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Long-term video tracking of cohoused aquatic animals: a case study with daily locomotor activity in the Norway lobster (Nephrops norvegicus)

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Dr. Lyndsay Troyer
Science Editor
JoVE, Journal of Visualized Experiments

Dear Dr. Troyer,

As agreed in the emails previously exchanged we are glad to accept your invitation to submit the methodological article entitled "Long-term video tracking of daily locomotor activity in a group of co-housed lobsters: A case study with the Norway lobster (*Nephrops norvegicus*)" to your Journal.

The video tracking presented in our paper provides an efficient, fast and cheap protocol that is able to provide individual tracking of cohoused animals over a long period of time. We tested the protocol using the Norway lobsters (*Nephrops norvegicus*) that is a species with consistent burrow emergence rhythms and when cohoused forms dominance hierarchies. Hence, our protocol is of particular interest for all those researchers particularly engaged in investigating social modulation of behavior and circadian rhythms of lobsters and more in general aquatic animals.

We are excited by the unique opportunity provided by your journal to support our protocol with a video documentation of the methodological approach. We are looking forward to receive feedbacks from reviewers.

Best regards,

Jose A. Garcia & Dr. Valerio Sbragaglia

TITLE:

Long-term Video Tracking of Cohoused Aquatic Animals: A Case Study of the Daily Locomotor Activity of the Norway Lobster (*Nephrops norvegicus*)

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KEYWORDS:

OpenCV, Python, video analysis, space occupancy, daily activity rhythms, tracking

SUMMARY:

Here we present a protocol to individually track animals over a long period of time. It uses computer vision methods to identify a set of manually constructed tags by using a group of lobsters as case study, simultaneously providing information on how to house, manipulate, and mark the lobsters.

ABSTRACT:

We present a protocol related to a video-tracking technique based on the background subtraction and image thresholding that makes it possible to individually track cohoused animals. We tested the tracking routine with four cohoused Norway lobsters (*Nephrops norvegicus*) under light-darkness conditions for 5 days. The lobsters had been individually tagged. The experimental setup and the tracking techniques used are entirely based on the open source software. The comparison of the tracking output with a manual detection indicates that the lobsters were correctly detected 69% of the times. Among the correctly detected lobsters, their individual tags were correctly identified 89.5% of the times. Considering the frame rate used in the protocol and the movement rate of lobsters, the performance of the video tracking has a good quality, and the representative results support the validity of the protocol in producing valuable data for research needs (individual space occupancy or locomotor activity patterns). The protocol

presented here can be easily customized and is, hence, transferable to other species where the individual tracking of specimens in a group can be valuable for answering research questions.

INTRODUCTION:

In the last few years, automated image-based tracking has provided highly accurate datasets which can be used to explore basic questions in ecology and behavior disciplines[1]. These datasets can be used for the quantitative analysis of animal behavior[2,3]. However, each image methodology used for tracking animals and behavior evaluation has its strengths and limitations. In image-based tracking protocols that use spatial information from previous frames in a movie to track animals[4-6], errors can be introduced when the paths of two animals cross. These errors are generally irreversible and propagate through time. Despite computational advances that reduce or almost eliminate this problem[5,7], these techniques still need homogeneous experimental environments for accurate animal identification and tracking.

The employment of marks that can be uniquely identified in animals avoids these errors and allows the long-term tracking of identified individuals. Widely used markers (*e.g.*, barcodes and QR codes) exist in industry and commerce and can be identified using well-known computer vision techniques, such as augmented reality (*e.g.*, ARTag[8]) and camera calibration (*e.g.*, CALTag[9]). Tagged animals have previously been used for high-throughput behavioral studies in different animal species, for example, ants[3] or bees[10], but some of these previous systems are not optimized for recognizing isolated tags[3].

The tracking protocol presented in this paper is especially suitable for tracking animals in one-channel imagery, such as infrared (IR) light or monochromatic light (particularly, we use blue light). Therefore, the method developed does not use color cues, being also applicable to other settings where there are constraints in the illumination. In addition, we use customized tags designed so as not to disturb the lobsters and, at the same time, allow recording with low-cost cameras. Moreover, the method used here is based on frame-independent tag detection (*i.e.*, the algorithm recognizes the presence of each tag in the image regardless of the previous trajectories). This feature is relevant in applications where animals can be temporarily occluded, or animals' trajectories may intersect.

The tag design allows its use in different groups of animals. Once the parameters of the method are set, it could be transferred to tackle other animal-tracking problems without the need for training a specific classifier (other crustaceans or gastropods). The main limitations of exporting the protocol are the size of the tag and the need for attachment to the animal (which makes it not suitable for small insects, such as flies, bees, *etc.*) and the 2D assumption for the animal movement. This constraint is significant, given that the proposed method assumes the tag size remains constant. An animal moving freely in a 3D environment (*e.g.*, fish) would show different tag sizes depending on its distance to the camera.

The purpose of this protocol is to provide a user-friendly methodology for tracking multiple tagged animals over a long period of time (*i.e.*, days or weeks) in a 2D context. The methodological approach is based on the use of open source software and hardware. Free and open source software permits adaptations, modifications, and free redistribution; therefore, the generated software improves at each step[11,12].

The protocol presented here focuses on a laboratory set up to track and evaluate the locomotor activity of four aquatic animals in a tank for 5 days. The video files are recorded from a 1 s time-lapse image and compiled in a video at 20 frames per second (1 recorded day occupies approximately 1 h of video). All video recordings are automatically postprocessed to obtain animal positions, applying computer vision methods and algorithms. The protocol allows obtaining large amounts of tracking data, avoiding their manual annotation, which has been shown to be time-intensive and laborious in previous experimental papers[13].

We use the Norway lobster (*Nephrops norvegicus*) for the case study; thus, we provide species-specific laboratory conditions to maintain them. Lobsters perform well-studied burrow emergence rhythms that are under the control of the circadian clock[14,15], and when cohoused, they form dominance hierarchy[16,17]. Hence, the model presented here is a good example for researchers interested in the social modulation of behavior with a specific focus on circadian rhythms.

The methodology presented here is easily reproduced and can be applied to other species if there is a possibility to distinguish between animals with individual tags. The minimum requirements for reproducing such an approach in the laboratory are (i) isothermal rooms for the experimental setup; (ii) a continuous water supply; (iii) water temperature control mechanisms; (iv) a light control system; (v) a USB camera and a standard computer.

In this protocol, we use Python[18] and OpenCV[19] (Open Source Computer Vision Library). We rely on fast and commonly applied operations (both in terms of implementation and execution), such as background subtraction[20] and image thresholding[21,22].

PROTOCOL:

The species used in this study is not an endangered or protected species. Sampling and laboratory experiments followed the Spanish legislation and internal institutional (ICM-CSIC) regulations regarding animal welfare. Animal sampling was conducted with the permission of the local authority (Regional Government of Catalonia).

