Analixity: An open source, low-cost analysis system for the elevated plus maze test, based on computer vision techniques

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ABSTRACT

Manual analysis of behavioral tests in rodents involves inspection of video recordings by a researcher that assesses rodent movements to quantify parameters related with a behavior of interest. The assessment of the researcher during the quantification of such parameters can introduce variability among experimental conditions or among sessions of analysis. Here, we introduce Analixity, a video processing software for the elevated plus maze test (EPM), in which quantification of behavioral parameters is automatic, reducing the time spent in analysis and solving the variability problem. Analixity is an adaptable multiplatform open-source system. Analixity generates an Excel file with the quantified behavioral variables, such as time spent in open and closed arms and in the center zone, number of entries to each zone and total distance traveled during the test. For validation, we compared results obtained by Analixity with results obtained by manual analysis. We did not find statistically significant differences. In addition, we compared the results obtained by Analixity with results obtained by the commercial software ANY-maze. We did not find statistically significant differences in the quantification of parameters such as time spent in open arms, time spent in closed arms, time spent in center zone, number of closed arms, open arms entries, and anxiety index. We concluded that Analixity is an open-source software as reliable and effective as a commercial software.

1. Introduction

The studying of animal behavior has led researchers to the development of automated methods to quantify parameters related to stress, anxiety, fear, etc. Usually, researchers perform a test with an animal model for a short period of time (Peters et al., 2016) and the behavior of interest is video recorded for later analysis, either manual or automated. Manual analysis involves observation of video recordings by a trained researcher that assesses animal movements to quantify parameters that describe a behavior. The researcher percipience takes part during the quantification of parameters, and it can be a source of variability, particularly if quantification is made in different moments or if there are several researchers involved in the analysis. In addition, manual analysis is time consuming because typically, the researcher needs to watch the video recordings more than twice, with a slow playback speed for a better quantification. Test automation aims to decrease the time spent by the researcher, as well as to standardize the criteria used to quantify parameters.

Nowadays, there are many software development efforts to analyzing animal behavior such as EPM3C, OF3C, NORT-3D (Bioseb, www.bioseb.com), LABORAS (Metris, www.metris.nl), Ethovision, Track3D (Noldus, www.noldus.com), ANY-maze (Stoelting Co., www.anymaze.com), Zantiks MWP, Zantiks AD (Zantiks, www.zantiks.com) and SMART Video Tracking (Panlab, www.panlab.com).

Implementation of these tools for behavioral recording and analysis of animal models requires in some cases, buying a complete setup including software, hardware, mazes, fish tank, etc. ln other cases, a camera and license or a license-only must be purchased. For laboratories with small budgets, the software license acquisition can be unattainable. Moreover, there is no certainty that the software requirements can be adapted to laboratory conditions or that it can be used for analysis of videos recorded previously. Other fact to consider is that, proprietary software works like a black box, i.e., researchers do not know the procedures performed by the software for quantification of parameters. Furthermore, most commercially available software for behavioral analyses are not multi-platform.

Video recording of animal behavioral is a noninvasive method that prevent animal distractions, and allow direct observation of the subjects (Crispim-Junior et al., 2017; Ellens et al., 2016; Heijkoop et al., 2017; Yao et al., 2017). With the recent advances in video/image processing techniques and the increasing availability of technology, several groups have developed their own open-source software for video analysis and measurement of parameters (Butensky et al., 2017; Hewitt et al., 2017; Peters et al., 2016; Telonis & Margarity, 2015), providing transparency in the processes for parameter quantification and, at same time decreasing the cost for data analysis. After video recording, video processing techniques such as vision-based tracking systems have become a standard for analysis. Here, we introduce Analixity, a scalable video processing software, with one completely functional module for EPM. Analixity was developed on Python programing language linked to OpenCV libraries for video processing tasks, therefore Analixity is a multiplatform open source system, that is, there are Windows, MacOs and Linux versions. Analixity run file is available at the following link: Executable Windows.

