Journal of Neuroscience Methods 270 (2016) 9–16

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

COLORcation: A new application to phenotype exploratory behavior models of anxiety in mice

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HIGHLIGHTS

- *COLORcation* analyses exploration patterns and provides a heat map of mouse activity.
- *COLORcation* provides new parameters to track activity and locomotion of the test animals.
- The results demonstrate the use of *COLORcation* in different anxiety paradigms.

ARTICLE INFO

Article history:
Received 10 February 2016
Received in revised form 31 May 2016
Accepted 2 June 2016
Available online 4 June 2016

Keywords:
Behavior
Mouse
Open field
Anxiety
Analysis

ABSTRACT

Background: Behavioral analyses in rodents have successfully delineated the function of many genes and signaling pathways in the brain. Behavioral testing uses highly defined experimental conditions to identify abnormalities in a given mouse strain or genotype. The open field (OF) is widely used to assess both locomotion and anxiety in rodents. In this test, the more a mouse explores and spend time in the center of the arena, the less anxious it is considered to be. However, the simplistic distinction between center and border substantially reduces the information content of the analysis and may fail to detect biologically meaningful differences.
New method: Here we describe *COLORcation*, a new application for improved analyses of mouse behavior in the OF.
Results: The application analyses animal exploration patterns in detailed spatial resolution (e.g. 10×10 bins) to provide a color-encoded heat map of mouse activity. In addition, *COLORcation* provides new parameters to track activity and locomotion of the test animals. We demonstrate the use of *COLORcation* in different experimental paradigms, including pharmacological and restraint-based induction of stress and anxiety.
Comparison with existing method(s): COLORcation is compatible with multiple acquisition systems, giving users the option to make the most of their raw data organized text files containing time and coordinates of animal locations as input.
Conclusion: These analyses validate the utility of the software and establish its reliability and potential as a new tool to analyze OF data.

1. Introduction

Mouse behavioral profiling relies on a defined set of experiments which aim to find differences between mouse strains or genotypes (Beckers et al., 2009). Most such experiments provide a quantitative or qualitative score that can be used as a proxy for specific neurological functions (Crawley, 2008). The open field (OF) test is widely used to assess both locomotion and anxiety in rodents (Prut and Belzung, 2003; Crawley, 2008). In this test, the animal is placed in an arena and its location is monitored over time. Initially the assay was done by manual monitoring of animal location in an arena subdivided by square markings on the floor. Nowadays, automated video-tracking systems provide location coordinates throughout the experiment (Gould et al., 2009), however many analyses do not take full advantage of this extensive raw data.

Here we describe *COLORcation*, a program analyzing OF data as a batch and enabling description of group behavior by heat maps

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http://dx.doi.org/10.1016/j.jneumeth.2016.06.003
0165-0270/

instead of multiple tracking images of single animals as used in most studies. *COLORcation* analyses raw data from video tracking software, virtually dividing the arena into bins used as spatial units for analysis. This spatial annotation provides detailed localization information throughout the experiment rather than general regional assessments. *COLORcation* also calculates velocity at any given time point, and can then ignore pausing events (rest time) to accurately report the median velocity of animal movements throughout a trial. Finally, *COLORcation* provides the user with a summary file containing all calculated parameters for each animal and per group.

2. Materials and methods

2.1. Animals

Animal procedures were in accordance with Weizmann Institute of Science animal care committee regulations. 2–3 months-old C57BL6 male mice (body weights 20–23 g) were used. The mice were kept at 24.0 ± 0.5 °C in a humidity-controlled room under a 12-h light–dark cycle (lights on at 7:00 PM) with free access to food and water.

2.2. Open field

We assessed motility and anxiety-like behaviors in the open field (OF) paradigm (Neufeld-Cohen et al., 2010b). Animals were tested over 10 min in a 50 × 50 cm rectangular OF arena, an illumination of 120 lux and background noise of 65 dB. The total distance moved (cm), the time spent (global, center, border; in seconds), center/border ratio, mean speed (cm/s), and percentage of time spent moving versus rest in the different user-defined areas were recorded using VideoMot2 software (TSE System, Germany).

2.3. Pharmacological treatment

The benzodiazepine receptor inverse partial agonist FG7142 (*N*-methyl-ß-carboline-3-carboxamide; Sigma–Aldrich, cat# E006-100MG) was used for pharmacological induction of anxiety as described previously (Evans and Lowry, 2007). The drug was dissolved in 1 ml ethanol (96%) and subsequently diluted in 10 ml 1x PBS and injected intraperitoneally (IP) at a final concentration of 5 mg/kg of body weight 30 min before the behavioral test. This dosage induced anxiety-related behaviors without affecting general locomotion or inducing seizures (Little et al., 1984; Löscher and Stephens, 1988). We compared two groups of animals, FG7142-treated ($n = 8$) versus vehicle control ($n = 10$).