1. Animal Maintenance and Sampling

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 the photoreceptors[23]. Exposure of *N. norvegicus* to sunlight must be avoided. After sampling, the lobsters are supposed to be housed in an acclimation facility similar to the one reported on previously[17,24], with a continuous flow of refrigerated seawater (13 °C). The animals used in this study are male at the intermoult state with a cephalothorax length (CL; mean ± SD) of 43.92 ± 2.08 mm (N = 4).

1.1. Keep the individuals in isolated compartments to avoid any damages due to individual fights (see **Figure 1a-d**).

1.2. Feed them about 3x a week at random times to not interfere with the circadian rhythms.

NOTE: In this experiment, mussels (approximately 4 g per lobster) were used as food. Mussels were bought from frozen food suppliers and were suitable for human consumption.

1.3. Use blue light (425 - 515 nm) to simulate light hours according to the spectral sensitivity of the species[25] and the environmental conditions at 400 m deep[26] (see **Figure 1c,d**).

NOTE: The facility used here has a vertical ceiling of two blue (478 nm) fluorescent lamps that produced a light intensity of 12 lx at 1 m of distance from the lamps. See **Figure 1a** for the ceiling lamps' position and see the **Table of Materials** for the manufacturer's and technical lamps' characteristics.

1.4. Adjust the photoperiod of the acclimation facility to 12/12 light/darkness hours or simulate the natural photoperiod of the local latitude.

1.5. Regulate the facility temperature to 13 °C and monitor 2x daily to check the temperature of the inflowing seawater is around 13 °C (see **Figure 1e**).

1.6. Regulate the inflow of seawater at a rate of about 4 L/min to maintain good oxygenation.

NOTE: The seawater circulates in an open circuit (no filters and additional pumps are used). The water supply depends on the main aquarium plant services.

[Place **Figure 1** here]

2. Tag's Construction

NOTE: The tag used here can be changed according to the characteristics of the target animal or other specific considerations.

2.1. Cut four circles of 40 mm in diameter from a black plastic sheet.

2.2. Cut from a white PVC plastic sheet two equilateral triangles with 26 mm sides.

2.3. Cut from a white PVC plastic sheet two circles of 26 mm in diameter.

2.4. Mark the center of the white triangles and circles and make a 10 mm hole in it.

2.5. Glue the four white shapes to the center of the four black circles.

[Place **Figure 2** here]

3. Experimental Setup

NOTE: The experimental arena is supposed to be in an experimental chamber independent from but in close proximity to the acclimation facility.

3.1. Set up an experimental chamber where the air temperature can be controlled and maintained at the same temperature as the seawater in the experimental arena.

3.2. Modify a fiberglass tank (1,500 x 700 x 300 mm) to be used as an experimental arena. Add four burrows using PVC flexible pipes at the bottom of the tank and stick sand on the surface where the lobsters are supposed to move (**Figure 3b-e**). For more details, see Sbragaglia *et al.*[17] and Aguzzi *et al.*[27].

3.2.1. Provide the experimental arena with submergible blue LEDs (472 nm, simulating light hours) and IR LEDs (850 nm, dark conditions) (see also **Figure 3a**)[17,24].

NOTE: LED light is used due to its low heat impact and the availability of usable electronic control and free hardware. An isolated facility with an environmental and seawater temperature of 13 ± 0.5 °C was used.

3.2.2. Always keep the IR LEDs switched on.

NOTE: The IR is needed to video record in dark conditions and in light conditions. It is not necessary to switch it off.

3.2.3. Connect the blue LEDs with an apparatus to manage the photoperiod. See the suggestions in the **Table of Materials**, and for more details, consult Sbragaglia *et al.*[17] (also shown in **Figure 3a**).

NOTE: Illumination in video- or image-automated analyses is a critical factor. Regular illumination without shadows all over the arena avoiding water surface reflections makes the posterior video or image analysis easier. In the context of this protocol, only 12/12 light/darkness conditions were used. Light and darkness were gradually achieved within 30 min, and a light-controller script is added as **Supplementary File**.

3.2.4. Place the chilled seawater inlet at one corner of the tank and the corresponding outlet at the opposite corner.

3.2.5. Regulate the seawater input at a flow rate of about 4 L/min.

3.2.6. Surround the tank with a black curtain in order to provide a full isolation from other light (**Figure 3a**).

3.3. Place the tripod to which the web camera is fixed to the side of the experimental arena and position the video camera above (130 cm) and at the center of the experimental arena(75 cm x 32.5 cm (see **Figure 3a**).

3.4. Check whether the video camera is in the centered position (see step 3.3) to make sure it has not been moved involuntarily.

3.5. Connect the web camera to a computer that is placed outside the curtain (**Figure 3a**).

3.5.1. Install the software to manage the time-lapse recording with the video camera.

NOTE: Time-lapse recordings depend on the movement's speed of the species. Also, see the **Table of Materials** for the camera, fisheye lens, PC, and software characteristics and manufacturers used here.

3.5.2. Adjust the parameters of the video recording according to the characteristics of the species.

NOTE: Considering the mobility rate of *N. norvegicus*, a 1 s time-lapse recording was used here, and the video was saved every 24 h.

3.5.3. Make sure to create a timestamp (including the date) in the time-lapse video (as this can help for the future manual scoring of the behavior).

[Place **Figure 3** here]

4. Experimental Trial and Animal Preparation

NOTE: All steps with animals must be done in the acclimation facility and under red light conditions according to the spectral sensitivity of the Norway lobster[25]. When moving the animals between the acclimation and the experimental facility, avoid any exposure of the lobsters to light, using an opaque black bag to cover the icebox.

4.1. Prepare an icebox previously separated into four submerged compartments with water at about 7 °C.

4.2. Prepare the four tags previously constructed and a fast glue, like cyanoacrylate.

4.3. Prepare a tray with crushed ice.

4.4. Select the four lobsters to be tagged in the acclimation facility and put each of them in a compartment of the icebox.

4.5. Wait for 30 min and, then, start the tagging procedure.

4.5.1. Take a lobster and put it on the crushed ice for 5 min to immobilize it and facilitate the tagging operation.

4.5.2. Dry the upper part of the lobster's cephalothorax with adsorptive paper and put a drop of fast glue on it.

4.5.3. Place the tag horizontally on top of the animal's cephalothorax, in contact with the glue, and wait enough time for it to harden (for about 20 s).

4.5.4. Return the lobster to its compartment in the icebox and proceed with the other three animals in the same way.

4.5.5. Put the lobsters back in the cell where they were previously and wait for 24 h to be sure that the tag is properly glued on.

