НАУЧНЫЕ СООБЩЕНИЯ

COMPUTER METHODS
FOR DETERMINING *MNEMIOPSIS LEIDYI* MOTILITY CHARACTERISTICS

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The paper considers capacities of common computer programs for analyzing the behavioral reactions to light of ctenophores, marine planktonic animals, under laboratory conditions. We showed that most image analysis programs do not automatically determine body contours of translucent objects, such as ctenophores. We developed a new method for determining basic motility characteristics of *Mnemiopsis leidyi* using ImageJ, Davinci Resolve video filters, and scripts written by the us. The developed method allows automatic calculating of such parameters as average and maximum speed, changes in direction of movement (locomotion vectors), and the percentage of time animals spend in certain regions of interest (ROI). The average speed of ctenophore movement was estimated in millimeter scale with high precision. The method is applicable for studying the behavior of various translucent marine animals. Computer scripts are available by request from the authors.

Keywords: *Mnemiopsis leidyi*, ctenophore, migration, motility characteristics, image analysis tools, ImageJ, wrMTrck_Batch, regions of interest (ROI), threshold

The species *Mnemiopsis leidyi* A. Agassiz, 1865 (Fig. 1) belongs to a distinct phylum Ctenophora. These planktonic predatory marine animals have eight meridional rows of locomotory ciliary comb plates (ctenes). Ctenophores are the largest animals swimming by means of cilia. Synchronous movement of cilia is coordinated by nervous system (Ringelberg, 2009 ; Schnitzler et al., 2012).

Ctenophores can sense the direction of gravity and use it to modify their behavior and often assume a vertical position with their oral–aboral axis parallel to the gravity vector. A recent molecular-genomic study showed co-expression of photoprotein and opsin genes in developing photocytes of *Mnemiopsis* that suggests that light production and light reception are linked (Ruppert et al., 2004). Changes in the level of illumination in a water column are considered to be the key factor regulating diel vertical migrations. There are some evidences of diel vertical migrations of *Mnemiopsis leidyi* (Kideys & Romanova, 2001 ; Mutlu, 1999 ; Wilson-Leedy & Ingermann, 2007 ; Zaika & Sergeeva, 1991) but the mechanisms and factors determining and triggering diel vertical migrations remain unclear (Haraldsson et al., 2014 ; Schnitzler et al., 2012).

The main reason for daily diel vertical migrations of most planktonic organisms from lighted surface layers to deeper waters is to avoid visually hunting predators (Hays, 2003 ; Ringelberg, 1995, 2009). Light, temperature, and food concentration can also affect migration patterns (Haney, 1988 ; Ringelberg, 1995).

Fig. 1. An adult Black Sea ctenophore *Mnemiopsis leidyi*
Рис. 1. Взрослая особь черноморского гребневика *Mnemiopsis leidyi*

The behavioral responses to light of marine animals could be studied by registration and analysis of their motility characteristics in a water column under laboratory conditions. Most of recent computer programs that provide general characteristics of the animals' movement [such as speed, distance, and time spent in regions of interest (hereinafter ROI)] use threshold filters to determine the contours of an object on video records (Franco-Restrepo et al., 2019). With the threshold method, moving contrasting objects over their entire surface are painted in black on a white background (the color scheme is converted into a binary) (Alsaif & Hamid, 2018). However, there are some issues for photo and video recording and further analysis of the movement of translucent animals because it is impossible to automatically select their fuzzy body contours. *Mnemiopsis leidyi* body is almost transparent since it consists of a relatively thick, jelly-like mesoglea (gel composed of water, cells, and collagen) sandwiched between two epithelial layers (Ringelberg, 1995). On the other hand, ctenes can possess light diffraction which can also complicate the automatic determination. This may be one of the reasons for the lack of experimental research in the behavioral reactions of this species.

The aim of the present work is to develop methods for movement registration and automated computer assessment of basic locomotion characteristics of the ctenophore *Mnemiopsis leidyi* in laboratory aquariums, such as average and maximum speed, changes in direction of movement (locomotion vectors), and the percentage of time animals spend in certain ROI, on the example of the investigation of the light effect on their behavior.

