Barcelona, 10 August 2018

Alisha DSouza, Ph.D.

Senior Review Editor

[JoVE](http://www.jove.com/)

Dear Ph.D. Alisha DSouza,

Please find herewith enclosed our revision of the manuscript JoVE58515 now entitled (reviewer suggestion) “Long-term video tracking of cohoused aquatic animals: a case study with daily locomotor activity in the Norway lobster (*Nephrops norvegicus*)” for which the reviewers have recommended major and minor revisions.

We appreciate very much the comments made by the editor and reviewers. As requested, we are including below a list of changes made to the manuscript in response to the suggestions made by the editor and reviewers. Also, we have answered the concerns addressed by both of them, and we have included the modifications they suggested.

We hope you will find our revised manuscript to be of a quality that deserves publication in JoVE — Journal of Visualized Experiments.

Sincerely,

José A. García

**Changes required by the JoVE Scientific Review Editor:**

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

We modified the introduction text following all suggestions from the editor and reviewers, and we added new paragraphs for this purpose.

Essentially we expose the following advantages of the method:

- The use of a moderate tag size that allows better recognition rates with low cost cameras with respect to alternative methods based on QR and Barcodes.

- The use of one channel signal. That makes the method usable in settings where it is not possible to acquire color images due to biological constraints.

- The frame independent detection of tags. Each tag is recognized at each frame, this makes the method more robust against intersecting trajectories with respect to previous tracking methods

Pérez-Escudero, A., Vicente-Page, J., Hinz, R.C., Arganda, S., de Polavieja, G.G. idTracker: tracking individuals in a group by automatic identification of unmarked animals. *Nature Methods*. **11** (7), 743–748, doi: [10.1038/nmeth.2994](https://doi.org/10.1038/nmeth.2994) (2014). Similarly, that makes the method more robust when there are partial or total occlusions of the animal.

And the protocol has the following limitations, which are now also mentioned in the introduction:

- Only can be applied to animals that can hold the proposed tags. The method could be used with mice, crustaceous, and other small animals, but it it’s not suitable for small insects.

- The method assumes 2D movement of the animals. Animals can freely move in the plane, but we assume that they do not move vertically. The method uses thresholds designed for a specific tag size according to the distance of the camera to the animals. Although the method can tolerate small changes, it is not suitable to be used in a 3D environment where animals closer to the camera will show significant changes in the tag size. That makes the method not suitable for certain applications dealing with freely moving fish.

We added this information in the Introduction and the Discussion according to the reviewer comments.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

1) Example: 1.4 should be a note.

2) Example not in imperative voice: Line 147-148

We changed all sentences according to these instructions.

• **Protocol Detail:** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples of missing details:

1) 1.1: Mention lobster sex, age, weight. Please expand the description of the housing. What kind of water is used?

2) 1.2: Feed with what?

3) 1.3: Mention lighting during and level (e.g. in watts/cm2, or lumens)

4) 1.5: What kind of water? Sea water? What is the source?

5) 2.2: What kind of fiber?

6) 3.8: Help how? Do you assist directly by hand?

We added new information and changed all sentences according to these instructions.

• **Protocol Highlight:** The current highlighting needs minor revisions. Please highlight ~1-2.5 pages (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

5) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.

We took into account all instructions and changed all highlight sentences according to these suggestions.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We modified the paper discussion following the editor and reviewers suggestions. We comment similarities with other existing methods, future developments, and the limitations on the animal tracking.

• **Figure/Table Legends:**

1) Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

2) Please add legends for the videos.

We added legends for videos, expanded all poor legends and modified two figures to improve the paper’s quality. Also, we eliminated a figure without added value, and we created a new figure that better explains the software script steps.

• **References:**

1) Please move the in-text http weblinks (line 125, 143, into the reference list, and use superscripted citations.

We changed all http weblinks to the reference list or to materials list, and used superscript for the citations.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

We changed all wrong abbreviations following these instructions.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. 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]."

We used original figures for this paper.

**Comments from Peer-Reviewers:**

**Reviewer #1:**

Dear Authors, dear Editor,

please find here my comments:

Manuscript Summary:

I have read this protocol paper with attention and interest. This paper is a protocol paper that has merit because it details every steps rarely described in video tracking methodological paper. This paper has some originalities as the capacity to track individually in long-term animals which can be inactive during long period, a problem observes in numerous video tracking, which are frequently based on the previous motion to determine each ID. The solution to avoid light reflection is also interesting.