4.5.6. Transfer the lobsters from the acclimation facility to the experimental chamber using the same icebox that was used for the tagging procedure.

4.6. Launch the video recording and wait for 5 min before introducing the tagged lobsters. Obtain an averaged background image from the initial 100 frames.

NOTE: Waiting a minimum of 1 min is mandatory to obtain background frames without tagged lobsters; they are needed for video processing.

4.7. Introduce the animals one by one in the experimentation tank inside their respective compartment, keeping the water in it (**Figure 4**).

4.8. Wait for them to get out; if they do not come out, help them gently by tilting the compartment.

[Place **Figure 4** here]

5. Video Analysis Script

5.1. Perform the analysis after completion of the experiment.

5.1.1. Launch the computer vision script for video analysis.

5.1.2. Launch Java program to calculate the positions and distance covered by the lobsters and insert the data in the database.

NOTE: This program is a Euclidean distance-based algorithm[28].

5.1.3. Launch SQL script to binning data as desired time interval (ex. 10 min).

6. Computer Vision Script for Video Analysis

NOTE: The script avoids fisheye image correction because it does not introduce a relevant error in the experimental setup. Nonetheless, it is possible to correct this with OpenCV[29] camera calibration functions based on vector and matrix rotation methods[30,31].

6.1. Select the Python[18] program language.

6.2. Select the OpenCV[19] image and video processing library.

6.3. Load a video.

NOTE: Video formats .avi or .mp4 were used in this experiment, but this is not mandatory. It depends on the FourCC[32] codecs installed in the operating system.

6.4. Perform the following steps for each frame *Fi* in the video.

6.4.1. Subtract the background[20] ***B*** (average of the last 100 frames, obtained from step 4.6) from the current frame ***Fi***, and update the background image ***B*** as ***Fi***. Use the function *BackgroundSubtractorMOG2* from the OpenCV[19] library (see the scripts in the **Supplementary File**).

6.4.2. Determine the set of regions of interest (ROIs) ***R*** from the pixels with relevant motion indicated by the background subtractor. Use the method *apply* from *BackgroundSubtractorMOG2* in the OpenCV[19] library (see the scripts in the **Supplementary File**). In the set, include the animal detections from the previous frame, to take into account nonmoving animals.

6.4.3. Perform the following steps for each ROI ***Ri***:

6.4.3.1. Apply the dilate function and compute the contours[33] of ROI ***Ri***. Use the functions *dilate* and *findContours* from the OpenCV[19] library (see the scripts in the **Supplementary File**).

6.4.3.2. Compute the hull area[34] ***hi*** in the number of pixels. Use the function *convexHull* from the OpenCV[19] library (see the scripts in the **Supplementary File**).

6.4.3.3. Compute the radius[35] ***ri*** of the ROI ***Ri***. Use the function *minEnclosingCircle* from the OpenCV[19] library (see the scripts in the **Supplementary File**).

6.4.3.4. Compute the solidity ***si*** of the ROI ***Ri***. Solidity is the ratio of the contour area (obtained in step 6.4.3.1) to its convex hull area (obtained in step 6.4.3.2) of the *Ri*.

6.4.3.5. Compute the aspect ratio ***ai*** of the ROI ***Ri***. Aspect ratio is the ratio between the width and the height of the *Ri*-bounding rectangle. The bounding rectangle is computed using the function *boundingRect* from the OpenCV[19] library.

6.4.4. Select a reduced set of ROIs as a candidate to contain the animals, by adjusting the properties for hull area, radius, solidity, and aspect ratio.

6.4.4.1. Check if ***hi*** is less than *500.0* or greater than *100000.0*. If so, discard the ROI ***Ri***. Otherwise, keep the ***Ri*** as a candidate ROI for the animal location.

6.4.4.2. Check if the ***ri*** is less than *40.0*. If so, discard the ROI ***Ri***. Otherwise, keep the ***Ri*** as a candidate ROI for the animal location.

6.4.4.3. Check if the ***si*** is less than *-4.0* discard the ROI ***Ri***. Otherwise, keep the ***Ri*** as a candidate ROI for the animal location.

6.4.4.4. Check if the ***ai*** is less than *0.15* or greater than *4.0*. Is so, discard the ROI ***Ri***. Otherwise, keep the ***Ri*** as a candidate ROI for the animal location.

NOTE: The use of ROIs reduces the computational cost, focusing the tag search on the animal's body region. Animal detections from previous frames are included to avoid wrong detections when the animals are not moving.

6.4.5. Analyze the animal ROIs to determine the tag identities. Execute de following steps for each ROI ***Ri*** and for each internal ROI ***Pi***, and extract the internal ROIs ***P***.

6.4.5.1. Binarize the grayscale image ***Pi*** using the *Otsu*[36] *thresholding* algorithm.

6.4.5.2. Compute the contours[33] of ***Pi***, as in step 6.4.3.1.

6.4.5.3. Compute the hull area[34] ***hi*** and the aspect ratio ***ai***, as in steps 6.4.3.2 and 6.4.3.5.

6.4.5.4. Compute the shape *moments*[37,38] ***mi*** of ***Pi***. Use the function *moments* from the OpenCV[19] library (see the scripts in the **Supplementary File**).

6.4.5.5. Select a reduced set of ROIs as a candidate to contain the tags, using the following criteria.

6.4.5.5.1. Check if ***hi*** is less than *150.0* or greater than *500.0*. If so, discard the ROI ***Pi***. Otherwise, keep the ***Pi*** as a candidate ROI for the tag location.

6.4.5.5.2. Check if the ***ai*** is less than *0.5* or greater than *1.5*. If so, discard the ROI ***Pi***. Otherwise, keep the ***Pi*** as a candidate ROI for the animal location.

6.4.5.5.3. Check if the ***mi*** is greater than *0.3*. If so, discard the ROI ***Pi***. Otherwise, keep the ***Pi*** as a candidate ROI for the animal location.

6.4.6. Classify the tag ROIs. Approximate a polygon[39] using the OpenCV[8] library for each selected ROI ***Pi***[19].

6.4.6.1. Check if there are exactly three vertices in the approximated polygon; assign the tag to the **triangle** class. Otherwise, assign the **circle** class to the tag region.

NOTE: Approximated polygon is stored using a matrix with the vertices.

6.4.6.2. Check the central pixel of the ROI **Pi**. If it is a **black** pixel, assign the ***Pi*** to the **holed** class. Otherwise, assign the **Pi** to the **white** class.

NOTE: The shape center is deduced from the moments calculated in step 6.4.5.4. Search the black pixels in an area of a 4-pixel radius around the center.

6.5. Save the frame data: frame date, frame time, shape class, x center shape coordinate, and y center shape coordinate.

6.6. Continue with the next frame or end the process (see **Figure 4** as a visual example of the script execution). See **Figure 5** below as a visual example of the working script steps and watch **Video 1** as an example of script functioning.