MATERIAL AND METHODS

Experimental design. Adult *Mnemiopsis leidyi* were sampled from the natural habitats in the Martynova Bay (Sevastopol, the Black Sea) and then transported to the laboratory, where those were held in 200-L (length 92 cm, width 55 cm, and height 40 cm) transparent tanks equipped with a flowing seawater system. For 12 h, ctenophores were acclimated to laboratory conditions. The water temperature in the aquariums corresponded to that in the sea. During the investigation, ctenophores were kept in still water because we experimentally found that even a minimal duct masks the animals' own movements. For each experiment ($n = 21$), three animals of different size were placed in the aquarium. Adult *Mnemiopsis* were divided into three size groups: large (with body length over 80 mm), medium-sized (with body length 60 to 80 mm), and small (body length less than 60 mm). Animals moved freely in a water column almost without contact with each other.

Experiments were conducted in a dark room. A light source was placed on a side wall of the aquarium, so that the upper third of the tank was illuminated. Light irradiance was measured with an irradiance sensor QSL-2101 (Biospherical Instruments Inc.). Four lighting regions (ROI) were distinguished in a water column: a region with relatively high illumination, 12 μmol quanta·m^{-2}·s^{-1} (1); with medium illumination, 5 μmol quanta·m^{-2}·s^{-1} (2); with low illumination, 2 μmol quanta·m^{-2}·s^{-1} (3); and a dark region, 0.5 μmol quanta·m^{-2}·s^{-1} (4) (Fig. 2). Control experiments were carried out in a completely dark room with no light source, *Mnemiopsis* speeds were evaluated visually, and the animals were almost motionless.

The movements of ctenophores were recorded in time-lapse mode (1 frame in 2 seconds; 300 frames in 10 minutes) using a camera mounted perpendicular to a front wall of the aquarium. We used a Nikon D5300 camera with a Nikon AF-S Micro-Nikkor 40mm lens and a Hoya 52mm PL polarizing filter. After turning on the light, the experiment was carried out during the time intervals as follows: 1–10, 31–40, and 51–60 minutes.

Fig. 2. Regions of interest (ROI): 1, a region with high level of illumination; 2, with medium level of illumination; 3, with low level of illumination; 4, a dark region

Рис. 2. Выделение зон интереса (ROI): 1 — зона с высоким уровнем освещения; 2 — со средним уровнем освещения; 3 — с низким уровнем освещения; 4 — неосвещённая нижняя область

Hardware. We used computer equipment with the following parameters: MacBook Pro (13-inch, 2017, two Thunderbolt 3 ports). Processor: 2.3 GHz, Intel Core i5. RAM: 8 GB, 2133 MHz, LPDDR3. Graphics processor: Intel Iris Plus Graphics 640, 1536 MB. Operating system: macOS High Sierra 10.13.6.

Video analysis. The images were processed with the free access Davinci Resolve software (https://www.blackmagicdesign.com). Time-lapse frames for each experiment were combined in a video file with a frequency of 25 frames *per* second. The contour of an individual animal was manually selected on the first frame – using the "tracking" mask in the Davinci Resolve. For each animal in the aquarium, the contour determination process was repeated.

Sometimes the contours of the "tracking" masks can "jump" to the designated body contouring, incorrectly determining the trajectory of the animal movement. In such cases, the position of the mask has to be corrected manually.

The contrast of the image is adjusted using two color filters ("Brightness and Contrast" and "Gamma Correction") so that the animal is painted in white on a black background. Only one animal was visualized on the video record (Fig. 3).