We would like to thank the reviewer for his/her interest and the comments provided to improve the paper.

Major Concerns:

If the manuscript gives details for a protocol, it misses details of how works the video tracking systems introduced here, and the references in the field is few developed. This paper stays focused to the case study of lobster tracking, I think introduction and particularly the discussion is too limited to lobsters, and need to be developed for the video tracking aspect.

We expanded the introduction and discussion to develop the aspect suggested by the reviewer. The paper is focused on the protocol related to the actual problem faced in our lab, the lobster track. We conjecture that similar accuracies could be obtained tracking other animals with similar movement patterns, although we tested the method with the data available in the lab. Nevertheless, we added in the introduction and discussion specific comments about other applications, and the limitations of the method regarding to its use with other particular species.

In addition, we have expanded the explanation about the video tracking system. We also included the following references with the implementation details of the algorithms that might be unfamiliar for non-image processing experts:

1. Bradski, G. OpenCV Library. *Dr. Dobb’s Journal of Software Tools* (2000).
2. Piccardi, M. Background subtraction techniques: a review. *2004 IEEE International Conference on Systems, Man and Cybernetics (IEEE Cat. No.04CH37583)*. **4**, 3099–3104 vol.4, doi: 10.1109/ICSMC.2004.1400815 (2004).
3. Sankur, B. Survey over image thresholding techniques and quantitative performance evaluation. *Journal of Electronic Imaging*. **13** (1), 146, doi: 10.1117/1.1631315 (2004).
4. Yu-Kun Lai, Rosin, P.L. Efficient Circular Thresholding. *IEEE Transactions on Image Processing*. **23** (3), 992–1001, doi: 10.1109/TIP.2013.2297014 (2014).
5. OpenCV Team Structural Analysis and Shape Descriptors — OpenCV 2.4.13.7 documentation. at <https://docs.opencv.org/2.4/modules/imgproc/doc/structural\_analysis\_and\_shape\_descriptors.html?highlight=findcontours#void%20HuMoments(const%20Moments&%20m,%20OutputArray%20hu)>.
6. Slabaugh, G.G. Computing Euler angles from a rotation matrix. 7, doi: 10.1.1.371.6578.
7. Z. Zhang A flexible new technique for camera calibration. *IEEE Transactions on Pattern Analysis and Machine Intelligence*. **22** (11), 1330–1334, doi: 10.1109/34.888718 (2000).
8. www.FOURCC.org - Video Codecs and Pixel Formats. at <https://www.fourcc.org/>.
9. Suzuki, S., be, K. Topological structural analysis of digitized binary images by border following. *Computer Vision, Graphics, and Image Processing*. **30** (1), 32–46, doi: 10.1016/0734-189X(85)90016-7 (1985).
10. Sklansky, J. Finding the convex hull of a simple polygon. *Pattern Recognition Letters*. **1** (2), 79–83, doi: 10.1016/0167-8655(82)90016-2 (1982).
11. Fitzgibbon, A., Fisher, R. A Buyer’s Guide to Conic Fitting. 51.1-51.10, doi: 10.5244/C.9.51 (1995).
12. Otsu, N. A Threshold Selection Method from Gray-Level Histograms. *IEEE Transactions on Systems, Man, and Cybernetics*. **9** (1), 62–66, doi: 10.1109/TSMC.1979.4310076 (1979).
13. Hu, M.-K. Visual pattern recognition by moment invariants. *IRE Transactions on Information Theory*. **8** (2), 179–187, doi: 10.1109/TIT.1962.1057692 (1962).
14. Structural Analysis and Shape Descriptors — OpenCV 2.4.13.6 documentation. at <https://docs.opencv.org/2.4/modules/imgproc/doc/structural\_analysis\_and\_shape\_descriptors.html?highlight=cvmatchshapes#humoments>.
15. Krizhevsky, A., Sutskever, I., Hinton, G.E. Imagenet classification with deep convolutional neural networks. Advances in neural information processing systems. 1097–1105, at <http://papers.nips.cc/paper/4824-imagenet-classification-w> (2012).