[Place **Figure 5** here]

REPRESENTATIVE RESULTS:

We manually constructed a subset of the experimental data to validate the automated video analysis. A sample size of 1,308 frames with a confidence level of 99% (which is a measure of security that shows whether the sample accurately reflects the population, within its margin of error) and a margin of error of 4% (which is a percentage that describes how close the response the sample gave is to the real value in the population) was randomly selected, and a manual annotation of the correct identification of ROIs and the correct identification of the tag within each ROI was performed. Note that a single frame may contain a variable number of ROIs within an undetermined range because some lobsters may be concealed inside the burrows or one ROI contains two or more animals or false detections.

The total number of animals in the 1,308 frames was 3,852 (manually annotated ROIs). The method revealed 3,354 animal detections. A total of 701 (21%) of these detections were false positives (*i.e.*, the number of ROIs where the lobster was confused with the background). Of the total number of animals counted, 2,653 detections (79%) were correctly matching (*i.e.*, the number of times the classifier correctly recognized the presence of a lobster in the detected regions; see also **Figure 6a, b**). With respect to the total 3,852 ROIs present in the 1,308 frames, the script detects 69% of the individuals.

Regarding the tag detection, the script identified 2,353 ROI candidates as tags (89% of the 2,653 detected regions with animals). The classifier successfully identified as class tag 1,808 of these tags (in which the candidate is classified as a circle, triangle, holed circle, or holed triangle) and missed 545 cases (23% of the 2,353 ROI candidates for tag). Related to the tag classification, 1,619 are correctly identified (89.5%, **Figure 6f**). Only 70 tags where wrongly classified (3.8% error, **Figure 6e**), and the remaining 119 (6.6%) corresponded to false positives (internal ROIs identified as tag that corresponded to animal parts, such as claws; **Figure 6d**).

[Place **Figure 6** here]

After the video analysis was completed, the obtained positions (X, Y) data can be used to evaluate different behavioral patterns of the lobsters. For example, we plotted a space occupancy map using two-dimensional kernel density estimation with an axis-aligned bivariate normal kernel, evaluated on a square grid[41,42] with the best performance are automated estimated by the statistical algorithm. A higher color intensity represents the areas were the lobsters spent a higher percentage of their time (**Figure 7**). **Video 2** gives a visual example of animal tracking.

Another example is represented by the daily activity rhythms of the lobsters, plotted as millimeters and covered at 10 min binned time intervals (**Figure 8**). We removed the data corresponding to the first 24 h of the experiment, which corresponded to the animals' environmental adaptation process.

[Place **Figure 7** here]

[Place **Figure 8** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Facility acclimation views. (**a**) Tank shelves. (**a1**) Seawater input. (**a2**) Fluorescent ceiling lights. (**b**) Detail of blue light illumination. (**c**) Animal cell detail. (**d**) Detail of an isolated facility control panel. (e) Temperature setting for one of the entrances.

Figure 2: The four tags used for the individual tagging of the lobsters. Circle, circle-hole, triangle, triangle-hole.

Figure 3: Experimental setup. (**a**) Diagram of the assembly of the experimental tank and video acquisition. (**b**) General view of the experimental tank. (**c**) Bottom view of the experimental tank, indicating the artificial burrows. (**d**) Top view, showing the bottom of the experimental tank. (**e**) Detail of one of the burrow entrances.

Figure 4: Raw video frame. An example of a representative frame from one of the time-lapse videos collected during the experiments. At the upper right corner, we show the time stamp with the date, time, and frame. Notice the differences in the tank illumination in the image's lower

corner.

Figure 5: Relevant steps of the video-processing script. (**1**) Evaluate the background subtraction motion over the mean of the last 100 frames. (**2**) Result of the background subtraction algorithm. (**3**) Apply a dilate morphological operation to the white-detected areas. (**4**) Apply a fix, static, main ROI; the yellow polygon corresponds to the bottom tank area. (**5**) Calculate contours for each white-detected region in the main ROI and perform a structural analysis for each detected contour. (**6**) Check structural property values and, then, select second-level ROI candidates. (**7**) Binarize the frame using an Otsu thresholding algorithm; the script works only with second-level ROIs. (**8**) For each binarized second-level ROI, calculate the contours of the white regions and perform a structural analysis for each detected contour. (**9**) Check the structural property values and, then, select internal ROI candidates. (**10**) For each contour in the internal ROI candidate, calculate the descriptors/moments. (**11**) Check if the detected shape matches with the model shape and approximate a polygon to the best match candidates. (**12**) Check the number of vertices of the approximate polygon and determine the geometric figure: circle or triangle. (**13**) Calculate the figure center and check if black pixels occur; if yes, it is a holed figure. (**14**) Visual result after frame analysis.

Figure 6: Representative views from frames showing the most common experimental situations during video analysis. (**a**) Wrong animal detection, a background area is detected. (**b**) Animal misdetection. Two animals are close together and only one is detected. (**c**) Shape misdetection. The animal is detected (blue rectangle) but the tag is not detected. (**d**) Fake shape detection. Two shapes are detected, one is a claw. (**e**) Incorrect classification of a shape. A triangle is classified as triangle-hole. (**f**) Ideal situation. All animals are detected, and the tags are correctly identified.

Figure 7: Space occupancy map. The chart only shows the bottom tank area that is the animal displacement area (see the yellow polygon in **Figure 5**). The areas were the different tagged lobsters spent more time appear colored; a higher color intensity means more occupancy time.

Figure 8: Daily activity rhythms of the lobsters plotted as millimeters and covered at 10 min binned time intervals. Grey bands indicate the hours of darkness at 12/12 light/darkness, with the sunset time starting at 7.00 a.m. and the sunrise time starting at 7.00 p.m.

Figure 9: Detail of frame binarization errors. A red circle shows how lobsters and tags are detected as a unique object.

Video 1: Desktop record of an example of a running video analysis script. The video shows in 2 min and 27 s 1 h of real-time footage (3,625 frames). Notice that there is no error accumulation for the animal and tag misdetections and unidentified events while the recording is being made.

Video 2: Video of the animal tracking after the locomotor analysis. We used X, Y image pixel coordinates obtained from the video analysis and stored them into the database, to draw the animal track in the recorded videos as an example of the video analysis script. The longer the track, the faster the animal moves and the more distance traveled. In this case, 30 s of video corresponds to 12 min of real-time.

