

**Application Manual**

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# **EthoVision<sup>®</sup> XT**

## **Version 18**

**Noldus**  
Information Technology

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January 6, 2025

For EthoVision XT 18

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# Chapter 1 ---

## General Information

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For more manuals, from the Windows **Start** menu choose **Apps > Noldus > EthoVision XT 18 Other Documentation**.

# Setting up the experiment in EthoVision XT

## CREATE THE EXPERIMENT

1. Start EthoVision XT and choose **File > New from template**.
2. In the **Select a template option** window, select **Apply a pre-defined template**. Next, follow the instructions in the guided setup.

For more information, see **Set Up an Experiment** in the EthoVision XT Help. For details on connecting the camera, see **Camera Installation** in the EthoVision XT Help, or watch the video tutorial **Set Up the Camera (Help > Video Tutorial)**.

3. Enter the name of the experiment and click **OK**.
4. Before you can actually acquire data, check the settings listed in the rest of this section.

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

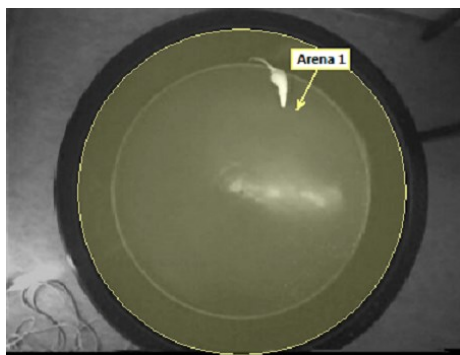
Under **Video Source**, and **Tracked Features**, make sure that the options selected corresponds to your needs. To adjust the camera settings, click the video icon in the camera row.

**NOTE** The option **Deep learning** is only available if your PC has a graphic processor (GPU) that supports additional software (CUDA). See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.

## ARENA SETTINGS

Choose **Setup > Arena Settings > Open Arena Settings 1**.

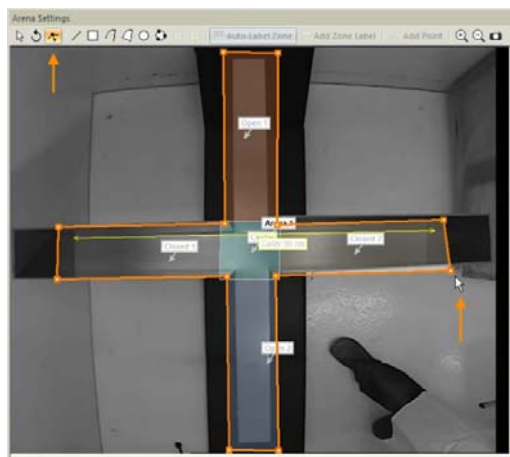
1. Click **Grab** to grab an image of the empty enclosure from the camera image, or click **Browse** to select a video file and then click **Grab**.
2. Click **1. Draw Scale to calibrate** and calibrate your arena.
3. Click **2. Select Shape and Draw Arena**. Check that the arena covers the whole area in which you want to track the animal. For open fields and other enclosures, remember to include enough of the walls so that the animal is still tracked when it rears, but exclude any bright reflective rims that might interfere with tracking. Make sure the label stays inside the arena.



**TIP** If you need to rotate the outline of the arena, drag around all elements (1), and then click the **Rotation mode** button and move the outline until it matches the arena in the video image (2).



**TIP** If you need to reshape the outline of the arena, click the **Point edit mode** button and move the vertices of the outline.



4. To draw zones, click **3. Select Shape and Draw Zones**. See more details in the chapter about your test.

For details, see **Arena Settings** in the EthoVision XT Help.

## TRIAL CONTROL SETTINGS

In the Trial Control Settings you can define conditions for the start and stop of data acquisition. For details, see the EthoVision XT Help.

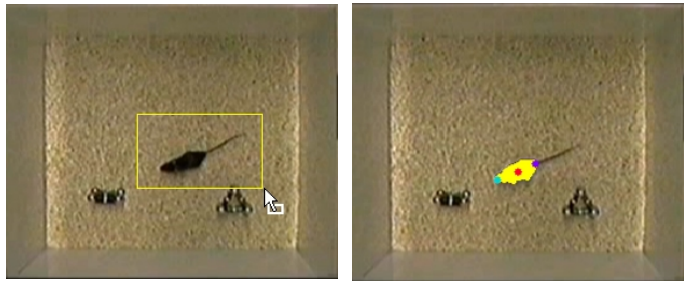
Choose **Setup > Trial Control Settings**.

## DETECTION SETTINGS

Choose **Setup > Detection Settings > Detection Settings 1**.

1. If you work with pre-recorded video, select a video file. If you work with the live camera image, release the animal in the arena.
2. Click **Automated Setup**.

3. Select:
  - **Rodent** for tracking rats and mice.
  - **Adult fish** for tracking fish from the top view.
  - **Other** for any other case like tracking insects, crustaceans, or when tracking fish from the side view.
4. When the subject does not move a lot, and is far from the enclosure walls and other objects, draw a rectangle around it. EthoVision XT detects the subject.
  - If the subject's body is well detected (marked in yellow), click **Yes**.
  - In other cases move the **Finetune** slider or click **No** and adjust the **Advanced detection** settings.



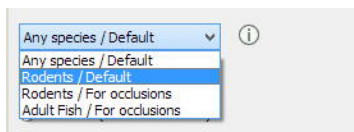
### **Note**

The **Automated Setup** may not work in all situations. If the subject is not detected correctly, see below.

### **Advanced detection settings**

1. Under **Video**, choose the sample rate.
2. Under **Method**, check that either **Dynamic subtraction** or **Differencing** is selected as the detection method. Differencing is the preferred option if other methods do not work well, or if you work with hooded animals.
3. Select the tracking method. The options here are depending on your experiment settings, including the number of body points you want

to track. The optimal method is automatically selected after choosing the type of animal in the Automated Setup.



For more information, see **Tracking methods** in the EthoVision XT Help.

4. When using Dynamic subtraction, move the slider to define the animal's contrast. The animal must be fully detected in all parts of the arena and the noise must be minimal.

When using Differencing, set the **Sensitivity** slider. The slider determines what difference in contrast from the background is seen as the animal.

#### ***For more information***

- Choose **Help > EthoVision XT Help** and search for **Advanced Detection Settings**.
- Choose **Help > Video Tutorial** and watch the videos under **How Subject Detection Works**.

## **TRIAL LIST**

Choose **Setup > Trial List**.

Enter your independent variables such as rat ID, first or second phase of the experiment, treatment (with values *drug* vs. *control*), dose, type of object, name of the experimenter, etc. If you want, you can pre-define all your trials here, or you can enter the independent variable values as

you carry out the trials. You can also prepare the trials in Excel, randomize them and then paste the values into your Trial List.

				System	User-defined	User-defined	User-defined	User-defined
Label				Acquisition status	Animal ID	Treatment	Novel Object	Familiar object
Description				The current status of acquisition per arena		(Exemplary)	(Exemplary)	(Exemplary)
Type					Numerical	Text	Text	Text
Format					x			
Predefined Values				Unknown; Postpon	All values	Control; Treated	object 1; object	object 1; object
Scope				Arena	Subject	Subject	Subject	Subject
Trial	Arena	Subject	No.					
Trial 1	Arena 1	Subject 1	1	Planned		1 Control	object 1	object 2
Trial 2	Arena 1	Subject 1	2	Planned		2 Treated	object 2	none
Trial 3	Arena 1	Subject 1	3	Planned		3 Control	none	object 1
Trial 4	Arena 1	Subject 1	4	Planned		4 Treated	object 1	object 2

Figure 1.1 An example of the Trial List with four planned trials.

Furthermore, you can define a list of trials for batch acquisition. See **Acquire a series of trials** in the EthoVision XT Help.

## PROTOCOLS ON THE WEB FEATURING ETHOVISION XT

Researchers are encouraged to publish test protocols to improve reproducibility of animal experiments.

Browse to

<https://www.bio-protocol.org/en/searchlist?content=ethovision>

There you find protocols created with or mentioning EthoVision XT.



# Acquire the data

## PROTOCOL

Each test described in this manual has one or more specific protocols. See the chapter that applies.

**IMPORTANT** Always record your video with EthoVision XT or MediaRecorder. Do not record video with Pylon Viewer or other software! The resulting video file may not be compatible with EthoVision XT.

## ACQUIRE THE TRACKS

### Procedure

1. Check that you have selected the correct **Video Source** in the Experiment Settings (see page 15).
2. Choose **Acquisition > Open Acquisition**.
3. **OPTIONAL** Skip this step if you have entered the values of the independent variables already in the Trial List.

Open the **Independent Variables** tab of the **Analysis Results and Scoring** pane at the bottom of your screen and enter the values of the independent variables. You can also do this during acquisition.

Analysis Results and Scoring			
Trial Status Independent Variables Dependent Variables Manual Scoring			
Trial	Acquisition status	Rat ID	Treatment
Trial 1	Acquired	1	drug
Trial 2	Acquired	2	saline
Trial 3	Acquired	3	drug
Trial 4	Ready for start		

4. If you did not plan any trials in the Trial List, click the **New trial** button in the **Playback Control** window (**Ctrl+F3**).



5. If you planned two or more trials: select in the **Acquisition Settings** window whether you want to track only the next planned trial, or all planned trials.

If you carry out batch acquisition and track live, also specify the **Inter-trial interval**.

6. **OPTIONAL** In the **Acquisition Settings** window, select **Auto-start data analysis**.

Do this to start calculation of analysis results immediately after acquisition. If you choose this option, first create an Analysis profile and optionally a Data profile and a Track Smoothing profile (see page 24). Choose whether to carry out analysis only for the profiles highlighted in blue in the Experiment Explorer, or for all possible profile combinations. In the latter case, first delete all profiles you do not need for analysis.

7. Start the trial (either choose **Acquisition > Start Trial** or click the **Start trial** button in the **Playback Control** window or press **Ctrl+F5**).



8. If you do live tracking, release the animal in the arena.
9. Data acquisition starts when the **Start track** condition in your **Trial Control Settings** becomes true.
10. The trial stops automatically when the **Stop track** condition specified in the Trial Control Settings becomes true or when the time defined in the Trial Control Settings under **Maximum trial duration** has expired. If you planned a series of trials, the next trial will start.

To stop the trial manually, either choose **Acquisition > Stop Trial** or click the **Stop trial** button in the **Playback Control** window or press **Ctrl+F6**.



If you chose to simultaneously record a video file, the video file that has been created is stored on your PC in the **Media Files** folder of your experiment.

### ***After testing***

After each test, remove any debris (bedding, feces, etc.) from the arena and clean all interior surfaces, walls and floor thoroughly, first using water and soap, or a perspex cleaner (see also Table 1), then with water. Allow at least 5 minutes between tests to ensure that any residual odor will have dissipated before the next animals are introduced into the test apparatus.

**Table 1** *Acceptable techniques and substances for cleaning acrylic (perspex)*

Electronic beam	High intensity visible light
Gamma radiation	Alkaline substances (in low concentrations)
Ethylene oxide gas	Low-temperature hydrogen peroxide gas plasma
Hydrogen peroxide up to 3%)	Diluted solutions of isopropyl alcohol
UV radiation	

**Do not use** wet ethylene oxide, steam, strong acids, hydrocarbons, ethanol, methanol, or solvents containing acetone.

# Analyze the data

## DATA PREPARATION

### *Track editing*

Choose **Acquisition** > **Edit Tracks**.

You can fix tracking errors and swap back nose-points and tail-points that have been swapped in tracking. Normally you do not need to edit your data.

### *Track smoothing*

Choose **Acquisition** > **Track Smoothing Profile**.

Select one or both options to remove noise and artifacts due to for example body wobbling. For more information, see **Smooth the Tracks** in the EthoVision XT Help.

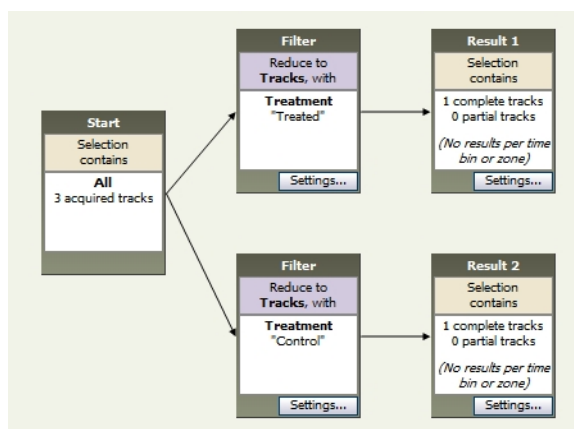
### *Select data for analysis*

Choose **Analysis** > **Data Profile**. The data you select on this screen will be analyzed or visualized (see the next sections).

- To analyze data per zone, in the Result box, click **Settings** and select **Results per zone**. Use this function for example to analyze the velocity of the subject in the different zones.
- To analyze some tracks, not others, under **Filter**, choose one of the variables available. For example, **Treatment** to analyze *Treated* animals only. Insert the box in the Data profile sequence.
- To analyze segments of tracks based on the subject's behavior, under **Nesting** choose one of the options. For example, to select all data points when the subject was moving, choose **Movement**. Insert the box in the Data profile sequence.
- To analyze the tracks split in segments of equal duration, in the Result box, click **Settings** and select **Time bins**. Use this function for

example to analyze a 10-minute recording in ten segments of one minute each.

- To create groups of tracks and compare results, under **Common Elements** click **Result** and add a **Result** box. You can link each Result box with a specific Filter (see above). This way you can compare the results of different selections, or visualize groups of tracks that result from different treatments, etc.



### ***For more information***

See the EthoVision XT Help.

## **VISUALIZE THE DATA**

You can visualize your tracks in three ways:

### ***Plot the tracks***

Choose **Analysis > Results > Plot Tracks**.

You can view your tracks on a still image of the background. Tracks can be shown in different colors according to the values of independent variables (for example, blue for animals treated with saline and red for drug-treated animals). Sample points can be shown in different colors

according to the values of dependent variables, for example red when the animal was moving fast.

To set the track colors, in the **Track Plot Settings** pane, click the **Colors** tab.

### ***Plot integrated data***

Choose **Analysis > Results > Plot Integrated Data**.

Look at a track with the video file in the background. When you plot integrated data you can also view Time Event plots of your independent variables. Just like with plotting tracks, you can show tracks or sample points in different colors.

### ***Plot heatmaps***

Choose **Analysis > Results > Plot Heatmaps**.

Make heatmaps of the location of your animals during the tracks.

For more information, see **Visualize Data** in the EthoVision XT Help.

## **CALCULATE THE STATISTICS**

### ***General procedure***

1. Choose **Analysis > Analysis Profile > New**.

Select the variables you are interested in. For information about the variables suggested, see the chapter about the test of your choice and the EthoVision XT Help.

2. Choose **Analysis > Results > Statistics & Charts**, then click **Calculate**.

The **Trial Statistics** tab shows the results per trial. The **Group Statistics** tab shows the statistics and charts from the results over all trials or the groups defined in your Data profile (see page 24).

### ***Adjust the table layout (optional)***

Sometimes you need to adjust the layout of your results table, for example to export the results to a statistics software. Click the **Layout** button on the toolbar and specify which headers should be on the rows

and which on the columns. For details and examples, search for *table layout* in the EthoVision XT Help.

### ***Batch calculations***

It is also possible to carry out multiple calculations at once with different filters, data profiles or analysis profiles. To do so:

1. In the **Statistics & Charts** window, click **Batch**.
2. Select the profiles from the lists and click **Add**.
3. Repeat the previous step for other combinations.
4. Click **Calculate**. Choose the profiles from the lists on the toolbar to view the analysis result for that combination.

For more information, see **Calculate Statistics** in the EthoVision XT Help.

### ***Advanced analysis with JavaScript custom variables***

In the Analysis profile you can now create fully custom dependent variables with JavaScript code. With JavaScript you have an almost endless range of possibilities to extract information from your tracks. JavaScript variables can be of type continuous, event, or state.

See **JavaScript** in the EthoVision XT Help.

**NOTE** If you are not familiar with programming, we can create custom analysis variables for you. Please contact Noldus for information.

## **EXPORT THE RESULTS**

To find out if your independent variables (like treatment, dose, etc.) have a significant effect on the dependent variables, you can export your data to a statistical package.

1. Choose **Analysis > Export > Statistics**.
2. Choose **Trial Statistics** or **Group Statistics**.

You can also export the raw track data. Choose **Analysis > Export > Raw Data**.

## **Chapter 2** ---

# The Open Field Test

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# Introduction

## THE OPEN FIELD FOR RODENTS

The open field test was first published 1932 by C. S. Hall and E.L. Ballachey to determine the fearfulness of rats. Today, the test is often used with rats and mice to investigate their locomotory behavior and their willingness to explore. Also, the test is one of the most commonly used methods to investigate anxiety in rats and mice. It is based on the natural tendency of rats and mice to spend more time in the corners and the periphery than in the center. Changes in these measures are often used to assess the sedative or stimulant effects of pharmacological agents.

The test arena consists of a circular or rectangular empty open space with walls to prevent the animals from escaping. The arena is divided into a border zone and a center zone. In addition, the arena can be subdivided into a number of other zones, like north, east, south and west zones, or corner zones.



**Figure 2.1** *A rat in a square open field.*

Common behaviors to be measured are:

- Time spent in the border zone and center.
- Time until the border of the center zone is first crossed.
- Walking distance in each of the zones.
- Percentage of time the animal is mobile/immobile.
- Walking speed in each of the zones.
- Frequency of rearing.

### **References**

- Hall, C., Ballachey, E. L., 1932. A study of the rat's behavior in a field. A contribution to method in comparative psychology. *University of California Publications in Psychology*, Vol 6, 1-12.
- Gould, T.D., Dao, D.T., Kovacsics, C.E., 2010. The Open Field Test. *Neuromethods*, Vol 42, 1-20.
- De Visser, L., Van Den Bos, R., Kuurman, W.W., Kas, M.J.H., Spruijt, B.M., 2006. Novel approach to the behavioural characterization of inbred mice: automated home cage observations. *Genes brain and behavior*, Vol 5(6), 458-466.

For the complete list of publications, see the results in [Google Scholar](#).

On the downloads section of the Noldus website, [my.noldus.com](http://my.noldus.com), you find a number of sample experiments, including the open field.

## **THE OPEN FIELD FOR ZEBRAFISH**

### ***Open field***

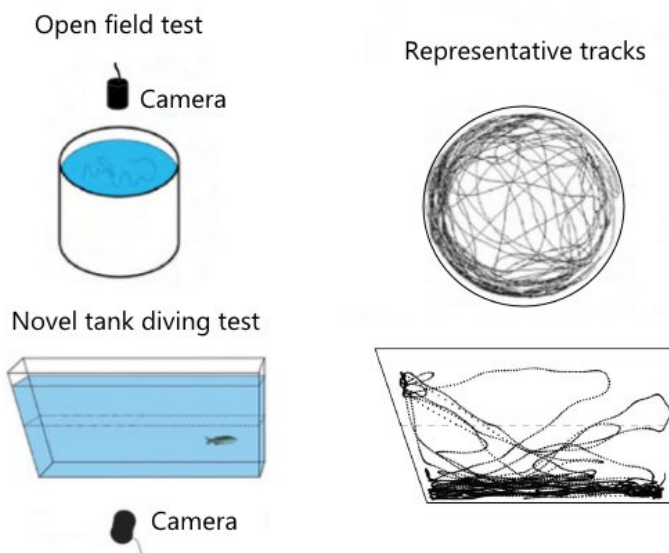
The open field test (Figure 2.2, top) is used to evaluate exploratory and affective behaviors. Fish are filmed with a video camera positioned overhead. The bottom of the tank is divided into two virtual zones, center and periphery (a circular zone with inner boundary 5 cm from the testing tank wall), which enables quantification of regional activity.

In addition to standard movement parameters, primary behavioral measures included the distance traveled, number of visits and time spent in central and peripheral zones, and the ratio of time spent in the center zone over total testing time.

### ***Novel tank diving test***

The novel tank test (Figure 2.2, bottom), is used to assess exploratory and affective domains. The novel tank is a trapezoidal tank maximally filled with treated water and divided into two equal, horizontal regions (top/upper and bottom/lower zones).

Primary behavioral measures include the number of visits and time spent in central and peripheral zones, the ratio of time spent in the top zone over total testing time, latency to enter the top zone, time spent in the top zone, number of transitions to top zone.



**Figure 2.2** *Open field and novel tank diving test for zebrafish. Redrawn from Kalueff et al. Trends in Pharmacological Sciences, 35: 63 - 75.*

Similar to behavioral tests used in mammalian models, affective phenotypes are inferred from modulation of thigmotaxis (center/periphery in the open field test) and geotaxis (top/bottom in the novel tank diving test).

### ***References***

Kalueff A.V., A.M. Stewart, R. Gerlai (2014). Zebrafish as an emerging model for studying complex brain disorders. *Trends in Pharmacological Sciences*, Vol. **35** (2), 63 - 75.

### ***Example videos***

[https://www.youtube.com/watch?v=7vhK8fq\\_W4A](https://www.youtube.com/watch?v=7vhK8fq_W4A).

# Physical setup

The following suggestions are meant to optimize video tracking:

- The camera should have a good view of the entire region the animal can be in. The arena should fill the field of view.
- The lighting should be as even as possible throughout the arena. Make sure there are no shadows in the corners. Bright lights are not necessary but should you need to supplement the light, consider red (or infra-red) light, as the animals will be less sleepy. Regardless of the light level chosen, the investigator should use a light meter to record the lighting level and report this level in any publication.
- When working with multiple arenas, make sure that the background light intensity is the same across arenas. In the picture below, there is more background light in the arenas on the left than in the arenas on the right. This is usually not a problem, as long as the contrast between each animal and its arena background is much bigger than the difference in brightness between arena.