2. Material and methods

2.1. Animals

Animal housing, care and handling were in accordance to the Official Mexican Standard NOM-062-ZOO-1999 (Technical Specifications for the Production, Care and Use of Laboratory Animals). In addition, we followed the U.S. National Institutes of Health (NIH) guidelines. Five-month-old male Wistar rats were housed in vivarium conditions under 12-h light/dark cycles (lights on from 07:00–19:00 h), with water and food provided ad libitum.

Three-month-old male C57BL/6 J mice (23 −25 g) were housed in standard cages (30 × 20 × 12 cm; 4 animals per cage) and maintained on a 12-h light/dark cycle (lights on from 19:00 h to 07:00) in a temperature (23 ± 1 °C) and relative humidity (55 ± 5%) controlled room with food and water ad libitum. Behavioral tests were performed during the dark period.

2.2. Elevated plus maze test (EPM)

The EPM was developed to studying the fear-induced behavior in rat (Handley & Mithani, 1984). Nowadays is widely used in rodent behavior studies involving anxiety (Garcia et al., 2005; Li et al., 2014; Sorregotti et al., 2013; Yeung et al., 2016), learning and memory (Carobrez & Bertoglio, 2005), thigmotaxis (Filgueiras et al., 2014) and, behavior modeling (Arantes et al., 2013; Costa et al., 2014; Tejada et al., 2009). We used the EPM to quantify parameters related to anxiety behavior in rat. The EPM was a plus-shaped surface elevated 50 cm above the floor. The open arms were 50 cm long and 10 cm wide; while the closed arms were 50 cm long, 10 cm wide and 70 cm high. The arms were connected by a square surface (10 ×10 cm), in the central area (see Fig. 1).

The EPM for mice consisted of two perpendicular open arms (30 ×5 cm) and two enclosed arms (30 ×5 cm) with 15 cm high walls, connected by a square surface (5 ×5 cm), made of white plexiglass.
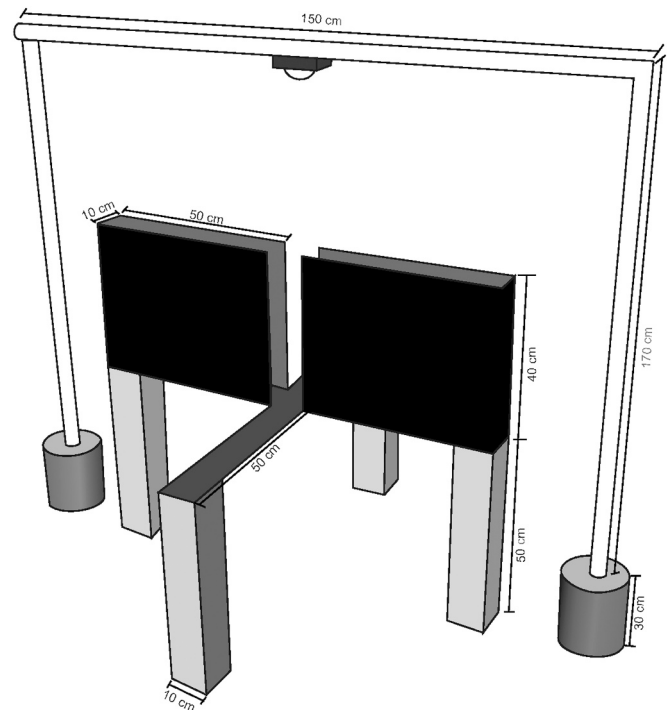

Fig. 1. A webcam connected to a personal computer was placed perpendicular to the elevated plus maze.

2.3. Video recording equipment

For video acquisition the following setup was used (see **Error! Reference source not found.**):

- Asus ROG G501VW computer
  - Intel Core i7 6700HQ Processor
  - DDR4 2133 MHz SDRAM 8 GB
  - NVIDIA GeForce GTX 960 M with 2 G/4 G GDDR5 VRAM
  - 1TB HDD 5400 RPM
  - 128 GB SSD
  - Windows 10 Pro Operating System

- WebCam Logitec HD Webcam C525

It is important to note that during the recording of each video, the open arms must be oriented vertically and the closed arms horizontally.