To reduce the number of surveys and increase statistical significance, several animals were placed in experimental aquariums at once. Animal trajectories may overlap while moving, and animals overshadow each other; in this case, automatically detected object contours "jump" from animal to animal. In one video file, the movement of several animals was monitored using several "tracking" masks. Video files were saved as separate uncompressed AVI files for each animal (one mask – one animal – one video file) (Fig. 3) and analyzed in the ImageJ program (https://imagej.nih.gov/ij) using the installed wrMTrck_Batch plugin (http://www.phage.dk/plugins/wrmtrck.html) (Mutlu, 1999) which allows saving TXT files with X-Y coordinates of objects on each frame.
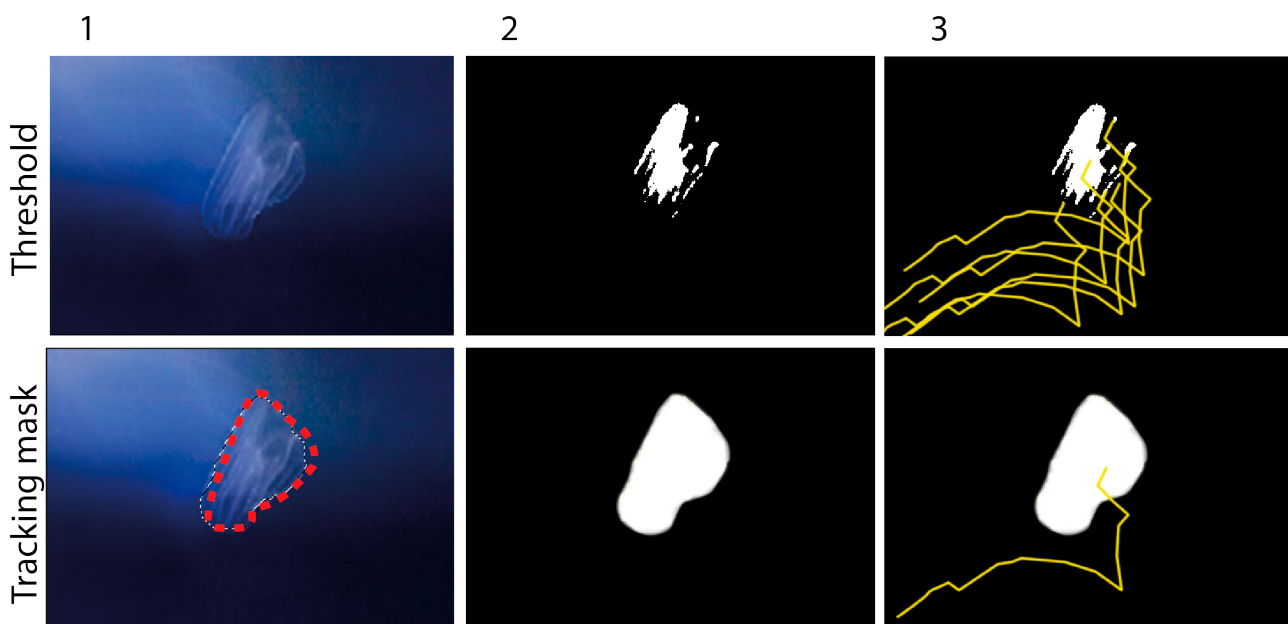

Fig. 3. Two methods for tracking *Mnemiopsis leidyi* movement during a video recording (enlarged video fragment is shown). With the threshold filter: 1, color video scheme converted to 8-bit; 2, application of the threshold filter; 3, plotting of the track of one of the objects. With the developed "tracking" mask method: 1, designation of the borders of a ctenophore body using a "tracking" mask in the Davinci Resolve program on the first frame of the video; 2, use of filters and conversion of the color scheme into a binary; 3, determination of a moving object and construction of its motion track for the entire time of movement in the AnimalTracker plugin

Рис. 3. Два метода отслеживания перемещения гребневика *Mnemiopsis leidyi* в течение видеозаписи (приведён увеличенный фрагмент). С помощью фильтра threshold: 1 — цветовая схема видеофайла преобразована в 8-битную; 2 — применение фильтра threshold; 3 — построение трека одного из объектов. С помощью разработанного метода «следящей» маски: 1 — обозначение границ тела гребневика с помощью «следящей» маски в приложении Davinci Resolve на первом кадре видео; 2 — применение фильтров и перевод цветовой схемы в бинарную; 3 — определение подвижного объекта и построение трека его движения за всё время в плагине AnimalTracker

The scripts processing TXT data files. The scripts processing TXT data files were written to obtain the motion characteristics (such as speed, distance, and time spent in ROI) of the monitored objects (obtained in ImageJ). Bash (version 3.2.57(1)-release) (x86_64-apple-darwin17) and the programming language Python (version 3.6.7) were used. The software pipeline was designed and tested for Linux and MacOS operating systems.