**Figure 2.3** *Minimize the shadows at the corners of open fields using even, diffuse light, in any case not pointing to the floor since it would create shadows.*

- The lighting should be diffuse, so as not to cast strong shadows (which might be tracked instead of the animal). Even, diffuse lighting is important to optimize tracking of the nose.
- The background should contrast with the animal. If necessary use a different setup for light and dark colored animals. Matt, gray walls often give good results.
- Depending on circumstances, pools of urine can cause problems with the tracking because they reflect light. An absorbent base can help.
- Make sure the sides of the box are not reflective (e.g. sanding). Alternatively, exclude the sides from the arena, but make sure that the animal is tracked well in the entire border zone.
- Always place the apparatus in the same position in the room, cues from overhead might influence the behavior of the animal. For the same reason the experimenter should not be visible to the animal during the trial (automated tracking makes this possible).
- Minimize disturbance from audio sources, especially from ultrasound sources such as ventilation fans, CRT monitors, or strip-lights. You can mask audio disturbance by using the same background sounds, for example of a radio, in the test room as in the room where the animals are normally housed.
- When you handle the animals, be careful to minimize stress.
- If you use multiple arenas, make sure that the animals in the arena do not influence each other. The animals should, for example, not be able to hear or smell each other.
- In EthoVision XT, choose **Help > Video Tutorial** and watch the video chapter **Set Up Your Test Environment**. That provides useful tips about the camera position, how to improve lighting and contrast with the background.
- If you intend to use the **Deep learning** method to track the subject's nose point, note that there are additional requirements and limitations. See the EthoVision XT Help.

# The Open Field experiment in EthoVision XT

Create an experiment. For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help.

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

- To detect freezing behavior, select **Activity analysis** under **Analysis options**. Click the video button next to it and watch the video tutorial to learn about Activity analysis.
- If you work with rats or mice, you can let EthoVision XT automatically detect behaviors like *Rearing*, *Grooming*, and *Sniffing*. Select **Behavior recognition** under **Analysis options** and choose the options that apply to your experimental setup. The **Behavior recognition** option is only available if you have the Rat Behavior Recognition module or the Mouse Behavior Recognition module of EthoVision XT.

## MANUAL SCORING SETTINGS

Choose **Setup > Manual Scoring Settings**.

If you used the template for an open field experiment, two start-stop behaviors have been predefined in the manual scoring settings. These are the behaviors *rearing* and *grooming*. Depending on your research question, you can add other behaviors that can be manually scored, like defecating, which is a measure for anxiety, and urinating and vomiting.

Note that with the Rat (or Mouse) Behavior Recognition module you can have EthoVision XT detect a number of behaviors automatically.

For more information, see **Manual Scoring Settings** and **Behavior Recognition** in the EthoVision XT Help.

## ARENA SETTINGS

Choose **Setup > Arena Settings > Open Arena Settings 1**.

We assume that you followed the procedure in **ARENA SETTINGS** on page 15.

### **Arena**

Check that the arena covers the whole area in which you want to track the animal. Remember to include enough of the walls so that the animal is still tracked when it rears, but exclude any bright reflective rims that might interfere with tracking. Make sure the label stays inside the arena.

**TIP** If you create an experiment from scratch, the arena is not yet defined. Click **2. Select Shape and Draw Arena** and choose the drawing tool. If you want to have the open field divided in quadrants, choose one of the **subdivided** tools. Depending on the shape of your open field, then draw the outline of the arena and select the number of sectors.



Each sector is automatically defined as zone.

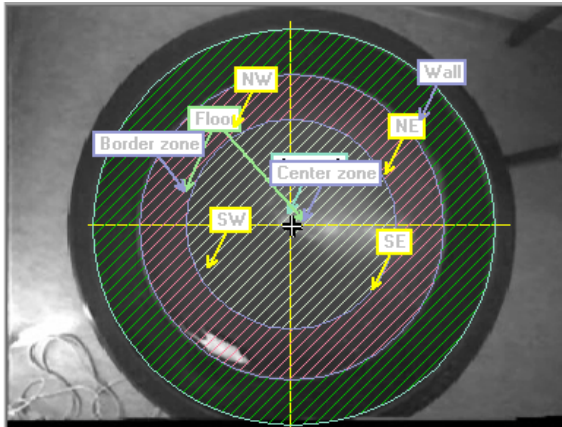
### **Zone group “Borders”**

Check that the ‘Wall’ zone covers only the wall of the open field (see Figure 2.4). If necessary, adjust the center zone and the border zone.



### Zone group “Quadrants”

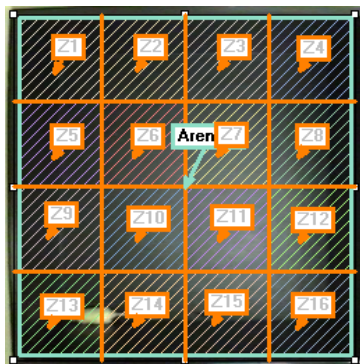
Check that the zones ‘NW’, ‘NE’, ‘SE’, and ‘SW’ divide the ‘Floor’ zone in four equal quarters.



**Figure 2.4** Circular open field with ‘Border’ zone and ‘Quadrants’ zone.

### Zone group “Squares”

Check that the zones divide the arena in equal squares.



**Figure 2.5** Rectangular open field with a subdivided arena.

### ***Arena settings for Rat/Mouse behavior recognition***

When you selected **Behavior recognition** in the Experiment Settings (only available when you have the Rat or Mouse Behavior Recognition Module of EthoVision XT), defining a few special zones and points significantly increases behavior recognition accuracy.

- **Feeder zones.** To detect eating at the feeder.
- **Drinking spout points.** To detect drinking.
- **Wall zones.** To detect rearing to wall and to discriminate this from unsupported rearing.

For the exact procedure, see **Zones and points for Behavior recognition** in the EthoVision XT Help.

## **TRIAL CONTROL SETTINGS**

In the Trial Control Settings you can define conditions for the start and stop of the data acquisition.

Choose **Setup > Trial Control Settings**.

### ***Default Trial Control***

The templates for the open field contain two Trial Control Settings profiles:

- **Default.**
- **Track duration 10 (or 30) min** – When you use this profile, tracking starts automatically 2 seconds after the animal has been placed in the arena. The track stops automatically after 10 or 30 minutes.

If you use Behavior Recognition or Activity Analysis, the default Trial Control rule is slightly different. See **Trial Control** in the EthoVision XT Help.

### ***To stop acquisition after some specific time***

Open a Trial Control Settings profile, click the **Settings** button in the **Condition - Time (1)** box, and set the time you require.

Traditionally, the open field test had a duration of between 2 and 10 minutes. This short length of time was chosen in the past because the data were acquired manually. It also emphasizes the exploratory behavior and response to novelty, rather than baseline activity of the rodents. Technology like video tracking with EthoVision XT allows for much longer periods of monitoring.

## DETECTION SETTINGS

Choose **Setup > Detection Settings**.

We assume that you followed the procedure in **DETECTION SETTINGS** on page 17.

### *Sample rate*

Under **Video**, set the sample rate. Common values are, expressed in number of images analyzed per second:

- For rats: 5 samples/s.
- For mice: 12.5 samples/s.
- For nose-tail tracking of all rodents: 25 to 30 samples/s.
- For adult zebrafish: 5 samples/s.

Remember, however, that the optimal sample rate is much depending on a variety of factors, including the characteristics of the setup, and even the animal's strain and behavior. Faster animals need a higher sample rate. This is also valid for fish. If the fish shows very rapid movements, then set the sample rate to the maximum; in other cases a sample rate of 5 fps may be enough.

### *To detect freezing behavior*

Open the **Activity** section in the **Detection Settings** pane. This option is only available if you selected **Activity analysis** under **Analysis options** in the Experiment settings. Enter the **Activity** threshold for detecting a change in the pixels in the arena. Start with the default value and check the purple-colored pixels in the video image. Adjust the threshold in

such a way that when the animal is completely still, those pixels should be almost completely absent.

### ***Rat or Mouse Behavior Recognition***

If you selected **Behavior Recognition** in the Experiment settings (only available when you have the Rat or Mouse Behavior Recognition module of EthoVision XT), remove the tail from the detected body contour. To do so, in the **Detection Settings** pane:

1. Under **Subject Contour**, select one or more pixels for the first **Erosion** field until the tail is no longer detected. The detected body area (in yellow) looks now smaller.
2. Increase the **Dilation** filter until the detected body area is the same as before.

Next, open the **Behavior** section in the **Detection Settings** pane. Wait until the rat has a normal, straight, posture (so not bending or rearing) and is moving forward. You should see the hips of the rat moving. Click **Grab** and check that the body contour is correct. You can also enter the values manually if you know them from previous experiments. However, in this case make sure that the conditions are equal, like lighting, size of the animal and distance between the animal and the camera. The detected body must have a minimum length of 60 pixels (sum of nose-point to center-point and center-point to tail-base) for **Behavior Recognition**. The detected body must also be smaller than half the arena size.

For more information, see the EthoVision XT Help.

# Acquiring data

## PROTOCOL

A typical open field test is carried out in the following way:

1. Transport the subject to the testing room at least 1 hr. prior to testing, to allow them to acclimate to the experimental room. This time period should be extended if the transfer involves excessive changes in the environment.
2. Clean the open field, even if it is not dirty prior to the initial trial. This way all animals experience the residual smell of the disinfectant equally.
3. In EthoVision XT, click the **Start Trial** button. EthoVision XT waits until the subject is detected in the arena.
4. Place the subject in the open field. Commonly, the subject is placed in the center. However, placing adjacent to a wall is sometimes used to avoid immediate exposure to a stressful situation. Also, in case of a large open field, the position adjacent to the wall may be easier to reach.
5. The researcher should leave the room, or position himself or herself as far as possible away from the arena and field of view of the subject. Also the tester should remain quiet throughout each trial.
6. Tracking starts after the subject is detected in the arena (see also Trial Control Settings on page 38). Make sure that tracking does not start because your arm is detected instead of the animal. To do so, for example, make a door in the open field, or increase the period that the subject needs to be detected before tracking starts (see page 38). If you use multiple arenas, tracking starts in each arena separately, depending on when the subject is detected in that arena. The subject is allowed to explore freely for a standardized time. Traditionally track duration was between 2 and 10 minutes, but video tracking with EthoVision XT allows longer tracking (see also page 38).

7. After the test has ended, take the subject out of the arena and put it back in its home cage.
8. Optionally, count the fecal boli in each zone of the open field.
9. Clean the open field and allow it to fully dry prior to introducing the next test subject.

### **Notes**

- **TIP** When tracking white animals, then wear dark clothes and when tracking dark animals then wear white clothes. This prevents EthoVision XT from detecting your arms while you release the animals in the arena.
- See also **ACQUIRING TRACKS** on page 21.

## **SCORE BEHAVIORS MANUALLY**

Once you have defined your behaviors of interest in the Manual Scoring Settings (page 35), you can score those behaviors in two ways:

- **Live.** That is, during the test. On the Acquisition screen, click the Manual Scoring tab and score behaviors when these occur.
- **Offline.** If you have recorded video, choose **Acquisition > Manual Scoring**. Review the video and add or edit behavior scores.

For more information, see **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

# Data Analysis

## ANALYSIS PROFILES

The template experiment contains four analysis profiles:

- **Distance & Time** – With the variables **Distance Moved** and **Velocity**, the mean velocity and the total distance moved and the group means with their standard errors are calculated. The total distance moved is a measure of the activity of the test animal.
- **Movement** – With this analysis profile the behavior of the animals is divided into moving and not moving. The frequency, duration and latency to either of these states and the group means with their standard errors are calculated. Like total distance moved, movement is a measure for activity.
- **In zones** – With this analysis profile, the frequency the animal visited the zones defined in the arena settings and the group mean with its standard error are calculated. The data profile also contains the total time spent in the zones and the latency until the animal first enters either of the zones.
- **Behaviors** – With this analysis profile, the frequency, duration and the group means with their standard errors of the manually scored behaviors are calculated. In the template experiment, the behaviors rearing and grooming are predefined. Possibly, you added additional behaviors like defecating, or urinating (see page 35). You can add these behaviors to the analysis profile and calculate for example their frequency and duration.

## ANALYSIS PER ZONE

*To calculate distance or speed in a specific zone*

1. Choose **Analysis > Data Profile > New**.

Click on the **Settings** button on the **Result** box, select **Results per zone** and subsequently select the zones you want to analyze.

2. Next, choose **Analysis > Analysis Profile** > open the analysis profile and select the variable (distance, velocity etc.).
3. Choose **Analysis > Results > Statistics and Charts**.

#### *To calculate time spent in a specific zone*

1. Choose **Analysis > Analysis Profile > New**.

Choose **In zone**, select the zones you are interested in.

2. Choose **Analysis > Results > Statistics and Charts**.

## **TIME VARIATION OF BEHAVIOR**

Define *Results per time bin*. In this way, the dependent variables are calculated over each time interval.

1. Choose **Analysis > Data Profile > New**.
2. Click the **Settings** button on the **Result** box, select **Results per time bin** and specify the time interval.
3. Choose **Analysis > Results > Statistics and Charts**.

## **ZONE CROSSINGS**

In addition to the predefined analysis profiles you can create an analysis profile for zone crossings. This way you can calculate the number of crossings from the border zone to the center zone.

1. Click the **Add** button next to **Zone transition**.
2. Select the zone sequence.
3. Depending on which option you select (**Allow intermediate zone visits** or **Only count direct transitions**) you can get different results.

For more information, see the **Zone transition** in the EthoVision XT Help.



## **FREEZING BEHAVIOR**

If you want to quantify freezing behavior, which is a measure of the fear the animals experience, create an analysis profile for **Activity state**. Activity is defined as the amount of pixels in the arena that change at any time. Click the **Add** button next to **Activity state** and define the number of states and their thresholds (for freezing, the important threshold is **Inactive below**). On the **Statistics** tab, choose the statistics. With this analysis profile the behavior of the animals is divided into **the states you specified**.

Next, use the **Plot Integrated Data** function to visualize the activity states. Adjust the settings in the Analysis profile until the state “Inactive” matches the freezing behavior of the animal in the video.

The frequency, duration and latency to all those states is calculated in the next step (see Calculating statistics).

For more information on Activity, see the EthoVision XT Help.

## **STRETCH-ATTEND**

Stretch-attend postures can be detected with the **Body elongation state** variable in the Analysis profile.

For more information, see **Body elongation state** in the EthoVision XT Help.

## **RAT OR MOUSE BEHAVIOR RECOGNITION**

To analyze the automatically detected behaviors, in the Analysis profile under **Rat or Mouse Behavior Recognition** choose the behaviors you are interested in.

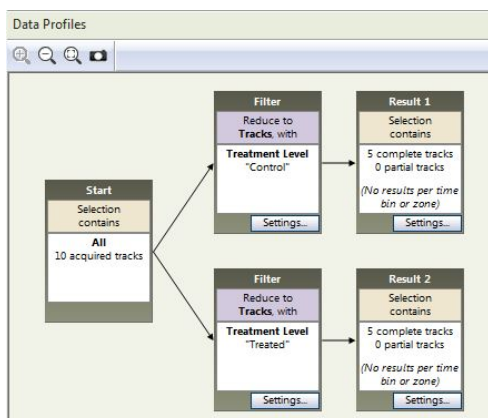
Furthermore:

- If you want to analyze multiple behaviors as one, click **Merged Behavior**.

- If you want to view the probability (that is, a measure of detection uncertainty) associated with the behaviors detected, click **Behavior probability** and specify the behaviors you are interested in.

## COMPARING GROUPS

1. Prerequisite: You can define groups of tracks based on independent variables (see page 19).
2. Choose **Analysis > Data Profile > New**.
3. Create a structure like that in the following picture.



**Figure 2.6** *An example of data selection to compare two groups of tracks.*

To create a new branch, click the button next to **Result** and connect the **Start** box with the new **Result** box.

## VISUALIZING DATA

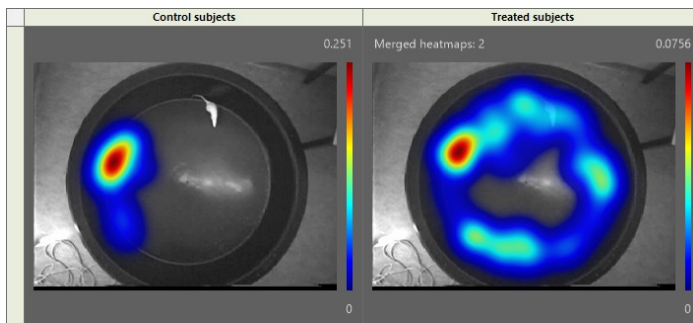
Choose **Analysis > Results > ...**

### ***Plot tracks***

You can view your tracks on a still image of the background. Tracks can be shown in different colors according to the values of independent variables (for example, blue for animals treated with saline and red for drug-treated animals). Sample points can be shown in different colors according to the values of dependent variables, for example red when the animal was moving fast.

### ***Plot heatmaps***

You can make heatmaps of the location of the subject.



### ***Plot integrated data***

You can look at a track with the video file in the background. When you plot integrated data you can also view Time Event plots of your independent variables.

## Chapter 3

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# The Morris Water Maze Test

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# Introduction

## THE MORRIS WATER MAZE TEST

The Morris water maze is a behavioral procedure designed to test spatial memory. It was developed by neuroscientist Richard G. Morris in 1984, and since its initial description it has become a very popular paradigm for the study of learning and memory in rodents (D'Hooge and De Deyn, 2001).

In the water maze paradigm, a rat or mouse is placed into a small circular pool of water which contains a hidden platform (the Atlantis platform). The platform is either fixed (a few millimeters below the water surface) or adjustable. In the latter case, the platform is submerged at the start of the trial (about 15 centimeters, depending on the device) and remains submerged until the subject finds the location of the platform. The platform is then automatically raised. Visual cues, such as colored shapes, are placed around the pool to aid the animal in learning to locate the platform. After sufficient training, a capable rat can swim directly from any release point to the platform. Next, animals are treated and the effects on spatial learning and memory are evaluated based on how they now perform in the Morris water maze.



**Figure 3.1** *A rat in a Morris water maze.*

## THE SAMPLE EXPERIMENT

EthoVision XT comes with a sample experiment that shows you how a Morris water maze test is carried out with EthoVision XT. It includes a number of videos from a true research project and shows interesting patterns of spatial behavior depending on the age of the subject.

- If the sample experiment was installed during installation of EthoVision XT, you can open it directly: Choose **File > Restore Backup** and browse to C:\Users\Public\Documents\Noldus\EthoVision XT\Experiments\Sample Experiments. Select the file with extension EVZ and click **Open**.
- If you do not find the sample experiment, download it from MyNoldus.com. First log in or register, then choose **Downloads > EthoVision XT > Sample Experiments**.

## REFERENCES

D'Hooze, R. and De Deyn, P.P. (2001). Applications of the Morris water maze in the study of learning and memory. *Brain Research Reviews*, **36**, 60-90.

Gallagher, M., Burwell, R. and Burchinal, M. (1993). Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. *Behavioral Neuroscience*, **107**, 618-626.

### *EthoVision XT and the Morris water maze test*

For the complete list of publications, see the results in [Google Scholar](#).

# Physical setup

The following suggestions are meant to optimize video tracking:

- The camera should have a good view of the entire region the animal can be in. The arena should fill the field of view.
- There must be maximum contrast between the background and the animal. If you use dark rats in the water maze, you can color the water with milk powder (400 g, pre-dissolved in 2 l). It is also possible to track white rats or mice with dark coloring in the water: add 300 g of tempera black nontoxic powdered paint to a 45-l pool.
- If you have reflections in your image, EthoVision XT may confuse those reflections with your subject, and track the reflections rather than your animal. Place four (or more) bulbs round the pool, below the level of the water surface or other indirect lighting above the water maze (see Figure 2). 'Globe' type bulbs are ideal (twice the diameter as standard incandescent light bulbs). They should be close enough to the pool wall so that there is no direct line of sight between the bulbs and the camera lens. The light is reflected off the walls and ceiling, so that it only reaches both the lens and water surface indirectly.



**Figure 3.2** *Lighting a water maze. Left: view from side, right: view from above.*

- The water temperature should be  $\pm 26^\circ\text{C}$ , which is cold enough to encourage the animals to seek the platform, but not too cold to induce hypothermia.

# The Morris water maze test in EthoVision XT

Create an experiment. For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help.

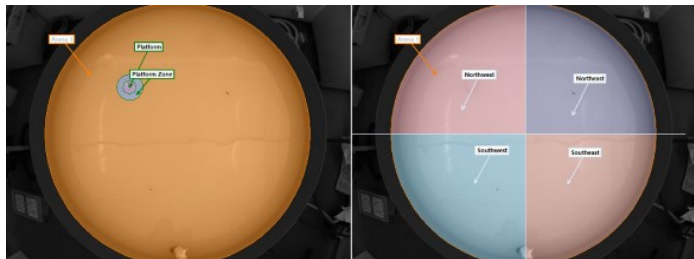
## ARENA SETTINGS

Choose **Setup > Arena Settings > ...**

We assume that you followed the procedure in **ARENA SETTINGS** on page 15.

The zone groups in your experiment depend on what zone template you chose when creating the experiment.

- **Platform, quadrants** – This zone template creates one arena settings profile with a zone group *Platform* with zones *Platform* and *Platform Zone* (the zone around the platform) and a zone group *Quadrant* with a zone for each quadrant.

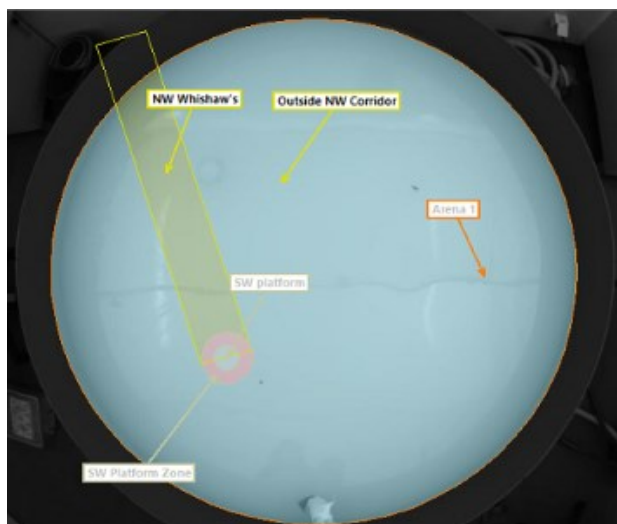


- **Platform, quadrants, corridors, border** – This zone template creates four arena settings profiles, one for each quadrant. The *South-West Platform* profile, for example, contains a zone group *Platform* with zones *Platform* and *Platform Zone* (the zone around the platform), a



zone group *Quadrant* with a zone for each quadrant, a zone group *Thigmotaxis* with zone *Border Zone*, and a *Whishaw's corridor* for the other quadrants than the one with the platform.