2.4. Restraint stress experiment

The mice were tested in the OF under four conditions ($n = 16$–18 for each condition): (i) unstressed: no additional stressor other than the challenge of the test ("NAIVE"); (ii) following an habituation procedure that included handling and transferring the mice to the experimental room over 3 days before the test ("HABITUATED"); (iii) immediately following 15 min of acute restraint stress ("IMMEDIATE"); and (iv) 24 h following the noted acute restraint stress ("DELAYED"). Acute restraint stress was performed as described in Neufeld-Cohen et al. (2010a).

2.5. Comparison of COLORcation and Ethovision

Animals ($n = 10$; C57BL6 mice, 2 months old) were tested over 10 min in the same rectangular OF arena (50 × 50 cm) described above (provided by TSE) under illumination of 120 lux and background noise of 65 dB. Movies of 10 min were acquired and analyzed off-line using the Ethovision XT11 software (Noldus Information Technology, The Netherlands). The animal movements were based on the center-point tracking. The total distance traveled (cm), the distance traveled in the center area (cm; 25% of the total area), the time spent in the center area (in seconds), and the number of visits in the center area were calculated and both individual and group heat maps generated. Individual raw tracking data were exported and analyzed using *COLORcation* with the same center area definition and the corresponding parameters, individual and group heat maps generated.

Table 1
List of *COLORcation* output parameters.

Parameter	Description and units
File name	Source file
Group	Source file's folder/group
Center	Time spent in center (s)
Walls	Time spent in walls (s)
Corners	Time spent in corners (s)
PauseCenter	Pausing time in center (s)
PauseWalls	Pausing time in walls (s)
PauseCorners	Pausing time in corners (s)
VelocitiesLow	Lower quartile (.25) of velocity (cm/s)
VelocitiesMid	Median velocity (cm/s)
VelocitiesHigh	High quartile (0.75) of velocity (cm/s)
VelocitiesMean	Average velocity (cm/s)
MeanPauseTime	Average pausing time (s)
NumOfPause	Number of pauses (s)
DistCovered	Distance covered (cm)
DistCent	Distance covered in center (cm)
DistWall	Distance covered in walls (cm)
DistCorn	Distance covered in corners (cm)
1st Center loc	Time to visit center (min)
NumVisitsCenter	Number of visits in center region
NumVisitsWalls	Number of visits in walls regions
NumVisitsCorners	Number of visits in Corner regions
AccelerationLow	Lower quartile (0.25) of acceleration (cm/s^2)
AccelerationMid	Median acceleration (cm/s^2)
AccelerationHigh	High quartile (0.75) of acceleration (cm/s^2)
AccelerationMean	Average acceleration (cm/s^2)

2.6. Algorithm and overview of the procedure

COLORcation is a MATLAB based analysis tool which reads the individual raw tracking files of animal locations to assemble a database of the given experiment. Any organized excel file (.xlsx) containing the *x*, *y* coordinates of animal locations over time can be used as an input. In such case the user will need to provide his data frame rate, field size and pixel-to-cm ratio. We used TSE VideoMot2 raw data files in this paper, which already contain the calibration index and the video frame rate, both required for the analysis. Additional input parameters are user-provided, including the initial mouse position in the arena and the field size used in the assay (in cm). Given files containing coordinates of animal locations over time, *COLORcation* matches each coordinate to a corresponding spatial bin and then calculates the total time spent in each bin of the arena. The average time spent in each bin is then used to produce a heatmap representation for the group. Bins are then assigned to a region—center, walls and corners and the average time spent in each region is calculated as well. Additional parameters such as velocity (in cm/s) and acceleration (in cm/s^2) (lower/median/upper quartiles; average), mean pausing time, number of pauses, distance covered in each region, latency to explore the central compartment and number of visits in every compartment are also calculated. Finally, the analysis tool summarises all parameters (Table 1) for each mouse and group into an Excel summary file. Cluster analysis is done using the "Clustergram" function in MATLAB with euclidean distance calculation. The program can be run on any PC after installation of the MATLAB runtime environment.