Input:
- path to the directory containing files with motion coordinates;
- file path with coordinates describing ROI;
- path to the directory for generating results.
  Output:
- files (TXT) for each track containing the original coordinates, ROI, difference of coordinate values between frames, instantaneous speeds, and motion vectors;
- a file that combines the results of all tracks (TXT);
- a file containing average speeds for each track (TXT).
  The bash-script includes:
- receiving input data;
- definition of files with coordinates in the directory (*raw.txt);
- running a Python script for each file;
- combining results.
  The Python script implements the following computational steps:

$$X = \{x_i\}; \; i = \overline{1,l}; \; Y = \{y_i\}; \; i = \overline{1,l} \; , \tag{1}$$

where X and Y are set of coordinates for each frame;

l is a number of frames.

Calculation of difference of coordinate values between frames (Δx, Δy):

$$\forall i, \lfloor \Delta x_{i+1} = x_{i+1} - x_i, \; \Delta y_{i+1} = y_{i+1} - y_i \rfloor \; . \tag{2}$$

Calculation of motion vectors V (Fig. 4):

$$V = \begin{cases} \forall i, \lfloor |\Delta x_{i+1}| > |\Delta y_{i+1}| \to V[1] = "H", |\Delta x_{i+1}| > |\Delta y_{i+1}| \to V[1] = "V" \rfloor \\ \forall i, \lfloor |\Delta x_{i+1}| > 0 \to V[2] = "R", |\Delta x_{i+1}| < 0 \to V[2] = "L" \rfloor \\ \forall i, \lfloor |\Delta y_{i+1}| > 0 \to V[3] = "D", |\Delta y_{i+1}| < 0 \to V[3] = "U" \rfloor \end{cases}, \tag{3}$$

$$Z = \{z_j\}; \; j = \overline{1,m}; \; z_j = \{[zxs_j, zxf_j], [zys_j, zyf_j]\} \; , \tag{4}$$

where Z is the set of analyzed ROI;

zxs and zys are the zone beginning coordinates;

zxf and zyf are the zone end coordinates.

Calculation of ROI for each frame:

$$\forall i, j, \lfloor zxs_j < x_i \le zyf_j, \; zys_j < y_i \le zyf_j \to ROI = j \rfloor \; . \tag{5}$$

Instantaneous speed:

$$s_{i+1} = \sqrt{\Delta x_{i+1}^2 + \Delta y_{i+1}^2} \; . \tag{6}$$

Average speed:

$$avrs = \frac{\sum_{k=2}^{l} s_k}{l - 1} \; . \tag{7}$$

The program is patented: Kuleshova O. N., Bayandina Iu. S. CtenophoraTrack. Certificate of state registration of the computer program 2020661100 Russian Federation; no. 2020617476; declared 13.07.2020; publ. 18.09.2020, Bull. no. 9.
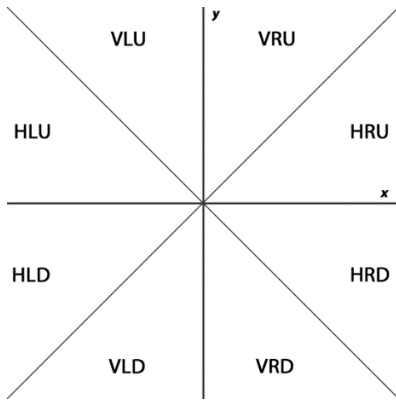
Fig. 4. Diagram of motion vectors in the X-Y coordinate system; letter combinations characterize motion vectors: H, horizontal movements; V, vertical; R, right; L, left; U, upward; D, downward movements