2.4. Behavioral tests

Rat testing in light phase. Video recordings were acquired using the Logitech webcam software with a 1280 × 720 pixels image resolution at 24 frames per second, in Windows media video format RGB color space. The webcam was placed perpendicular over to EPM, on a custom polyvinyl chloride (PVC) pipe support. A dedicated room for behavioral tests was illuminated with fluorescent light. Each rat (n = 13) was placed at the center of the EPM and recorded for 300 s. The parameters of interest for quantification were: the time spent in each area, i.e., open arms, closed arms and center zone, and the number of entries to open and closed arms. Open/closed arm entries were counted when the rat moved from the center zone to the open/closed arm and its 4 legs were in the arm.

Mouse testing in dark phase. Each mouse (n = 4) was placed in the center square of the EPM facing the open arm and allowed to freely explore the maze for 300 s. All these tests were performed in the dark phase, from 15:00 h to 18:00 h, under red light. After each animal was

recorded, the EPM was cleaned with 50% isopropyl alcohol and wiped with damp and dry cloths.

2.5. Video processing

Fig. 2 summarizes the image processing techniques applied to each frame to make rat segmentation. First, we changed the color space to grayscale. Second, we selected regions of interest (ROI) to analyze only the areas of interest in the EPM, i.e., the user must select and define the areas corresponding to closed arms, open arms and center zone. Third, we applied gamma correction to the selected ROI in order to darken the light reflection on the closed arms of the EPM without changing the white areas on the image. Sometimes, the tail of the rat introduces an error in quantification. To avoid such an error, we transformed the tail as background by using a Gaussian filter. Fourth, for rat segmentation, we used a thresholding technique. The resultant binary image leaves the rat-corresponding pixels in white color and background pixels in black color. Fifth, the rat centroid (only in white pixels) is calculated. Last, the centroid obtained on the latter step is used to determine the rat position and to update the time counter of the specific area, e.g., open/close arm, center. In addition, Analixity generates a tracker sheet showing the rat walking trajectory. Supplemental material shows in detail each step mentioned above.

2.6. Video resolution

We performed tests using several video resolutions. We found significant differences in the quantification of parameters in low resolution 320×240 pixel vs higher resolution 640×320 pixel or upper video recordings. To use Analixity, we recommend video recordings with at least 640×320 pixel resolution. Lower resolutions can cause up to 30% of error per parameter quantified.

3. Results

3.1. Quantification of parameters: Manual analysis vs Analixity

A trained researcher performed manual analysis of each video recording of white rats on a black EPM (n = 13) during the light phase, and black mice on a white EPM (n = 4) during the dark phase under red light. We quantify five parameters: time spent in open arms (TSO), time spent in closed arms (TSC), time spent in center zone (TSX), number of entries to open arms (EOA) and number of entries to closed arms (ECA). Data obtained by the researcher were compared to data obtained by ANY-maze or Analixity. All results are expressed as mean ± SEM. We performed a Kruskal-Wallis test for each parameter. We did not find statistically significant differences between Analixity, manual quantification and ANY-maze for TSO, TSC, TSX, EOA, ECA and Anxiety Index (AI). AI was calculated to provided information about the state of anxiety of the animal, with values that range from 0 to 1. A higher AI indicates a higher level of anxiety: AI = 1 − ([TSO / test duration] + [EOA / EOA+ECA] / 2) (Figs. 3 and 4).

A video comparison of tracking performance and processing time is

shown in Video-1.mp4. A mouse in red light environment is shown in Video-2.mp4.