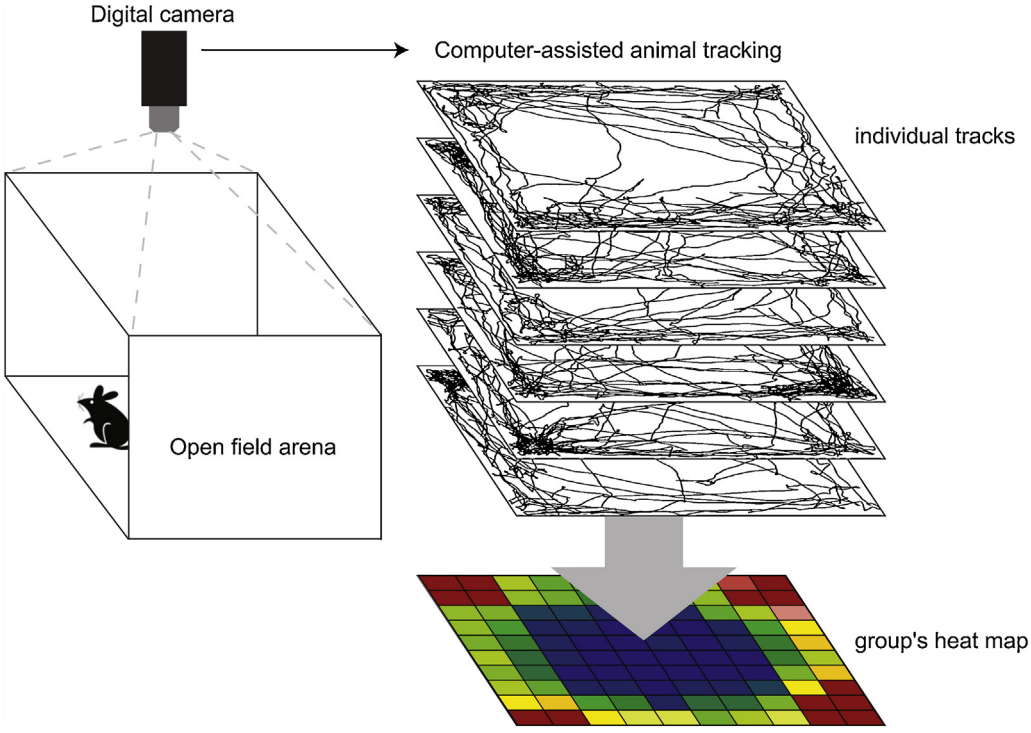

Fig. 1. Schematic representation of the *COLORcation* method. The animal is placed in an open field arena and its location is monitored over time. Automated videotracking generates individual tracking files for each animal. *COLORcation* performs a batch analysis of the tracking files and calculates the average time spent by the group in each segment in the arena. The data are then summarised as heat maps.

2.7. Statistical analysis

The data were analyzed either using unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis test. A two-way ANOVA, followed by a Sidak multiple comparison test was used to examine the influence of treatment and time on distance and velocity measures, using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com). The results are expressed as mean ± standard error of the mean (SEM). Statistically significant *P*-values are shown as $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

3. Results

3.1. COLORcation—*mode of operation*

An open field experiment requires analysis of the exploratory behavior of animal over a course of several minutes in a test arena. Most commonly used video tracking modules produce a raw tracking file indicating the exact location of the examined animal at each sampled time point in a single analysis. *COLORcation* collates the data from a series of tracking files, and generates a combined spatial representation for the entire group. This is done by division of the arena to a defined number of bins (100 bins over 10 rows and 10 columns). The algorithm then assigns every tracking coordinate to the appropriate bin, and then sums the amount of time spent (in seconds) in each bin. Finally, a heat map representation of group behavior is obtained by plotting the average time spent in each bin (Fig. 1**).**

After generating averaged representations of group localization we sought to extract additional parameters from the data. First, we used the coordinate files to calculate velocity of the test animal at any given time. Supplementary Fig. 1 shows the velocity and location of two animals over a course of five seconds. As depicted in the figure, one of the animals did not move during the plotted period, however, since the animal is recognized by the image analysis software, small movements can be misinterpreted as walking bouts. We therefore implemented a velocity cutoff (2.5 cm/s) to divide tracks to stationary and motile periods. If an animal was stationary for more than 2 s, this period was designated as a pause.

3.2. Pharmacological induction of anxiety

We chose a pharmacological experiment for the first evaluation of the potential of *COLORcation* in analyzing anxiety-related behaviors, using the GABA-A partial inverse agonist FG7142, a β-carboline described for its anxiogenic effect (Evans and Lowry, 2007). Dose-response tests indicated that a dose of 5 mg/kg efficiently enhanced avoidance of an illuminated OF center area without affecting the distance traveled on the borders or movement speed. Moreover, this dosage did not produce detrimental side-effects such as seizures. As expected, our results show that the FG7142 injection induced a significant increase of anxiety behavior. First, we used the VideoMot2 software (TSE systems, Germany) to monitor mouse activity in the OF and calculate standard anxiety indices (Fig. 2A–E). FG7142-treated animals exhibited a significant reduction of the number of visits in the center (Vehicle: 21.70 ± 2.79; FG7142: 11.88 ± 2.67; $t=2.49$, $P=0.0238$), with a decrease in distance traveled in the center (Vehicle: 415.7 ± 63.23 cm; FG7142: 181.3 ± 47.48; $t=2.83$, $P=0.0120$) and a lower average movement speed (Vehicle: 9.76 ± 0.43 cm/s; FG7142: 8.42 ± 0.43 cm/s; $t=2.16$, $P=0.0455$). On the other hand, the time in center, the latency to first visit the center and the total distance traveled were not significantly altered by the treatment ($P>0.05$) (Supplementary Fig. 2).