DISCUSSION:

The performance and representative results obtained with the video-tracking protocol confirmed its validity for applied research in the field of animal behavior, with a specific focus on social modulation and circadian rhythms of cohoused animals. The efficiency of animal detection (69%) and the accuracy of tag discrimination (89.5%) coupled with the behavioral characteristics (*i.e.*, movement rate) of the target species used here suggest that this protocol is a perfect solution for long-term experimental trials (*e.g.*, days and weeks). Moreover, the protocol offers the basic advantage of being easy to use and faster in its development and customization with respect to other techniques, such as automatic learning algorithms and neural networks[43]. The tracking techniques used here represent the final refinement of an experimental activity started with a slightly different approach[44].

A critical step in the protocol is the tag design; it should be considered that the implementation of other tag designs could improve the performance of the Otsu binarization. For example, one of the sources of error reported here was the misdetection between the black outside circle in the tag and the white internal geometric form (see **Figure 9** with a binarized frame with a detail of this error). It is possible to improve the binarization process, increasing the diameter (2 - 3 mm) of the black circle outside the white internal geometric form, or checking the colors (white/black). We do not consider the use of image morphological functions like *erode* or *dilate* when trying to correct this error, given that these operations modify the structural properties of the tag imaged, being, therefore, not possible to maintain the threshold values of the script. In conclusion, it is advisable to adapt the tag design to the target animal species anatomy. That would involve the adjustment of the script threshold values and the structural properties according to the new design.

[Place **Figure 9** here]

The most relevant source of errors was the missed detection of the ROIs (both external and internal). The video analysis script presented here is only able to keep track of individuals that are not moving or are hidden for a period of fewer than 100 frames; to avoid problems with this, we stored the last position of an individual until it is detected again. This parameter might influence the results of missing immobile or hidden animals. This fact must be taken into account when using this protocol with species showing different mobility rates than the ones presented here for the lobsters. The video frame rate and analysis script should be modified and adjusted to the species used according to its specific behavior.

One major challenge was to obtain a monochromatic blue (472 nm) and IR (850 nm) illumination, to avoid the possibility of retinal damage and adjust the light environmental conditions to the animal's habitat[23,45]. Therefore, the color component in video recording is not relevant and video recordings were performed in grayscale. The system helps to program different light time periods and modifies the illumination system according to the target species' characteristics changing the

LED's light spectrum. Another customization to consider in the protocol presented here is the movement rate of the target animal. For this specific case, the frame rate used was 1 frame per second, producing video files of about 1 h length corresponding to 24 h of experimental recording. These two customizations (grayscale and frame rate) allowed us to obtain video files with a reduced size that were easy to work with and reduced the storage capacity and machine time for tracking.

A limitation of the described method is that it has only been tested with the species presented here; however, there are no specific reasons concerning the application of this protocol to other species that allow the carrying of identification tags. Another significant limitation is that the protocol is not suited to track the movements of animal appendices (*e.g.*, chelae). For example, decapod crustaceans use chelae movements to display dominance among conspecifics. Future implementations are aimed at improving this aspect.

The proposed protocol resembles previous existing commercial software[46] and published methods SwisTrack and idTracker[7,47]. The commercial software[46] uses background subtraction to detect animals, similar to the scripts presented here. Although it covers a wider spectrum of applications, it is programmed using a commercially interpreted program language[48], which is not an open source solution and is economically costly. The SwisTrack[47] method uses the OpenCV library, similarly to the approach presented here. Nevertheless, it is coded in C++. We used Python code, which is usually easier to adapt to the particular needs of each environment. IdTracker[7] is a strong proposal coded in a commercially interpreted program language[48] but targets nonmarked animal applications. The correct outcomes of the tracking can be compromised when animals are occluded for a long period of time as occurs in the experimental conditions presented here. The method presented here processes each frame independently and is not influenced by the previous trajectory of the animal. Therefore, an error in a specific frame does not propagate to future frames. This fact is relevant in this application but also constraints the method presented here to a specific set of animals (those that allow manual tagging).

Another aspect to consider is that we have used free software during the development of the protocol, including the postprocessing and storage of the data generated by the video analysis script, as well as the code used to control the lighting system. The processed data are stored in a free relational database system (MySQL). These processed data can be obtained through queries in Standard Query Language (SQL) according to the desired format. The reader can modify and adapt the proposed open code and freely adapt it to particular needs.

With regard to the method toxicity, the only delicate step is the gluing of the tag to the animal. We used cyanoacrylate glue due to its low toxicity, its wide medical use[28], and its wide use in aquaria for fragging corals and fixing the fragments with glue[29]. The major concern about its use is the vapor toxicity for humans. We reduced the exposition to the minimum. The Health and Safety Executive and the United States National Toxicology Program have concluded that the use of ethyl cyanoacrylate is safe[49].

Future applications of this protocol are the automation of the detection of other behaviors of burrowing crustaceans (*e.g.*, fights, burrow dominance). We also plan to improve the algorithm to obtain real-time video analysis and to use Convolutional Neural Networks[50] for improved animal detection.

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DISCLOSURES:

The authors have nothing to disclose.

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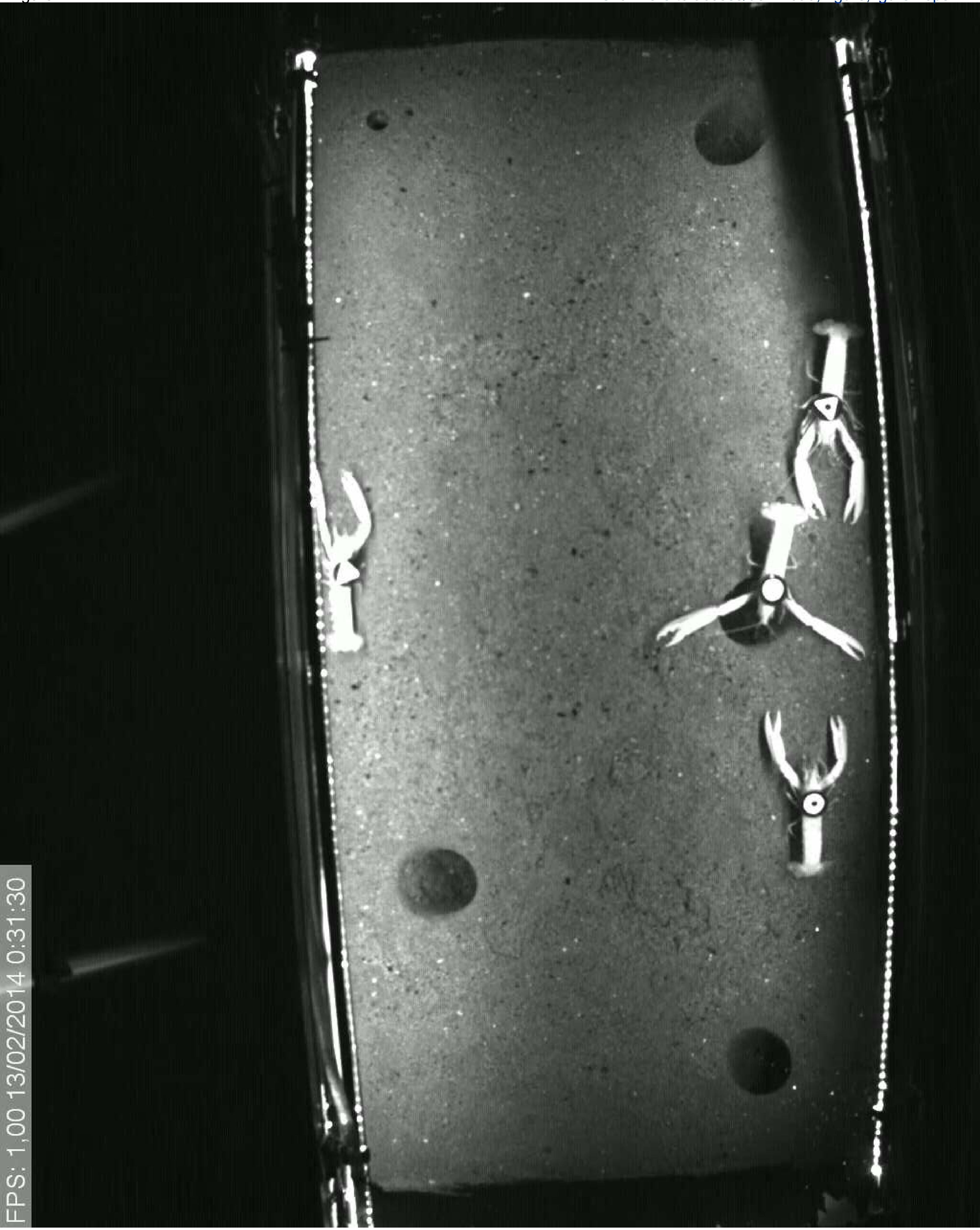
FPS: 1,00 13/02/2014 0:31:30