The advantages and limits of the present method over alternative methods (color methods, colored tags method, barcodes, QR tags, etc.), with references, are missing. Add also some reviews in the video tracking field, notably about aquatic animal video tracking is for me required, notably in more biological papers (see journal as Fish & Fisheries, Behavior Research Methods, Nature Methods or Protocols, etc.).

We expanded the discussion to develop the aspect suggested by the reviewer

We highlighted the limitations of the method, and commented the differences with the use of color tags, barcodes and QR tags. Particularly, we focus our development to the use of grayscale images. This constraint is imposed by the biological features of the animals. We agree with the reviewer that color based segmentation approaches would improve the performance in the general case. The advantages of using the designed tags with respect to barcodes and QR tags have also been added to the text. Our method allows using simpler hardware at the expense of recognizing a reduced set of tags.

We also added the following relevant references:

1. Dell, A.I. *et al.* Automated image-based tracking and its application in ecology. *Trends in Ecology & Evolution*. **29** (7), 417–428, doi: 10.1016/j.tree.2014.05.004 (2014).
2. Berman, G.J., Choi, D.M., Bialek, W., Shaevitz, J.W. Mapping the stereotyped behaviour of freely moving fruit flies. *Journal of The Royal Society Interface*. **11** (99), doi: 10.1098/rsif.2014.0672 (2014).
3. Mersch, D.P., Crespi, A., Keller, L. Tracking Individuals Shows Spatial Fidelity Is a Key Regulator of Ant Social Organization. *Science*. **340** (6136), 1090, doi: 10.1126/science.1234316 (2013).
4. Tyson L Hedrick Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinspiration & Biomimetics*. **3** (3), 034001 (2008).
5. Branson, K., Robie, A.A., Bender, J., Perona, P., Dickinson, M.H. High-throughput ethomics in large groups of *Drosophila*. *Nature Methods*. **6** (6), 451–457, doi: 10.1038/nmeth.1328 (2009).
6. De Chaumont, F. *et al.* Computerized video analysis of social interactions in mice. *Nature Methods*. **9**, 410 (2012).
7. Pérez-Escudero, A., Vicente-Page, J., Hinz, R.C., Arganda, S., de Polavieja, G.G. idTracker: tracking individuals in a group by automatic identification of unmarked animals. *Nature Methods*. **11** (7), 743–748, doi: 10.1038/nmeth.2994 (2014).
8. M. Fiala ARTag, a fiducial marker system using digital techniques. *2005 IEEE Computer Society Conference on Computer Vision and Pattern Recognition (CVPR’05)*. **2**, 590–596 vol. 2, doi: 10.1109/CVPR.2005.74 (2005).
9. Koch, R., Kolb, A., Rezk-salama (eds, C., Atcheson, B., Heide, F., Heidrich, W. *CALTag: High Precision Fiducial Markers for Camera Calibration*.
10. Crall, J.D., Gravish, N., Mountcastle, A.M., Combes, S.A. BEEtag: A Low-Cost, Image-Based Tracking System for the Study of Animal Behavior and Locomotion. *PLOS ONE*. **10** (9), e0136487, doi: 10.1371/journal.pone.0136487 (2015).

Discuss of the weakness of other methods (video tracking with UV, IR, VIE, notably papers with fish and decapods) used to track aquatic animals in the night/dimlight/darkness. Discuss of the question of multitracking, in comparison of other available methods (EthoVision, Swisstrack, IDtracks, all a series of methods applied on zebrafish for example). Moreover, in the discussion, future applications or directions of the introduced method could be better developed, notably to increase the range of potential readers, not only the researchers in lobsters.

We extended the introduction and discussion to fulfill the reviewer’s concerns. Essentially we expose the following advantages of the method:

- The use of a moderate tag size that allows better recognition rates with low cost cameras with respect to alternative methods based on QR and Barcodes.

- The use of one channel signal. That makes the method usable in settings where it is not possible to acquire color images due to biological constraints.

- The frame independent detection of tags. Each tag is recognized at each frame, this makes the method more robust against intersecting trajectories with respect to previous multitracking methods Pérez-Escudero, A., Vicente-Page, J., Hinz, R.C., Arganda, S., de Polavieja, G.G. idTracker: tracking individuals in a group by automatic identification of unmarked animals. *Nature Methods*. **11** (7), 743–748, doi: [10.1038/nmeth.2994](https://doi.org/10.1038/nmeth.2994) (2014).Similarly, that makes the method more robust when there are partial or total occlusions of the animal.