We then used *COLORcation* to analyze the OF raw data obtained as a txt file from VideoMot2. *COLORcation*-generated heat maps (Fig. 2F and G) show that FG7142-treated mice avoided the central OF area to a greater extent than vehicle-treated littermates. This is also evident from the parameters computed by *COLORca-*

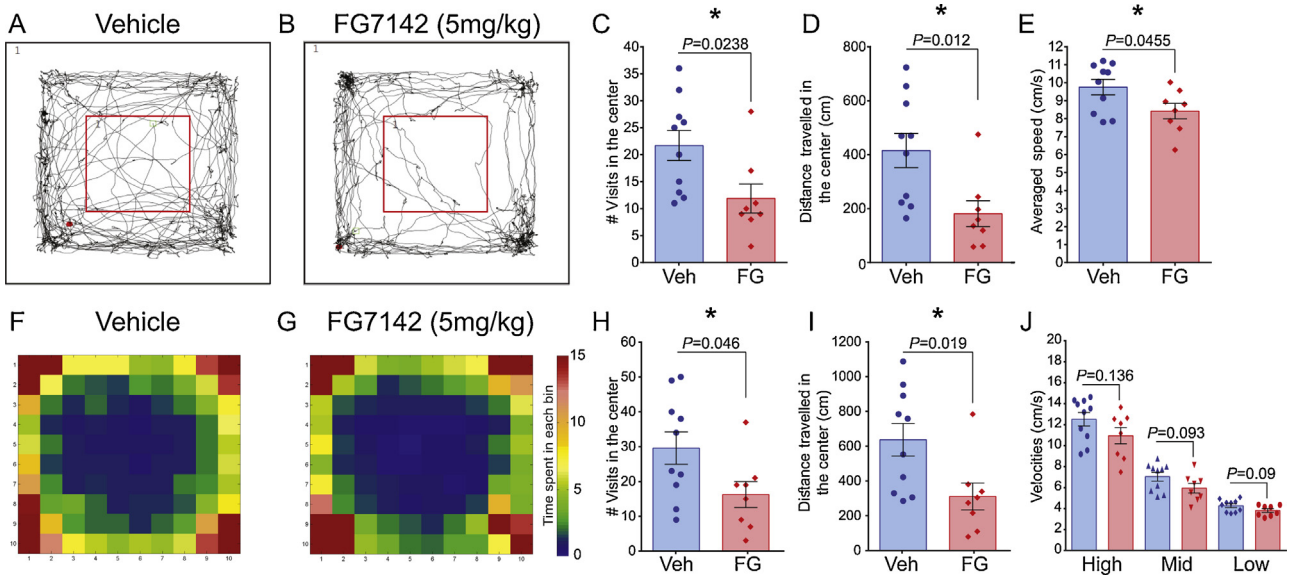

Fig. 2. (A and B) Representative 10 min OF tracks of mice treated with vehicle or FG7142. The red squares depict the center area of the open field. (C–E) Assessment of anxiety-related behaviors in the OF in mice treated with the FG7142 and their vehicle-treated littermates. This analysis was generated using the VideoMot2 software (TSE systems). (F and G) *COLORcation* heat maps of activity in the OF. Each arena is divided into 100 bins and the color defined as the average time spent in seconds in each bin reveals increased avoidance of the OF center in the FG7142-treated mice. (H–J) *COLORcation* assessment of anxiety-related behaviors in mice treated with FG7142 and their vehicle-treated littermates showing a significant decrease in the exploration of OF center. Blue bars represent the vehicle group: $n = 10$; red bars represent the FG7142 group: $n = 8$. OF indexes are represented by average ± SEM bar graphs and dots for individuals' values distribution within a group. * indicates $P < 0.05$, ** indicates $P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tion (Fig. 2H and I), with significant decreases in the number of visits to the center (Vehicle: 29.60 ± 4.64; FG7142: 16.25 ± 3.74; $t = 2.16$, $P = 0.0465$) and distance traveled in the center area (Vehicle: 637 ± 93.39 cm; FG7142: 311 ± 77.32 cm; $t = 2.59$, $P = 0.0194$), in agreement with the anxiogenic nature of the drug. Note that the region considered "center" in both analyses is not identical, while in VideoMot2 the central region is drawn by the user (25% of the total area), in our *COLORcation* settings the central area represents 36% of the total area (inner 6 × 6 bins). Hence the numbers of visits and distance traversed in the center are higher for both groups when analyzed by *COLORcation*. However, running *COLORcation* with the same area definitions results in values which are consistent with VideoMot2 (Supplementary Table 1). Additional parameters were calculated (time spent, number of visits, distance traveled and number of pauses along the walls or in the corners) and were not significantly different after FG7142 treatment (Supplementary Fig. 3). *COLORcation* also performs a distance–velocity analysis providing the distance traveled and changes in velocity over time. Importantly, the velocity measurement – unlike most standard tracking softwares – does not include the pausing of the mice and is based only on actual movement events. The software separates the velocities and the accelerations to higher, medium and lower quartiles allowing accurate monitoring of movement speeds. This feature may help