Whishaw's corridor is an 18-centimeter wide path from the starting location to the platform. This corridor is designated as the correct route and if a rat deviates from this route at any point it receives a maximum of one error on that trial (Whishaw's error). To be able to determine whether the animal deviates from this route, you should define the rest of the arena as an *Outside corridor* zone. A Whishaw's corridor is defined for each release point you use. By creating separate Arena Settings for each corridor, you can select the appropriate Arena Settings for data acquisition depending on the release point for that trial.

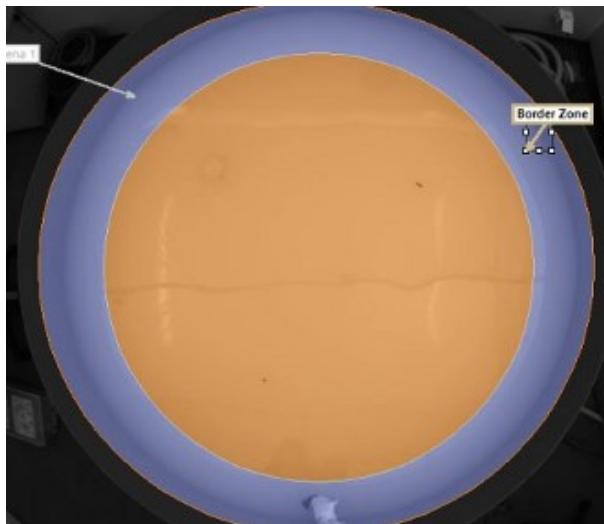


**Figure 3.3** Part of the South-West Platform arena settings profile. The zones in the zone groups **Platform** and **Whishaw's Corridor NW** are displayed.

To resize and reshape a zone, click first the Point edit mode button on the toolbar, then move the corners of the zone outline. Hold **Shift** down to keep the same aspect ratio of the shape. Hold **Ctrl** to enlarge/reduce the shape size in all directions.

### ***Zone for analyzing thigmotaxis***

To quantify thigmotaxis, that is, persistent swim along the wall of the pool, create an additional zone group, and draw a circle which should be the internal margin of the zone. Place the zone label between this circle and the outline of the arena.



## **TRIAL CONTROL SETTINGS**

In the Trial Control Settings you can define conditions for the start and stop of the track.

Choose **Setup** > **Trial Control Settings** > Open one of the following:

- **Default (no max duration).** When you use this profile, tracking stops when you click the **Stop trial** button.
- **Max Track duration 1 min** – When you use this profile, tracking starts automatically 2 seconds after the animal has been placed in the maze. The track stops automatically when the center-point of the animal has been in the zone *Platform* for at least 5 seconds, or when 60 seconds have elapsed since the start of the track.

- **Max Track duration 2 min** – When you use this profile, tracking stops when the center-point of the animal has been in the zone *Platform* for at least 5 seconds, or after 2 minutes if the animal does not find the platform within that time.

### *Using an adjustable platform*

If you use an adjustable platform you can define an action to raise the platform when the animal swims around the platform. Note that you need the Trial & Hardware Control module to be able to control the platform.

## **DETECTION SETTINGS**

Choose **Setup > Detection Settings > Detection Settings 1**.

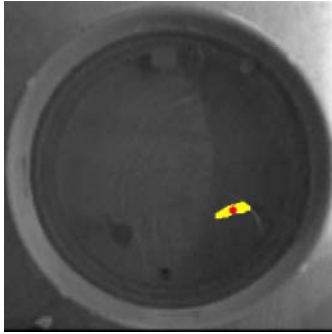
We assume that you followed the procedure in **DETECTION SETTINGS** on page 17.

Check in the **Video** Section that the sample rate is set to 5 samples/second (for rats) or 12.5 samples/second (for mice).

### *Advanced detection settings*

If detection of the subject is not optimal after using the Automated setup function (page 17), do the following:

1. Check that either **Dynamic subtraction** or **Differencing** is selected as the detection method. **Differencing** is the preferred option if you work with hooded animals.
2. **When using Dynamic subtraction** – Move the slider to define the animal's contrast. The animal must be fully detected in all parts of the arena and the noise must be minimal. Specify the **Current frame weight**. A low value (for instance, 10-20) usually works well.



When using **Differencing** – Set the **Sensitivity slider**. The slider determines what difference in contrast from the background is seen as the animal.

For more information, see **Configure Detection Settings** in the EthoVision XT Help.

## **TRIAL LIST**

Choose **Setup > Trial List**.

### ***Defining Independent Variables***

Enter your independent variables such as:

- *Subject ID* with the ID of your animals as predefined values.
- *Phase* with Training and Probe as predefined values.
- *Treatment* with Treated and Control as predefined values.
- *Dose* with numerical values (e.g. 0.1 mg/kg, 0.5 mg/kg, etc.)
- *Day after treatment* (with values 1, 2, etc.)
- *Name of the experimenter*, etc.

### ***Making a list of trials***

If you want, you can pre-define all your trials here. Click the **Add Trials** button. You can also prepare the trials in Excel, randomize them and then paste the values into your Trial List.

Specify the values of the independent variables in advance, or enter them as you carry out the trials.

Furthermore, you can define a list of trials for batch acquisition. See **Batch Data Acquisition** in the EthoVision XT Help.

	System	System	System	User-defined	User-defined	User-defined	User-defined
Label	Trial Duration	Arena settings	Acquisition status	Animal ID	Release quadrant	Platform Position	Treatment
Description	The duration of this trial	The arena settings used for acquisition	The current status of acquisition per arena	(Exemplary)	(Exemplary)	(Exemplary)	(Exemplary)
Type	Duration		Numerical	Text	Text	Text	Text
Format	HH:mm:ss		x				
Predefined Values			Unknown; Postpon	NE; NW; SE; SW	NE; NW; SE; SW	Control; Treated	
Scope	Trial	Trial	Arena	Subject	Subject	Subject	Subject
Trial	Arena	Subject	No.				
Probe trial	Arena 1	Subject 1	1	Planned	0 SE	SW	Treated
Trial 1	Arena 1	Subject 1	2	Planned	1 SW	SE	Control
Trial 2	Arena 1	Subject 1	3	Planned	2 NW	NW	Treated
Trial 3	Arena 1	Subject 1	4	Planned	3 NE	NE	Control
Trial 4	Arena 1	Subject 1	5	Planned	4 SE	SW	Treated

**Figure 3.4** *The predefined Trial List in the water maze template experiment.*

# Acquiring data

## PROTOCOL

In a typical water maze test, an animal is put in the maze at a predefined position at the rim of the pool, facing the wall. Lower the animal gently into the water (e.g. in a paper cup), avoiding stress as much as possible.

Before you start data acquisition, make sure the appropriate arena settings profile, depending on the starting location of the animal, is selected in the **Acquisition Settings** window.

### *Training trials*

In training trials, the animal starts swimming and the trial stops when the animal has found the platform or when the maximum trial duration has been reached.

### *Probe trials*

After the training trials, a probe trial is conducted in which the escape platform is removed from the pool and the animal allowed to swim for 60 sec. Thus typically each animal is tested  $n$  times (training) + 1 time (probe). Each test corresponds to a trial in EthoVision XT.

### *Reversal training trials*

In reversal learning tasks, after one location has been thoroughly trained, the platform is moved to a different quadrant of the pool. Because it is hidden, it is not apparent that anything has changed until the animal fails to find the platform in its usual place. The focus is on how the animal reacts to this change and how quickly it learns the new location.

In EthoVision XT, mark different types of trials with an independent variable in the Trial List. For example *Type*, with values *Training* and *Probe*. Or, a variable *Platform Position* with values *SouthWest*, *NorthWest*, etc.

See also **ACQUIRING TRACKS** on page 21.

# Data Analysis

## DATA PREPARATION

### *Track smoothing*

Choose **Acquisition** > **Track Smoothing Profile**.

- Select **Lowess** smoothing to remove the effect of body wobble.
- When the animal sits still on the platform, yet the body center-point still moves slightly due to system noise. This results in an overestimation of, for example, the total distance moved. Select **Minimal distance moved** to only measure the total distance moved over the time periods in which the animal was really swimming.

## ANALYSIS PROFILES

Choose **Analysis** > **Analysis Profile**.

The template experiment contains five analysis profiles:

- **Latency to reach platform** –This profile contains the variable **Latency to platform** to calculate how long it takes the animal to find the platform. You can use the variable **Distance to zone** to calculate Gallagher’s index (Gallagher et al., 1993). The variable **In quadrants** calculates the frequency and duration of visits to each quadrant.
- **Path shape** – The path shape can be determined by using the variables **Turn angle**, **Angular velocity** and **Meander**.
- **Whishaws Corridor** (only present if you chose a zone template with corridors when creating the experiment)– In this profile, the variable **Inside Whishaw’s Corridor** calculates the time the animal spent inside any of Whishaw’s corridors. The variable **Outside Whishaw’s Corridor** calculates the time the animal spent outside any of Whishaw’s corridors.

- **Distance & Time** – In this profile, the total **Distance Moved** and the mean **Velocity** and the group means with their standard errors are calculated.
- **Heading** – In this profile, the mean **Heading** to each of the platforms is calculated.

## SWIM PATTERNS

### *Thigmotaxis (wall-hugging swim)*

This is a persistent swim along the wall of the pool that could include sporadic swims toward the center of the pool. To quantify thigmotaxis, calculate the time spent in the Thigmotaxis zone defined as on page 54, relative to the total track duration.

### *Random search*

This is swimming over the entire area of the pool in straight swims (zig-zag pattern), or in wide circular swims.

Divide the pool into quadrants or even more, smaller zones, then count the number of times each zone was visited, the time in zones. If zone visits/time are uniformly distributed across zones, the search pattern could be said to be random or at least covering the whole pool.

### *Scanning*

The search path is restricted to a limited, often central, area of the pool.

Do the same as above, but now covering only the area/zones towards the center (see Figure 1C in the paper Janus C. 2004. *Learning and Memory* 11: 337-346). Relate this to the time in the external zones.

### *Chaining/serial visits*

This is circular swimming (in anticlockwise or clockwise direction) at a fixed distance from the wall, in which the escape platform was located.

To quantify chaining, draw a specific circular crown zone. Calculate the time spent in this zone versus the time in the rest of the arena, or calculate rotations occurring in that zone.



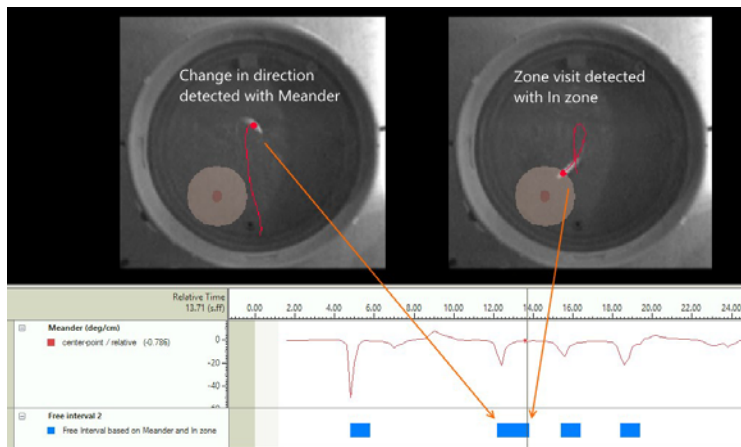
### ***Focal search***

This is searching in a restricted area of the pool, usually the target quadrant. The path is characterized by a directional, straight swim to a specific area followed by dense concentration of superimposed loops and turns there.

To detect rapid changes in direction, use the **Multi condition** dependent variable to define your criterion, for example Absolute Meander > 10 degrees/cm. This marks the time when Meander is greater than the threshold value.

You can precisely capture the instances when the subjects turns and heads back to the target zone. Combine Meander with a **In zone** variable in a **Free Interval**, to select the time from when the rapid change in direction occurs, to when the subject enters the target area. In the Arena Settings, define a zone around the platform or any other target point, and name it *Focal zone*. In the Analysis profile, define a **Free interval** from Interval start “Meander: Absolute Meander > 10 degree/cm” to Interval stop “In zone: Center point is in *Focal zone*”.

In the figure below, the peaks in the **Meander** variable mark the sudden changes in direction. The color bars represent the **Free interval** marking the time between the directional change and the zone visit.



### ***Direct swim path***

This is the direct swim path to the location containing the escape platform.

Define an **In zone** variable based on the Whishaw's corridor (page 59) to calculate the time spent in and outside this corridor before reaching the platform. See also the EthoVision XT Help.

### ***Floating***

A state of inactivity without forward movement. The plotted path is short, often with thick or tight sections caused by non-directional drift.

Quantify floating with **Movement** (Not moving) or a **Multi condition** that includes **Movement** (Not moving) and **In zone** (Center point is *not* near the wall of the pool; define a dedicated zone if necessary).

## **GALLAGHER'S PROXIMITY SCORE**

### ***Cumulative search error (for training trials)***

Gallagher *et al.* (1993; see page 50) proposed the Cumulative search error to provide information about the spatial distribution of the animal's search during training trials. This was obtained by sampling the position of the animal in the water maze (10 times per second), and calculating the averages of distance to the platform for 1-second periods (see Figure 6 in the original paper). The averages are then summed up.

In EthoVision XT, you have two options:

- Calculate the Total distance from the platform.

In the Analysis profile, choose the dependent variable **Distance to zone**, with the Statistic **Total**. This equals the "true" Gallagher's Cumulative search error when sample rate = 10 samples/s. In most cases the Total distance from the platform highly correlates with the "true" search error.

- Calculate the "true" Gallagher's Cumulative search error.
  - a In the Data profile, select time bins (1 second).

- b In the Analysis profile, define the dependent variable **Distance to zone**, with the Statistic **Mean**.
- c Choose **Analysis > Results > Statistics and Charts**, click **Calculate**, then export the results (**Analysis > Export > Statistics**).
- d In Excel, open the export file and sum up the average values per trial.

### ***Average search error (for probe trials)***

According to Gallagher *et al.* (1993), the Average search error is obtained by calculating the average distance of the animal to the platform location over 1-second intervals, and then averaging the values (Average proximity; see Figure 9 in the original paper).

In EthoVision XT:

In the Analysis profile, choose the dependent variable **Distance to zone**, with the Statistic **Mean**. This equals the Gallagher's Average search error in all cases.

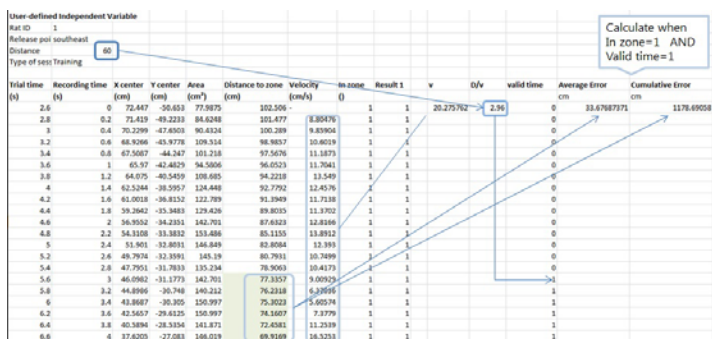
### ***Data correction***

For both probe trials and training trials, Gallagher *et al.* (1993) made a correction so that trial performance was relatively unbiased by differences in distance to the goal from the various start locations at the perimeter of the pool (see page 621 in the paper).

If you want to apply this correction to your data:

1. Measure the distance  $D$  between the release point and the platform. In the EthoVision XT Trial List, define an independent variable  $D$  and for each trial enter the appropriate value depending on the release point used in that trial. Values will be used later in the Excel export file.
2. In the Analysis profile, define the dependent variables **Distance to zone**, **In zone (not in platform)** and **Velocity**.
3. Choose **Analysis > Export > Raw Data**. and choose **Excel** as **File type**.
4. In Excel, create macros/formulas that calculate (see the next page):
  - The average velocity  $v$  when **In zone** =1.
  - The time needed to reach the platform from that starting point:  
 $t = D/v$ .

- Insert a new column **Valid time**, assign the value 1 if Recording time in the same line is higher than t.
- Calculate the average distance to zone (platform) for when **In zone=1** and **Valid time =1** (use the Excel function **AVERAGEIFS**).
- Calculate the total distance to platform when **In zone=1** and **Valid time =1** (use the Excel function **SUMIFS**).



**Figure 3.5** Example of Excel file for calculating corrected Total distance (see text).

## Chapter 4 ---

# The Novel Object Recognition Test

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The NOR test in EthoVision XT .....	69
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# Introduction

## THE NOVEL OBJECT RECOGNITION TEST

The Novel Object Recognition (NOR) test was first described by Ennaceur and Delacour (1988). Rats or mice are exposed first to two identical objects and then one of the objects is replaced by a new (novel) object. The time spent exploring each of the objects is measured. The test has become popular for assessing visual memory in rodents in general and to test the effects of amnesic drugs in particular.

The test is based on spontaneous behavior with no reinforcement such as food or shock. Non-amnesic animals will spend more time exploring the novel object than the familiar one. An absence of any difference in exploration time can be interpreted as a memory defect or, in case an amnesic drug is tested, a non-effective drug. This chapter describes how a NOR test can be conducted with EthoVision XT.



**Figure 4.1** *A mouse exploring novel objects.*

On the downloads section of the Noldus website [my.noldus.com](https://www.noldus.com), you find a number of sample experiments, including one that features a NOR test.

## THE SAMPLE EXPERIMENT

To see how a Novel Object Recognition test is carried out in EthoVision XT, see also the sample experiment **Novel Object Recognition test XT180** on the downloads section of the Noldus website ([my.noldus.com](http://my.noldus.com)). Download this file and save it on your computer. In EthoVision XT, choose **File > Restore Backup** and select the file. For more information, see the document **Description of sample experiments of EthoVision XT.pdf**.

## REFERENCES

Ennaceur, A.; Delacour, J. (1988) A new one-trial test for neurobiological studies of memory in rats, 1: Behavioral data. *Behavioral Brain Research*, **31**, 47-59.

### *EthoVision XT and the novel object test*

Benice, T.J.; Raber, J. (2008). Object recognition analysis in mice using nose-point digital video tracking. *Journal of Neuroscience Methods*, **168**, 422-430.

McDowell, K.A.; Hutchinson, A.N.; Wong-Goodrich, S.J.E.; Presby, M.M.; Su, D.; Rodriguiz, R.M.; Law, K.C.; Williams, C.L.; Wetsel, W.C.; West, A.E. (2010). Reduced cortical BDNF expression and aberrant memory in Carf knock-out mice. *The Journal of Neuroscience*, **30**(22), 7453-7465.

Siegel, J.A.; Park, B.S.; Raber, J. (2011). Long-term effects of neonatal methamphetamine exposure on cognitive function in adolescent mice. *Behavioural Brain Research*, **219**, 159-164.

Simmons, D.A.; Rex, C.S.; Palmer, L.; Pandeyarajan, V.; Fedulov, V.; Gall, C.M.; Lynch, G. (2009). Up-regulating BDNF with an amphetamine rescues synaptic plasticity and memory in Huntington's disease knock-in mice. *PNAS*, **106**, 4906-4911.

For a complete list of publications, see the results of [Google Scholar](https://scholar.google.com).

# Physical setup

The following suggestions are specifically to optimize video tracking:

- For general information, see the section **Physical setup** in the chapter **The Open Field test** on page 28.
- The objects should contrast with the animal. If necessary use a different setup for light and dark colored animals.
- Make sure that the objects do not move due to the activity of the animal.
- Either make sure the sides of the box are not reflective (for example, sand the box to make it matte), or exclude the sides from the arena. If your objects are close to the sides, you will need to track the animal when it is against the side.



# The NOR test in EthoVision XT

Create an experiment from a template (**Ctrl+T**). Choose **Open field** (**round** or **square**) as Arena template, and **Novel object zones** as Zone template.

For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help.

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

Under **Video Source**, and **Tracked Features**, make sure that the options selected corresponds to your needs.

If you want to use Deep learning technique to track the subject's nose, under **Body point detection technique** choose **Deep learning**. Note that in order to use this technique there are additional requirements. The PC must have a powerful graphics card which supports additional software (CUDA). See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.

## MANUAL SCORING SETTINGS

These settings enable you to record behaviors manually, using keystrokes. This is handy for example to record exploratory behaviors like sniffing.

Choose **Setup > Manual Scoring Settings**.

If you created the experiment from the template with novel object zones, the Manual Scoring Settings already includes one behavior, **Sniffing object**. This is defined as "start-stop", which means that you press a key (default: q) to score the start of sniffing, and then you press the same key once again to score the end of sniffing.

For more information, see **Manual Scoring Settings** in the EthoVision XT Help.

**NOTE** With the Rat or Mouse Behavior Recognition add-on, you can have EthoVision XT detect sniffing and other behaviors, without the need to score them manually.

## ARENA SETTINGS

Choose **Setup > Arena Settings > Open Arena Settings 1**.

To set up your Arena Settings:



1. Click the **Grab Background Image** button to grab an image of the empty enclosure from the camera image.



2. Click **1. Draw Scale to calibrate** and calibrate your arena (for details, see the EthoVision XT Help).

**TIP** Draw the double-arrow in such a way it points to two opposite walls of the open field, at the level where the animal moves (thus not at the top of the walls!). When done, enter the distance in real world units between the tips of the arrow.

3. Click **2. Select Shape and Draw Arena**. Check that the arena covers the whole area in which you want to track the animal. Remember to include enough of the walls so that the animal is still tracked when it rears, but exclude any bright reflective rims that might interfere with tracking. Make sure the label stays inside the arena.

**TIP** If the arena is not yet defined, and you want to divide it in equal zones, click **2. Select Shape and Draw Arena**. Choose the tool that applies   depending on the shape of your open field and draw the outline of the arena.

4. If you used the Zone template **Novel object zones**, click **3. Select Shape and Draw Zones**.

Click the layer Zone group **Objects**. Check that the ‘object 1’ zone and ‘object 1 boundary’ zone cover the object (Figure 4.2). If the predefined zones do not have the correct shape, delete the zones and draw new ones.