And the protocol has the following limitations:

- Only can be applied to animals that can hold the proposed tags. The method could be used with mice, crustaceous, and other small animals, but it is not suitable for insects.

- The method assumes 2D movement of the animals. Animals can freely move in the plane, but we assume that they do not move vertically. The method uses thresholds prepared to a specific tag size according to the distance of the camera to the animals. Although the method can tolerate small changes, it is not suitable to be used in a 3D environment where animals closer to the camera will show significant changes in the tag size. That makes the method not suitable for certain applications dealing with freely moving fish.

We also introduced some differences with respect to the three papers suggested by the reviewer. Our protocol is essentially based on non expensive Hardware and open source Python libraries, which are easier to modify and adapt than closed solutions. Nevertheless, we also highlight the advantages of these previous works in settings were our protocol faces strong limitations (e.g. tracking in the 3D general case scenario needed in the zebrafish example).

Finally, we expanded the future works section. We conjecture that the use of Convolutional Neural Networks could be the following step to improve the results of the classifier, although we do not have experimental evidence.

Your system works with lobsters, but which other types or groups of animals could benefit from your video tracking system? Is it possible with other arthropods, fish, terrestrial animal, small or large species? Translucent, colored or cryptic species? In 2D or 3D environment? What about the occlusion cases, source of frequent error in classical video multitracking system? The biological model allow to avoid this problem? And with species where occlusions happen, what can you say about your video tracking system?

We expanded the discussion to develop the aspect suggested by the reviewer

As mentioned, the introduction and discussion now mention the limitations of the method, and clear the concerns made by the reviewer. Essentially, the protocol can be applied to other species where it is possible to glue the designed tags. That makes the method non suitable for small insects, but we hypothesize that it could be easily applied to medium size large animals (mice, etc.). The second limitation is the 3D environment, which has not been tested. We suggest not using the method in a fully 3D environment, given that animals closer to the camera could experience strong changes in the scale of the tag images. The configuration used would not be useful in these cases.

In the case of occlusions or intersection trajectories of animals, we suggest that our method is especially suitable. The proposed algorithm detects each tag independently, and it is able to handle frames that come from occluded regions very well. An error in a specific frame due to an occlusion does not propagate to future frames, given that the identification is performed using specific features of the frames.

If the lamps under water are very interesting approach to avoid reflection (it would be interesting to mention that), how do you manage the temperature impact induced by these lamps, particularly for the IR lamp where IR light is largely absorbed by water?

We added an explanation note in step 2.2.1 to make clear this point, but the lightning system does not have impact on the temperature, given that we used refrigerated (12-13 ºC) seawater for renovation purpose only. Also, we used an isolated facility with temperature control (13º C) to put in the experimental set up. Besides, IR lamps are LED’s lights (850 nm) with low heat impact in the seawater.

At this stage, as there are numerous questions and unclear points, and the quality of some figures and legends is insufficient, I recommend this manuscript as a major revision.

Minor Concerns:

Title: too "lobster", not sufficiently "videotracking": I suggest something like: Long-term video tracking of cohoused aquatic animals: a case study with daily locomotor activity in the Norway lobster (Nephrops norvegicus).

We accept the suggested change in the title.

1.2. Feed them at about 3 times per week: miss a lot of details or references: which type of food, which relative quantity of food (g/ g body weight), we have not details about the lobsters: size, age or life stage, sex.

We added new information in 1.2 and an explanation for this aspect in a note:

*Note: The following protocol is based on the assumption that researchers can sample N. norvegicus in the field during the night to avoid damage to photoreceptors**13. Exposure of N. norvegicus to sunlight must be avoided. After sampling, lobsters are supposed to be housed in an acclimation facility similar to the one reported with a continuous flow of refrigerated seawater (13 o C). The animals used in this study are male at intermoult state with a cephalothorax length (CL; means±s.d.) of 43.92±2.08 mm (N=4).*

1.3. Suggest the type of lamp, which position and power? How many Lux?

We used SERA T8 blue moonlight fluorescent bulb 36 watts, that produce 12 lux approx. a 1 m in front of the tube. This kind of lamps are discontinued, now we use led blue moonlight lamps, see SERA website for more information. We added into the text and explanation about this fact.

1.3. Blue light only justified by the spectral sensibility? If this the case, why not use other wavelengths? I think this information is not complete, see the real light condition in natural environment of lobsters.