users to distinguish between different exploration strategies or detect hyper/hypolocomotion phenotypes. Fig. 2J shows that the anxiogenic treatment did not affect high, mid or low velocity movements. A two-way ANOVA of the distance traveled/min revealed a time-dependent decrease of activity in both vehicle and FG7142-treated animals [$F_{(9,144)} = 4.11$, $P = 0.0001$]. However, the treatment versus time interaction was not significantly influencing distance [$F_{(9,144)} = 1.11$, $P = 0.35$] and velocity performances [$F_{(9,144)} = 0.91$, $P = 0.51$]. While there were differences in total distance traveled, this did not reach significance [Vehicle: 4410 ± 322.2 cm; FG7142: 3500 ± 286 cm; $F_{(1,16)} = 4.23$, $P = 0.0564$] (Supplementary Fig. 4).

3.3. Restraint-stress-induced alteration in the OF exploration

In a second experiment, we examined the possibility of using *COLORcation* to identify stress-related changes that are not detectable with standard methodology. To this end we used the restraint-stress protocol to induce anxiety (Gregus et al., 2005). This protocol has milder effects and higher variability than pharmacological induction of anxiety (Buynitsky and Mostofsky, 2009). Four groups of mice under different levels of stress (naive, habituated, immediate, delayed) were tested in the OF. VideoMot2 software analyses (ANOVA for the center/border exploration) did not identify any differences between the groups in any of the revelant anxiety indices; including time in center, number of visits to the center, relative distance in the center and latency to first visit to the center. Similarly, the groups did not differ significantly in total distance traveled and movement speed ($P > 0.05$ for all parameters; Supplementary Fig. 5). In contrast, the *COLORcation* heat map representation of these four groups (Fig. 3A–D) suggests that the immediate stress was the most anxiogenic condition (increase in OF center avoidance is shown as decrease of OF bin's color temperature in the center) while it did not significantly affect exploratory parameters (time spent, number of visits, distance traveled, number of pauses) per se (Supplementary Fig. 6).

Further analyses took advantage of *COLORcation*'s quantitative spatial representation to perform a two dimensional hierarchical clustering of the groups average localization data in each bin (Fig. 3E). The analysis clustered the different groups according to similarities and differences in the overall exploration pattern (*Y* axis) as well as the spatial distribution of exploration (*X* axis) highlighting the peripheral region preference of stressed animals compared to the habituated and naive groups. The between-groups clustering (*Y*-axis) identified that the immediate stress condition differed from all others and that the habituated and naive groups were the most similar (i.e. separated by the lowest Euclidian distance) thus corresponding to the graded levels of stress induction.

