Chapter 12

Phenotyping of Zebrafish Homebase Behaviors in Novelty-Based Tests

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Abstract

Various novelty-based assays used to quantify zebrafish (*Danio rerio*) behavior show a striking similarity to behavioral responses in rodents. Exposed to the open field test, zebrafish establish overt homebases demonstrating clear preference for a particular area of the tank. This behavior aims to establish a "safe zone" that zebrafish can familiarize themselves with and feel secure in, and is similar to homebase behaviors of various laboratory rodent species. Here we outline a simple protocol for homebase phenotyping in zebrafish.

Key words: Zebrafish, homebase behavior, exploration, open field test, cognitive maps, spatial orientation.

1. Introduction

Animal exploratory behavior provides a robust source of quantifiable endpoints used in neuroscience and behavioral research (1, 2). Traditional exploration-based paradigms include the elevated plus maze (3), light–dark box (4), and the open field test (OFT) (5–7), which have been extensively studied in rodents (6, 8–10).

The OFT paradigm has also provided important insights into animal motor and affective phenotypes (13, 14). Although the OFT has recently been applied to zebrafish (15–17), this research

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has primarily utilized larvae (18). While larval zebrafish represent a popular and useful model in neuroscience research (19–21), they are not without some limitations. For example, larvae do not exhibit the rich behavior of their adult counterparts (21), and their behavior and cognitive abilities cannot be fully translated to adult subjects' behavior. Additionally, they lack fully developed neuromediatory and endocrine systems (22), as well as some neural circuits and projections (23). Our method, therefore, will focus on using adult zebrafish to characterize their neurophenotypes.

Homebase behavior is a naturally occurring phenomenon, as animals often select a home site to which they repeatedly return after exploring the surrounding territory (24). Perhaps even more importantly, laboratory rodents use these homebases as strategic "reference points" to orient and organize their exploration (24–26). For example, mice and rodents visit many places in a novel environment, but typically choose one or two zones to spend most of their time, also displaying the highest grooming and rearing activity (26).

Taken together, this emphasizes the fact that homebase formation represents an important aspect of animal exploration. Our observations suggest that homebase behavior exists in zebrafish, and may therefore play a role in the spatial organization of zebrafish locomotor behavior. Here we present the methodology to analyze and quantify this interesting behavioral phenotype in zebrafish (also *see* (27) for details).

2. Methods and Materials

2.1. Animals and Housing

Adult wild-type short-fin zebrafish (6–8 month-old; ≈50:50 male:female ratio) can be obtained from a local commercial distributor, and should be given at least 10 days to acclimate to the animal facility. Animals can be housed in groups of approximately 20–30 fish per 40-L tank. Tanks should be filled with deionized water, with room and water temperatures maintained at ≈25°C and water pH at 7.0–8.0. Illumination can be provided by ceiling-mounted fluorescent light tubes on a 12–12 or 10–14 h cycle, consistent with the zebrafish standard of care (29).

2.2. Apparatus

The zebrafish homebase paradigm can be established using several different novel OFT tanks. For example, in our experiments, OFT1 represented a large rectangular plastic opaque tank (12.3 height × 38.7 width × 47.3 cm length) divided into nine zones. OFT2 was a white plastic cylinder (23.6 height × 22.8 cm diameter) divided into nine zones, and OFT3 was a white square tank (14.0 height × 29.0 width × 37.0 cm length) with textured surface and rounded corners divided into eight sections (*see* **Figs. 12.1 and 12.2** for details). These three apparatuses