Рис. 4. Схема категориальных векторов направления движения в системе координат X-Y, буквенные обозначения характеризуют сочетание векторов движения: H — движение по горизонтали; V — по вертикали; R — вправо; L — влево; U — вверх; D — вниз

Statistics. To compare the data, analysis of variance ANOVA was applied. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

The developed method was successfully applied to determine the visible contours of ctenophores in a water column under various illumination. Using the macros and scripts, we analyzed the individual characteristics of the movement of the Black Sea ctenophores *Mnemiopsis leidyi*, such as average and maximum speed, changes in direction of movement (locomotion vectors), and the percentage of time animals spend in certain ROI. The average speed of ctenophore movement was calculated. The speed of large individuals is (0.54 ± 0.36) mm·s^{-1} ($n = 21$); of medium, (0.46 ± 0.38) mm·s^{-1} ($n = 21$); and of small, (0.84 ± 0.63) mm·s^{-1} ($n = 21$). During the first hour, the average speed was significantly higher for small-sized ctenophores compared to that of large and medium-sized animals. Most of the time, large individuals were in a highly lit area, avoiding an unlit one; medium and small animals migrated in a water column.

DISCUSSION

The type of software chosen for determining characteristics of animal movement depends on physical characteristics of the object and shooting conditions, as well as the quality of the resulting video material and its size.

Most programs used for determination of locomotion characteristics of biological objects were developed for investigating the behavior of mammals (Ringelberg, 1995) and fish (Larsen et al., 2015), for studying sperm motility (Tamm, 2014) and characteristics of other objects (Franco-Restrepo et al., 2019 ; Gulyás et al., 2016). In the most of software available, the threshold filter is used for distinguishing a moving object on the background. This filter replaces each pixel in the image with a black pixel (if the image intensity is lower than some fixed constant) or with a white one (if the image intensity is higher than this constant). For the precise determination, the object should be contrast to the background, have clear contours and uniform color, and be appropriately illuminated.

It was experimentally found that the methods of the threshold filter (such as the method based on the histogram shape, the method based on clustering, the method based on entropy, *etc.*) do not automatically determine body contours of translucent ctenophores in a water column. When converting the color scheme into a binary applying the threshold filter, individual parts of ctenophores remain distinguishable; programs count these parts as separate objects falsely identifying glare and irregular sections of lighting as moving objects; and the constructed track "jumps" from region to region (Fig. 3).

To estimate the effect of the illumination level on the change in a ctenophore behavior, it is necessary to determine contours of animal in heavily lit and darkened regions.

Applying the developed method with a "tracking" mask in Davinci Resolve, for better accuracy, it is possible to determine manually the body contour of an individual ctenophore, which differs it from special programs for tracking the movement of biological objects, and to track its movements during video recording in semi-automatic mode. The use of additional threshold algorithms is not required since the background is removed.

Video files processed in this way can be exported to almost any program for motion analysis. We tested programs for our purposes. Biological motion analysis programs have a number of benefits and limitations (Table 1).

