
Novel Object Recognition, Metric Change and Temporal Order Tests

Avoid behavioral testing on days when, as a part of normal animal husbandry, home cages are scheduled for changing. Cage change typically causes an increase in general activity and stress levels [44-46]. Researchers should fully describe any special circumstances pertaining to housing and care of test subjects in their experimental methods. To ensure consistency of experience prior to the test session, subjects are brought to the testing room or a common staging area, in their home cages, at least 1 hr prior to the start of behavioral testing. Individual mice can then be transported singly, in clean cages, into the testing apparatus. Test room lighting, temperature, and noise levels should be consistent for all subjects. Behavioral testing equipment described in this chapter is usually cleaned thoroughly with a solution of mild soapy water at the end of a test session. Prior to running the first subject on a day of testing, the experimenter wipes the behavioral equipment with 70% ethanol. After each subject completes its test session, fecal boli and urine are removed, surfaces are wiped with 70% ethanol, and the test chamber is allowed to dry completely before starting another subject. Most studies use a minimum of 12-15 animals per experimental group to insure sufficient power for statistical analysis [22]. See <http://www.ncbi.nlm.nih.gov/books/NBK5221/> for more details.

Before starting this protocol, please ensure you read the following:

1. To achieve the best results it is critical that only one person does all of the handling and behaviour testing with any one set of mice.
2. All procedures should be done in a well-lit room.
3. Allow at least one hour of adaptation to the test room before starting the test at Day 8-Day 10. All mice should be in their home cage initially, but the test subject can be moved to a transfer cage just prior to testing and for the intersession periods. The transfer cage and the home cage should have a filter top in place to reduce noise and scent transfer. All mice should acclimate to testing room for at least an hour. The order the mice are tested in should be recorded each day by noting the identity of the mouse in your notes.
4. Transfer the mice from the transfer cage to the arena: pick up the tail of the mouse from the home cage gently. You can either put the mouse into a beaker, or let it rest on your hand/arm, and then release the mice to the edge of the arena or transfer the mouse directly to the edge of the arena. After finishing the test, pick up and transfer the mouse either using the beaker or use your hand to support it, and then introduce the mouse back to the transfer cage.
5. Analyze data manually (the exploration time should exclude the time when sit immobile on top of the objects). Actively crawling all over the objects and exploring them is considered exploration (Goodrich-Hunsaker et al., 2008) These authors state that exploration included such behaviours as touching and sniffing the object with nose, vibrissae or forepaws, biting the object, and even crawling all over the objects.
6. Person analyzing should be blinded to genotype. It is recommended that 2 blinded researchers analyze all data to ensure consensus.
7. Behavioral testing must be performed at the same time each day.

8. Circular arena set up: The arena should have 4 visual cues at the level of the arena floor and placed off center (Borthwell et al., 2012; Hunsaker et al., 2009)
9. Releasing point for the mice: For object exploration tasks, mice should be released from the edge of the table/arena so that they have to actively approach the objects to reach them. If you are using a cup during the intersession, make sure you move mouse slowly back to the starting position. If you are placing the mouse in the chamber, do so gently. With either method they may engage in escape behaviour initially, and this should not be scored as exploration. Note that for anxiety testing you should place the mouse in the middle of the apparatus.

Please perform handling/testing in the following order:

- **Days 1-7:** Handle each mouse for ~5 min per day. Pick up one mouse, let him run on your arm while still holding on to tail, do not pet the mouse, let him explore your arm and habituate to you. Be calm, let mouse sit on your hand and become comfortable. Put mouse back in the transfer cage, and move on to the next mouse. At the end of the period move mouse 1 into the transfer cage and mouse 2 into the home cage, start handling with mouse 3. Repeat with all cage mates. Pick up each mouse a second time, but for a shorter time.
- **Day 7:** Acclimation to empty arena (15 min) for each mouse after a short handling period. If you are going to use a beaker, allow the mouse to habituate to the beaker during what would normally be the handling period.
- **Day 8:** Novel object recognition test (See detailed instructions below)
- **Day 9:** Metric/Temporal test (See detailed instructions below)
- **Day 10:** Temporal/Metric test (See detailed instructions below)

1 NOVEL OBJECT RECOGNITION PROCEDURE

This task should be performed in a well-lit room (~ 150 Lux) with no shadows in the testing arena. The arena should have 4 distinct visual cues at the bottom of each compass direction (N, S, E, W).