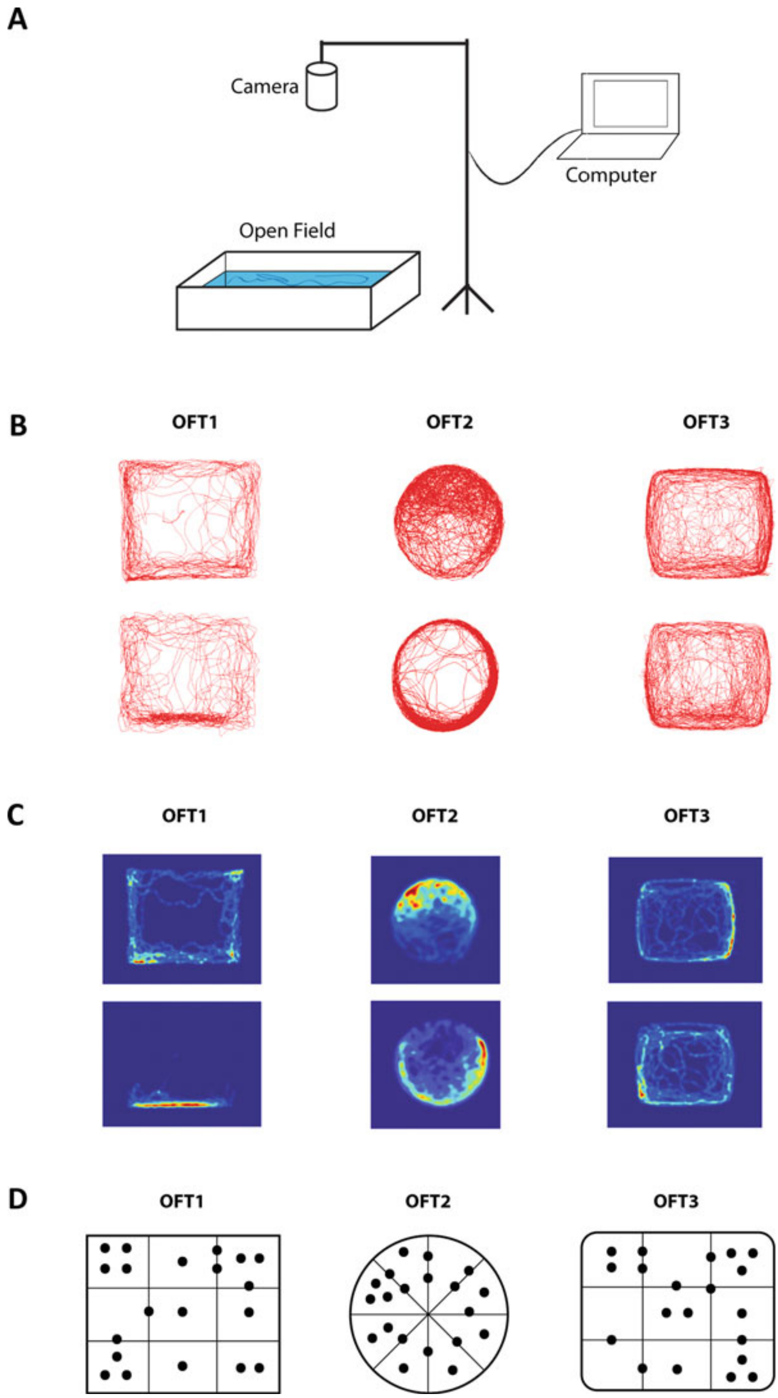

Fig. 12.1. Experimental setup and representative homebase behavior demonstrated in three different 30-min open field tests (OFT1-3). OFT1 was the large rectangular arena, OFT2 was the circular arena, and OFT3 was the small *square* arena. **a**. A typical experimental setup to record zebrafish homebase behavior in the open field test. **b**. Traces generated by Noldus Ethovision XT7 software for three different zebrafish. Note clear spatial preferences of zebrafish OFT behaviors. **c**. Density maps generated for the same fish by Noldus Ethovision XT7 (*see* Section 3.3d for details). D. Summary of homebase topography for all fish ($n = 20$ per OFT) tested here. Each homebase is shown as a *black dot*. Note that each fish was able to establish a clear homebase, typically encompassing one or, less frequently, two sectors (two-sector homebases are shown as *dots* on the border between the two respective sectors).