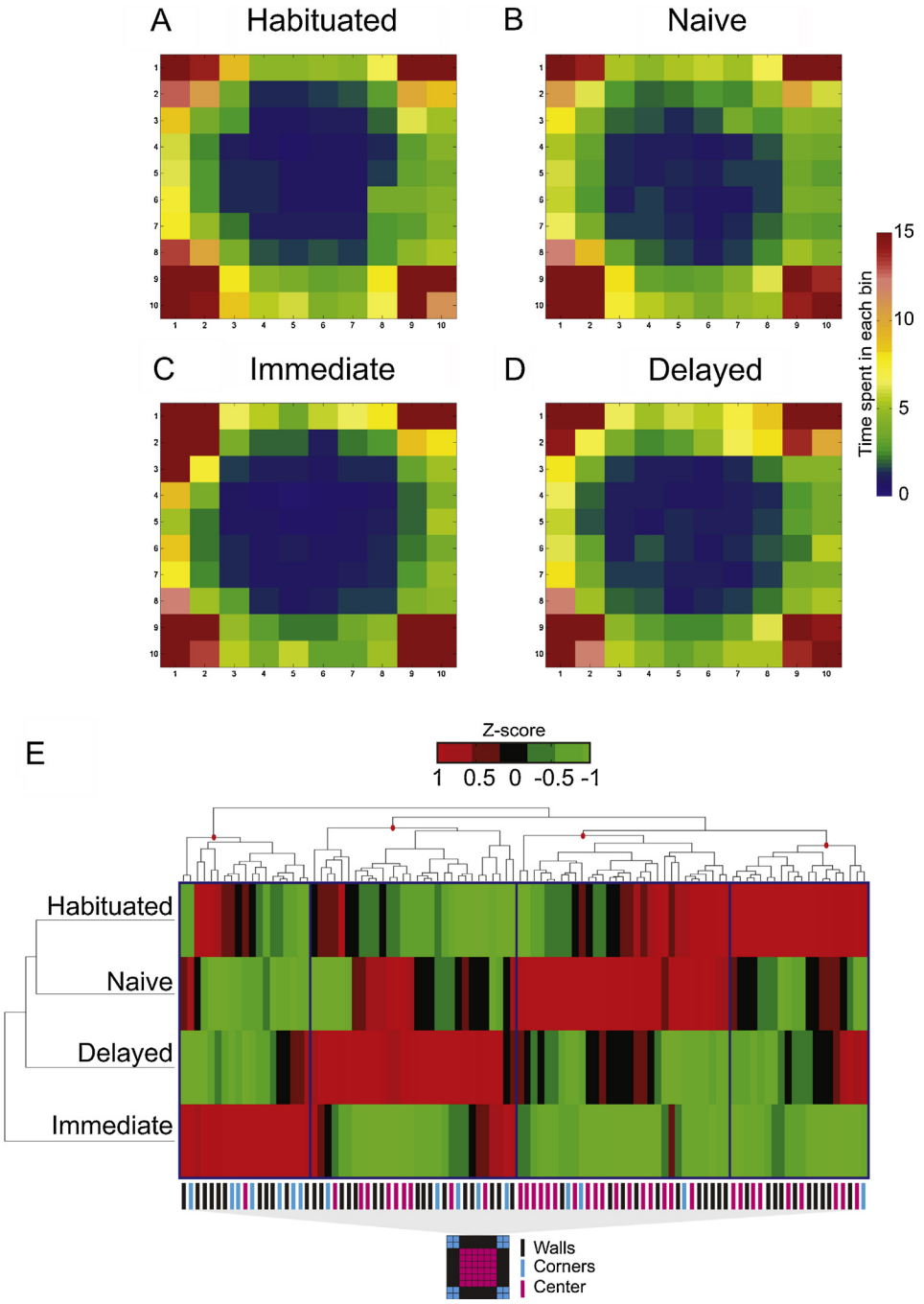

Fig. 3. (A–D) *COLORcation* heat maps of activity of habituated, restraint-stressed (immediate or delayed) and naïve mice ($n = 16–18$ for each condition) showing a trend to avoid the OF center in "Immediate" and "Delayed" mice compared to the "Habituated" and "Naïve" mice. Each arena is divided into 100 bins and the color defined as the average time spent in seconds in each bin. (E) Hierarchical clustering according to the groups and the spatial bins in the "restraint-stress" experiment. The analysis reflects the stress experienced in each group and clusters them accordingly, defining the "Immediate" most polar to "Habituated" group. Moreover, it illustrates the bias toward peripheral regions of the OF arena in the "Delayed" and "Immediate" groups. The color scale represents the *Z*-score (standardized mean time spent in the bin).

Collectively this clustering analyses indicates, as expected, that the more the mice are stressed the more they prefer to explore peripheral regions. Thus, *COLORcation* could reveal differences between experimental groups that were not apparent using standard analysis methods.

3.4. *Compatibility of* COLORcation *with Ethovision raw data*

We performed a separated experiment to verify the compatibility of *COLORcation* with Noldus Ethovision, one of the most widely used animal tracking platforms. For this purpose 10 animals were subjected to a 10 min OF session and the video files recorded for off-line analysis. We used Ethovision XT11 to measure anxiety-related parameters, produce individual and group heat maps and export raw tracking data. Using the same area definition (25% of the total area) both Ethovision XT11 and *COLORcation* produces similar results (see individual values in Table 2) for parameters such as the total distance traveled (Ethovision, 2191 ± 260.6 cm; *COLORcation*: 2199 ± 262.1 cm; $t = 0.0194$, $P = 0.984$), the distance traveled in the center (Ethovision, 127.1 ± 29.27 cm; *COLORcation*: 135.7 ± 31.23 cm; $t = 0.0199$, $P = 0.843$), the time spent in the center (Ethovision, 9.67 ± 2.50 s; *COLORcation*: 9.70 ± 2.54 s; $t = 0.0098$,