That is not only justified by the spectral sensibility we also use blue light due to the fact that this light spectrum reaches 400 m deep in seawater. We added an explanation sentence with a reference.

1.5. the goal of the flow rate? Create water current? From a circular system of filter or completely new water?

We added more information and rewrote the sentence. Seawater circulates in open circuit (new water), flow rate references renewal rate.

Lines 112-113. "See Fig.1 at the end of this point that shows a schematic representation of the experimental set up" rewrite or remove a part of this sentence.

We removed this sentence.

2.2. "more details". Too short for a sentence.

We added an explicative note and decomposed it in more steps (now step 3.2).

2.2.1. Use IR lamps underwater has an impact on water temperature, how to manage that?

We think that this aspect is unclear, we added an explicative note. It has no impact on the water temperature, we used refrigerated (12-13 ºC) seawater for renovation, also we used an isolated facility with temperature control (13º C) to put in the experimental set up. Besides, the IR lamps are LED’s lights (850 nm) with low heat impact on the seawater temperature (now step 3.2.1).

2.2.1. "…Reported here6" not clear, reported in your paper or in your reference?

We rewrote the sentence (now step 3.2.1).

2.3. webcam: we need more details: resolution, type, frame rate, suggested model, computer program needed?

We added a note with the specifications and reference to materials list. See Excel file with material list. Resolution 1 Megapixel, 1 frame per second (see now 3.5.1. protocol step).

Location is imprecise, notably the height is connected to deformation of image.

We added precise location in text and figure 3.

Have you resolved completely the reflection problem? Have you some problem of reflection or is it very minor?

Yes, we solved this problem. But, we found situations where the light is overexposed due to underwater situation lamps and this fact generates an incorrect image binarization. That makes impossible the tag identification in these specific situations.

Do you perceive directly the lamps at the screen?

We only perceive a line with some little lights spots. See figure 4 in the text, but it’s possible eliminate this with LED’s with a minor illumination angle.

2.5.3.: to create a data and time stamp: could you suggest a program or reference to do that? A comment perhaps to avoid the time stamp in the arena image, as you work by subtraction method.

Yes, we suggest two software solutions. See material list and now step 3.5.1, this kind of software for video surveillance offers different ways to timestamps the frames.

Data and time stamp are situated in the top left corner, and possible variations in the frame background calculating the mean background subtraction method are not taken into account.

3.2. Fast glue: comment the potential toxicity of the glue. What type of glue do you use, with cyanide? Provide comments about water stability of tags and glue. Provide comments about any skin reaction of lobster (perhaps inexistent in this species)

We used cyanoacrylate gel. We changed the text to add comments about your suggestions. The glue has no effect on the lobsters. There is only a marginal effect on the human manipulation of the glue due to the vapors emitted, but is considered a regular glue as the ones used in domestic homes and medical applications (now step 4.2).

3.2. More details concerning the tags.

OK, We changed the text to add some steps about tags construction.

3. You put the lobster in crushed ice: could please comment if the goal is just to immobilize the lobster or if you anesthetized the lobster? This is an important question concerning animal welfare, and because if question of animal welfare concerned essentially vertebrate species, and now cephalopods, some debates about decapod welfare begins to be discussed. Please add some references concerning this procedure on lobster or close species. What the procedure of recovery?

It is an important issue and we would like to stress the fact that animal welfare is strictly monitored at the Marine Science Institute where we conducted the experiments and that we got all the permissions to perform what is presented. In particular, we put the lobsters on crushed ice for few minute to immobilize them and facilitate handling. Now it is better explained in the manuscript. The animals were not anesthetized and according to welfare protocol in the place where we conducted experiments there were no need of specific welfare concerns.

3.5.5. Note: avoid exposure …: why? Please some references

Certainly, it’s not correctly explained. We used red light to carry out all operations. We changed the text and added a reference (see now steep 4).

Line 195: wait 1 min. minimum => wait 1 min minimum

OK. We corrected the text (now line 292).

Line 203: Fig.2 shows tag's examples: to replace above when you introduce the tags.

OK. We modified and changed this text.

5.3. All video format works or do you suggest to work with specific format (mpg4, AVI, others). Do you suggest some resolution level? Are there some constraints of resolution or computer memory?