1. **Object Set 1 (5 min):** Transfer the mouse from the transfer cage to the arena with 2 identical objects placed at 40 cm apart.
2. **Interphase (5 min):** Introduce the mouse back to transfer cage, not the home cage. Remove objects and wipe/dip in 70% EtOH, wipe arena with 70% EtOH, replace objects with Object Set 2 at 40 cm apart. This must be completely dry before the mouse is re-introduced.
3. **Object Set 2 (5 min):** Transfer the mouse to the arena and allow mouse to explore objects for 5 min.
4. **Interphase (5 min):** Pick up and introduce the mouse back to transfer cage. Remove objects and wipe/dip in 70% EtOH, wipe arena with 70% EtOH, replace objects with one object from Object Set 1 and one Novel Object at 40 cm apart.
5. **Test Phase (5 min):** Transfer the mouse from transfer cage and introduce it to the arena and allow mouse to explore objects for 5 min.

2 METRIC CHANGE PROCEDURE

This task should be performed in a well-lit room with no shadows in the testing arena. The arena should have 4 visual cues at the bottom of the walls.

1. Transfer the mouse from the transfer cage using the beaker and introduce it to the edge of the arena with 2 identical objects (Object Set 3) placed at 40 cm apart.

2. **Acclimation Phase (15 min):** allow mouse to explore and habituate to objects for 15 min.
3. **Intersession (5 min):** Pick up mouse and place in the transfer cage. Remove objects and wipe/dip in 70% EtOH, wipe arena with 7% EtOH, reposition objects to 20 cm apart. Place subject gently back into the chamber at the same position they initially started in.
4. **Test Phase (5 min):** Transfer the mouse from the transfer cage back to the arena and allow mouse to explore objects for 5 min.

3 TEMPORAL ORDER PROCEDURE

This task should be performed in a well-lit room with no shadows in the testing arena. The arena should have 4 visual cues at the bottom of the walls.

1. **Object Set 4 (5 min):** Transfer the mouse from the transfer cage and introduce it to arena with 2 identical objects placed at 40 cm apart.
2. **Interphase (5 min):** Gently return the subject to the transfer cage. Remove objects and wipe/dip in 70% EtOH, wipe arena with 70% EtOH, replace objects with Object Set 5 at 40 cm apart.
3. **Object Set 5 (5 min):** Transfer the mouse from the transfer cage and introduce it to the arena and allow mouse to explore objects for 5 min.
4. **Interphase (5 min):** Gently transfer the mouse back to the transfer cage. Remove objects and wipe/dip in 70% EtOH, wipe arena with 70% EtOH, replace objects with Object Set 6 at 40 cm apart.
5. **Object Set 6 (5 min):** Transfer the mouse from transfer cage and introduce it to the arena and allow mouse to explore objects for 5 min.
6. **Interphase (5 min):** Pick up mouse in large beaker covered with tinfoil and introduce the mouse back to transfer cage. Remove objects and wipe/dip in 70% EtOH, wipe arena with 70% EtOH, replace objects with Test Set (Object 4 at left, Object 6 at right) at 40 cm apart.
7. **Test Phase (5 min):** Transfer the mouse from transfer cage to the arena and allow mouse to explore objects for 5 min.

4 CATEGORICAL SPATIAL PROCEDURE

This task should be performed in a well-lit room with no shadows in the testing arena. The arena should have 4 visual cues at the bottom of the walls.

1. Transfer the mouse from the transfer cage using the beaker and introduce it to the edge of the arena with 2 highly similar objects (Object Set 7) placed at 40 cm apart.
2. **Acclimation Phase (15 min):** allow mouse to explore and habituate to objects for 15 min.
3. **Intersession (5 min):** Pick up mouse and place in the transfer cage. Remove objects and wipe/dip in 70% EtOH, wipe arena with 70% EtOH, swab the locations of the objects. Place subject gently back into the chamber at with their position swabbed.
4. **Test Phase (5 min):** Transfer the mouse from the transfer cage back to the arena and allow mouse to explore objects for 5 min.