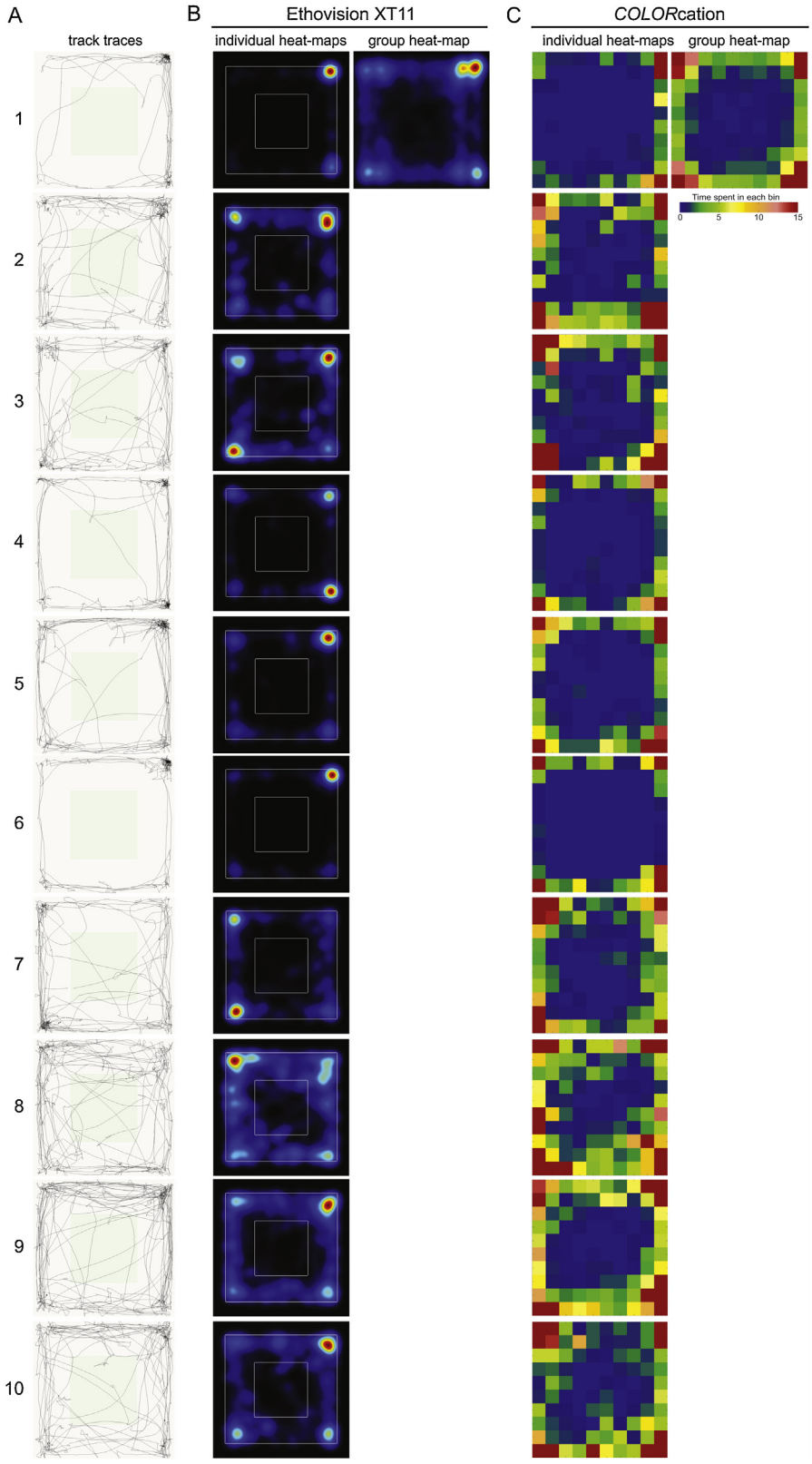

Fig. 4. (A) Individual tracking traces from mice exploring an OF arena for a period of 10 min. The center area is depicted by a light green square. (B and C) Individual and group heat maps generated with the Ethovision XT11 and *COLORcation* softwares. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$P = 0.992$) and the number of visits in the center (Ethovision, 8.5 ± 2.06; *COLORcation*: 8.7 ± 2.12; $t = 0.0674$, $P = 0.946$) which were non-significantly different. Indeed, these data confirm that Ethovision users can analyze their raw data with *COLORcation*. Both programs generate a group heat map representation which aims to emphasise the regional preferences (Fig. 4). However, there is a

Table 2
Comparison of Ethovision XT11 and *COLORcation*.