3.2. Development of a user-friendly interface

We developed a PyQt5-based intuitive user interface. PyQt5 is an API designed to binding with Python. Fig. 5 shows the Analixity graphic user interface, and in a graphic way, the steps to analyze the video recordings. Fig. 5a shows the start-up window. In this window, the user can select the process to be performed, e.g., ROI selection, processing, or results visualization. The first step is ROI selection. Fig. 5b shows selection of the video recording file. Fig. 5c shows how the ROIs are selected. The user must adjust the predefined areas according to their own maze size. As mentioned above (see Methods), in the recorded video the open arms must be oriented vertically, and the closed arms horizontally. This allows the correct matching of the values for each ROI with the correspondent parameter column in the database generated by the software.

After finishing ROI selection, a file containing the coordinates of the ROIs selected is generated (Fig. 5d, e). Fig. 5f shows the processing table In this module, the user must fill-in the video length in seconds, select the video file to be processed, select whether the color of the EPM (background) is darker or lighter than the animal (Fig. 5g.1) and select the file containing the coordinates of the ROIs (Fig. 5g.2). Then, a new window of Settings opens, and the user must select gamma, blur, and binary values (Fig. 5g.3). First, set gamma, blur, and binary to 1, then double-click on the black screen and slide blur to 4. Next, slide binary until most of the animal is visualized in white. If there are some parts of the EPM still in white, adjust blur until only the animal is seen in white. Without closing the Settings window, return to the main window by clicking on it. The selected settings will be stored in a settings file that can be used for all videos recorded in the same lighting conditions. Once in the main window, select Processing and wait until analysis is finished. Then, close the video window, press Esc to close the Settings window and press Excel to generate the database. Fig. 5h shows the result tab. Some commercial software assume that video recording speed is the same in all cases. However, recording speed can change from one video to another depending on the recording camera used, light conditions and video acquisition software. To avoid errors, Analixity performs a verification of video recording speed.

Supplemental material shows all image processing techniques used in this work. Configuration of critical variables for image processing allows adaptability to many scenarios. In Tutorial.mp4 we show how to use Analixity. In addition, we provide detailed documentation in our GitHub repository: source code, executable files for Windows, Linux and MacOS, programming changes and how to compile from source code; all available upon request.

4. Discussion

4.1. Advantages of our method

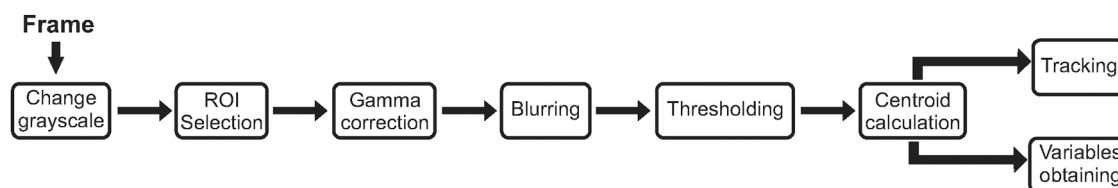The main advantage of our method is portability, i.e., it can be