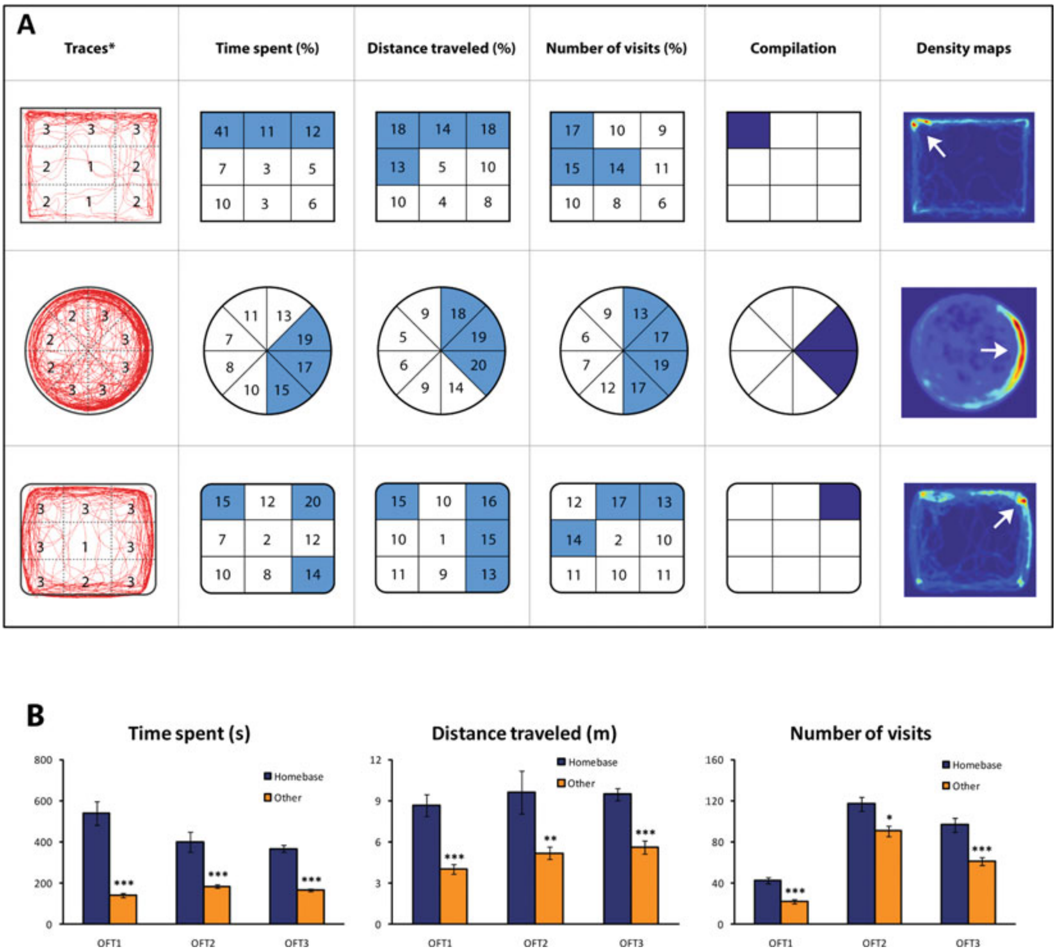

Fig. 12.2. Methodology of homebase identification in three representative zebrafish observed in three different open field test (OFT) tanks for 30 min (see details of the OFT tanks in legend to **Fig. 12.1**). **a** – Summary of the algorithm used in this study to identify zebrafish homebases. Briefly, the traces were generated by Noldus Ethovision XT7 and scored manually by two experienced observers, using a 0–3 scoring system. Time spent, distance traveled, and number of visits (frequency in zone) were calculated using the video-tracking software for each zone of OFT arenas, and expressed as percent of total. Potential homebases were identified and mapped based on top three percentages. These maps were then compiled to establish the overlap of all four levels of analysis. Density maps were generated by video-tracking software (for time spent data) and used as an additional tool to visualize and reconfirm zebrafish homebase behaviors (indicated by *white arrows*). Note a good correlation between different homebase-related behaviors and their spatial patterning (that enables a precise identification of zebrafish homebases). **b** – Confirmation of zebrafish homebases (identified using the method described above) based on calculation of average time spent, distance traveled, and the number of visits per a homebase sector vs. a nonhomebase sector of the OFT arena. Note striking and highly significant differences in zebrafish behavioral activity between homebase and nonhomebase OFT zones.

differed in size, color, shape, and texture and were selected to reveal differences in zebrafish homebase activity that may be potentially associated with distinct OFT environments. Note, however, that these OFT designs may vary according to the experimenter's preference.