Figure 5

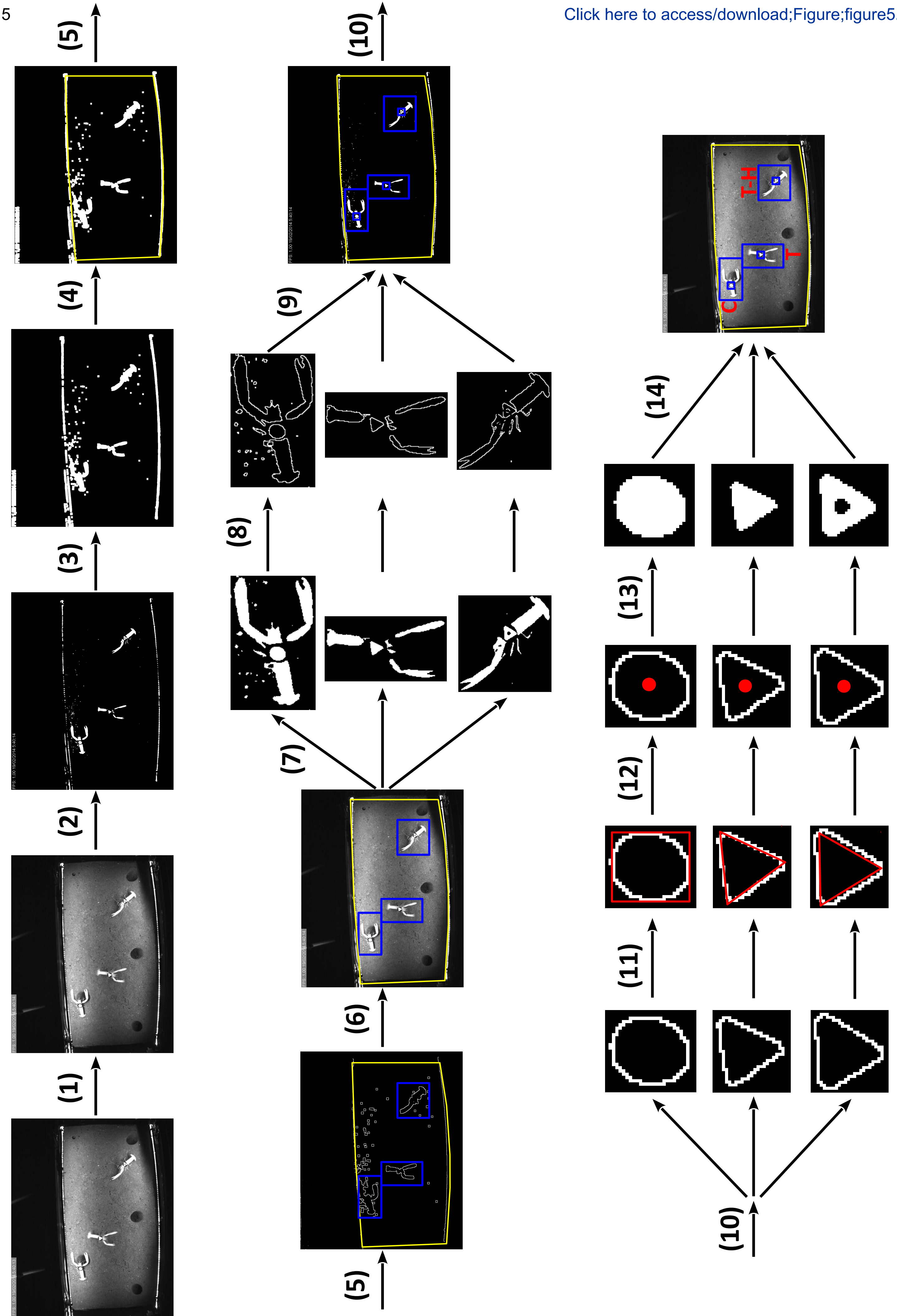

Figure 6

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