Table 1. Comparison of programs for analyzing the movement of biological objects

Таблица 1. Сравнение программ анализа движения биологических объектов

Program	Types of analyses	Benefits	Limitations
EthoVision XT	Average speed, confidence intervals, area of objects, distances, and other motion characteristics	Real-time motion tracking. Ability to track multiple animals. Real-time track control	High cost. Need to purchase additional plugins. Specified types of analysis. Inability to save intermediate data
MouseMove	Distance, average speed, track curvature, and ROI	Free access. Plotting distance diagram, speed changes, *etc.*	Processes only one file at a time. Decreases the resolution of the source file
AnimalTracker	Total track length, average speed, trajectories, and percentage of time spent in ROI	Free access. Intuitive interface. Semi-automatic application of video filters and detection of moving objects. Flexible ROI setup. Saving coordinates	Processes only one file at a time
wrMTrck	Total distances, average speed, area, perimeter, and trajectories of objects. Coordinates, curvature of a track, *etc.*	Free access. The set of input parameters of the objects (their areas and changes over time, number of frames on which the object is monitored, maximum and minimum speeds, *etc.*). The movement of many objects in one video. Saving image files with motion tracks and background, video files with numbered objects	Processes only one file at a time. Does not determine the location of objects in ROI. Does not determine the motion vector
wrMTrck_Batch		All the benefits of wrMTrck. Batch processing of files. Automatic separation of the background and the use of the threshold filter. Automatic saving of the resulting tables with average values for all video files and for each file separately	Does not determine the location of objects in ROI. Does not determine the motion vector. Does not draw graphs of speeds and distances. Tracks are saved in raster format

EthoVision XT (information on acquisition options: https://www.noldus.com/animal-behavior-research/products/ethovision-xt) is an automatic, independent system designed to detect and analyze the movement of biological objects. EthoVision XT tracks the movement of one or more animals on video (in real time or pre-recorded). It has a variety of functions and types of motion analysis; it calculates average speed and confidence intervals, detects a change in direction of movement,

and builds speed diagrams. For analysis, most types of video files (MPEG-1, MP4, AVI, *etc.*) are available. There are many additional plugins for specific research tasks (for example, connecting to a microscope and tracking several objects), but each plugin is supplied for a fee. The basic version of the program has limited functionality. In addition to the high cost of the program and its plugins, the disadvantages include the inability to save intermediate data, such as X-Y coordinates, instant speeds, *etc.* In the program, image tracks cannot be saved as separate files. There is no option to remove the background automatically.

MouseMove is a free access program developed in the LabVIEW 12.0 system [to download the program, see electronic supplementary material to (Samson et al., 2015)]. To run MouseMove.exe, installing an additional LabVIEW file is required. Creating a folder with video for analysis, *inter alia* a background video without animals, is pre-necessary. Then, it is required to perform the preparatory action algorithm developed by the authors in the ImageJ program. MouseMove accepts video in AVI with M-JPEG compression at 25 frames *per* second, a resolution of 640×480, but the program reduces the resolution down to 320×240 pixels. It provides such motion characteristics, as speed, distance, acceleration, curvature of the track, laterality (the number of turns to the left and right; the L / R ratio), and the time spent in ROI. MouseMove can be applied to analyze locomotor and social behavior of various animal species. However, the program has limitations on the input resolution of the analyzed video and cannot process certain types of behavior.

AnimalTracker (available for download at http://animaltracker.elte.hu/) is a plugin installed in the ImageJ program. It has three modules that work together: Tracker module processes video, facilitates the process of binarization of the image, and allows to get a TXT file with X-Y coordinates; TrackAnalyzer module visualizes the trajectories created by Tracker module; and ZoneDesigner can be configured to analyze different ROI. AnimalTracker calculates the following characteristics: total time of movement, time of absence of movement, distance and velocity vectors, their standard deviations, and maximum and minimum speed. Results can be saved as TXT files and spreadsheets. This software is convenient for determining locomotor and some characteristics of social and cognitive behavior. It is easy to use and involves the completion of several steps in a semi-automatic mode. However, the software recognizes only one animal at a time and does not allow processing several files at once.

WrMTrck (available for download at http://www.phage.dk/plugins/wrmtrck.html) is an ImageJ plugin written by Jesper Søndergaard Pedersen to determine movement parameters of *Caenorhabditis elegans* (Maupas, 1900) (Nussbaum-Krammer et al., 2015). Installing the plugin and using it do not require any additional actions from the researcher. Applying the plugin, it is possible to set the most suitable parameters for analyzing motion characteristics of various objects – minimum and maximum dimensions of the object in pixels, maximum speed of the object moving between frames, changing the visible area, and minimum number of frames during which the object moves. The parameter settings file can be saved and used for further analysis. The plugin monitors the movement of several objects at once on the frame and calculates the number of moving objects, determines characteristics of the movement of the objects separately and their average values. Moreover, processed videos can be saved in AVI; images can be saved in TIFF, JPEG, GIF, *etc.* The plugin does not track the location of objects in ROI and does not determine the motion vector.