We added a note into the text, in the step 6.3 with an explanation about these questions. Any video format that uses public codecs would suffice. The resolution is determined by the camera specifications, although it has been shown to be sufficient.

5.4.3.x: it is required to define clearly ROI, hull area, radius ri, solidity si and aspect ratio ai. Firstly, this is unclear and difficult to understand, secondly a figure is welcome to explain this terms. References is also needed. For me, ROI is unclear, is it the blue rectangle located on the detected lobster?

We modified the text, added a new figure to explain the script steps and added an image processing book reference (now 6.4.3.x steps).

We define three regions of interest (ROI = Region of Interest), depicted as yellow or blue rectangles in for example the figure 5. Yellow rectangle is fixed and represents the tank bottom area (only in this area script search animals), the big blue rectangles represent a possible animal detection and little blue square represents tags candidates to be classified.

5.4.4.x. You need to discuss the value in connection with the lobster sizes and the arena size. Can we have the size of lobster (real and/or relative in term of arena size or pixels). Could you please describe in a note how the lobster is identified as one individual, and how the blue rectangle is shaped around it? How your system detects the vertex in tag analysis? Need more details and/or references of the method; again, the same comments to detect the central point.

We use pixels as image base unit, we added a note. Also, in the new figure 5 (view previous comment reply) we think this aspect is explained (lobster identification).

We worked with well-known image operations; we added an image processing book reference to detail the vertex analysis. We used a specific library for that purpose (see code). We used the notion of bounding box from object recognition field.

5.4.6.1: need a space line

OK. We corrected the text.

Line 309: remove "instructions"?

OK. We corrected the text.

Lines 335: kernel method: could you please communicate the smoothing degree of your analysis to drawn the kernel polygons/curves?

To draw polygons in the figure we used two-dimensional kernel density estimation with an axis-aligned bivariate normal kernel, evaluated on a square grid. The smoothing degree is proportionate by the grid size. In our case is a pixel square degree and uses an optimal estimated bandwidth (150x80 pixel approx.) to calculate polygons, that it is calculated by the used R function (see Venables, W. N. and Ripley, B. D. (2002) Modern Applied Statistics with S. Springer, equation (5.5) on page 130). We also added this information to the text and references (now line 455).

The determination of blue rectangle could help to detect some behavioural display, as in the video where one lobster spreads its arms. Could you please add a comment in the discussion about this possibility?

We agree with this possibility, and we are exploring it at this moment. We added comment about this in the discussion.

Line 337: the accuracy of tags discrimination: details your calculation, there are different ways in literature to do that. Have you some inversions of individual identification? How many cases of detection without identification? Do you use only the information in ROI, or do you taking account of the identification of other individuals. For instance, you have not the ID of one individual, but you can deduce it because you know the ID of the three others; or you can deduce because you know the ID of this individual in a close other frame. Have you some correction process to limit these errors?

We explained this aspect in results section (lines 436 - 451). Also, in the review process we added a new sentence to make clear the calculated accuracy following the reviewer suggestions.

Although it could be a useful improvement, we used only information from the detected ROIs and we did not consider the other individuals previously identified to identifier an individual. We identified the animals using tags; we searched and found the tags in each frame using image morphological properties. The occlusion is not considered due to last mentioned, if an undetected animal (is occluded or into the burrow) in a frame, in the next few frames after this situation will be detected and identified again. The movement rate along the time is not affected for these situations.

Is the error of detection connected with the cases where the lobster is in the hole? Is it easy to identify the individual if the lobster is partially out the burrow?

No, is not the case. If the animal is into the burrow (partially or completely) neither is detected and its tag not is identified, because the tag is not visible. We store the last animal position detected and assign this to each frame while the animal remains occluded.

Fig.2 legend is incomplete!

We modified and expanded the legend.

Fig.3; legend so poor that we are not convinced of the utility of the figure, particularly that this is a repetition of the video. Please comment a few your figure.

Yes we agree with the reviewer. We deleted this figure and added the legend for video 1.

Fig.4. Legend insufficient, with missing information and some errors. (e) correct detection: I don't think so! (f) ????? (b) shape misdetection: sure?

We modified and expand the legend to correct these errors (now figure 6).

Fig.5: poor legend. Moreover, this is not a complete image of the arena, it is important to communicate this information! The edges would be show because your images seem have some perspective deformation.

We modified and expanded the legend to correct these errors (now figure 7).