5. If necessary, draw additional zone groups/zones.

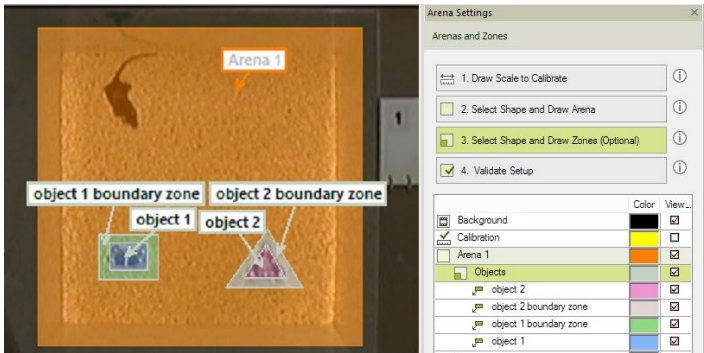
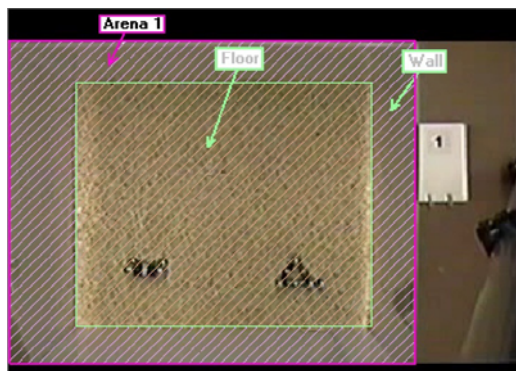


Figure 4.2 Zones of the zone group “Objects”.

**Additional zones**

- **Zone group Floor and Wall.** Check that the ‘Floor’ zone covers the whole floor area of the open field (see Figure 4.3).

- **Zone group Halves.** Check that the 'West' and 'East' zones divide the 'Floor' zone in two equal halves. Make sure that the vertical line crosses the entire arena.



**Figure 4.3** *Open field with 'Floor' zone and 'Wall' zone.*

## TRIAL CONTROL SETTINGS

In the Trial Control Settings you can define conditions for the start and stop of the track.

Choose **Setup > Trial Control Settings**. The template contains the following profiles:

- **Default.** When you use this profile, tracking starts automatically two seconds after the animal has been placed in the arena. Tracking stops when you click the **Stop trial** button.
- **Track duration 10 min.** When you use this profile, tracking starts automatically two seconds after the animal has been placed in the arena. Tracking stops automatically after 10 minutes.

### *To stop tracking when exploration exceed a specified time*

You can also stop tracking when the subject has spent some time around an object. For example, when the protocol specifies that in pre-test trials the animal must explore an object for at least 30 seconds.

Make a new Trial Control Settings, and immediately before the **Stop track** box, insert a **Condition** box based on the variable *In zone*, or another variable, or a combination of variables using **Multi condition**. In the Condition settings, specify that **Cumulative duration** in the boundary zone for that object must be greater than N seconds, for the nose point.

If you use Multi condition, you can define “exploration” as a combination of variables. For example: *In Zone* for the noise point in the “object zone” is true + *Head directed to Zone* “object” is true + Velocity lower than 5 cm/s is true. The trial stops only when the cumulative time that the three criteria are met reaches the specified value.

#### ***To stop tracking automatically when no exploration occurs***

To stop tracking automatically even when the animal does not explore the object, add another condition in parallel with the first one, which specifies to stop tracking after some time (e.g. 10 minutes), and combine the two conditions with an **Operator** box of type “ANY”.

## **DETECTION SETTINGS**

Choose **Setup > Detection Settings > Detection Settings 1**.

#### ***Advanced detection settings***

If detection of the subject is not optimal after using the Automated setup function (page 17), do the following:

1. Check that the sample rate is set to at least 25 samples/second when tracking the nose and tail.
2. Check that either **Dynamic subtraction** or **Differencing** is selected as the detection method. Differencing is the preferred option if you work with hooded animals.
3. **When using Dynamic subtraction**, move the slider to define the animal's contrast. The animal must be fully detected in all parts of the arena and the noise must be minimal. Specify the **Current frame weight**. A low value (for instance, 1 to 10) usually works well. A novel

object test normally has a duration of a few minutes in which the background does not change much.



**When using Differencing**, set the **Sensitivity** slider. The slider determines what difference in contrast from the background is seen as the animal.

4. When detection still is not satisfactory, select **Rodents / For occlusions** as the tracking method and repeat step 2 above.

**NOTE** If you want to use Deep learning technique to track the subject's nose, note that there are additional requirements and limitations. Among other things, you need a powerful graphics card. See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.

## TRIAL LIST

Choose **Setup** > **Trial List**.

### *Defining Independent Variables*

Enter your independent variables such as:

- *Subject ID* with the ID of your animals as predefined values.
- *Phase* with Acclimation, T1, T2 as predefined values, where:
  - Acclimation to mark trials where the animal is free to explore the arena, with no objects present.
  - T1 to mark trials where the animal is presented with a pair of identical objects.
  - T2 to mark trials where one of the familiar objects is changed for another new (novel object test).

- *Treatment* with Treated and Control as predefined values.

If you want, you can predefine all your trials here, or you can enter the independent variable values as you carry out the trials. You can also prepare the trials in Excel, randomize them and then paste the values into your Trial List.

	System				
Label	Acquisition status	Animal ID	Treatment	Novel Object	Familiar object
Description	The current status of acquisition per arena		(Exemplary)	(Exemplary)	(Exemplary)
Type		Numerical	Text	Text	Text
Format		x			
Predefined Values	Unknown; Postpon	All values	Control, Treated	object 1; object	object 1; object
Scope	Arena	Subject	Subject	Subject	Subject
Trial	Arena	Subject	No.		
Trial 1	Arena 1	Subject 1	1	Planned	
Trial 2	Arena 1	Subject 1	2	Planned	
Trial 3	Arena 1	Subject 1	3	Planned	
Trial 4	Arena 1	Subject 1	4	Planned	

**Figure 4.4** An example of the Trial List with four planned trials.

Furthermore, you can define a list of trials for batch acquisition. See **Batch Data Acquisition** in the EthoVision XT Help.

# Acquiring data

## PROTOCOL

A typical NOR test consists of three phases:

- **Acclimation.** The animal is placed in an empty arena for 10 minutes to habituate to the environment. Tracking in this phase is optional. After 10 minutes, the animal is taken out of the arena and is put back in its home cage.
- **T1.** After 15 minutes in the home cage, the animal is put back in the arena in which two identical objects have been placed. The animal is put at a position midway between the objects and such that its nose points towards the wall. The animal is tracked for 3 minutes and is then taken out of the arena and put back in its home cage.

Define a Trial Control Settings profile for this phase, then assign this profile to the trials of type T1.

- **T2.** One of the objects in the open field is replaced with a novel object (sometimes the other object is also replaced with an identical one) and the animal is put in the arena again. Tracking is done for 3 minutes.

Define a Trial Control Settings profile for this phase, then assign this profile to the trials of type T2.

See also **ACQUIRING TRACKS** on page 21.

## SCORE BEHAVIORS MANUALLY

To score sniffing and other behaviors defined under Manual Scoring Settings, open the Manual Scoring tab on the Acquisition screen. There you find the key codes and the buttons for the behaviors.

To score an instance of the behavior or its end, either press the key or click the corresponding button on the screen.



### **Notes**

- **IMPORTANT** If you do tracking from video, make sure you de-select **DDS** in the Playback Control window.
- You can also score behaviors *after* tracking. See **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

# Data Analysis

## DATA PREPARATION

### *Track editing*

Choose **Acquisition > Edit Tracks**.

You can fix tracking errors and swap back nose-points and tail-points that have been swapped in tracking. Normally you will not need to edit your data.

### *Track smoothing*

Choose **Acquisition > Track Smoothing Profile > open MDM Filter 2 mm**.

In this profile, the **Minimal Distance Moved** filter is used (option set to **Direct**, with a threshold of 2 mm). When the animal sits still, the body points may still move slightly due to system noise. This results in an overestimation of, for example, the total distance moved. With the minimal distance moved filter, you measure the total distance moved over the time periods in which the animal was really walking. Adjust the threshold if necessary, based on your setup and sample rate.

### *Review video and behaviors*

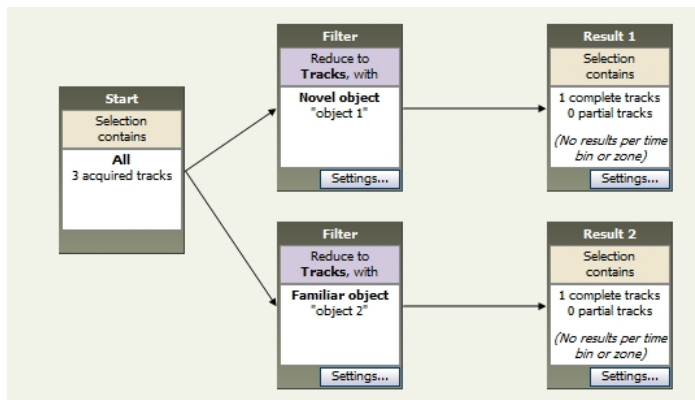
If you have scored behaviors manually, you can review the video and if necessary edit the data. Choose **Acquisition > Score behaviors** manually. For details, see **Score behaviors manually** in the EthoVision XT Help.

## SELECTING DATA

Choose **Analysis > Data Profile**.

You can select (**Filter**) your tracks according to your independent variable values (for example, *Treated* animals only) and also select parts of tracks (**Nesting**).

Figure 4.5 shows a data profile to compare 'Novel Object' and 'Familiar Object' trials which uses Filters. This way you create groups of tracks to obtain group statistics for each group.



**Figure 4.5** An example of data selection to compare two data sets in your experiment.

## ANALYSIS PROFILES

The template experiment contains four analysis profiles:

- **Touching objects.** This analysis profile contains an In zone variable Nose touching objects to calculate the frequency, duration, and latency of when the nose-point of the animal touches the objects. The variable *Distance to objects* is used to calculate the mean and standard error of the animal's nose-point to the objects. The Manually-scored behavior *Sniffing object* calculates the frequency and duration, with standard errors, of the instances when you scored *Sniffing object* during the trials.
- **In halves.** In this analysis profile, the In zone variable *In Halves* is used to calculate the frequency, duration and latency of when the center-point of the animal was in either half of the arena.

- **Head directed to zone.** This analysis profile contains two **Head directed to zone** variables, one for the novel object and one for the familiar object. For each object, the center plus a 0.10 cm radius is used as the Point of interest. The frequency and duration for **Head direction to zone** is calculated when the nose-point is in the zone of the corresponding object.
- **Distance & Time.** With the variables **Distance Moved** and **Velocity**, the total distance moved and the mean velocity are calculated.

All analysis profiles also contain settings to calculate the group mean and standard errors of the statistics for trial groups.

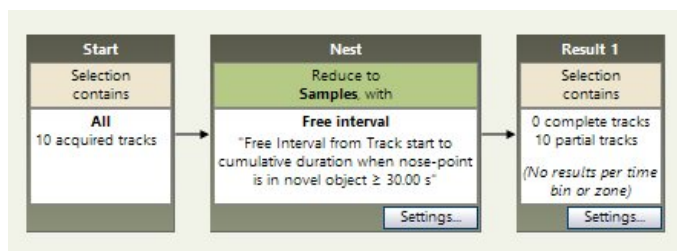
## EXPLORATORY BEHAVIOR

You can quantify exploratory behavior with the Analysis profiles described above, or you can create your own Analysis profiles.

There is usually variation between subjects in the time spent exploring an object. To account for this variation, you can select the part of each track up to when the animal explored an object for a specific time, and then analyze the behavior of the subject in that part.

To do so, use the Free Interval function in the Data profile.

1. Choose **Analysis > Data Profile > New**.
2. Under **Nesting**, click the button next to **Free Interval**.
3. Select the following:
  - As a **Start criterion: Time** (select **Track start**).
  - As a **Stop criterion: Dependent variable**. Select the variable **In zone**, and the statistic **Cumulative duration**. In the settings, specify which zone the animal explored, and with which body point (for example, Novel object, nose point). Set the total exploration time.
4. Click **OK** and insert the resulting box as in the figure below.

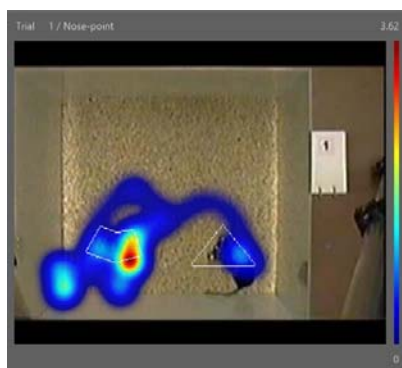


5. In the Analysis profile, choose the endpoints (distance moved, time spent in zones etc.).
6. Choose **Analysis > Results > Statistics and Charts**. The results are calculated for the time until exploration reaches the duration specified.

For more examples, see **Free interval** in the EthoVision XT Help.

## HEATMAPS

Heatmaps facilitate identification of “hotspots” and clustering of data points. Choose **Analysis > Results > Plot Heatmaps**. Next, click **Plot Heatmaps** on the toolbar.



**Figure 4.6** A heatmap of a trial with two objects, unfamiliar (left) and familiar (right).

## Chapter 5

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# The Elevated Plus Maze Test

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# Introduction

## THE ELEVATED PLUS MAZE TEST

The elevated plus-maze was designed to provide measures of anxiety that were relatively uncontaminated by changes in overall motor activity, and has been extensively validated by Pellow et al. (1985) using behavioral, physiological, and pharmacological measures.

The open and closed arms are considered to evoke the same exploratory drive in the animals, therefore avoidance of the open arms is considered to be a result of the induction of higher levels of fear (Rodgers and Dalvi, 1997). It is thought that the aversion of animals to explore the open arms of the maze is caused by fear of open and/or elevated spaces.



**Figure 5.1** *An elevated plus maze.*

The plus-maze is sensitive to the anxiolytic effects of neurotoxic lesions of serotonergic neurons and to the anxiogenic effects of drugs (Pellow and File, 1986), drug withdrawal (File and Andrews, 1991), and

predator odor (Zangrossi and File, 1992). For a discussion of the additional, ethological measures that can be taken in this test, see Rodgers et al. (1995) and Fernandes and File (1996). Unlike the social interaction and light/dark tests, the elevated plus-maze does not rely on aversion to bright light, and it has been found repeatedly that behavior in the maze is independent of light level (e.g., Becker and Grecksch, 1996).

For a review of the protocol, see Walf and Frye (2007).

### ***What to measure?***

- Principal component analysis of the conventional plus-maze (File, 1991) has shown that the percentage of time spent on the open arms and the number of entries onto the open arms are the best measures of anxiety (these are increased by anxiolytic and decreased by anxiogenic treatments).
- The number of closed arm entries is the best measure of locomotor activity.
- There are marked strain differences in baseline scores and even between the scores of different batches of animals. If the scores are high it will be hard to detect an anxiolytic effect. If they are very low it will be difficult to detect an anxiogenic effect.
- The plus-maze was originally developed for male rats, but it can be used with female rats. However, while in male rats anxiety is the main factor measured, in females it is activity (Fernandes et al., 1999). The plus maze has also been validated for mice (Lister, 1987; Walf and Frye, 2007). For a discussion of the factors controlling measures of anxiety in the mouse, see File (2001).

### ***Quantifying head dips***

EthoVision XT can detect when the subject's nose is outside the open arms.

However, you can also score head dips manually. First define head dips in the **Manual Scoring Settings**. Next, during acquisition, press the assigned keyboard key when a head dip occurs.



## THE SAMPLE EXPERIMENT

To see how a plus maze test is carried out in EthoVision XT, see also the sample experiment **Elevated plus maze XT180** on the downloads section of the Noldus website ([my.noldus.com](http://my.noldus.com)). Download this file and save it on your computer. In EthoVision XT, choose **File > Restore Backup** and select the file. For more information, see the document **Description of sample experiments of EthoVision XT.pdf**.

## REFERENCES

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- Becker, A. and Grecksch, G. (1996). Illumination has no effect on rats' behavior in the elevated plus maze. *Physiol. Behav.*, **59**, 1175-1177.
- Fernandes, C. and File, S.E. (1996). The influence of open arm ledges and maze experience in the elevated plus-maze. *Pharmacol. Biochem. Behav.*, **54**, 31-40.
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Pellow, S. and File, S.E. (1986). Anxiolytic and anxiogenic drug effects in exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. *Pharmacol. Biochem. Behav.*, **24**, 525-529.

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Rodgers, R.J. and Dalvi, A. (1997). Anxiety, defense and the elevated plus-maze. *Neurosci. Behav. Rev.*, **21**, 801-810.

Violle, N., Balandras, F., Le Roux, Y., Desor D., and Schroeder, H. (2009). Variations in illumination, closed wall transparency and/or extramaze space influence both baseline anxiety and response to diazepam in the rat elevated plus-maze. *Behav. Brain Res.*, **203**, 35-42.

Walf, A.A. and Frye, C.A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols*, **2**, 322 - 328. doi:10.1038/nprot.2007.44.

Web:[www.nature.com/nprot/journal/v2/n2/pdf/nprot.2007.44.pdf](http://www.nature.com/nprot/journal/v2/n2/pdf/nprot.2007.44.pdf)

Zangrossi, H. and File, S.E. (1992). Behavioral consequences in animal tests of anxiety and exploration of exposure to cat odor. *Brain Res. Bull.*, **29**, 381-388.

### **Videos**

<https://www.youtube.com/watch?v=4rSxfiU6-w>

[http://www.dailymotion.com/video/x2e25p\\_4tgnodox16\\_animals](http://www.dailymotion.com/video/x2e25p_4tgnodox16_animals)

# Physical setup

The following suggestions are specifically to optimize video tracking:

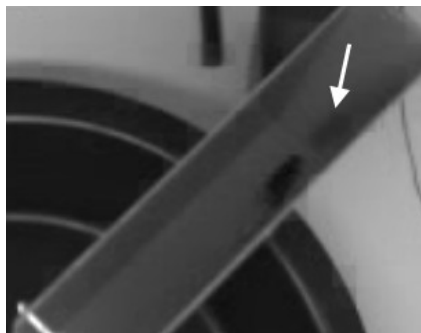
- Place the plus maze in such a way that its apparent size is maximized. You can obtain this by rotating the plus maze (or the camera) until the closed arms lie along one of the diagonals of your video window (see Figure 5.2).



**Figure 5.2** *Turn the plus maze to maximize its apparent size in the video image from above.*

- The lighting should be as even as possible throughout the arena. Bright lights are not necessary but should you need to supplement the light, consider red (or infra-red) light, as the animals will be less sleepy.
- The camera should have a good view of the entire region the animal can be in. Center the plus maze in order to have the image of the arms as symmetrical as possible. This minimizes inaccuracy of calibration due to perspective.

- The lighting should be diffuse, so as not to cast strong shadows (which might be tracked instead of the animal; see Figure 5.3). Even, diffuse lighting is important to optimize tracking of the nose.



**Figure 5.3** *Lighting should also adjusted to minimize the shadow made by the animal in the arena, which in this example (indicated by the arrow) may result in incorrect detection.*

- A plus maze with transparent walls may help reducing shadows and make easier to observe closed arm rears. There is little practical difference between the two mazes in terms of their ability to detect differences in anxiety-related behavior. However, the transparent design may reduce the sensitivity for the detection of anxiolytic drug effects because it decreases the anxiogenic potential of the open arm, therefore leading to the reduction of anxiety-related behavioral baseline (Violle *et al.*, 2009 and references therein).
- The background should contrast with the animal. If necessary use a different setup for light and dark colored animals. In the closed arms, the contrast between animal and background is generally lower. Take this into account when positioning lights and choosing the background color (Figure 5.4 and Figure 5.5).

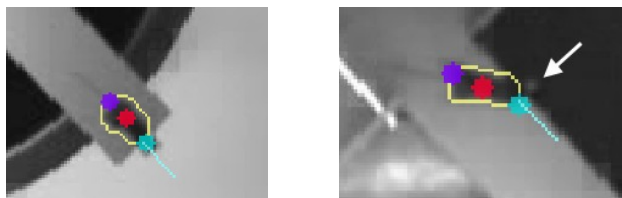


**Figure 5.4** *In this example, the closed arm at the top-right corner receives little light. This makes detection more difficult when the animal walks in that arm.*

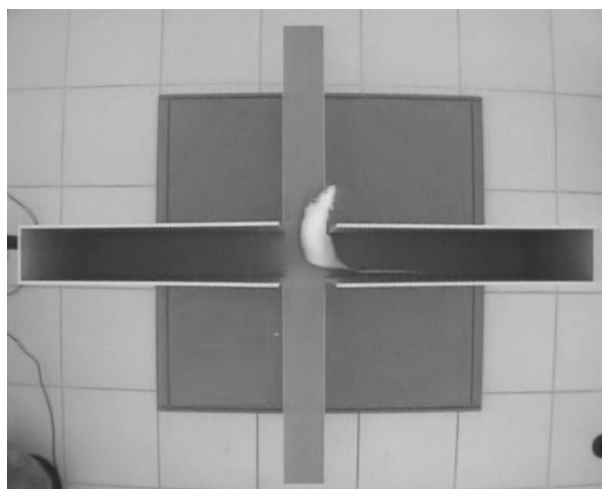


**Figure 5.5** *An example of good contrast between animal and background, using a white mouse. Furthermore, the transparent walls also help reducing shadows.*

- Make sure that the color of the floor is in good contrast with the color of the animal, and in minimal contrast with the arms. This makes it possible to track the animal's nose off the edge of the open arms (see Figure 5.6).



**Figure 5.6** *Left: CORRECT. The gray floor contrasts with the black mouse. When the mouse dips its head off the edge of the open arms, its nose is still detected. Right: WRONG. When the floor is too dark, the head dip (indicated by the arrow) is not found.*



**Figure 5.7** *An example of good contrast between floor and animal when testing white rats.*

- Both the plus maze arms and the floor should not be reflective. If necessary, place an opaque rubber mat on the floor.
- If there are barriers above the closed arms, remove them. If that is not possible, in the Detection Settings use the Dilation-Erosion filter (see page 97) to prevent EthoVision to “see” the animal cut in two. See also **Contour Settings** in the EthoVision XT Help.
- Depending on circumstances, pools of urine can cause problems with the tracking. An absorbent base can help.
- Always place the apparatus in the same position in the room, cues from overhead might influence the behavior of the animal. For the same reason the experimenter should not be visible to the animal during the trial (automated tracking makes this possible).
- If you want to use Deep learning technique to track the subject’s nose, note that there are additional requirements and limitations. Among other things, you need a powerful graphics card. See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.