WrMTrck_Batch (available for download with WrMTrck at the link above) is a modification of the wrMTrck plugin allowing to batch process files. The plugin automatically separates moving objects from the background and records additional parameters, such as X-Y coordinates, changes in shape and area of the object. After processing video files, WrMTrck_Batch automatically saves the resulting tables with the obtained characteristics in TXT format, motion tracks in PNG, and object movement frames for each video file separately in TIF. The plugin does not impose additional restrictions on the resolution of imported video files.

To determine the motion characteristics of *Mnemiopsis leidyi*, we chose the ImageJ program with the installed wrMTrck_Batch plugin: it allows to batch process video files and automatically save tables with coordinates for each moving object. This plugin was successfully tested by us to determine the motion characteristics of the Black Sea turbot sperm (Baiandina & Khanaychenko, 2019).

In its original version, wrMTrck_Batch plugin does not allow to obtain all the necessary movement characteristics. To process TXT data files, scripts were written. These scripts automate the calculation of animal motion characteristics (those are described in the "Material and methods" section). According to X-Y coordinates, the macros calculate and write into a new TXT file the values of instant and average speed for each track (one observation for one animal), average speed for all tracks, categorized values of direction vectors between all frames for each track, as well as finding an object in ROI for each frame and each track (ROI are set based on the experimental design by X-Y coordinates).

Thus, in our experiments, ctenophore movement was analyzed according to the scheme as follows:

1) video recording of the movement of ctenophores in aquariums in time-lapse mode;
2) processing of photographic materials in the Davinci Resolve – creating videos, determining individual animals with a "tracking" mask, and saving processed video clips as separate files;
3) running ImageJ with wrMTrck_Batch plugin – obtaining a separate folder with TXT files with the coordinates of movements of each animal on each frame;
4) running scripts for rendering new TXT files with summary tables of motility characteristics of each animal.

Conclusion. Automatic determination of motion parameters of a translucent ctenophore is almost impossible without preliminary "manual" processing of video files. The developed method allows automatic calculating of movement parameter set. The described algorithm helps to avoid many routine actions for processing each video file separately. It takes less than 1 min (for a computer with the configuration described in the paper) to create a summary table of the motion characteristics of 63 processed video records. The method was successfully applied for determining light-induced behavior motility characteristics of adult *Mnemiopsis leidyi* (Baiandina et al., 2022) in laboratory experiments and could be applied for behavioral study of various translucent marine animals.

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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КОМПЬЮТЕРНЫЕ МЕТОДЫ
ОПРЕДЕЛЕНИЯ ХАРАКТЕРИСТИК ДВИЖЕНИЯ *MNEMIOPSIS LEIDYI*

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Рассмотрены возможности основных современных компьютерных программ для анализа поведенческих реакций гребневиков, морских планктонных животных, в лабораторных условиях. Показано, что автоматическое определение контуров полупрозрачных объектов, таких как гребневики, недоступно в большинстве программ для анализа изображений. Разработан новый метод определения основных характеристик движения *Mnemiopsis leidyi* с помощью программы ImageJ, видеофильтров приложения Davinci Resolve и написанных авторами исполняемых программных кодов. Этот метод позволяет автоматизировать расчёт таких параметров, как средняя и максимальная скорость, изменение направления движения (векторы передвижения), процент времени, в течение которого животные находятся в определённых зонах интереса (regions of interest, ROI). Приведены значения средних скоростей движения гребневиков. Разработанный метод применим для изучения поведения различных полупрозрачных морских животных. Исполняемых программные коды доступны по запросу у авторов.

Ключевые слова: *Mnemiopsis leidyi*, гребневик, миграция, характеристики движения, анализ видеоизображений, ImageJ, wrMTrck_Batch, зоны интереса (ROI), threshold