2.3. Experimental Setup

The OFT should be filled with aquarium water to the level of about 12 cm. Apparatuses should rest on level ground with the same distance (e.g., 114 cm, as in our experiments) from the camera (**Fig. 12.1a**). Based on our experience, the standardized 12-cm water level allows enough room for the fish to move freely in the OFT apparatus, yet shallow enough to minimize extensive vertical movements (which may be misdetected by video tracking systems). OFTs should be positioned for optimal lighting while avoiding all glare from the room's light source. Use a light meter (e.g., 840006 by Sper Scientific, AZ) to ensure that all areas of the OFT apparatus are illuminated with the same intensity. Optimal and homogeneous lighting conditions are important for this protocol as shadows could influence zebrafish locomotion and spatial preference. In our experiments, the OFT lighting level was 500–700 lux, as detected by lightmeter applied to each zone/sector of the novel arena.

2.4. Computer-Aided Analysis

Analysis of recorded trials can be done on- or off-line using commercially available video-tracking software – for example, Ethovision XT7 (Noldus Information Technology, Netherlands); refer to **Chapter 1** by Cachat et al. for more details.

3. Procedure

3.1. Acclimation and Pre-treatment

Transport animals from their holding room to the experimental room for acclimation 1 h prior to testing. The water used in the OFT must be the same temperature as the holding room. If using filtered water drawn from a tap, note that temperature differences can evoke unwanted stress in animals. Therefore, filtered tap water may be drawn the night before, to acclimate to room temperature prior to testing. Alternatively, adjust temperature using hot water.

3.2. OFT Testing

Fill the tank with 12 cm of room-temperature filtered water. Begin video recording and promptly place the fish in the center of the OFT to begin the trial. Video-record for 30 min after placement of the fish. The trial duration may be modified (e.g., increased to several hours) according to researchers' needs and experimental goals. The experimenters should not be present in the room during the time of recording, to prevent disturbances to the fish. After recording, return fish to holding room. When changing water in between trials, make sure to place OFT back in the same place to avoid distorting its position relative to the camera (movement could interfere with proper zone alignment when computer-aided analysis is applied). Also ensure that the OFT and its environment are as homogeneous as possible. For example, remove from the vicinity of the tank any furniture items or other objects that zebrafish can perceive as additional visual cues. The role of external cues in zebrasfish homebase behavior is currently unclear, and merits further evaluation. For example, establishing homebases as "reference points" in homogenous OFT arenas may be a useful adaptive behavioral strategy used by zebrafish and other species. However, rodent homebase studies also indicate that rats and mice establish their homebases in relation to distal cues – often very subtle cues in the experimental room, such as a light switch on the wall (I. Whishaw, personal communication). Therefore, further research is needed on whether (and how) zebrafish rely on external cues in their homebase formation. For details on troubleshooting, refer to Notes 1–6.

3.3. Homebase Analysis

1. Transfer the videos to a computer for subsequent analysis using video-tracking software. Divide the OFT arenas into desired zones (**Figs. 12.1d**, **12.2a**) and set event rules to precisely and consistently register behavioral endpoints including time spent (s), distance traveled (m), and the number of visits to pre-defined zones. Fish tracks and density maps can also be generated to visualize zebrafish homebase behaviors based on swimming activity, location, and time spent (**Figs. 12.1b–c**, **12.2a**). For details on troubleshooting, refer to Note 7.
2. Identify zebrafish homebases using the following protocol (also *see* **Fig. 12.2** for details):
    a. Examine traces assigning a score of 0–3 for each zone. A score of 0 denotes no traceable activity within that zone, and 3 corresponding to very high activity. With scoring relative to each individual fish (*see* **Figs. 12.2a** and **12.3** for an example), consider each zone as a potential homebase based on tracing scores of 2 or higher. Note, however, that the score used here can be modified by the investigators. For example, more (or less) elaborate scoring system can be used, if necessary.
    b. Calculate the endpoints of distance traveled, number of visits, and time spent for each individual fish for each zone/sector of the OFT arenas. Express the total 30-min activity score for each individual fish for the entire OFT arena as 100%. From this, calculate the percent of activity (of total) for each zone of the OFT. Consider a zone a potential homebase based on three maximal percentages of the total distance traveled, time spent, and number of visits within that zone, as shown in **Fig. 12.2a**.
    c. Superimpose these four criteria for each tank in order to identify overlapping zones. In turn, overlap of all