Fig.5: how do you correct the image deformation? If not, what do you suggest? (Reply to place in text)

We modified the text to explain these aspects in the results section (now figure 7).

Fig.6: Photoperiod 12L:12D ???

Yes, we modified and expanded the legend to improve the figure 8 quality (figure number is changed).

Fig.5: this figure is not sufficiently clear. The nuance of gray level to legend the color is to avoid for the human eye. I suggest to replace with a gradient scale for each color!

We changed the figure and legend to improve the figure 7 quality (figure number is changed).

Fig.5: the superposition of kernel diagram of the four individuals do not help to understand the figure. Moreover, superposition of color nuances does not help also to distinguish the occupation of different individuals. Some shape (important lines cutting all the figure) is surprising, is it normal? I suggest to add four small images of kernel diagrams, one for each individual. The smoothing parameter could also help to understand the right lines.

We changed the figure and legend following your instructions and correct shape / lines figure 7 artifacts (figure number is changed).

Fig.6: the thickness of graph lines is too large, impossible to appreciate each individual activity because one overwrite the others. Please modify.

Following your suggestions, we changed the thickness of graph lines to improve the figure 8 (figure number is changed).

References:

Latin name of species in italic.

We corrected this, and we put the species names in italic.

Reference 9: incomplete and note in correct format

We corrected this error.

Reference 20: incomplete

We corrected this error.

Reference 21: incomplete reference. Is it really a reference? For me this paper is not referenced as a scientific peer-reviewed paper.

Is a seminar / conference, we modified and changed this reference.

**Reviewer #2:**

Manuscript Summary:

The submitted manuscript is properly written and meets the criteria of the Journal of Visualized Experiments editorial board. However, English could be improved to make contents clearer, particularly in Discussion section. In my opinion, present manuscript can be recommended for publication in JoVE after major revision.

Although, the proposed methodology is quite easy to reproduce, 69 % of successfully recognized N. norvegicus is not that high success. Also, it is not much clear what would be the success if apply other types of animals possessing other characteristics than described species. There are many questions related to proposed protocol application, however it is worth of reproduction and attention of JoVE's readers.

Major Concerns:

ABSTRACT:

Well written.

INTRODUCTION:

Generally clear but needs some extending; a description of the context of the method in the wider body of literature is missing.

We extended the introduction following your instructions. We also extended the discussion, to better explain the main advantages of the protocol with respect to other methods, and the main limitations when applied to other animals. This concern was also raised by the editor and reviewer 1.

In summary, the main advantages of the method are:

- The use of a moderate tag size that allows better recognition rates with low cost cameras with respect to alternative methods based on QR and Barcodes.

- The use of one channel signal. That makes the method usable in settings where it is not possible to acquire color images due to biological constraints.

- The frame independent detection of tags. Each tag is recognized at each frame, this makes the method more robust against intersecting trajectories with respect to previous tracking methods Pérez-Escudero, A., Vicente-Page, J., Hinz, R.C., Arganda, S., de Polavieja, G.G. idTracker: tracking individuals in a group by automatic identification of unmarked animals. *Nature Methods*. **11** (7), 743–748, doi: [10.1038/nmeth.2994](https://doi.org/10.1038/nmeth.2994) (2014).Similarly, that makes the method more robust when there are partial or total occlusions of the animal.

And the protocol has the following limitations, which are now mentioned in the introduction:

- Only can be applied to animals that can hold the proposed tags. The method could be used with mice, crustaceous, and other small animals, but it is not suitable for small insects.

- The method assumes 2D movement of the animals. Animals can freely move in the plane, but we assume that they do not move vertically. The method uses thresholds designed for a specific tag size according to the distance of the camera to the animals. Although the method can tolerate small changes, it is not suitable to be used in a 3D environment where animals closer to the camera will show significant changes in the tag size. That makes the method not suitable for certain applications dealing with freely moving fish.

PROTOCOL:

Generally well written.

OK.

RESULTS:

Clearly written.

OK.

DISCUSSION:

Generally well written but needs improvement of English.

As rewiever suggested we revised the English style.

Last paragraph: Another significant limitation is the disability locomotion detection of single organs such as chelae, antennas or walking legs. Very often, large crustaceans perform just single movements by mentioned body parts that can sometimes characterize their behavior much better than the travelled distance or preferred areas. The last are also very important ethological endpoints, however if you mention outcome of fights as a possibility for further development, you should take into account not just tracking of changing positions but also other behavioral patterns that could be of interest to biologists.

