
Ray DL, Clarac F, Cattaert D. 1997. Functional analysis of the sensory motor pathway of resistance reflex in crayfish. I. Multisensory coding and motor neuron monosynaptic responses. Journal of Neurophysiology 78(6): 3133-3143. http://jn.physiology.org/cgi/content/abstract/78/6/3133  
  
Le Bon-Jego M, Cattaert D. 2002. Inhibitory component of the resistance reflex in the locomotor network of the crayfish. Journal of Neurophysiology 88(5): 2575-2588. http://jn.physiology.org/cgi/content/abstract/88/5/2575

Response: Corrections made. Good references are added.

*Additional Comments to Authors:*  
This is good material, but may be best targeted at advanced students.  
  
  
**Reviewer #4:**   
*Manuscript Summary:*   
In this paper the authors present the interest of the crab leg preparation to demonstrate function of proprioceptors. The presentation is clear and the practical informations will be helpful for teaching. In addition, procedures used to stain sensory neurons and nerves are given with all details very useful for replication with students. I found the paper very interesting for the amount of practical details it provides.  
  
*Major Concerns:*  
Page 4 Figure 1: indicate cut line between carpopodite and propodite

Response: Corrections made.

Page 9, Figure 6  
Fig 6A: The limit of the removed cuticle section is hardly visible.   
The difference between figures 6B and 6C is not obvious.

Response: Corrections made. This is really Figure 5.

“2.1.3.1) Cut the apodeme of the bender muscle and remove the muscle carefully as not to pull the main leg nerve out of the leg cavity (**Figure 5B, C-** note the arrows where to bender apodeme is separated).”

Fig 6F: indicate the motor nerve with an arrow.

Response: Corrections made.

Page 17, Figure 13: Add indication of opening and closing movements of the joint

Response: Corrections made. This is now Figure 11.

Page 18, Figure 14, bottom: indicate the significance of colors?

Response: Corrections made.

Page 18, § 4.3: "? nerve can be correlated ?" (insert "be").

Response: Corrections made.

Change "Figure 11" into "Figure14"

Response: Corrections made.

Page 19: Figure 11 is indeed Figure 14

Response: Corrections made.

Page 20: Figure 12 is indeed Figure 15

Response: Corrections made.

Page 20: Figure 13 is indeed Figure 16

Response: Corrections made.

Page 20: Figure 14 is indeed Figure 17

Response: Corrections made.

Page 20, Line 2: (and Figure 8) Indicate in Figure 8 which area is magnified in Figure12 15  
Response: Corrections made.

*Minor Concerns:*  
Page 1, Long Abstract, last line : « ? receptoris ?» : insert a space « ?receptor is ?»

Response: Corrections made.

Page 8, §2.1.2, L2: suppress the full stop after carpopodite

Response: Corrections made.

*Additional Comments to Authors:*  
N/A  
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Your revision is due by **Jun 17, 2013**.  
To submit a revision, go to the [JoVE submission site](https://exchange.uky.edu/owa/redir.aspx?C=GSim4hwZ0U2r86a-GQZZexWoN61QK9AIMit7D6J2ET9dAG22YC2waxXU-JQMZHTjn3nFIBIeAO0.&URL=http%3a%2f%2fwww.editorialmanager.com%2fjove) and log in as an author. You will see a menu item called 'Submission Needing Revision'. You will find your submission record there.   
Sincerely,  
  
Susan Rasakham, Ph.D.   
Science Editor  
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[https://exchange.uky.edu/owa/14.2.342.4/themes/resources/clear1x1.gif](https://exchange.uky.edu/owa/redir.aspx?C=GSim4hwZ0U2r86a-GQZZexWoN61QK9AIMit7D6J2ET9dAG22YC2waxXU-JQMZHTjn3nFIBIeAO0.&URL=http://www.jove.com)