Fig. 2. For each frame in the video, we applied vision techniques for image enhancement: intensity transformation, contrast adjustment, filtering and thresholding. These techniques were applied for rat segmentation. Then, we obtained the centroid position of the rat on the binary image. Using the centroid location, we determined the rat location on the elevated plus maze, and therefore, we calculated the time spent in open/close arms and center zone and we reconstructed the rat trajectory.
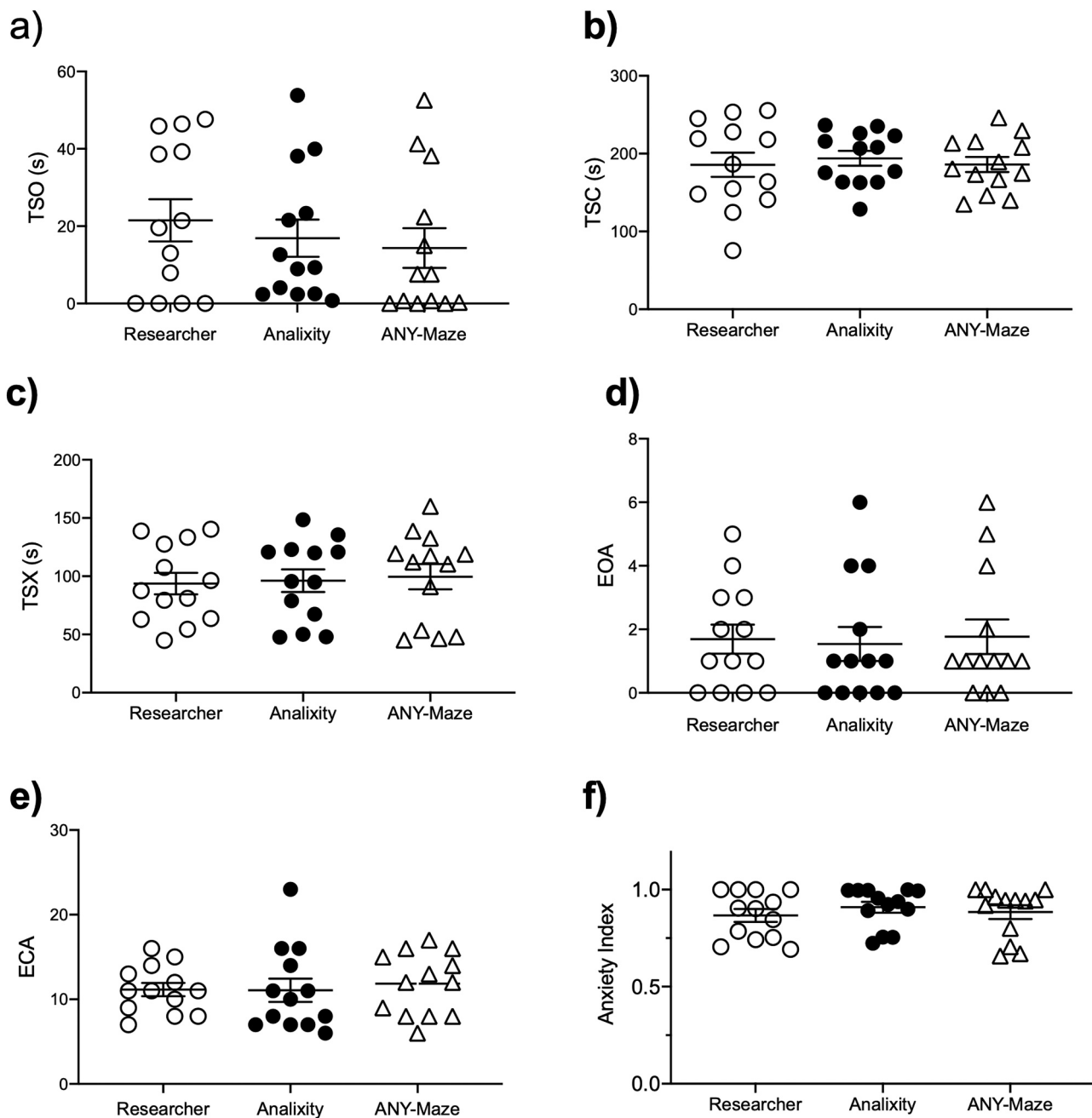
Fig. 3. Comparison of data (mean ± S.E.M) obtained by manual analysis, ANY-maze and Analixity in rats. a) time spent in open arms (TSO), b) time spent in closed arms (TSC), c) time spent in center zone (TSX), d) number of entries to open arms (EOA, e) number of entries to closed arms (ECA), and f) Anxiety Index. Data obtained using Analixity do not show statistically significant differences when compared against data obtained by manual analysis or ANY-maze software.

executed in any computer, no matter its operating system. Other important characteristic is that any generic webcam with at least 640 × 480 pixel resolution can be used, whereas that, for executing commercial systems, the user must purchase not only software but also hardware. An additional advantage is that our system reduces the time needed for analysis. To quantify five behavioral parameters from a 300 s length video, a researcher requires 30 min, whereas Analixity requires 2.5 min approximately.