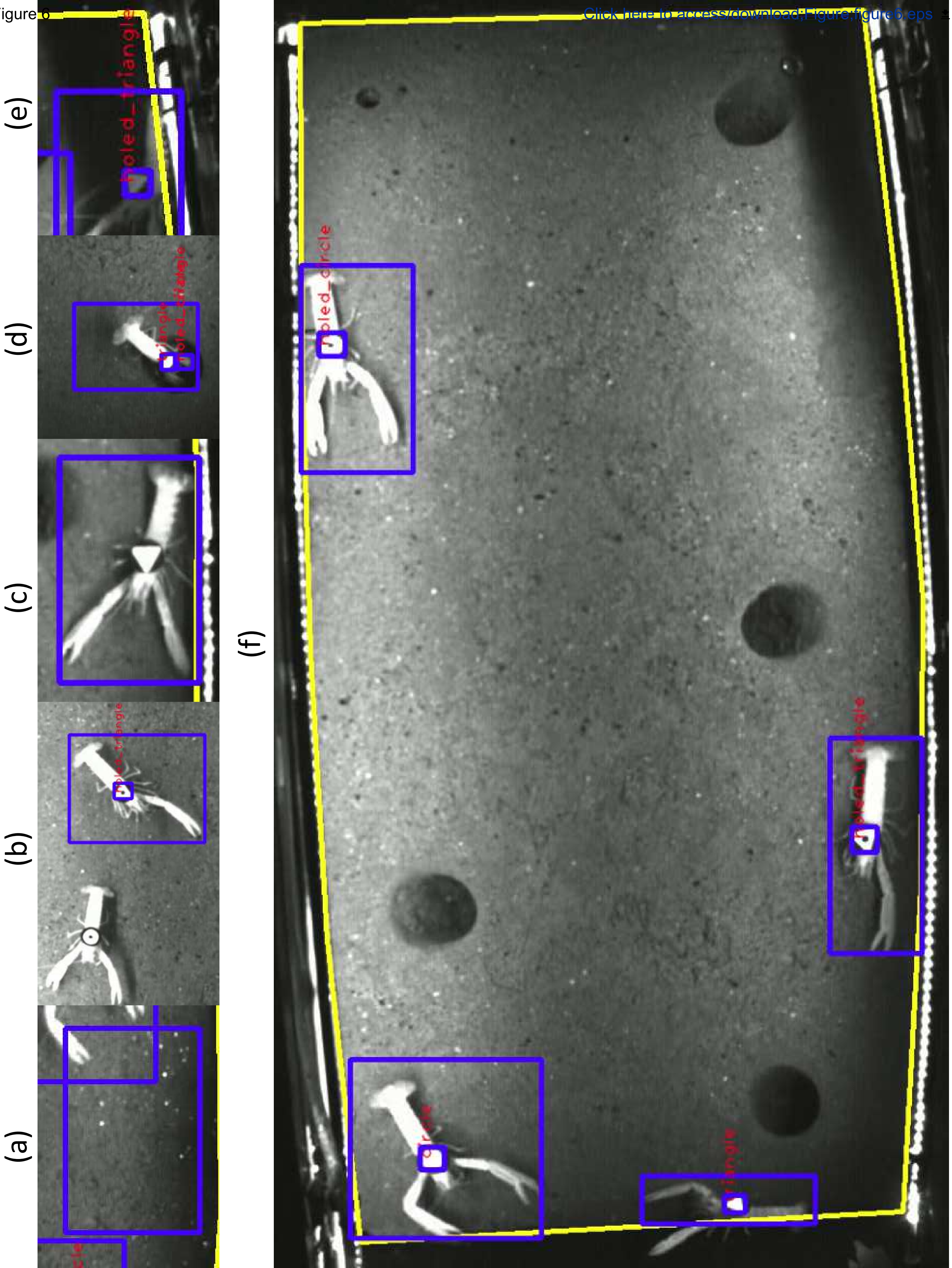
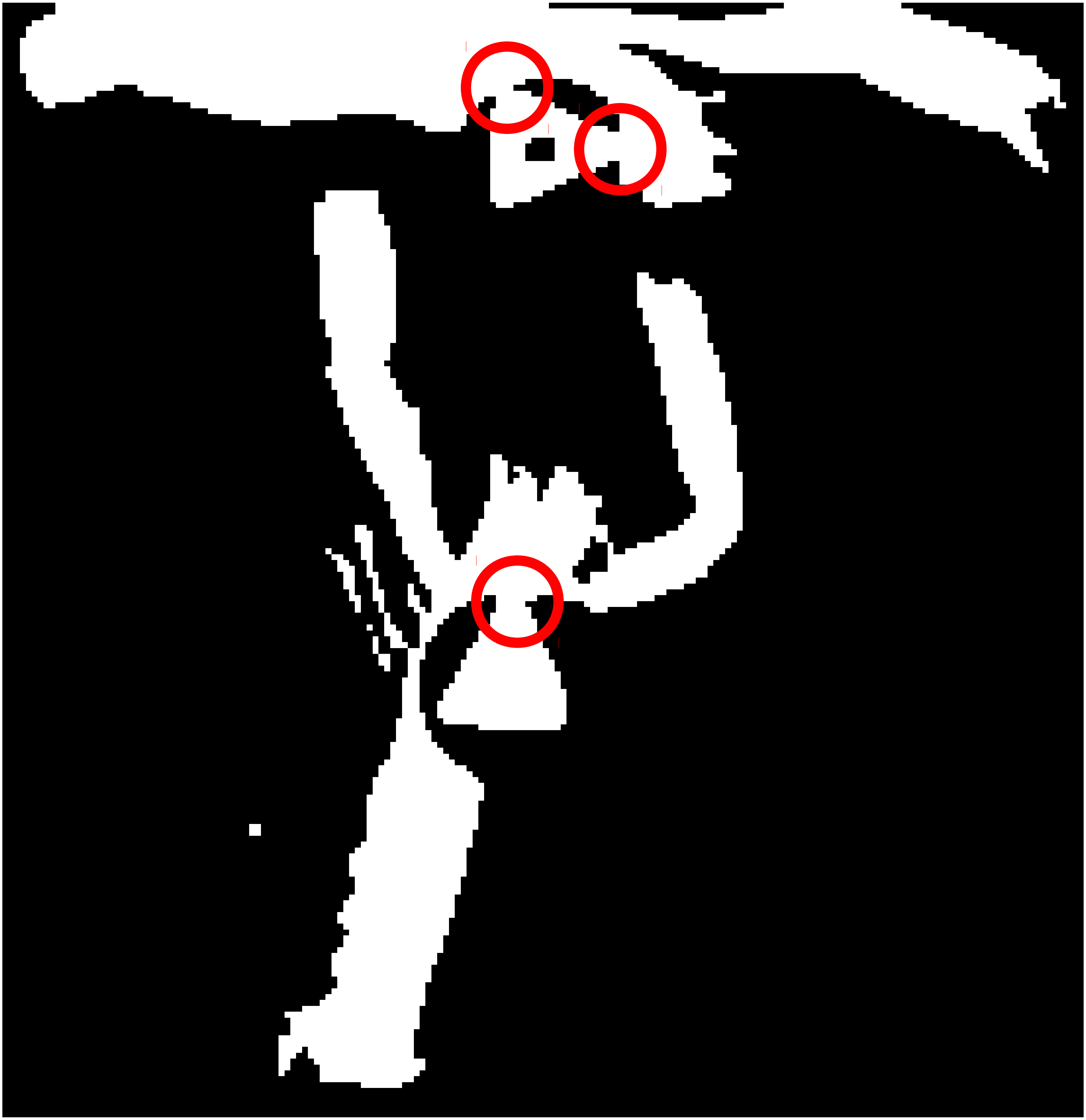