Parameter	Animal #	Ethovision XT11	*COLORcation*
Total distance traveled (cm)	1	1205.01	1206.338
	2	2104.94	2108.553
	3	2125.34	2124.447
	4	1465.74	1471.075
	5	2175.39	2175.513
	6	1078	1078.138
	7	2255.23	2275.635
	8	3010.1	3021.876
	9	3726.37	3737.531
	10	2767.34	2786.32
Distance traveled in center (cm)	1	11.9076	11.295
	2	159.14	164.284
	3	141.846	151.333
	4	48.0861	50.515
	5	82.2105	88.88
	6	0	0
	7	157.534	179.239
	8	302.042	321.495
	9	191.728	194.822
	10	176.578	194.76
Time spent in the center (s)	1	0.72	0.692
	2	12.24	11.692
	3	14.56	14.538
	4	3.28	3.153
	5	4.24	4.307
	6	0	0
	7	9.76	9.769
	8	24.8	24.692
	9	9.6	9.307
	10	17.52	18.923
Number of visits in center	1	2	2
	2	9	9
	3	11	12
	4	2	2
	5	4	4
	6	0	0
	7	9	9
	8	20	20
	9	12	12
	10	16	17

methodological difference between the two heatmaps, our method allows an absolute and accurate representation of time spent per bin while Ethovision's heat map reflect highlights hot spots visited by the mice. We believe that numerical representations of exploratory behavior as proposed in our program convey information regarding the variability within a group and group differences and will be valuable for future behavioral studies.

4. Discussion

Despite the introduction of computer-assisted videotracking for data collection in OF experiments, the parameters provided in most OF studies are still limited to those defined in the manual scoring era (Hall and Ballachey, 1932). Furthermore, the need to select representative tracks for data presentation does not capture the full complexity of a typical OF dataset. *COLORcation* captures and presents complex group datasets as activity heat maps, and calculates a series of informative parameters with appropriate statistical analyses. We demonstrated the utility of *COLORcation* in two different anxiety-induction paradigms, and found that *COLORcation*'s heat map representation and cluster analysis could detect group differences in a restraint-stress paradigm that did not reveal differences using standard parameters.

OF exploration can be defined by a range of kinematic indexes (location, speed, acceleration, path curvature and heading direction) and patterns such as walking/running, bounding, progressing, pivoting, circling, scanning, as well as rearing, leaning, stretch-attend, grooming and gnawing (Fonio et al., 2006). *COLORcation* does not quantify the latter parameters, although variations in non-locomotory behaviors may affect kinematic values such as distance traveled and movement velocity. The initial version of *COLORcation* was designed for robustness and compatibility with a wide range of OF designs, hence the focus is on location, distance, and speed parameters, with a user-defined option to include rearing data (vertical activity). Future versions might be expanded to include tools allowing the discrimination of locomotion segments (Kafkafi et al., 2001), for detection of more subtle and cognitive or neuropsychiatric phenotypes (Drai and Golani, 2001).

OF arena design can vary, with both rectangular and circular versions in use (Gould et al., 2009). There are advantages and caveats to both formats. Direct examination of the effects of size, color and shape of OF arenas on mouse exploration revealed highly similar temporal organisation of novelty-induced exploration in circular and rectangular arenas (Kalueff et al., 2006). *COLORcation* is suitable for rectangular arenas, in which the tracking arena can easily be subdivided for analysis in squares of equal dimensions. Circular arenas would have required inner–outer concentric binning, however two concentric circles will have different radii, thus the distances traversable in each bin would not be equal. This problem can potentially be overcome by normalising tracking data to the dimensions of each bin, thus it should be possible to develop a *COLORcation*-like application for circular arenas if desired.

New behavioral analysis toolboxes have recently been described, aiming to provide comprehensive phenotyping solutions for a variety of rodent assays (Patel et al., 2014). *COLORcation* is a specific analysis tool compatible with multiple acquisition systems, giving users new options to make the most of their raw data. *COLORcation* can use any organised excel file (.xlsx) containing coordinates of animal locations as input, and we verified its functionality with such raw data files exported from widely used platforms such as VideoMot2 (TSE) or Ethovision (Noldus). The program is freely available for academic use upon request from the corresponding author, and we hope it will prove to be of broad utility in neurobehavioral research.

Author contributions

SYD, MMT, MF and NP designed research, SYD wrote the code; MMT and NP performed animal experiments; NP and MF wrote the paper.

Conflict of interest

Authors report no conflict of interest.

Funding sources

This work was supported by the Israel Science Foundation (grant no. 1284/13), the European Research Council (project NeuroGrowth) and by an International Civil Volunteer Fellowship from the French Ministry of Foreign Affairs to N.P.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jneumeth.2016.06.003.

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