Most commercial systems for behavior analysis assume that video recordings are acquired under specific lighting conditions. However, having the same lighting conditions is not always possible, especially over the closed arms of the EPM. To ensure that processing is made according to actual lighting conditions in the recording room, Analixity allows choosing whether the background is white or dark and has a

video settings module for video processing. Gamma correction is a vision technique for contrast adjustment. Since it is a nonlinear technique, gamma correction allows making some regions lighter just in some video frames without affecting clearer areas in other frames. We compared the results obtained by processing our videos with two different contrasts adjustment techniques: histogram equalization and gamma correction. The video processing by gamma correction shows similar results to those obtained by a researcher. The video settings module has other parameters needed for video processing which can be adjusted by users according to their own lighting conditions. Analixity is an open source tool, therefore its source code is provided to users through GitHub platform, allowing them to explore and modify it according to their own hardware, space and lighting conditions; that is, procedures and internal operations of Analixity are known. According to our results, we did not
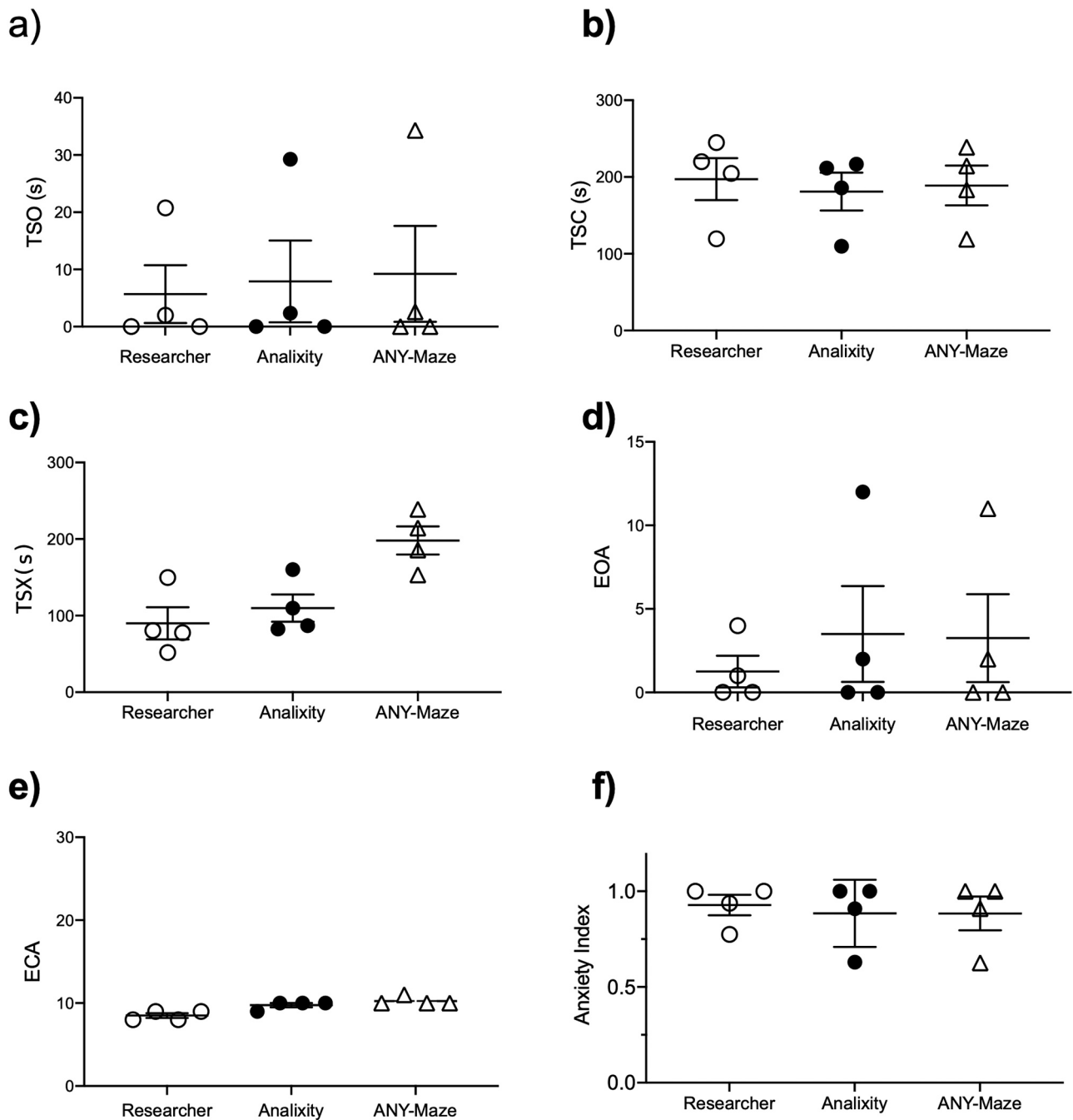
Fig. 4. Comparison of data (mean ± S.E.M) obtained by manual analysis, ANY-maze, and Analixity from recording of C57BL/6 mice. a) time spent in open arms (TSO), b) time spent in closed arms (TSC), c) time spent in center zone (TSX), d) number of entries to open arms (EOA, e) number of entries to closed arms (ECA), and f) Anxiety Index. Data obtained using Analixity do not show statistically significant differences against data obtained by manual analysis or ANY-maze software.