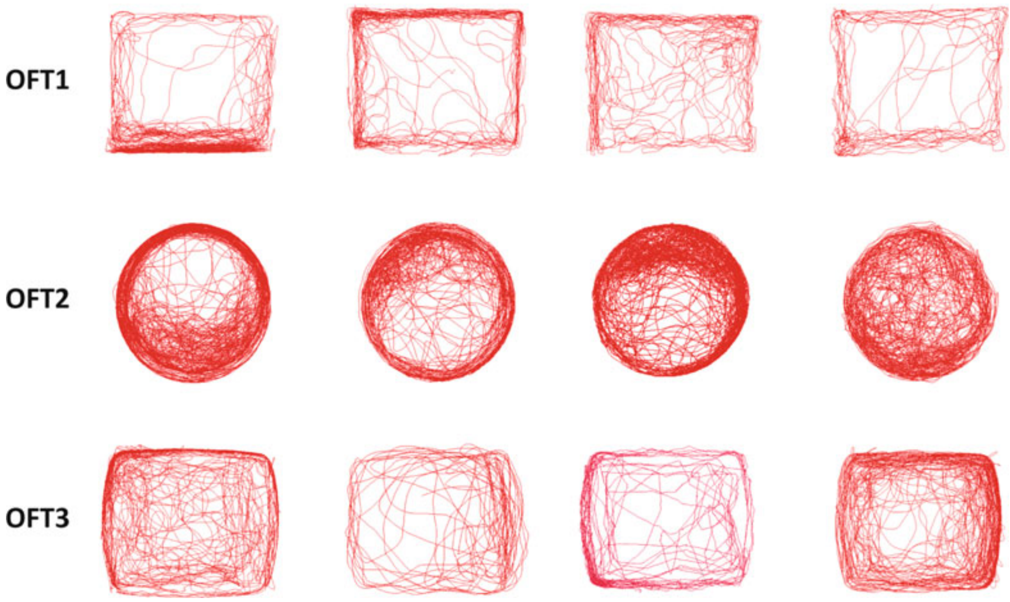

Fig. 12.3. Examples of traces recorded in three different open field tests. Note the individual differences in homebase formation.

homebase-specific loci defines that area as the final homebase for the particular trial.

d. For additional confirmation, generate density maps using Noldus Ethovision XT7, using the *EthoVision Heatmap Generator*, an add-on downloadable through the company's website (http://www.noldus.com/restricted/ethovision-heatmap-generator). Set the time interval equal to that of the recording, to generate a color gradient ranging from yellow to red based on the time spent in location (**Figs. 12.1c** and **12.2a**). This option will usually provide a good method to visualize zebrafish homebase behavior and will strongly correlate with homebase areas detected using either criteria (**Fig. 12.2a**).

For details on troubleshooting, refer to Notes 8–9.

3.4. Statistical Analyses

1. Homebase data can be analyzed using the chi-square (χ^2) or Wilcoxon-Mann-Whitney U-test. The *t*-test can also be used for normally distributed data. The U-test is useful when comparing the behavior exhibited in the homebase vs. the nonhomebase area. The χ^2 test can be performed to analyze the spatial distribution of homebase-related behaviors, comparing *actual* percentages of time spent, number of visits and distance traveled in each zone (of total 30-min scores) with *theoretical* random (by-chance) distribution of

these. First, calculate χ^2 data for each endpoint, each OFT tank, and each individual fish. Once all homebases are identified (as described above), generate three combined homebase topographic maps for all three OFT tanks, with dots representing each individual homebase (**Fig. 12.1d**).

Additionally, the χ^2 test can be applied to compare actual spatial distribution of all homebases (established in the respective OFT) with random by-chance distribution. In all our experiments, significance was set at $p<0.05$. Furthermore, *n*-way Analysis of Variance (ANOVA) can also be utilized. For example, one-way ANOVA is appropriate for comparing homebase behaviors in more than two different OFT types or more than two experimental groups, while one-way ANOVA with repeated measures would be suitable for comparing OFT types across test minutes. *N*-way ANOVA can be applied, for example, for the comparison of OFT type, time, drug, dose, sex, etc. Additionally, these analyses must be followed by a post-hoc test (e.g., Tukey or Dunnett tests).

2. To further reconfirm the homebase behavior, assess the average *per zone* activity for homebase-specific (vs. non-homebase) areas, based on percentages of time spent, distance traveled and number of visits, calculated as described above. Use U-test or ANOVA to analyze this data. For details on troubleshooting, refer to Note 10.