# The Elevated Plus maze experiment in EthoVision XT

Create an experiment. For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help.

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

Under **Video Source**, and **Tracked Features**, make sure that the options selected corresponds to your needs. To adjust the camera settings, click the video icon in the camera row.

If you want to use Deep learning technique to track the subject's nose, under **Body point detection technique** choose **Deep learning**. Note that in order to use this technique there are additional requirements. Among other things, you need a powerful graphics card. See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.

## MANUAL SCORING SETTINGS

Choose **Setup > Manual Scoring Settings**.

If you used the template for an open field experiment, two start-stop behaviors have been defined:

- *Head dipping*, to record manually the head dips over the edge of the open arms.
- *Rearing*, to record manually the posture of the animal with forelimbs lift off the floor. Note that EthoVision XT does not detect rearing automatically in an Elevated plus maze. You must score rearing manually in order to record it.



You can define more behaviors, like scanning. Stretched posture can in principle be detected automatically by using the Elongation variable in EthoVision.

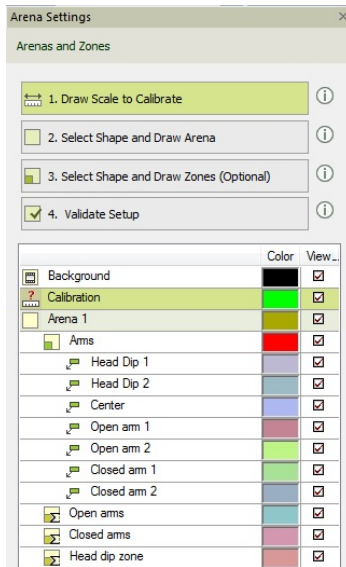
### ***Notes about Head dipping***

- By head dipping we mean here protruding the head over the ledge of an open arm and down towards the floor. This response can occur while the animal's body is in the closed arms, central square or open arms.
- You can also let EthoVision count the number of times or the total time that the nose point is found within the head-dipping area (provided that this has been chosen in the template). This also quantifies the risk-assessment behavior.
- For a more reliable scoring of head dips, you could place a camera in front of each open arm, to film the arm's side-view. You can record video from multiple cameras using the software Noldus MediaRecorder.
- If you film the side-view of the open arm, you can in principle record head-dips automatically with EthoVision by defining a zone immediately below the arm's floor. For these videos you need to create additional Arena Settings and Detection Settings.

For more information, see **Set Up an Experiment > Manual scoring settings** in the EthoVision XT Help.

## ARENA SETTINGS

Choose **Setup > Arena Settings > open Arena Settings 1.**



**Figure 5.8** *Arena Settings.*

We assume that you followed the procedure in **ARENA SETTINGS** on page 15.

### **Arena**

Check that the arena covers the whole area in which you want to track the animal.

Remember to include enough space around the open arms (so that the animal is still tracked when it dips its head off the edge of those arms) and the inner side of the walls (to track the entire animal when it rears).

Exclude any bright reflective rims that might interfere with tracking. Make sure the label Arena 1 stays inside the arena.

To adjust the contour of the arena to the plus maze in the video image, do the following:

- Click the **Normal mode** button on the tool bar and drag the arena contour until it is centered on the plus maze.
- Click the **Point edit mode** button. Click one of the corners of the arena and drag the mouse to move the corner to the position you require.



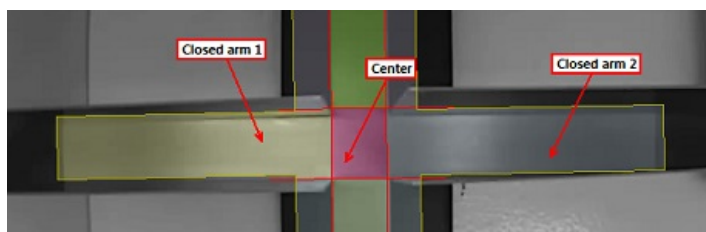
If you use video files, select the aspect ratio correction that applies. For more information, see **Adjust the video aspect ratio** in the EthoVision XT Help. If you use the live camera image or the video was recorded with EthoVision XT in the same experiment, you can skip this step.

### **Zone group Arms**

Check that each arm zone covers the corresponding sector of the plus maze.

To make sure that a zone is limited to the actual arm, move the label of that zone until the arrow points outside the zone. The color of the zone should change. If the color changes for a wider area of the plus maze, it means that the zone label also pointed to that area. Use the edit functions to move and reshape the zone.

For details, see **Move, rotate and resize a shape** in the EthoVision XT Help. Do not forget to place the arrow of the zone label back in original position.



If the zones “closed arms” predefined in the template overlap with the open arms of the plus maze, rotate the entire arena and zones.

To do so, drag around all the shapes, so they are selected. Next, click the **Rotation mode** button. Click in the middle of the arena and drag the mouse to rotate it.



Click the mouse pointer icon on the toolbar to exit the rotation mode.



### ***Zone group Open arms, Closed arms, Head dip zone***

These are cumulative zones. They are defined automatically from the sum of the open arms, the closed arms and the head dip zones, respectively.

## **TRIAL CONTROL SETTINGS**

Choose **Setup > Trial Control Settings**.

In the Trial Control Settings you can define conditions for the start and stop of the track.

The template contains two trial control settings profiles:

- **Default.**
- **Track duration 5 mins** – When you use this profile, tracking starts automatically 2 seconds after the animal has been placed in the plus maze. The track stops automatically after five minutes.

## **DETECTION SETTINGS**

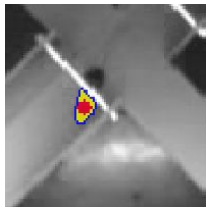
Choose **Setup > Detection Settings**.

In the **Detection Settings** window, check in the **Video** Section that the sample rate is set to:

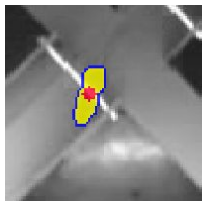
- **Rats:** 5 samples/second.
- **Mice:** 12.5-15 samples/second.
- **Nose-tail tracking:** 25-30 samples/second.

See **Configure Detection Settings** in the EthoVision XT Help for details on the advanced detection settings. If you use hooded animals, use the detection method **Differencing**.

If the plus maze has barriers above the arms, the animal is not always well detected, like in this example.



To improve detection, open the **Advanced** section in the **Detection Settings** pane. Under **Subject Contour**, select one or more pixels for the **Dilation** filter and the second **Erosion** filter. Leave the first Erosion filter to zero. Select more pixels for **Dilation** than for **Erosion**, for example 3 vs. 1, until the entire animal's contour is detected.



## TRIAL LIST

Choose **Setup > Trial List**.

Do one of the following:

Click **Add Variables** and enter your independent variables such as *Animal ID* and *Treatment* (with possible values Treated, Control, Sham etc.), Dose, Name of the experimenter, etc. Each trial will receive only

one value of each of those variables; for example, one trial must be either Control or Treated or Sham etc.

Add Trials...		Add Variable		
	System	System	User-defined	User-defined
Label	Trial Duration	Acquisition status	Animal ID	Treatment
Description	The duration of the trial	The current status of acquisition per arena	(Exemplary)	(Exemplary)
Type	Duration		Text	Text
Format	HH:mm:ss			
Predefined Values		Unknown; Postpon		Control; Treated
Scope	Trial	Arena	Subject	Subject

### ***Making a list of trials***

If you want, you can pre-define all your trials here. Click the **Add Trials** button and specify the number of trials (=recording sessions of one animal), and the values of the independent variables for each trial (see Figure 5.9).

You can also prepare the trials in Excel, randomize them and then paste the values into your Trial List.

Furthermore, you can define a series of trials for batch acquisition. See **Acquire a series of trials** in the EthoVision XT Help.

- Test animals only once, unless the intention is specifically to study the different form of anxiety evoked by a second trial. If the animal falls off, it is best to exclude its scores.

Add Trials... Add Variable			
Label	System	System	User-defined
	Trial Duration	Acquisition status	Animal ID
Description	The duration of the trial	The current status of acquisition per arena	(Exemplary)
Type	Duration		Text
Format	HH:mm:ss		Text
Predefined Values		Unknown; Postpon	Control; Treated
Scope	Trial	Arena	Subject
Trial	Arena	Subject	No.
Trial 1	Arena 1	Subject 1	1
Trial 2	Arena 1	Subject 1	2
Trial 3	Arena 1	Subject 1	3
Trial 4	Arena 1	Subject 1	4
Trial 5	Arena 1	Subject 1	5
Trial 6	Arena 1	Subject 1	6
Trial 7	Arena 1	Subject 1	7
Trial 8	Arena 1	Subject 1	8
Trial 9	Arena 1	Subject 1	9
Trial 10	Arena 1	Subject 1	10
Trial 11	Arena 1	Subject 1	11
Trial 12	Arena 1	Subject 1	12
	Planned	1	Treated
	Planned	2	Control
	Planned	3	Sham
	Planned	4	Sham
	Planned	5	Treated
	Planned	6	Control
	Planned	7	Treated
	Planned	8	Control
	Planned	9	Sham
	Planned	10	Control
	Planned	11	Sham
	Planned	12	Treated

**Figure 5.9** An example of the Trial List with twelve planned trials.

### Independent variables

- You can also enter the independent variable values as you carry out the trials. You can edit the independent variables for trials already acquired, for example to enter a-posteriori data, like whether the animal in that trial entered the open arms or not. This way you can quickly create groups of tracks based on the result of the trial, and use such groups in analysis.
- You can add a new independent variable at any time.

# Acquiring data

## PROTOCOL

The subject is placed at the junction of the four arms of the maze, facing an open arm. Each subject is generally tested once for 5 minutes.

For extensive protocol information, see Walf, A.A. & Frye C.A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature protocols* 2(2): 322-328. doi:10.1038/nprot.2007.44

See also **ACQUIRING TRACKS** on page 21.

## SCORE BEHAVIORS MANUALLY

To score head dipping and other behaviors defined under Manual Scoring Settings, open the Manual Scoring tab on the Acquisition screen. There you find the key codes for the behaviors.

To score an instance of the behavior or its end, either press the key or click the corresponding button on the screen.

### Notes

- **IMPORTANT** If you do tracking from video, make sure you de-select **DDS** in the Playback Control window.
- You can also score behaviors *after* tracking. See **Acquire Data > Score behaviors manually** in the EthoVision XT Help.



# Data analysis

## DATA PREPARATION

### *Data editing*

Choose **Acquisition > Edit Tracks**. You can fix tracking errors and swap back nose-points and tail-points that have been swapped in tracking. Normally you will not need to edit your data.

### *Smoothing data*

Choose **Acquisition > Track Smoothing Profile** and open **MDM Filter 0.2 cm**. In this profile, the **Minimal Distance Moved** filter is used with a value of 2 mm and option **Direct**.

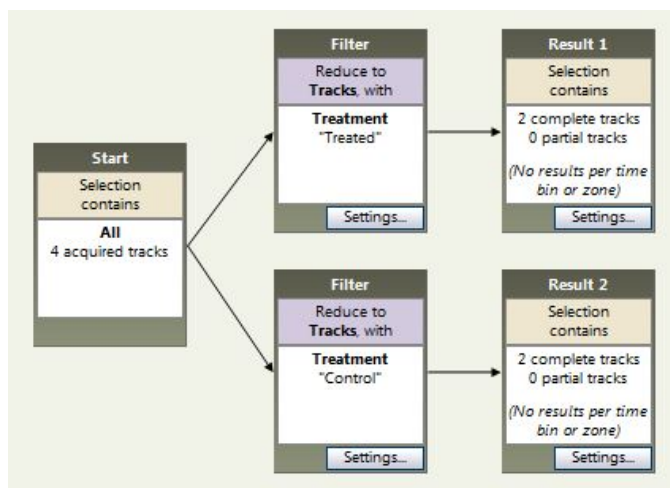
### *Review video and behaviors*

If you have scored behaviors manually, you can review the video and if necessary edit the data. Choose **Acquisition > Score behaviors manually**. For details, see **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

## SELECTING DATA

Choose **Analysis > Data Profile**. You can select your tracks according to your independent variable values (for example, Treated animals vs. Controls) and also select parts of tracks (data nesting). Figure 5.10 shows the Data Profile Treated vs. Control from the template to compare 'Treated animals' and 'Control animals' trials. This way you create groups of tracks to obtain group statistics for each group.

For more information and to create your own data selection, see the EthoVision XT Help.



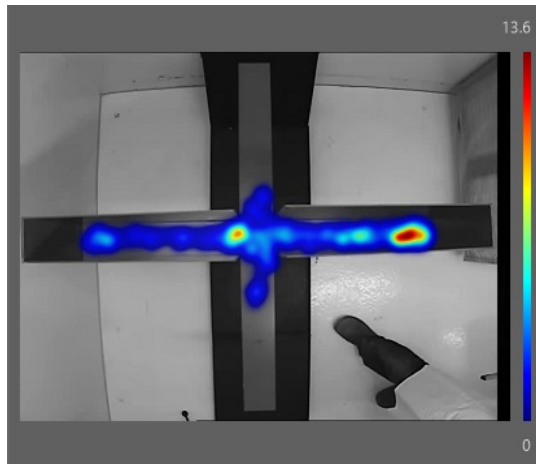
**Figure 5.10** An example of data selection to compare two data sets in your experiment, based on the independent variable “Treatment”. Two groups are formed: Treated and Control. Trial Statistics will be displayed per trial, and Group Statistics will be displayed per group of trials, according to the treatment level assigned to them.

If you have more treatment groups (e.g. Sham), to create more treatment groups click the Common Elements - Result button in the Components pane, and place the new box somewhere on your screen. Create a new branch by connecting the first (Start) box to this new box. Click the button next to “Treatment” and select the new treatment level. Insert the box in the new branch.

## VISUALIZING DATA

You can visualize your tracks in three ways:

- **Plot tracks.** You can view your tracks on a still image of the background. Tracks can be shown in different colors according to the values of independent variables (for example, blue for animals treated with saline and red for drug-treated animals). Sample points can be shown in different colors according to the values of dependent variables, for example red when the animal was moving fast.
- **Plot integrated data.** You can look at a track with the video file in the background. When you plot integrated data you can also view Time Event plots of your independent variables. Just like with plotting tracks, you can show tracks or sample points in different colors.
- **Heatmaps.** You can make heatmaps of the location of your animals during the test.



## ANALYSIS PROFILES

The template experiment contains three analysis profiles:

- **Time in arms** – This analysis profile contains two In zone variables:
  - In closed arms to calculate the frequency and duration of when the animal was in the closed arms.
  - In open arms to calculate the frequency, duration and latency of when the animal was in the open arms. Here, latency (the time to the first visit in any open arm) is used as an indication of anxiety.

For all variables, the animal is considered to be in a zone when all its body points are found in the zone simultaneously.
- **Behaviors** – In this analysis profile, four variables have been defined.
  - *Head dipping* and *Rearing* provide statistics of the behaviors manually scored.
  - *Body elongation* is used to quantify the time that the animal shows stretching behavior.
  - *Nose in Head Dip Area* is an **In zone** variable to calculate the frequency and duration of when the nose point of the animal was in the head dip areas.

The last two variables are present when you set the experiment to track the three body points or you select the zone template with the head dip area.
- **Velocity and distance** – With the variables **Distance Moved** and **Velocity**, the total distance moved and the mean velocity and the group means and their standard errors are calculated. Movement is based on a velocity threshold, and quantifies the time that the animal has moved significantly.

For more information, see the EthoVision XT Help.

### ***False positives in arm entry statistics***

Sometimes the center point of the animal fluctuates around the border line between the center and the arm zones. This may be caused by jitter

or exploration behavior, and results in false positives when calculating the number of zone entries.

To prevent this from happening, set the Zone exit threshold for the *In zone* variable.

### ***To set a Zone exit threshold***

1. In the Analysis profile, add the **In Zone** variable. Specify the arm zones you are interested in, and the body points that define a zone entry (when tracking the center, nose and tail base points).
2. Under **Threshold**, enter a value for **Zone exit threshold**. This is the distance from the border of the arm zone that the animal must be in order to be considered outside the zone. Use the Zone exit threshold to filter out small movements of the body points that result from jitter or exploration behavior.
3. Visualize the data (**Analysis > Results > Plot Integrated Data**).

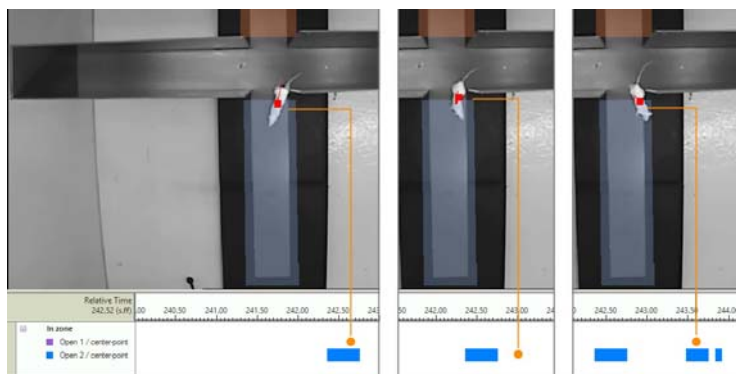
### ***Example***

In the following example we see that slight movements of the mouse's center point result in multiple open arm entries. In the Analysis profile, *In zone* is set with Zone exit threshold = 0 cm.

Left: Mouse enters the open zone.

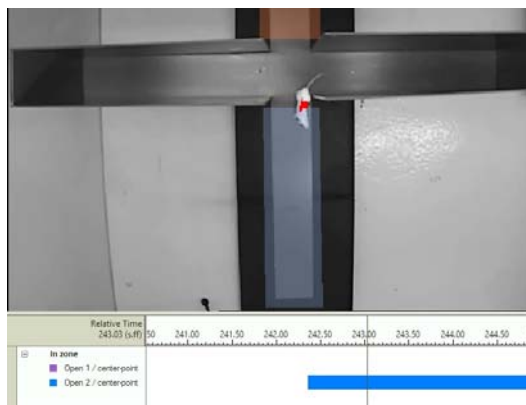
Middle: The center point is detected out of the zone.

Right: The center point is inside the zone; a second arm entry is scored.



To remove false positives of zone entries, in the Analysis profile, In zone is set with Zone exit threshold = 2 cm. This means that when the center point is outside the zone by less than 2 cm from the border, it is still considered in the zone.

Result:



This time, one arm entry is scored.

For more information on the Zone exit threshold, see **In zone** in the EthoVision XT Help.

## Chapter 6

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# The Sociability Cage Test

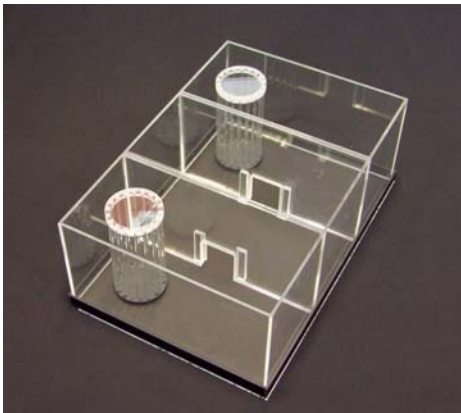
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# Introduction

## THE SOCIABILITY TEST (THREE-CHAMBER PARADIGM)

The three-chamber paradigm test known as Crawley's sociability and preference for social novelty protocol has been successfully employed to study social affiliation and social memory in several inbred and mutant mouse lines. Sociability testing is a more specific study of social behavior and only focuses on the behavior of one animal towards others. There are several variances to this test, all conducted in a three-chambered test apparatus.

In the first variant, the animal's interest in a social stimulus (a conspecific in a wired cage placed in one of the outer chambers) versus a neutral stimulus (an empty wired cage placed in the other outer chamber) is studied. In a preference test for social novelty, the two wired cages contain a familiar and an unfamiliar conspecific. A third variation involves testing the social preference for two different, but both unfamiliar conspecifics.



**Figure 6.1** *The Noldus Sociability test cage with three chambers and two wired cages for mice.*



In most cases the interest is measured by assessing the time spent in the same chamber or in close proximity to the familiar or unfamiliar other mouse.

### ***Representative results***

Typically, a wild type animal will spend significantly more time in the compartment with a strange mouse compared to the compartment with empty cage, indicating normal sociability, social motivation and affiliation.

In the social novelty test, the subject has a free choice between the first, already-investigated, now-familiar mouse, and a novel unfamiliar mouse. Usually, a wild-type animal recalls its previous contact with the familiar mouse, and, in this session, tends to spend more time with the newly encountered mouse, indicating intact social memory and predilection for novel experiences.

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# The Sociability test in EthoVision XT

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

Under **Video Source**, and **Tracked Features**, make sure that the options selected corresponds to your needs. If you want to analyze exploration in detail, choose Center-point, Nose-point and Tail-base detection.

If you want to use Deep learning technique to track the subject's nose, under **Body point detection technique** choose **Deep learning**. Note that in order to use this technique there are additional requirements. Among other things, you need a powerful graphics card. See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.

## MANUAL SCORING SETTINGS

If your EthoVision XT license does not include the Behavior Recognition module, EthoVision XT cannot detect sniffing or rearing automatically. However, you can still score those and other behaviors with keystrokes.

Choose **Setup > Manual Scoring Settings**.

If you used the template for an open field experiment, four behaviors have been defined:

- *Sniffing R* and *Sniffing O*, to record manually sniffing at the receiver (unfamiliar mouse) and object (either familiar mouse or empty cage, depending on the test), respectively.
- *Rearing R* and *Rearing O*, to record manually sniffing at the receiver (unfamiliar mouse) the object cage (either familiar mouse or empty cage, depending on the test), respectively.