find statistically significant differences between Analixity and manual quantification. Analixity results show a high confidence value and the analysis is faster than manual quantification.

4.2. Method limitations

One disadvantage is that Analixity needs, at least the first time it is used, a lot of human intervention. However, if the maze, webcam, and lighting conditions do not change, then this intervention is performed just once. For vision processing, we used OpenCV, which is a set of libraries with many capabilities to managing video formats. Nevertheless, OpenCV has not full support for.wmv format. Even though OpenCV can open and process this format, the frame rate estimate is not reliable. For

this reason, the user must fill-in manually the video length in seconds. Analixity estimates the correct frame rate of the video. For an optimal selection of gamma, blur and binary values, it is recommended to perform manual quantification for at least one video and then, compare the results obtained by Analixity to different gamma, blur and binary values. We must note that, as it still depends on human intervention in its initial settings and video treatment, likewise commercial systems, Analixity does not eliminate subjectivity, but standardizes it and helps to reduce intra-laboratory variability. However, it is true that homogeneity of experiments between different laboratories cannot be guaranteed.
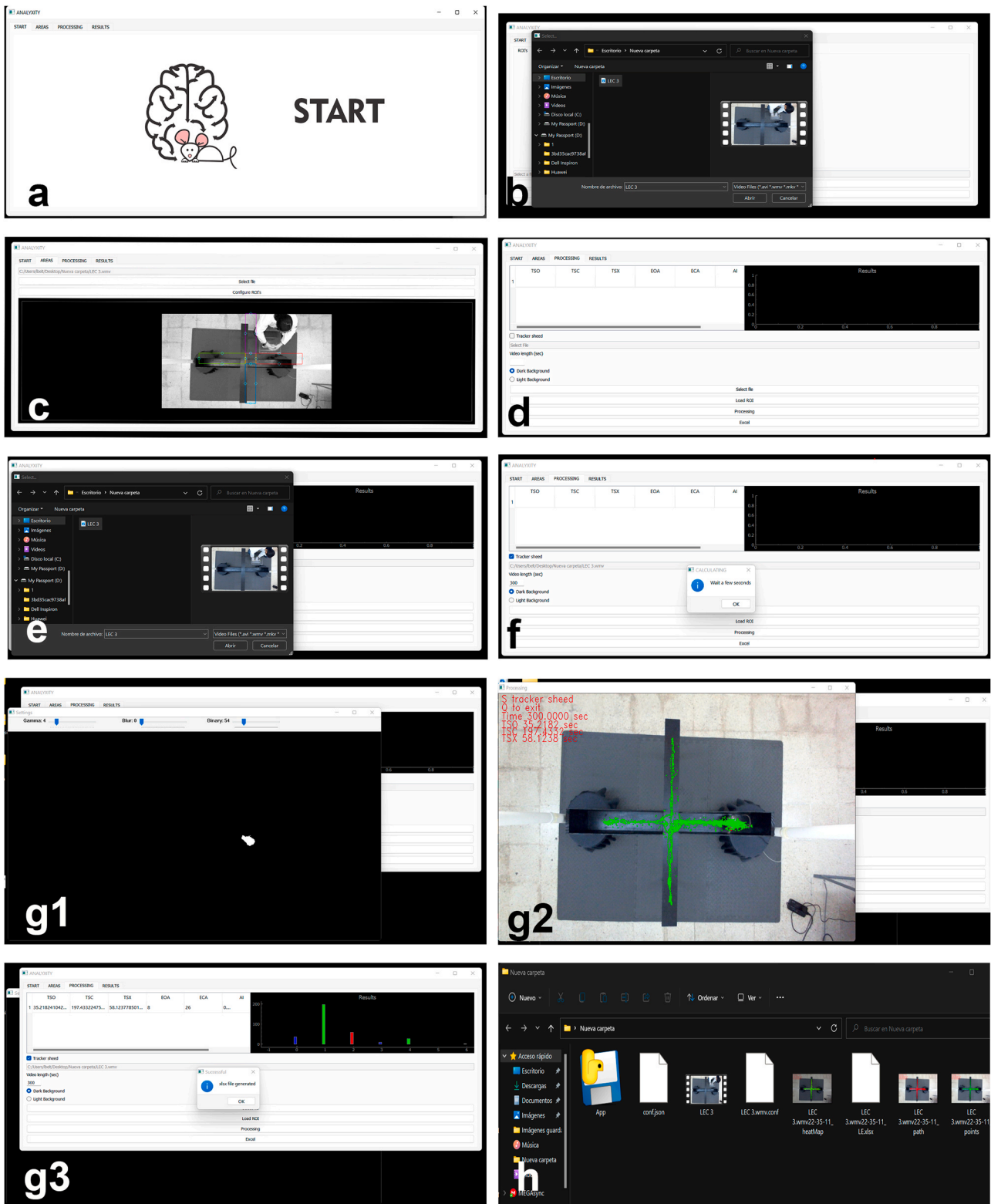
Fig. 5. PyQt5-based intuitive user interface. (a) Analixity start-up window. The user selects the process to be performed: ROI selection, processing, or results visualization. (b) Select video file for analysis. (c) Select ROIs. (d,e) Store ROI coordinates in configuration file on hard drive with the same name as the video selected. (f) Processing tab. (g) The user must