Figure 9



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Tripod 475	Manfrotto	A0673528	Discontinued
Articulated Arm 143	Manfrotto	D0057824	Discontinued
Camera USB 2.0 uEye LE	iDS	UI-1545LE-M	https://en.ids-imaging.com/store/products/came
Fish Eye Len C-mount f=6mm/F1.4	Infaimon	Standard Optical	https://www.infaimon.com/es/estandar-6mm
Glass Fiber Tank 1500x700x300 mm			
Black Felt Fabric			
Wood Structure Tank			5 Wood Strips 50x50x250 mm
Wood Structure Felt Fabric			10 Wood Strips 25x25x250 mm
Stainless Steel Screws			As many as necessary for fix wood strips structur
PC			2-cores CPU, 4GB RAM, 1 GB Graphics, 500 GB HI
External Storage HDD			2 TB capacity desirable
iSPY Sotfware for Windows PC	iSPY		https://www.ispyconnect.com/download.aspx
Zoneminder Software Linux PC	Zoneminder		https://zoneminder.com/
OpenCV 2.4.13.6 Library	OpenCV		https://opencv.org/
Python 2.4	Python		https://www.python.org/
Camping Icebox			
Plastic Tray			
Cyanocrylate Gel			To glue tag's
1 black PVC plastic sheet (1 mm thickness)			Tag's construction
1 white PVC plastic sheet (1 mm thickness)			Tag's construction
4 Tag's Ø 40 mm			Maked with black & white PVC plastic sheet
3 m Blue Strid Led Ligts (480 nm)			Waterproof as desirable
3 m IR Strid Led Ligts (850 nm)			Waterproof as desirable
6m Methacrylate Pipes Ø 15 mm			Enclosed Strid Led
4 PVC Elbow 45^{o} Ø 63 mm			Burrow construction
3 m Flexible PVC Pipe Ø 63 mm			Burrow construction
4 PVC Screwcap Ø 63 mm			Burrow construction
4 O-ring Ø 63 mm			Burrow construction
4 Female PVC socket glue / thread Ø 63 mm			Burrow construction
10 m DC 12V Electric Cable			Light Control Mechanism
Ligt Power Supply DC 12V 300 w			Light Control Mechanism

MOSFET, RFD14N05L, N-Canal, 14 A, 50 V, 3-Pin, IPAK (TO-251)	RS Components	325-7580	Light Control Mechanism
Diode, 1N4004-E3/54, 1A, 400V, DO-204AL, 2-Pines	RS Components	628-9029	Light Control Mechanism
Fuse Holder	RS Components	336-7851	Light Control Mechanism
2 Way Power Terminal 3.81mm	RS Components	220-4658	Light Control Mechanism
Capacitor 220 µF 200 V	RS Components	440-6761	Light Control Mechanism
Resistance 2K2 7W	RS Components	485-3038	Light Control Mechanism
Fuse 6.3x32mm 3A	RS Components	413-210	Light Control Mechanism
Arduino Uno Atmel Atmega 328 MCU board	RS Components	715-4081	Light Control Mechanism
Prototipe Board CEM3,3 orific.,RE310S2	RS Components	728-8737	Light Control Mechanism
DC/DC converter,12Vin,+/-5Vout 100mA 1W	RS Components	689-5179	Light Control Mechanism
2 SERA T8 blue moonlight fluorescent bulb 36 watts	SERA		Discontinued / Light isolated facility

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Barcelona, 10 August 2018

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE

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 (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 (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 photoreceptors13. 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 (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.

Barcelona, 9 October 2018

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear Ph.D. Vineeta Bajaj,

Please find herewith enclosed our revision of the manuscript JoVE58515R1 “Long-term video tracking of cohoused aquatic animals: a case study with daily locomotor activity in the Norway lobster (*Nephrops norvegicus*)” for you have recommended some revisions.

We appreciate very much your comments and as requested, we are including below a list of changes made to the manuscript in response to your suggestions and comments.

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

Editor comments and suggestions:

Line 26: Please have a minimum of 6 keywords.

We added a new keyword.

Line 29: Please rewrite the summary to bring out the goal of the protocol in alignment with the highlighted steps. Max 50 words.

We modified the text according the instructions.

Lines 65 – 66: Please check this part?

We corrected the text.

Line 97 – 99: Please check this sentence.

We rewrote the sentence.

Line 146: Please do not use personal pronouns throughout the protocol section.

We modified the text according the instructions.

Line 196: Please do not use personal pronouns throughout the protocol section.

We modified the text according the instructions.

Lines 220 – 221: What is the correct position.

We modified the text to add this information.

Lines 227 – 229: Please do not use personal pronouns throughout the protocol section.

We modified the text according the instructions.

Lines 254 – 258: Some parts of the notes can be moved to the discussion section.

We modified the text changing this note to the discussion section.

Line 310: Citation.

We added a geometry general book as citation:

Audin, M. *Geometry*. Springer Berlin Heidelberg :, Imprint: Springer. Berlin, Heidelberg. (2003).

Line 314: Is this all done by scripting? If there is a graphical user interface, button clicks in the software, please provide the details.

Yes, all is done by scripting. We added all cited OpenCV functions calls in the steps.

Lines 316 – 319: Please do not use personal pronouns throughout the protocol section.

We modified the text according the instructions.

Line 330: Explain how you do the each step described under. Also provide all the scripts as a supplementary file.

The scripts are already provided as supplementary material. We added this information in the script step.

Lines 332 – 333: How?

We added the OpenCV function called, in order to better explain this step.

Lines 335 – 337: How?

We added the OpenCV function called, in order to better explain this step.

Lines 341 – 342: How? e.g., Click **Ri** to compute the dilate function.

We added the OpenCV function called, in order to better explain this step.

Line 344: How?

We added the OpenCV function called, in order to better explain this step.

Lines 346 – 348: How?

We added the OpenCV function called, in order to better explain this step.

Line 353: How?

We added the OpenCV function called, in order to better explain this step.

Lines 521 – 529: Please refer video 1 and 2 in the manuscript as well.

We added the OpenCV function called, in order to better explain this step.

Lines 587 – 599: Are these open access? If not, we cannot have commercial languages in our manuscript. Please use generic terms only.

We modified the text according the instructions, we avoid mentioning commercial software and idTracker and SwisTrack are already published in scientific journals.

Line 593: Please remove commercial language from the manuscript and refer it in the table of materials.

We modified the text according the instructions.

Video Analysis Python Script

Click here to access/download

Supplemental Coding Files

nephrops_tracking_script.py

Light Control Arduino Script

Click here to access/download
Supplemental Coding Files
ritfim_light_control_script.ino