4. Notes

1. *Zebrafish homebase formation and/or exploration centered in middle of OFT, or focused nonrandomly on one area of OFT.* Verify that lighting conditions are optimal. For example, use a light meter (e.g., 840006 by Sper Scientific, AZ) to ensure the standard lighting conditions. Record 6–8 points (corner, near walls, center) ten times. If necessary, relocate the OFT to obtain homogeneous lighting data. Glare from ceiling lights can cause a glare that may be aversive, forcing the fish to one particular area. Shadows cast by the positioning in the room or overhanging camera can also attract the fish, and affect their homebase responses. When using opaque arenas, uneven or additional objects near or under the tank can provide cues for the fish. Therefore, visual cues must be kept to a minimum, ensuring homogenous conditions of testing environments.
2. *Zebrafish display aberrant behavioral phenotypes*
Several factors due to strain variation may nonspecifically affect animal behavior. For example, low- and high-

anxiety zebrafish strains may display higher or lower baseline anxiety levels. Some of these phenotypes could therefore result in a modulation or ablation of homebase behavior. To rule out such nonspecific factors, a careful examination of zebrafish neurological and sensory phenotypes is recommended.

3. *Fish display excessive freezing or little locomotion*
   The presence of the experimenter in the room during testing may startle the fish, causing a heightened anxiety-like behavior. Also, differences in water temperature or excessive net stress prior to testing can also induce a state of decreased locomotion. Higher anxiety strains, such as the leopard strain (12), may also demonstrate decreased exploratory behavior.

4. *High variability of observed responses*
   Despite animals' inherent tendency to form homebases, high variability in observed responses is common in behavioral research. This may be explained by genetic influences or animal stress in the animal facility (improved husbandry could normalize zebrafish behavior). It is also important that the testing room conditions (temperature, soundproofing, lighting, etc.) be carefully controlled in the experiments. Additionally, an increase in the sample size could normalize aberrant results (based on our experience, significant zebrafish data can be obtained for $n = 20$ per group). Since many studies currently involve a battery of tests, this could also influence OFT performance. Use less stressful challenges before subjecting the fish to the OFT. Acclimate fish for at least 7 days before the tests as well. Excessive stress may create potential confounds. For instance, increased freezing may increase the duration of time spent in a particular area, but will not be indicative of a homebase.

5. *Role of memory and conditioned responses*
   Zebrafish show good learning and memory capacities, can recall training for up to 10 days (30), and display robust intra- and inter-session habituation (11). Because of this, re-testing zebrafish in a novelty-based paradigm such as the OFT should be avoided (refer to **Chapter 1** by Cachat et al. for more details). However, since the OFT invokes a robust behavioral phenotype in zebrafish, this test may be utilized to further dissect the effects of various experimental manipulations on anxiety and spatial memory.

6. *Fish leap out of OFT during trial*
   Some fish have the tendency to slowly meander up to the edge of the tank and subsequently "catapult" themselves out of the OFT. Some experiments may necessitate that the water level be filled to the top of the OFT, in which case

the loss of fish is unavoidable. Precautions to deter the fish (e.g., mesh wire over the OFT) may provide confounding cues. However, keeping a water level several centimeters below the OFT edge will generally prevent this problem.

7. *Software not detecting fish*
   This lack of object detection can be resolved by altering one or several setting as well as ensuring adequate lighting (*see* **Chapter 1** by Cachat et al. in this book for details).
8. *The endpoint of duration in zone does not correspond to the traces*
   Traces are representative of the path taken by the zebrafish. Therefore, a significant duration within a zone may not necessarily correspond to movement, but rather a prolonged bout of immobility (freezing), which would appear as a single, unnoticeable point on the trace map.
9. *Zebrafish appear to be forming homebases (through track analysis), but the behavior is not significant when endpoints are evaluated on a per zone basis*
   The zone sizes may be too large. For example, the OFT may be better divided into nine smaller zones instead of four large quadrants. Even more zones may be needed as the size of the OFT increases (due to the fact that zebrafish homebase size may remain the same despite an enlargement of the arena).