fill-in the video length in seconds, select the video file to be processed, select whether the background is darker or lighter than the animal (g.1), and select the file containing the coordinates of the ROIs (g.2). Once the file is selected, a new window of Settings opens, and the user must select gamma, blur, and binary values (g.3). (h) Module for displaying results.

5. Conclusion

We developed an open-source software for automatic quantification of behavioral parameters for the EPM test. For the parameters evaluated, our system reduces the time of analysis from 30 min to 2.5 min and does not show statistically significant differences against ANY-maze or manual analysis. Software systems do not have judgement, they have well specified rules that are applied in the same way to all study subjects, hence, different researchers can be part of the analysis process without introducing an error by variability.

Information Sharing Statement

Analixity for windows can be found at the next link: Analixity.zip.
Videos: Tutorial.mp4 and Video-1.mp4.
GitHub repository: source code, executable files for Windows, Linux and MacOS, programming changes and how to compile from source code; all available upon request.

CRediT authorship contribution statement

All authors contributed to the study conception and design. Conceptualization: LB-P and CM-V. Material preparation, data collection and formal analysis and investigation were performed by PG-G, TCA-M, FMM-G, and ML-J. The first draft of the manuscript was written by CM-V and PG-G and all authors commented on following versions of the manuscript. Supervision: LB-P. Funding acquisition: CM-V and LB-P. All authors read and approved the final manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.beproc.2021.104539.

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