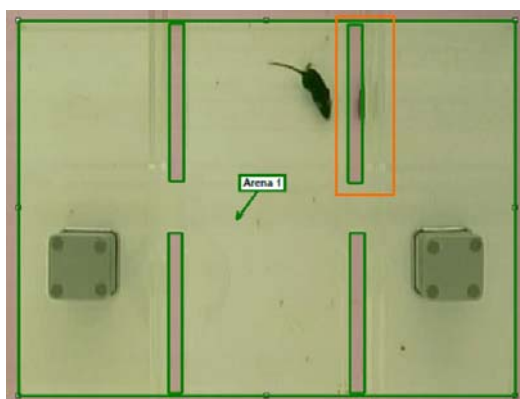
You can also rename the behaviors if you like.

## ARENA SETTINGS

Choose **Setup** > **Arena Settings**.

If you work with a Sociability test cage with backlight unit, you can reduce the intensity of the backlight before adjusting the arena settings. This makes the edges of the floor plate and walls more visible.

If the separation walls show reflections of the animal, this may hinder tracking. In the Arena Settings, click the Arena layer, and draw rectangles around the separation walls, like in the example below.



## DETECTION SETTINGS

Choose **Setup** > **Detection Settings**.

We assume that you followed the procedure in **DETECTION SETTINGS** on page 17.

Check in the **Video** Section that the sample rate is set to:

- For center point tracking: 5-8 samples/second (for rats) and 12.5-15 samples/second (for mice).
- For Nose-tail tracking (rats and mice). 25-30 samples/second.

## Notes

If detection of the subject is not optimal after using the Automated setup function, see **Configure Detection Settings** in the EthoVision XT Help for details on the advanced detection settings. If you use hooded animals, use the detection method **Differencing**.

If the animal moves from one compartment to the other, it may not be detected well, like in this example.



To improve detection, open the **Advanced** section in the **Detection Settings** pane. Under **Subject Contour**, select one or more pixels for the **Dilation** filter and the second **Erosion** filter. Leave the first Erosion filter to zero. Select more pixels for **Dilation** than for **Erosion**, for example 3 vs. 1, until the entire animal's contour is detected.

## TRIAL LIST

Choose **Setup > Trial List**. Click **Add Variables** and enter your independent variables such as the experimental phases and the ID code of your test subject.

Trial List			
Add Trials... Add Variable Add Videos... Import External Data...			
	User-defined	User-defined	System
Label	Phase	Subject ID	Arena settings
Description			The arena settings used for acquisition
Type	Text	Text	
Format			
Predefined Values			
Scope	Subject	Subject	Trial
Trial	No.		
Trial 1	1	Habituation	Arena Settings 1
Trial 2	2	Sociability	Arena Settings 1
Trial 3	3	Social discrimination	Arena Settings 1
Trial 4	4	Habituation	Arena Settings 1
Trial 5	5	Sociability	Arena Settings 1
Trial 6	6	Social discrimination	Arena Settings 1

# Acquiring data

## PROTOCOL

### *Sociability test*

For each test animal, three trials are carried out:

1. The first trial (for example 5 min) aims at habituating the test individual to the chamber. Tracking is required when one wishes to know whether the animal explores one chamber more than the other.
2. A second trial (5 min) is carried out with the empty tubes in the test chambers. The aim of this trial is to quantify the time spent in the two side chambers and near the tubes.
3. In the third trial (next 5 min), an empty tube is removed and replaced by a female animal restrained in an identical tube. This session is designed to observe differences between the time spent sniffing the social versus non-social object.

### *Social discrimination test*

For each test animal, three trials are carried out:

1. The first trial (10 min) is aimed at habituating the test individual to the chamber and empty tubes.
2. In the sociability test (next 10 min), an empty tube is removed and replaced by a female animal restrained in an identical tube. This session is designed to observe differences between the time spent sniffing the social versus non-social object.
3. In the social discrimination test (10 min), the other empty tube is removed and replaced by a novel female. This session is designed to test the ability of the test animal to distinguish between the two females.

See also **ACQUIRING TRACKS** on page 21.

## SCORE BEHAVIORS MANUALLY

To score sniffing and other behaviors defined under Manual Scoring Settings, open the Manual Scoring tab on the Acquisition screen. There you find the key codes for the behaviors.

To score an instance of the behavior or its end, either press the key or click the corresponding button on the screen.

### **Notes**

- **IMPORTANT** If you do tracking from video, make sure you de-select **DDS** in the Playback Control window.
- You can also score behaviors *after* tracking. See **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

# Data analysis

## DATA PREPARATION

### *Data editing*

Choose **Acquisition > Edit Tracks**. You can fix tracking errors and swap back nose-points and tail-points that have been swapped in tracking. Normally you will not need to edit your data.

### *Smoothing data*

Choose **Acquisition > Track Smoothing Profile** and open **MDM Filter 0.2 cm**. In this profile, the **Minimal Distance Moved** filter is used with a value of 2 mm and option **Direct**.

### *Review video and behaviors*

If you have scored behaviors manually, you can review the video and if necessary edit the data. Choose **Acquisition > Score behaviors manually**. For details, see **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

## ANALYSIS PROFILES

The template experiment contains four analysis profiles:

- **Distance and velocity**. For quantifying activity and exploration. Combine this with a Data profile that specifies **Results per zone**.
- **In chambers**. To calculate the time spent in each of the three chambers.
- **Behaviors**. To analyze behaviors scored manually (rearing and sniffing) or the time that the nose point was detected in the area around each cage (sniffing zone).



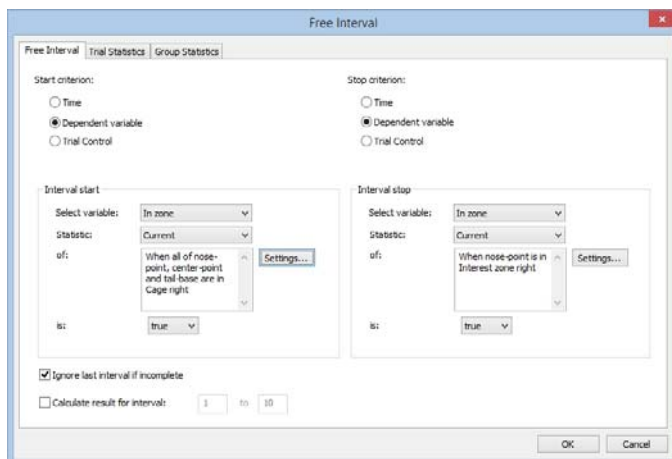
- **On cage.** To calculate how often and how long the center point of the subject was found in the zone “cage” (left and right). This correlates with the time the mouse spent on top of the cages.

Add other analysis profiles according to your research questions. Click one of the buttons in the **Dependent Variables** pane and open the **Trial Statistics** tab to select which statistic to calculate.

### Example

The researcher wants to know the time from the moment the animal is entirely in the right compartment to the first moment the animal's nose-point is close to the cage in the same compartment.

To calculate this time, in the Analysis profile choose **Free Interval**. Select an interval that goes from the time when the Current value of In zone, for zone *Cage right*, and for all body points, is “true” (Start criterion), to when the Current value of In zone for the zone “Interest zone right”, and for the nose point, is true (Stop criterion).



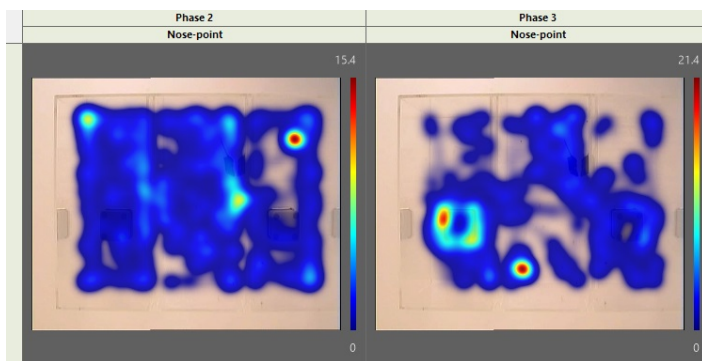
In the Statistics tab, select **Cumulative duration**.

For more information, see the EthoVision XT Help.

## VISUALIZING DATA

Choose **Analysis** > **Results** > ...

- **Plot tracks.** You can view your tracks on a still image of the background. Tracks can be shown in different colors according to the values of independent variables (for example, blue for animals in Phase 2 and red for animals in Phase 3). Sample points can be shown in different colors according to the values of dependent variables, for example red when the animal was moving fast.
- **Plot integrated data.** You can look at a track with the video file in the background. When you plot integrated data you can also view Time Event plots of your independent variables. Just like with plotting tracks, you can show tracks or sample points in different colors.
- **Plot Heatmaps.** Make heatmaps of the location of your animals during the different trials. In the example below: Left heatmap: Cages empty. Right: mouse restrained in the Left cage (right cage empty).



## Chapter 7

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# The Social Interaction Test

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# Introduction

## MEASURING SOCIAL INTERACTIONS

The Social Interaction test is widely used as a rodent model of anxiety-like behavior, under the assumption that anxiety is incompatible with social behavior (File and Hyde, 1978; File, 1980, 1988). So, an increase in anxiety generally results in a decrease in time spent on social interaction. The Social Interaction test is used to measure i) sensitivity to both anxiogenic and anxiolytic stimuli, ii) sensitivity to pharmacological, physiological and environmental manipulations and iii) ethologically relevant behaviors that do not require confounding manipulations such as food deprivation, pain or conditioned fear. Until recently, it has been difficult to automate the test with tracking systems. The features in EthoVision XT make this possible. This document describes the general procedure of how to do this.

In most cases for a social interaction test you need the **Social Interaction module**. This module allows you to track two or more subjects in one arena. This module also includes the Deep learning option for individual recognition in a 2-subject interaction test.

Here below you find an overview of the ways you can implement a social interaction test in EthoVision XT:

### ***Sociability test (1-subject tracking)***

If you intend to track a free-moving subject while a second subject is caged (social target) and barely visible from above, you can track only the free-moving animal and measure the behavior of that animal towards the cage defined as a zone. In that case you do not need the Social Interaction module. See **The Sociability Cage Test** on page 107.

### ***Social Interaction - with Deep learning-based recognition***

This solution, new in EthoVision XT 18, is based on artificial intelligence which discriminates between two subjects in the arena. One of the two subjects must be marked, e.g. its back being partially shaved. For this solution you can use a monochrome digital camera.

**NOTE** In order to apply artificial intelligence to animal tracking you need to have a compatible Graphics Processing Unit (GPU) installed on the EthoVision XT computer. See the EthoVision XT Help for more information.

### ***Social Interaction - marker-based tracking***

This option assumes that you can color-mark your subjects that interact in the arena, and you have a color camera compatible with EthoVision XT. Most of this chapter is based on this solution.

### ***Social Interaction - Live Mouse Tracker***

With the Live Mouse Tracker add-on module you can import and analyze Live Mouse Tracker data. Live Mouse Tracker allows long-term analysis of behavior of socially-grouped mice. With Live Mouse Tracker you do not have to color-mark the subjects, however each subject must have a RFID chip implanted. For more information, see the EthoVision XT Help.

## **THE SAMPLE EXPERIMENT**

To see an example of a social interaction test carried out in EthoVision XT, see also the sample experiment **Social Interaction XT180** on the downloads section of the Noldus website ([my.noldus.com](http://my.noldus.com)). Download this file and save it on your computer. Next, in EthoVision XT, select **File > Restore Backup** and select this file. For more information, see the document **Description of sample experiments of EthoVision XT.pdf**.

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***EthoVision XT and the Social interaction test***

For a list of selected publications, see the results of [Google Scholar](#).

# Physical setup

The following suggestions are aimed at optimizing video tracking of multiple, color-marked animals.

## LIGHTING CONDITIONS

- For general information on lighting condition, see the section **Physical setup** in the chapter **The Open Field Test**.
- Use a sensitive camera if possible. A low light intensity makes it difficult to separate different colors. When it is not possible to use a sensitive camera or strong illumination in your setup, try using fluorescent marker colors with UV lighting.
- For optimal color separation, illuminate your setup with lamps that approximate to day-light in color temperature, that is, have a wide spectrum range.

## MARKED VS. UNMARKED SUBJECTS

In some cases it is required that the identity of the animals that interact in the arena is known and followed, for example in resident-intruder or male-female interactions, or whenever one need to separate behavioral endpoints (e.g. speed, zone visited) for each individual. In such situations, we recommend to mark the animals (see below). EthoVision XT can also track unmarked animals, however identity switches may occur. It is not guaranteed that the correct identity of each unmarked animal is kept throughout the trial.

### *Markers for when using the Deep learning technique*

When using Deep learning, shave the back of one subject so that it looks different from the other. The shaved part should always be visible from the top. For more details, see the EthoVision XT Help.

### ***Color marker characteristics***

- Use a color scale (for example from a paint company) to find out which colors are most easily recognized by EthoVision in your setup and lighting conditions. Do this before applying color markers to your animals.
- Use colors that have different hue values (Figure 7.1). For example, use orange and green, pink and yellow, not red and orange. Avoid using red for marking, since it looks like blood.
- Note that marking your animals may stress them, and therefore affect their behavior. If necessary, ensure that you select a marking method that lasts for a longer period of time.
- Make sure that the marker is as round as possible, this will ensure that the relative movement of the center of gravity of the marker is the same in all directions when the edges of the marker change due to posture changes or otherwise.
- Make sure the marker is not too big; the marker can interfere with proper detection of the body contour. For example, make sure that a dark marker on a white animal does not cover the complete width of the animal because it can cause the body to be split in two (during tracking in EthoVision XT).
- See also **Tips for Color Tracking** in the EthoVision XT Help.



**Figure 7.1** *An example of color-marked rats in a social interaction test.*



# The Social Interaction test in EthoVision XT

## CREATE AN EXPERIMENT

### *For Live Mouse Tracker*

1. Choose **File > New** and in the dialog that opens select **Live Mouse Tracker** experiment.
2. For the rest of the procedure, see the EthoVision XT Help.

### *For all the other cases*

Choose **File > New From Template** and choose a predefined template in the guided setup. See below.

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

- Under **Video Source**, click the video icon in the camera row and adjust the camera settings if necessary.
- Under **Subjects**, specify the **Subject roles** (for example Resident or Intruder, or Subject 1 and Subject 2). Do not enter the ID of the individual animals.
- Under **Tracked Features**, select the option that corresponds to your needs. In most cases that is **Center-point, nose-point and tail-based detection**.
- Under **Body Point Detection Technique**, select **Contour-based** if you use color-marked animals, or **Deep learning** if your subjects look different (e.g. the back of one individual has been partially shaved).

**NOTE** You can use Deep learning to track two subjects per arena. The Deep learning technique works if you have a recent Graphics card and the driver is up to date.

## MANUAL SCORING SETTINGS

Choose **Setup > Manual Scoring Settings**.

Here you can define behaviors that you score manually, for instance by pressing a keyboard key.

In the template experiment, two start-stop behaviors, *Rearing* and *Grooming*, have been defined. Define other behaviors if needed, for example *Sniffing* and *Boxing*.

For more information, see **Set Up an Experiment > Manual Scoring Settings** in the EthoVision XT Help.

## TRIAL CONTROL SETTINGS

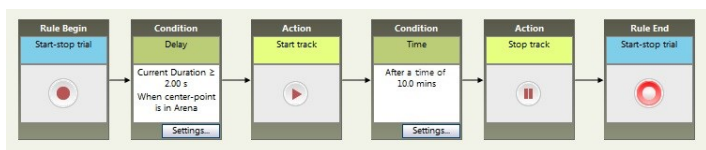
Choose **Setup > Trial Control Settings > New**.

In the Trial Control Settings, define conditions for the start and stop of the track.

- Starting condition. For instance, 'Start tracking 5 seconds after both animals are detected in the arena'. If you are tracking live, this 5-second delay enables you to put both animals in the arena and to take out your hand before tracking starts (5 seconds might not be enough; check beforehand how much time you need).

**TIP** To make sure that tracking starts when *both* individuals are in the arena, not before, click **Settings** in the **Condition** box before the **Start track** box. Select **Current Duration** from the **Statistic** list, then click **Settings** and then **Actors**. Select both subject names and select **All selected subjects** from the list.

- Stopping condition. In order to be able to compare the tracks in the analysis, they must have the same length. Either define a **Maximum Trial duration** or a **Stop track** condition based on time (see the next figure).



**Figure 7.2** Trial control settings that specify a track duration of 10 minutes.

## DETECTION SETTINGS

Choose **Setup > Detection Settings > Detection Settings 1**.

**IMPORTANT** Follow the instructions in the order described below!

If you selected **Deep learning** in the Experiment Settings (page 125), set the sample rate (below), and skip the next steps. The software is able to recognize the subjects automatically so you do not need to adjust subject contrast, size, contour filters etc. Go to **TRIAL LIST** on page 131.

### *Sample rate*

Click **Video**. Select a high sample rate: 25 (for PAL analog cameras) or 30 (for NTSC analog cameras and digital cameras) samples per second.

### *Automated setup*

Once the animals are in view, click **Automated Setup**. Select the type of animal you are going to test. Click **Next** and draw a rectangle around each subject. Make sure that you do this when the animal are not in close contact.

If detection of the subject is not optimal after using the Automated setup function, see **Configure Detection Settings** in the EthoVision XT Help for details on the advanced detection settings.

### *Subject Identification for color-marked subjects*

If you mark animals with colors, under **Subject Identification** make sure that **Marker-assisted identification** is selected.

1. Put the marked animals in the arena or play the video. Make sure to select a point in the video where the animals do not touch each other! Alternatively, place the animals in the arena one at a time.
2. In the **Subject Identification** section, select one of the subjects and click the **Identification** button. As a result, the **Identification [Subject name]** window opens.
3. Move the mouse pointer to the **Video** window so the pointer becomes an eyedropper. Move the eyedropper over the color marker of the subject you want to identify and click the left mouse button.



4. Fine-tune the color settings by adjusting the **Hue**, **Saturation** and **Brightness** in the **Identification [subject role name]** window. Change the range of color settings by changing the numbers or by resizing the **Hue** box on the vertical color bar, or resizing/moving the box in the color map (horizontally to adjust **Saturation**, vertically to adjust **Brightness**). As a result, the outline covers (almost) the complete marker (see Figure 7.3).
5. Next, play the video to see in the **Video** window whether the marker is detected correctly in different parts of the arena. If the marker 'dances' then your color settings are too sensitive. Go back to step 4 and make the box larger.
6. When you are finished fine-tuning the color settings, you continue with setting the **Minimal marker size**: increase the **Minimal marker size** until, first, noise is not detected anymore and, second, the marker is not detected anymore (as a result, the outline in the **Video** window disappears). Now enter a value for the **Minimal marker size** that is somewhere in between.

- Click **OK** when you are done and repeat steps 1-6 for the other subjects.



**Figure 7.3** The color of the marker after fine-tuning the color settings. Most of the marker is now selected as indicated by the black outline.

For details, see **Advanced detection settings for color marks** in the EthoVision XT Help.

Also watch the video **Identification Settings** in the EthoVision XT video tutorial.



### ***Set the subjects' contrast***

Make sure you have selected the correct contrast and check the following:

- When you use **Static Subtraction** or **Dynamic Subtraction**, move the slider to define the animal's contrast. The animal must be fully detected in all parts of the arena and the noise must be minimal. For **Dynamic Subtraction**, set the **Current frame weight** slider to a value between 1 and 5 to compensate for the changes in the background with time.

- When you use **Differencing**, adjust the position of the **Sensitivity** slider until the subjects are properly detected. From the **Background changes** list, select an option that reflects your conditions (usually, **Medium slow** works just fine).

### ***Contour erosion/dilation***

You need **Contour erosion/dilation** to get a smooth contour without the tail for accurate modeling and to remove individual pixels of noise.

To improve detection, open the **Advanced** section in the **Detection Settings** pane. Under **Subject Contour**, select one or more pixels for the first **Erosion** filter and the **Dilation** filter. Leave the second Erosion filter to zero. Increase the number of pixels for **Erosion** until the animal's tail is removed, then increase **Dilation** until the entire animal's body contour is detected.

### ***Subject Size***

Before you set the **Subject Size**, make sure the animals are properly detected and do not touch each other.

Click **Subject Size**, then click **Advanced**.

1. Under **Modeled subject size**, make sure the **Apply to all subjects** and **Fix** (for all subjects) options are selected.
2. Click the **Grab** button. This adjusts the **Average size** to the **Current size**.
3. Repeat clicking the **Grab** button, until the body fill completely fits the animal.

### ***Shape stability and Modeling effort***

The **Shape stability** setting is used when you track animals whose body can be occluded by, for example, cage bars or part of the body of another animal.

When you set the slider closer to **Occlusions**, EthoVision considers separate objects that are close together part of one animal. When you set the slider close to **Noise**, EthoVision considers separate smaller parts not part of the animal.

The **Modeling effort** setting is used when two animals touch and EthoVision loses the separate shapes. At this point, EthoVision tries to determine which part of the big 'merged' blob belongs to either animal, using a lot of processor load in the process.

When you set the slider close to **Performance**, EthoVision is only allowed a short time to determine which part of the 'merged' blob belongs to which animal. Therefore, modeling quality is low. When you set the slider to **Modeling**, EthoVision is allowed a longer time to determine which part of the 'merged' blob belongs to which animal. Therefore, modeling quality is good, but this costs a lot of processor load. If you track from video, video tracking slows down.

## **TRIAL LIST**

Enter your independent variables such as rat ID, treatment (drug vs. control), dose, name of the experimenter, etc. If you want you can predefine all your trials here, or you can enter the independent variable values as you carry out the trials. You can also prepare the trials in Excel, randomize them and then paste the values into your Trial List.

### **Notes**

- When using color markers, add a Marker color variable to the Trial List to specify the marker color you assigned to each Subject role. This is useful when you have defined, for example, the Subject name *Experimental* and *Stimulus*, or *Subject 1* and *Subject 2*, and you have randomly assigned the marker colors to the Subject roles.
- Furthermore, you can define a series of trials for batch acquisition. See **Acquire a series of trials** in the EthoVision XT Help.