We added a paragraph to the discussion in the text explained this fact.

Minor Concerns:

Line 48: …locomotor activity patterns).

We corrected the text.

Line 49: Rather protocol than routine here

We corrected the text.

Line 50: …individually tracking individuals in a group can be valuable for asking research questions modify to individual tracking of specimens in a group can be valuable for answering research questions.

We corrected the text.

Line 68: for a case study.

We corrected the text.

Line 105: Maintain photoperiod…

We corrected the text.

Line 108: Maintain water temperature

We corrected the text.

Line 120: Rather provide instead of endow

We corrected the text.

Line 143: ). See…

We corrected the text.

Line 158: °C

We corrected the text.

Line 160: It is not clear what material can be used for tagging. Some kind of a color construction paper or a plastic? What is the minimal/optimal size for the tag recommended?

We added new steps (2.x) and a note in the text to make this issue more understandable.

Line 173: Perhaps the glue should possess the minimal toxicity or marked as safe for aquatic environment. Is it so?

We added an explanation about this into the text but the glue has minimal toxicity.

Line 196: Avoid it's and don't in the research paper: it is, do not.

We corrected the text.

Line 324: correctly identified, manual identification indicated

We corrected the text.

**Reviewer #3:**

Manuscript Summary:

The authors presented an interested methodology to track animals during long-term experiments. Considering they used free software to do this, the system they proposed is useful, interesting and can be easily replicated for other species. The ms reported all the necessary details to reuse the methodology by other researchers. I don't have any major concerns, only minor comments to be addressed (see below).

Major Concerns:

None

Minor Concerns:

Maybe in the entire ms it would be better to use only a group of lobsters instead of cohoused.

We are not sure about the comment of the reviewer. We used a group of four cohoused lobsters to test the protocol presented here. Cohoused refers to the fact that the tracking needs to keep the positions of the animals while they are engaged in social behaviors such as fighting. We hope this help to clarify this point. Alternatively we are available to reply to other specific questions.

Line 42: please "used" instead of "presented".

We corrected the text.

Lines 51-52: this sentence could be removed from the abstract.

We removed this sentence.

Lines 75-79: If the authors are suggesting using the methodology also in freshwater animals they should provide more general requirements (not specific to marine species).

Yes, we agree with the reviewer. We removed “sea” from the paragraph.

Lines 319-323: maybe I have missed some details, but the total amount should be 100%: summing false positive, false negative and correct matching the overall percentage is 121%. Please clarify this issue.

As you suggested we clarified the issue, we extend this paragraph, and we added new details to clarify the text.

Lines 386-388: considering this, why not removing the initial data? Usually when manually taking the data, what happens in the acclimatization period is not recorded as useful data. How long could be the acclimatization period (5-10 min)?

In fact, we draw all figures related with the lobster behavior avoiding 24 initial hours. But, from a computer vision point of view all images / frames are important to test and to improve the analysis script. We rewrote this sentence and added a new figure to clarify this paragraph.

Line 413: a comma or a semicolon is missing after here.

We corrected the text.

**Reviewer #4:**

Manuscript Summary:

The publication presents novel findings on automated tracking mechanisms for marine invertebrates under controlled laboratory conditions. By using 'free software' as the basis for developing this protocol, the authors have crucially made this behavioural tracking platform accessible to all.

Major Concerns:

None.

Minor Concerns:

Suggest inclusion of details of Norway Lobster visual sensitivities (Re. frequency range of detection). If this precise detail is not available then acknowledge this / substitute with those of similar decapod crustaceans. If spectral sensitivity overlaps with the blue spectrum projected over the study arena the potential implications of this should be considered.

As the reviewer suggested we added details about visual sensitives in an explained note in step 1.3.

Suggest mentioning how the new technique builds upon previous (labor intensive) decapod behavioural tracking protocols undertaken in the past e.g. Edmonds, N.J., Riley, W.D., Maxwell, D.L. 2011. Predation by *Pacifastacus leniusculus* on the intra-gravel embryos and emerging fry of *Salmo salar*. Journal of Fisheries Management and Ecology, 18, (6) 521-524.

As the reviewer suggested we added in the introduction a new paragraph, and we cite the proposed work.