5. Anticipated Results

Using this protocol, the fish are expected to establish distinct homebases – particular areas where they spent most of the time, traveled more, and visited most frequently (**Figs. 12.1b–c**, **12.2** and **12.3**). These homebases will most likely be located near the walls of the tanks, and usually consist of one or, less frequently, several zones (**Fig. 12.1d**). While this protocol describes robust homebase behavior after 30-min OFT trials (27), we have also observed overt spatial preference in zebrafish OFT swimming activity after shorter 6-min trials (own unpublished observations), suggesting that zebrafish homebase formation may begin to emerge within several minutes of novelty exposure. Spatial distribution of the time spent, distance traveled, and number of visits are expected to show significant differences in the homebase relative to the area outside the homebase. The "combined" analyses of topographical maps of zebrafish homebases in each of the three OFT tanks (**Fig. 12.1d**) will show that the different OFT zones are chosen *at random* by different zebrafish for their homebases, without spatial preference of homebase location in relation to a particular OFT (**Fig. 12.3**). Furthermore, comparison of the distance traveled, frequency of visits, and time spent within the homebase zones would reveal similar temporal dynamics of homebase behavior across different OFT arenas. Essentially, zebrafish will generally maintain constant levels of activity in their homebases (**Fig. 12.4**), frequently visiting these strategic loci.

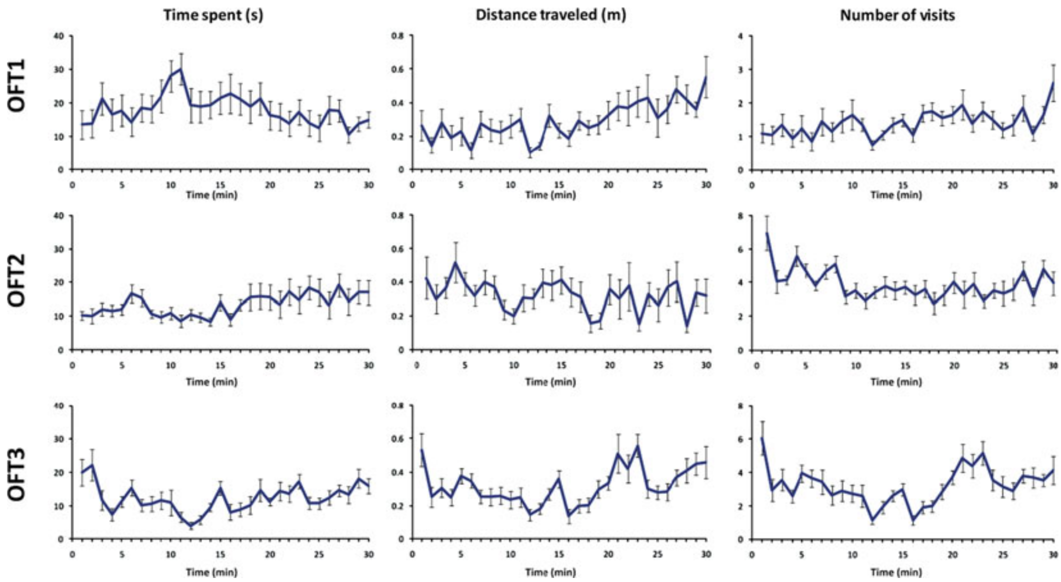

Fig. 12.4. Temporal dynamics of zebrafish homebase behaviors in three different open field tests for 30 min (distance traveled, time spent, and frequency of visits). Homebases were identified using our protocol (**Fig. 12.2a**) and reconfirmed, as shown in **Fig. 12.2b**. Note that zebrafish maintain active presence in their homebases throughout the test.

6. Summary

Here we described a simple method to identify and phenotype homebase behavior in zebrafish. Zebrafish homebase behavior (**Fig. 12.1–12.4**) is not determined by innate features of the OFT novelty, but rather actively established by animals exploring their environment, strikingly resembling homebase behavior in rodents (24, 31, 32). This new paradigm may also have a variety of important potential applications in biomedical research. For example, homebase analyses may be useful for screening pharmacological agents in zebrafish, since this behavior has already been demonstrated to be affected by different drugs in rodents (e.g., (28)). Furthermore, such analyses can be suitable for testing various inbred and mutant zebrafish strains, which may display aberrant behaviors including altered homebase phenotypes. Homebase behaviors are also highly relevant to exploration and cognition, and zebrafish models with abnormalities in either domain are likely to have impaired homebase behavior.

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