Trial List				
<div> Add Trials... Add Variable Add Videos... Import External Data... </div>				
	System	User-defined	User-defined	User-defined
Label	Acquisition status	Animal ID	Dose	Day
Description	The current status of acquisition per arena			
Type	Text	Numerical	Numerical	Text
Format		x.x mg/Kg	x	
Predefined Values		0.0 mg/Kg; 0.5	All valu	Yellow; Green
Scope	Arena	Subject	Subject	Subject
Trial	Arena	Subject	No.	
Trial 1	Arena	Control	1	
		Treated	2	
Trial 2	Arena	Control	3	
		Treated	4	
Trial 3	Arena	Control	5	
		Treated	6	
Trial 4	Arena	Control	7	
		Treated	8	
Acquired		1	0.0 mg/Kg	1 Green
		2	0.5 mg/Kg	Yellow
Planned		3	0.5 mg/Kg	Green
		4	0.0 mg/Kg	Yellow
Planned		5	0.5 mg/Kg	2 Yellow
		6	0.0 mg/Kg	Green
Planned		7	0.5 mg/Kg	2 Green
		8	0.0 mg/Kg	Yellow

**Figure 7.4** An example of Trial List for a social interaction test.



# Carrying out your trials

## PROTOCOL

### Animals

In many social interaction experiments, two animals from the same treatment group are tested together and scored as a single unit. According to Lapiz-Bluhm et al. (2008), this potentially can result in an artificial exaggeration of behavioral effects, that is, a treatment that only slightly impacts social behavior may have an apparently larger and more significant effect if the social behavior of both animals is affected. Their alternative approach test an experimental animal paired with a 'neutral' stimulus animal, whose behavior is as constant as possible. See **The Sociability Cage Test** on page 107.

### Habituation

The experimental animals are gently handled for 2 minutes on each of 3 days prior to the social interaction test. In addition, they are individually familiarized with the testing room and social interaction arena for 5 minutes on the 3 days prior to testing. The stimulus animals are habituated to the testing room and arena for 5 minutes per day for 3 days prior to testing. The stimulus animals are pre-exposed to the test arena in pairs, with a different, unfamiliar other stimulus animal on each pre-exposure day.

### *When using color marks*

To control for the effect that the color of the marker might have on the behavior of the other animal, it is recommended to randomly assign the marker color to stimulus and experimental animals. To take into account the combinations of animal type and marker color, you can either add a user-defined variable like *Marker color* to the **Trial List** or define two **Detection Settings**, one for each combination. In the latter case, make sure you set the appropriate Detection Setting as current before you start data acquisition.

### ***Social memory test (1-min confrontation)***

1. Let the focal animal establish a home-cage territory (e.g. 5 days).
2. Introduce the stimulus female into the home cage of a male mouse for a 1-min interaction.

At the end of the 1-min trial, remove the stimulus animal.

3. Repeat step 2 a number of times, with a 10-min inter-exposure interval.
4. “Dishabituation” trial: Introduce a different stimulus female to the same male mouse for one minute.

In EthoVision XT:

- If you use color markers, alternate different colors for the same subject (e.g. half resident mice marked orange, half marked green). If you use different colors for the resident animal, make different detection settings in such a way that **Subject 1** is always the resident animal. Before acquisition, make sure that you use the detection settings that apply to that animal.
- Mark different types of trials with an independent variable in the Trial List. For example *Type*, with values C1, C2 ... for confrontation with the first stimulus female and D for the second stimulus female.

### ***Scoring behaviors manually***

If you want to score the subject’s behavior manually, define them first under **Manual Scoring Settings** (page 126). After you have started the trial, watch the subjects in the video and in the **Manual Scoring** tab at the bottom of the screen, click the button to the category that applies.

Scoring	x					
	Trial Status		Independent Variables		Dependent Variables	
	Manual Scoring					
Trial		Subject	Fill color	Acquisition status	Rearing	Grooming
Trial 4	Control			Acquiring	a/a	b/b
	Treated				c/c	d/d

As soon as the behavior of the subject changes, click the button for the corresponding category.

For more information, see **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

# Data analysis

## DATA PREPARATION

### *Track editing*

Choose **Acquisition > Edit Tracks**.

You can fix tracking errors and swap back nose-points and tail-points that have been swapped in tracking. Normally you will not need to edit your data.

### *Track smoothing*

Choose **Acquisition > Track Smoothing Profile > open MDM Filter 2 mm**. In this profile, the **Minimal Distance Moved** filter is used with a value of 2 mm and option **Direct** selected.

### *Review video and behaviors*

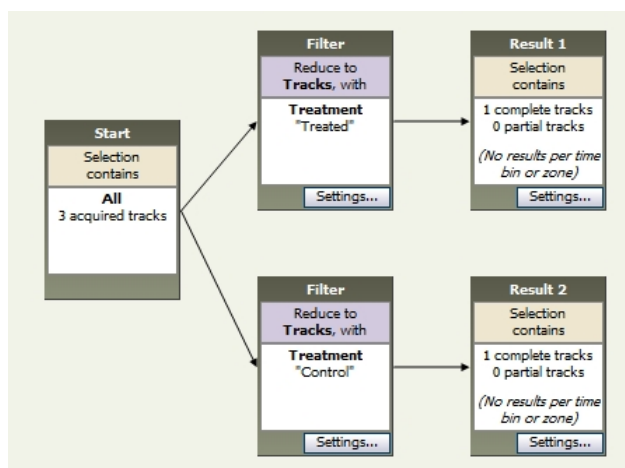
If you have scored behaviors manually, you can review the video and if necessary edit the data. Choose **Acquisition > Score behaviors manually**. For details, see **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

### *Selecting data*

Choose **Analysis > Data Profile**.

You can select (**Filter**) your tracks according to your independent variable values (for example, *Treated* animals vs. *Controls*) and also select parts of tracks (**Nesting**). Figure 7.5 shows a data selection to compare Treated and Control animals. This way you create groups of tracks to obtain group statistics for each group.

To create a group, click the button next to **Common elements - Result**. Connect the **Start** box to the new **Result** box. This creates a new group. Next, to specify a criterion for the new group, click an option under **Filter**, and insert the resulting **Filter** box between **Start** and the new **Result** box.



**Figure 7.5** A data profile to compare tracks of Treated animals and Control animals.

## ANALYSIS PROFILES

The template experiment contains a number of analysis profiles, depending on which arena / zone template you selected in the template wizard:

- **Distance & Time** – With the variables **Distance Moved** and **Velocity**, the total distance moved and the mean velocity are calculated.
- **Movement** – The variable Movement calculates how often and how long the center-point was moving.
- **In zones** – The variable **In zone** calculates how often and how long the subjects visited each zone in the Arena Settings. The latency to the first visit in a zone is also calculated for each subject.

- **Behaviors** – In this analysis profile, the frequency and duration of **Body elongation** and the manually scored behaviors *Rearing* and *Grooming* are calculated.

For all statistics also the group means with standard errors are calculated.

## SOCIAL INTERACTION IN DETAIL

The following variables are commonly used to study social interactions:

- **Distance between subjects** – Use this dependent variable to measure the average distance between the subjects.  
  
You can also use this dependent variable to determine the appropriate thresholds for the dependent variable **Proximity**.
- **Proximity** – When the nose-point of the experimental animal is in proximity relative to any of the body points of the stimulus animal, the experimental animal can be considered to be exploring or sniffing the other animal.
- **Body contact** – You can use this variable to quantify body contact, for example how often and for how long two animals are in contact with one another.
- **Relative movement** – Use **Relative movement** to determine how much time the experimental animal is actively seeking contact with the stimulus animal.
- **Net weighted movement** – **Net Weighted movement** can be used as an objective measure for the intensity of approach and avoidance behavior.
- **Weighted movement from** – **Weighted movement from** can be used as an objective measure for the intensity of avoidance.
- **Weighted movement to** – **Weighted movement to** can be used as an objective measure for the intensity of approach.

- **JavaScript state variables** - Additional variables in JavaScript code are available to quantify social approach, leaving, social contact and following behaviour. You can find those variables on the installation package under **Utilities > JavaScript custom variables**, also available on my.noldus.com or on the GitHub repository.

<https://github.com/noldus/EthoVision-JavaScriptCustomAnalysis>

See **Dependent Variables in Detail** in the EthoVision XT Help for details. Also see the analysis profile 'Social - Nose to Tail' in the sample experiment **Social Interaction XT180** which you can download from our web site.

## STATISTICS AND CHARTS

Choose **Analysis > Results > Statistics & Charts**, then click **Calculate**.

The **Trial Statistics** tab shows the results per trial. The **Group Statistics** tab shows the statistics and charts from the summarized results over all trials or the groups defined in your Data profile (see page 136).

## VISUALIZING DATA

Choose **Analysis > Results > Plot Integrated Data**.

Select a trial from the list on the tool bar. The video file and the Time Event Plot are displayed. You can select other data/analysis profile combinations from the lists on the tool bar.

Choose **Analysis > Results > Plot Heatmaps**.

There you can make heatmaps of the location of your animals during the test.

## Chapter 8

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# The Radial Arm Maze Test

Introduction .....	141
Physical setup .....	143
The Radial Arm Maze test in EthoVision XT .....	144
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Data analysis.....	154



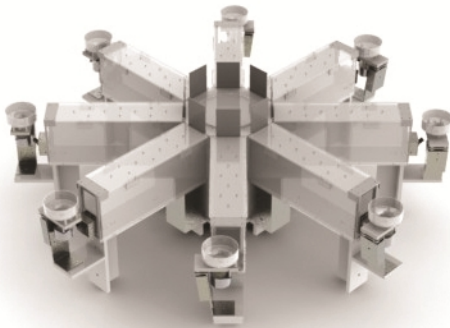
# Introduction

## THE RADIAL ARM MAZE TEST

The radial arm maze was designed to measure spatial learning and memory in rats (Olton and Samuelson, 1976; Olton, 1978, 1985). The original apparatus consists of eight arms, each about 4 feet long, and all radiating from a small circular central platform. At the end of each arm there is a food site, the contents of which are not visible from the central platform.

The design ensures that, after checking for food at the end of each arm, the rat is always forced to return to the central platform before making another choice. For example, when investigating working memory, all arms are provided with a food reward and the animal should visit each arm only once.

Correct performance of the radial maze task requires intact spatial memory abilities. Performance is affected by hippocampal impairment and a variety of pharmacological agents (e.g., Janitzky et al. 2011).



**Figure 8.1** *Example of an 8-arm radial maze.*

## REFERENCES

- Janitzky, K, Schwegler, H., Kröber, A., Roskoden, T., Yanagawa, Y. & Linke, R. (2011). Species-relevant inescapable stress differently influences memory consolidation and retrieval of mice in a spatial radial arm maze. *Behavioural Brain Research*, **219**(1), 142-148.
- Olton, D.S. (1978). Characteristics of spatial memory. In: Hulse S.H., Fowler H, Honig W.K. (Eds.), *Cognitive processes in animal behavior*, Hillsdale, NJ: Lawrence Erlbaum Associates, 341-373.
- Olton, D.S. (1985). The radial arm maze as a tool in behavioral pharmacology. *Physiol. & Behav.* **40**, 793-797.
- Olton, D.S., & Samuelson, R.J. (1976). Remembrance of places passed: Spatial memory in rats. *Journal of Experimental Psychology: Animal Behavior Processes*, **2**, 97-116.
- Wenk, G.L. (2004). Unit 8.5A Assessment of Spatial Memory Using the Radial Arm Maze and Morris Water Maze, in: *Current Protocols in Neuroscience*, John Wiley and Sons, Inc.

# Physical setup

The following suggestions are specifically to optimize video tracking:

- The camera should have a good view of the entire region the animal can be in. The arena should fill the field of view.
- There must be maximum contrast between the background and the animal. If you use white animals in the maze, make sure the floors of the arms are dark and vice versa. Preferably, the floor of the room should be the same color (and matted) as the maze, contrasting with the animal.
- Lighting should be uniform and even with no shadows.
- If you have reflections in your image, EthoVision XT may confuse those reflections with your subject, and track the reflections rather than your animal. To prevent reflections, place indirect lighting above or around the maze. 'Globe' type bulbs twice the diameter as standard light bulbs are ideal. They should be close enough to the maze so that there is no direct line of sight between the bulbs and the camera lens. The light should reflect off the walls and ceiling, so that it only reaches both the lens and radial maze indirectly.

# The Radial Arm Maze test in EthoVision XT

In EthoVision XT, you can set up a radial 8-arm maze experiment by using a predefined template in the guided setup.

Create an experiment. For details, see Chapter 1 of this manual, or see **Setup an Experiment** in the EthoVision XT Help.

## EXPERIMENT SETTINGS

1. Make sure that the radial maze is connected to the EthoVision XT computer.

To connect the radial maze to EthoVision XT, you must have the Trial and Hardware Control add-on and the USB-IO box. The procedure and the necessary cabling may differ between manufacturers of the radial maze. Please see the accompanying documentation.

2. Choose **Setup > Experiment Settings**.
3. Under **Video Source**, and **Tracked Features**, make sure that the options selected corresponds to your needs. To adjust the camera settings, click the video icon in the camera row.
4. If you want to use Deep learning technique to track the subject's nose, under **Body point detection technique** choose **Deep learning**. Note that in order to use this technique there are additional requirements. Among other things, you need a powerful graphics card. See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.
5. Under **Trial Control Hardware**, select **Use of Trial Control Hardware** and click **Settings**. Select **Noldus USB-IO box**.
6. In the Device Configuration window, for TTL ports 1 to 4, under **Device type**, select **Custom Hardware**. Next, type in *Doors1&2* for TTL

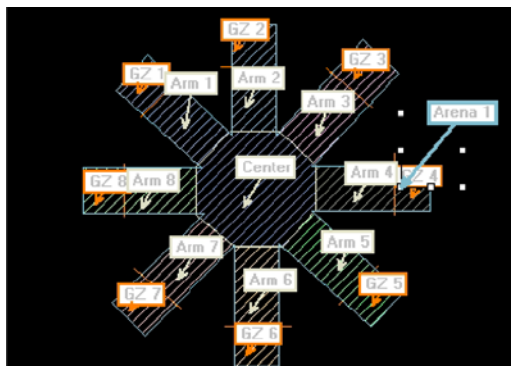
**Port 1, Doors3&4 for TTL Port 2, Doors5&6 for TTL Port 3 and Doors7&8 for TTL Port 4.**

Ports	Device type	Device ID
TTL Port 1	Custom Hardware	Doors1&2
TTL Port 2	Custom Hardware	Doors3&4
TTL Port 3	Custom Hardware	Doors5&6
TTL Port 4	Custom Hardware	Doors7&8

### ARENA SETTINGS

Choose **Setup > Arena Settings > Open Arena Settings 1.**

1. In the **Grab Background Image** window, click the **Grab** button to grab a background image of the radial maze from the camera image.
2. Click **1. Draw Scale to calibrate** and calibrate your arena. For details, see **Make the arena** in the EthoVision XT Help).
3. Click **2. Select Shape and Draw Arena** and check that the predefined arena has the correct shape and size. If necessary, adjust the contour of the arena to fit the radial maze.
4. If you used the Zone template, click **3. Select Shape and Draw Zones**. Check that the zones have the correct shape and size. If necessary, resize or add new zones.



5. If you are using automated doors, click **Arena - Hardware mapping** in the **Arena Settings** window to assign the doors to the arena.

Device type	Device name	Arena 1
Custom Hardware	1&2	Doors1&2
Custom Hardware	3&4	Doors3&4
Custom Hardware	5&6	Doors5&6
Custom Hardware	7&8	Doors7&8

## TRIAL CONTROL SETTINGS

In the Trial Control Settings you can define conditions for the start and stop of the track.

Choose **Setup > Trial Control Settings > Open one of the following:**

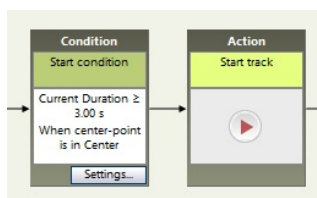
- **Default (no max duration)** - When you use this profile, tracking starts automatically 2 seconds after the animal has been placed in the Center zone. Tracking stops manually (for example when you click the **Stop trial** button).
- **Track duration 10 min** – When you use this profile, tracking starts automatically 2 seconds after the animal has been placed in the Center zone. The track stops automatically when 10 minutes have elapsed since the start of the track.

### *Controlling automated doors*

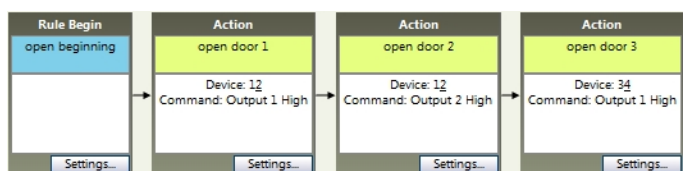
If you want to control the automated doors with EthoVision XT, you need to create new Trial Control Settings.

An example of Trial Control Settings for a protocol as described in Unit 8.5A of Current Protocols in Neuroscience (2004) to test basic working memory is the following:

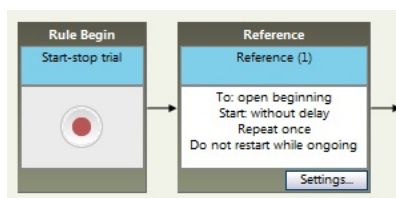
- **Start condition.** We assume that the animal is released in the Center zone with all doors closed. Tracking starts 3 seconds after the animal is put in the Center zone.



- *Opening all doors simultaneously.* You can create a Sub-rule with a Reference for this. In the Sub-rule, each door is opened. For each door, you need to create a separate Hardware Action box, with the corresponding Device name ('1&2') and appropriate Action ('Output 1 High' which opens door 1. The figure below shows part of the sequence of Sub-rule 'open beginning' in which doors 1-3 are opened.



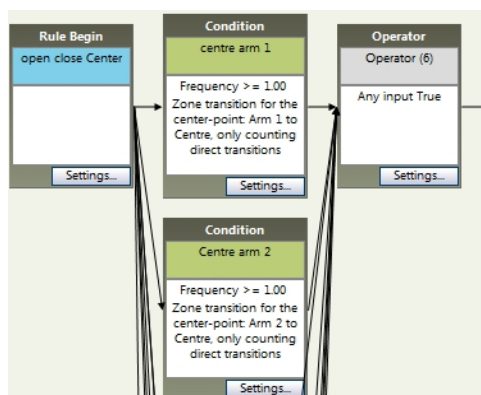
The figure below shows the Reference box to the Sub-rule. The reference is activated immediately after the Start trial command.



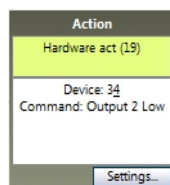
- *Closing and then opening all doors.* When the animal has visited the Goal zone of one of the arms and returns to the Center zone, all doors are closed and re-opened after 5 seconds.

You can create a Sub-rule with a Reference for this. In the Sub-rule, you need to create a Zone transition Condition for each arm separately. Next, all these 8 Zone transition Conditions need to be connected to an Operator 'Any input True'.

The figure below shows part of the sequence of the Sub-rule 'open close Center'. Here you see the Zone transition Conditions for arms 1 and 2. The Zone transitions Conditions of all 8 arms are connected to the Operator 'Any input True'.



Next, with a **Hardware Action** box, all 8 arms are closed. The figure below shows an example of closing door 4. Put the Hardware Action box for all doors in a sequence similar to opening doors as described in step 1 above.



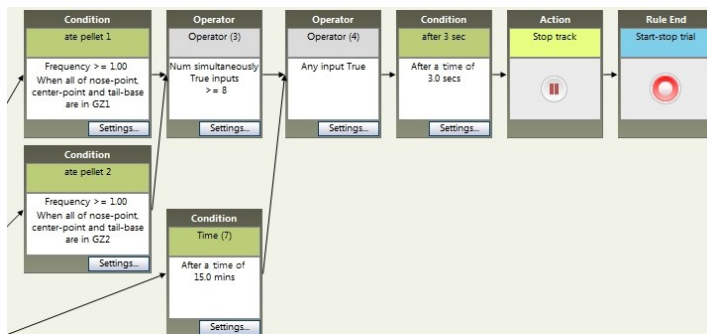


- *Stop condition.* The trial stops when the animal has visited the Goal zones of all 8 arms OR after 15 minutes have elapsed since the start of tracking.

For the first criterion (the animal visits the Goal zone of all arms) you use an In Zone Condition for each Goal zone. All eight In Zone Condition boxes are connected to the Operator 'Num simultaneously True inputs'. The figure below is an example and contains only two of the eight In Zone Goal zone Condition boxes.

For the second criterion (after 15 minutes) you need a Time Condition box as shown in the figure below.

The two criteria must be combined with OR logic. The Time Condition box and the 'Num simultaneously True inputs' Operator box are connected to a 'Any input True' Operator box (see figure below. In this example sequence, an extra delay of 3 sec is included before the trial stops).



## DETECTION SETTINGS

Choose **Setup > Detection Settings > Detection Settings 1**.

We assume you followed the procedure on page 17.

### *Advanced detection settings*

1. Under **Video**, check that the sample rate is set to 25 or 30 samples/second for multiple body-point tracking.
2. Click **Advanced**.
3. Under **Method**:

By default, **Static subtraction** is selected as the detection method and **Rodents / Default** as the method for nose-tail detection. The background in a radial maze test usually does not change much, so Static subtraction usually works well. Make sure the lighting is even and there are no shadows in the maze.

If after running some test trials, you get nose-tail swaps, try using the tracking method **Rodents / For occlusions**.

If you selected to use the Deep learning technique to track the subject's nose, next to **Deep learning** click **Define** and select a box around the subject. Make sure that the box includes the subject's nose. For details, see the EthoVision XT Help.

4. **OPTIONAL** Select Track noise reduction. Under **Smoothing**, set **Track noise reduction to On**.

In some cases better quality tracking can be obtained by reducing track noise during acquisition. This may especially be the case if you use Trial and Hardware Control. As an example, if the center point of an animal is detected in the center-zone of the maze, you want specific doors to open followed by the closing of doors as soon as the animal enters one of the arms. If the detected center point is moving rapidly because of noise, this may result in a doors opening and closing, every time the center point crosses the border of the zone. Track noise reduction may solve this problem.

5. Under **Subject size**, click **Advanced** and set a minimum subject size to prevent droppings from detected.

## TRIAL LIST

Enter your independent variables such as *Animal ID*, *Treatment*, *Baited arms*, Name of the experimenter, etc. (for blind trials, enter only the Animal ID). If you want, you can predefine all your trials here, or you can enter the independent variable values as you carry out the trials. You can also prepare the trials in Excel, randomize them and then paste the values into your Trial List.

Furthermore, you can define a list of trials for batch acquisition. See **Acquire a series of trials** in the EthoVision XT Help.

Label	System		Use-defined	Use-defined	Use-defined
	Acquisition status	Trial Control settings	Animal ID	Treatment	Baited arm
Description	The current status of acquisition per arena	The trial control settings used for acquisition			
Type			Numerical	Text	Text
Format			x		
Predefined Values			All values	Control; Treat	Arm 1; Arm 2; Arm
Scope	Arena	Trial	Subject	Subject	Subject
Trial	Arena	Subject	No.		
Trial 1	Arena 1	Subject 1	1	Planned	Test phase
Trial 2	Arena 1	Subject 1	2	Planned	Test phase
Trial 3	Arena 1	Subject 1	3	Planned	Test phase
Trial 4	Arena 1	Subject 1	4	Planned	Test phase
				1 Treated	Arm 1
				2 Control	Arm 4
				3 Treated	Arm 7
				4 Control	Arm 1

Figure 8.2 The predefined Trial List in the radial 8-arm maze template experiment.

### Baited arms

To mark the trials with the arms that were baited, create an independent variable *Baited arms* of *text* type, and define the various combinations as *Predefined Values*: for example, **1-3-6-8**, **1-3-5-7**, etc. For each subject to be tested, assign the correct value in the appropriate cell of the **Baited arms** column.

# Acquiring data

## PROTOCOL

The following protocol tests for basic working memory (the subject learns not to re-enter a baited arm).

### *Habituation (pre-training)*

In a typical radial 8-arm maze test, first a pre-training session is carried out in which the animal is allowed to freely visit all arms for 10 to 15 minutes per day. Also, the doors are regularly opened and closed to habituate the animal.

### *Training trials*

In this phase, the animal is exposed to where the food is, and this is what the later tested memory is based on. Animals are trained one session per day, for approx. one week.

Typically, all arms are baited in this phase. At the start of the trial, the animal is placed in the center of the maze, facing the same direction on every trial, everyday.

Stop the trial when (a) all eight arms have been visited; (b) 10 minutes passed since the start of the trial; or (c) 2 minutes since the animal's last arm entry.

### *Performance*

Among the variables commonly used for the analysis of the performance are (a) the number of errors in each session (entering an arm that has been visited previously counted as an error) and the total number of errors across eight sessions, and (b) the number of correct choices in the first eight arm entries of each session. See **Data analysis** on page 154.

### ***Reference memory errors***

While the previous protocol is primarily sensitive to impairments in working memory, an alternate protocol allows a disassociation to be achieved between working and reference types of memory.

In this protocol, four arms are baited for the training phase. The same maze arms are baited each day and, across sessions, the animal learns to ignore the other arms, which never contain a reward. This is the reference memory component of the task. In the test phase, food items are placed in the same arms as they were on the memory trials that day. It is recorded when the animal gets 100% correct on his memory trials. When the animal enters an arm that is not baited, it is marked as an error.

See Wenk, G.L. (2004). *Current Protocols in Neuroscience*, 8.5A.1-8.5A.12.

# Data analysis

## DATA PREPARATION

### *Track editing*

Choose **Acquisition > Edit Tracks**.

You can fix tracking errors but normally you will not need to edit your data.

### *Track smoothing*

Choose **Acquisition > Track Smoothing Profile** > open one of the two Track Smoothing Profiles:

- **No filter.**
- **MDM 0.2 cm** – In this Track Smoothing Profile, the Direct Minimal Distance Moved filter is used, with an MDM of 0.2 cm.

Use Smoothing when you want to eliminate small movements, such as body wobbling during locomotion, that might affect dependent variables such as total distance moved.

### *Selecting data*

Choose **Analysis > Data Profile**.

The template contains two data profiles:

- **All Data** – This data profile contains all data.
- **Treated vs. Control** – This data profile contains two Results containers with data for the treated animals and the control animals (see also **TRIAL LIST** on page 151). This way you create groups of tracks to obtain group statistics for both groups.

## ANALYSIS PROFILES

### *Default analysis profiles*

The template experiment contains three analysis profiles:

- **Distance and Velocity** – In this profile, the total Distance Moved and the mean Velocity are calculated for the center-point.
- **In Zones** – In this profile, the Frequency, Duration and Latency to First for all zones (arms and goal zones, see also **ARENA SETTINGS** on page 145) are calculated for the center-point. A visit to a previously chosen arm is considered a working memory error. Normal healthy young rats will perform this task almost perfectly every time, so will visit each arm and goal zone only once.
- **Zone transitions** – In this profile, the Frequency of zone transitions from the center zone to any arm is calculated for the center-point.

For all statistics also the group means with standard errors are calculated.

## TESTING WORKING MEMORY

### *Time taken to obtain all rewards*

The most basic test conducted using the radial-arm maze is to bait the ends of all of the arms with the reward and record the time taken to obtain all the rewards. The following procedure applies to trials where all baited arms were visited.

1. In the Analysis profile, choose **Target visits and errors**.
2. In the **Target zones** box, select all the zones that were baited.
3. Under **Calculate Statistics for**, select **Target first visits**.
4. Click the **Trial Statistics** tab and select **Latency to Last**.

### *Incorrect arm choices*

To obtain a reward, an animal must remember which arm(s) it visited previously and not re-visit those arms.

1. In the analysis profile, choose **Target visits and errors**.
2. In the **Target zones** box, select all the zones that were baited.
3. Under **Calculate Statistics for**, select **Target revisits**.
4. Click the **Trial Statistics** tab and select **Frequency**.

#### ***Percentage of correct choices***

One of the parameters described in the Unit 8.5A of Current Protocols in Neuroscience (Wenk, 2004) to calculate performance of all groups is: *The percentage of correct choices made in each test session in relation to the total number of arms entered.*

1. In the analysis profile, choose **Target visits and errors**.
2. In the **Target zones** box, select all the zones that were baited.
3. Under **Calculate Statistics for**, select **Target first visits** and **Target revisits**.
4. Click the **Trial Statistics** tab and select **Frequency**.
5. You can calculate the percentage of correct choices with:

$$\textit{Target first visits} / (\textit{Target first visits} + \textit{Target revisits}) * 100.$$

Where the statistics given by EthoVision XT are shown in italics.

## **TESTING REFERENCE MEMORY**

The radial-arm maze may also be used to test reference memory by only baiting some arms of the maze. In training trials, the reward is placed consistently from trial to trial for the same animal, but its placement is varied from animal to animal. In the test trials, the time to acquire the reward and the number of incorrect choices (i.e., entering an unbaited arm or re-entering a baited arm) are analyzed.

#### ***Time taken to obtain the rewards***

1. In the analysis profile, choose **Target visits and errors**.
2. In the **Target zones** box, select all the zones that were baited.



3. Under **Calculate Statistics for**, select **Target first visits**.
4. Click the **Trial Statistics** tab and select **Latency to Last**.

### ***Number of entries into unbaited arms***

1. In the analysis profile, choose **Target visits and errors**.
2. In the **Target zones** box, select all the zones that were baited.
3. Under **Calculate Statistics for**, select **Not-target first visits** and **Not-target revisits**.
4. Click the **Trial Statistics** tab and select **Frequency**.

**NOTE** Re-entries into baited arms are considered working memory errors.

Because the arms chosen to be baited differ between animals, make copies of the dependent variable Target visits and errors and edit the arms that are considered targets. Rename the variables (right-click the variable name and select **Rename**) and give them logical names (e.g. Baited arms 1-3-6-8).

Selected Dependent Variables	Description
Baited arms 1-3-5-7	Target visits and errors for the nose-point: Target first visits, Target revisits,
Baited arms 1-3-6-8	Target visits and errors for the nose-point: Target first visits, Target revisits,

In the analysis results, locate the variable that applies to specific subjects.

To easily locate which subjects were assigned to which baited arms in your results table, create an independent variable in the Trial List, which specifies which arms were baited (see page 151). This variable can then be included in the results table (in the **Statistics and Charts** screen, choose **Show/Hide > Independent Variable**).

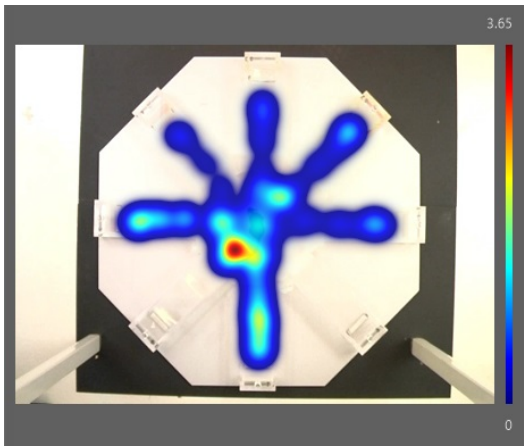
## **INTEGRATED VISUALIZATION**

Choose **Analysis > Results > Plot Integrated Data**.

Select a trial from the list on the tool bar. Choose a data profile and an analysis profile from the tool bar.

HEATMAPS

Choose **Analysis > Results > Plot Heatmaps**. Next, click **Plot Heatmaps**.  
Use heatmaps to visualize the location of the subject during the test.



**Figure 8.3** Heatmap of a 1-minute trial in a radial maze.

CALCULATING STATISTICS

Choose **Analysis > Results > Statistics & Charts**. Select an analysis profile from the list on the tool bar, then click **Calculate**.

Choose **Analysis > Export > Statistics**.

Choose whether you want to export the statistics per trial, or the combined statistics of groups of trials.

Statistics & Charts								
No filter		All Data		Zone transitions		Calculate		Batch
Trial Statistics		Group Statistics & Charts						
		Animal ID	Baited arm	Treatment	Center to arm 1		Center to arm 2	
					center-point /	Center > Arm 1	center-point /	Center > Arm 2
					Frequency	Latency to First s	Frequency	Latency to First s
Result 1	Trial 1	1	Arm 1	Treated	1	59.1200	1	16.4000

## **Chapter 9**

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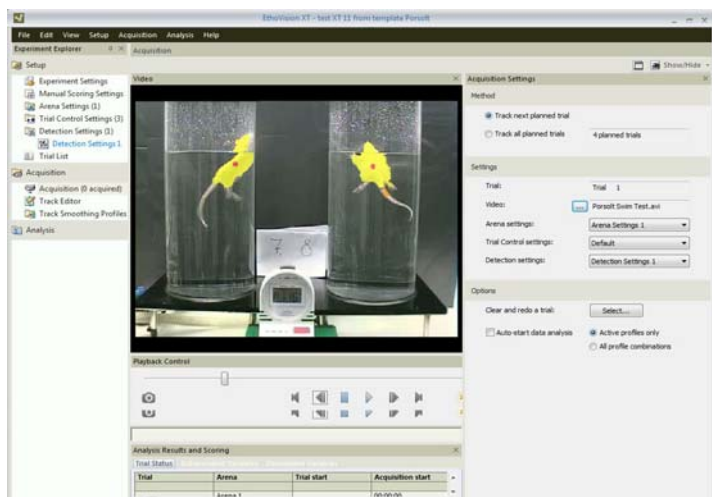
# The Porsolt Forced Swim Test

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# Introduction

## THE PORSOLT FORCED SWIM TEST

The Porsolt Forced Swim Test (Porsolt et al., 1977; Hédou et al., 2001; Juszcak et al., 2008) is the most commonly used test for assessment of depression in animal models. In the Porsolt test, a state of depression is induced by forcing a rodent to swim in a narrow cylinder or water tank from which it cannot escape.



**Figure 9.1** A Porsolt forced swim test carried out in EthoVision XT.

The procedure is generally divided into a pre-test session lasting 15 minutes, followed 24 hours later by a 5-minute test session. After a period of vigorous activity in the pre-test session, the animal adopts a characteristic immobile posture. Twenty-four hours later, after administration of antidepressants, the effect on immobility can be investigated by measuring the time of being immobile in the test session and comparing this with control animals.

## THE SAMPLE EXPERIMENT

To see how a Porsolt forced swim test is carried out in EthoVision XT, see the sample experiment **Porsolt Forced Swim test XT180** on the downloads section of the Noldus website (see [my.noldus.com](http://my.noldus.com)). Download this file and save it on your computer. Next, in EthoVision XT, select **File, Restore Backup** and select this file. See also the document **Description of sample experiments of EthoVision XT.pdf** for more information.

## REFERENCES

- Castagné, V., Moser, P., Roux, S., and Porsolt, R.D. (2011) Rodent Models of Depression: Forced Swim and Tail Suspension Behavioral Despair Tests in Rats and Mice. *Current Protocols in Neuroscience* 8.10A.1-8.10A.14.
- Juszczak, G.R., Lisowski, P., Sliwa, A.T., and Swiergiel, A.H. (2008). Computer assisted video analysis of swimming performance in a forced swim test: Simultaneous assessment of duration of immobility and swimming style in mice selected for high and low swim-stress induced analgesia, *Physiology and Behavior* **95**: 400-407.
- Hédou, G., Pryce, C., Di Iorio, L., Heidbreder, C.A., and Feldon, J. (2001). An automated analysis of rat behavior in the forced swim test, *Pharmacology, Biochemistry and Behavior* **70**: 65-76.
- Porsolt, R.D., Bertin, A., and Jalfre, M. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Therapie* **229**: 327-36.

### ***EthoVision XT and the Porsolt forced swim test***

For a list of selected publications, see the results of [Google Scholar](https://scholar.google.com).

# Physical setup

The following suggestions are meant to optimize the quality of tracking.

## *Camera view*

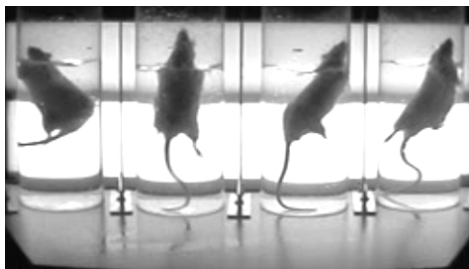
The camera should have a good, frontal view of the entire region the animals can be in. The complete body of the animal should be visible.

## *Number of animals*

You can test two or more animals simultaneously, when the Porsolt cylinder fit in the same camera view. For tracking multiple animals you need the **Multiple Arenas** add-on module.

## *Contrast*

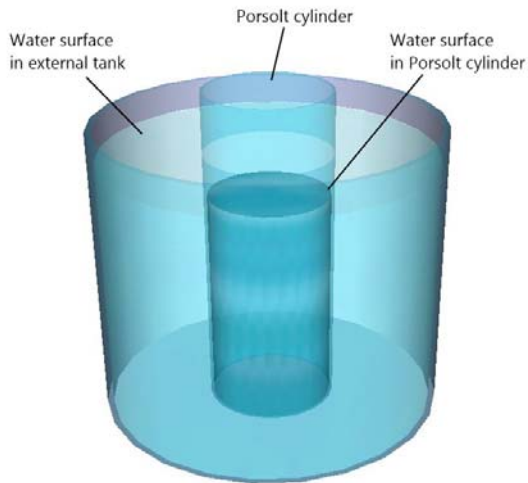
- Try to create as much contrast as possible between the animal and the background. This can be done by placing the cylinder or tank against a contrasting, non-reflecting background (so, a matt black background for white animals and vice versa).
- You can also use back-lighting to create maximum contrast. In this case, make sure you place a screen or curtain between the lights and the cylinders to avoid reflections.



**Figure 9.2** Backlighting allows to improve contrast between animal and background, and minimize reflections.

### ***Water surface***

You can reduce the contrast due to the water surface by placing the cylinders in a bigger tank with the water surface of the tank above those of the cylinders.



**Figure 9.3** Place the Porsolt cylinder in a water tank to remove or minimize the image of the water surface inside the cylinder.

# The Porsolt Forced Swim test in EthoVision XT

Create an experiment. For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help.

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

Under **Video Source**, and **Tracked Features**, make sure that the options selected corresponds to your needs. To adjust the camera settings, click the video icon in the camera row.

Under **Analysis options** select **Activity analysis**. Click the video button next to it and watch the video tutorial to learn about Activity analysis. Activity is a useful feature that helps you detect changes in the movement patterns of the subject.

## MANUAL SCORING SETTINGS

EthoVision determines the behavior of your subjects automatically on the basis of the intensity of the subject's movement (see **DETERMINING MOBILITY THRESHOLDS** on page 171). This means that you do not have to manually score the subject's behavior manually.

However, if you want to compare the data scored by EthoVision XT with your own ratings, define behavioral categories under **Manual Scoring Settings** (see below). Next, during acquisition watch the video and score those categories during your trial. You can also edit the data after acquisition.

### *Procedure*

Choose **Setup > Manual Scoring Settings**.



1. To add a behavior, click the **Add Behavior** button. Enter a name for that behavior (for example, *Struggling*) and specify **Mutually exclusive** as **Behavior type**. Specify the name of the behavior group (for example, Behavior Categories).
2. Add the remaining behaviors to the same group.

### Notes

- We advise you to create three categories for scoring the animal's mobility levels, which correspond to the three possible states of the Mobility dependent variable in EthoVision XT:
  - Immobile (corresponding to EthoVision XT's **Immobile** state);
  - Swimming (corresponding to **Mobile**);
  - Struggling/Climbing (corresponding to **Highly Mobile**)
- See **SCORE BEHAVIORS MANUALLY** on page 170.

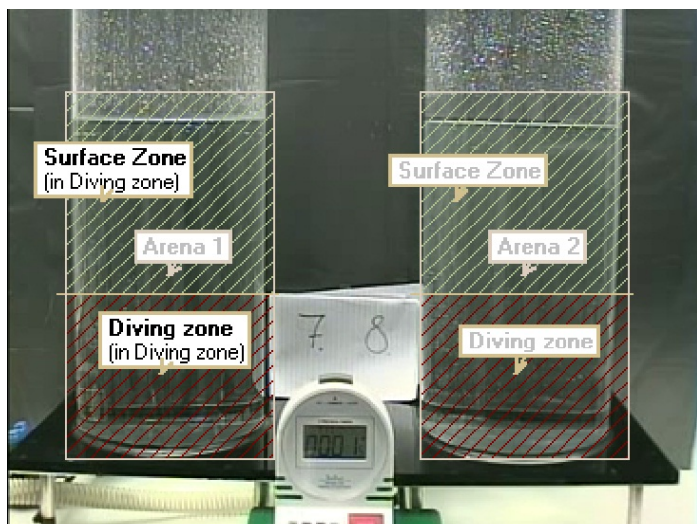
## ARENA SETTINGS

1. Choose **Setup > Arena Settings > Open Arena Settings 1**.
2. Click **2. Select Shape and Draw Arena** and check that the pre-defined arenas have the correct shape and size. If necessary, adjust the contour of the arena to fit the cylinders.

**IMPORTANT** If you work with multiple cylinders *and* use Activity analysis to quantify the animals' behavior, make sure that the arenas have the same size (you can check this under **Shape Size and Position** in the Arena Settings window).

3. Click **3. Select Shape and Draw Zones**. Check that the zones (if you used a Zone template) have the correct shape and size. If necessary, draw additional zone groups/zones.

For details, see **Arena Settings** in the EthoVision XT Help.



**Figure 9.4** Example of Arena Settings for the Porsolt Swim test with two arenas.

## TRIAL CONTROL SETTINGS

Choose **Setup** > **Trial Control Settings** > Open one of the following:

- **Default.** In this profile, tracking starts 1 second after the animal has been detected in the arena. Tracking stops manually (for example when you click the **Stop trial** button).
- **Max Track Duration 15 mins.** In this profile, tracking starts 1 second after the animal has been detected in the arena. Tracking stops automatically after 15 mins or when the animal has been in the Diving zone for over 1 second. You can use these Trial Control Settings for the pre-test session (See **PROTOCOL** on page 170).

If you need more time to put an animal in its cylinder, increase the number of seconds. This way you prevent that detection of your hand or arm starts tracking. The **Start Condition** works independently for each cylinder.

- **Max Track Duration 5 mins** – This profile is similar to the previous one, but now the maximum tracking duration is 5 minutes. You can use these Trial Control Settings for the test session.

When you work with multiple arenas, trial control is applied to each arena separately. Therefore, tracking may start at different times in different arenas, depending on when the animal is released.

## DETECTION SETTINGS

### *Important note*

It is important for stable detection, that the extremities of the animal (paws and tail) are either always included with the tracked body or always excluded from it. If the paws and tail are temporarily excluded and then included again in the detected subject during data acquisition, these changes affect the variables **Mobility** and **Movement**, while the animal might not actually be mobile or moving. On the other hand, the variable *Activity* is not affected by such changes, because it is based on the image change in the whole arena, not the change in the detected subject.

### *Detection Settings*

1. Choose **Setup > Detection Settings > Detection Settings 1**.
2. Under **Video**, choose a **Sample rate** of 5 (for rats) or 12.5 samples/second (for mice) or higher.
3. Click **Advanced**.
4. Under **Method**, choose **Dynamic subtraction**.

Under **Detection**, check you selected the correct contrast. Move the slider to define the animal's contrast. The animal's body must be fully detected in all parts of the arena and the noise must be minimal. Set the **Current frame weight** slider to a value between 1 and 5 to adjust the number of past images that contribute to the reference image.

5. If the limbs and the tail are not included in the detected body, and you want to include them, under **Subject Contour** select the **Dilation** filter, then the second **Erosion** filter. Leave the first **Erosion** filter to zero. Adjust **Dilation** and **Erosion** to achieve stable detection.
6. Under **Subject Size**, click **Edit**. Define a **Minimum Subject size** to prevent small objects like droppings from being detected.
7. If you intend to use **Activity analysis** to score the behavior of the subject automatically, click **Activity**. Enter the **Activity threshold** for detecting a change in the pixels in the arena. Start with the default value and check the purple-colored pixels in the video image. Adjust the threshold in such a way that when the animal is immobile, those pixels are almost completely absent.

Set **Compression artifacts filter** to **On** if you work with pre-recorded video.

## **TRIAL LIST**

Choose **Setup > Trial List**.

Enter your independent variables such as rat ID, Session type (with values *Pretest* or *Test*), Treatment (with values *Drug* vs. *Control*), Dose, Time of drug administration, Name of the experimenter, etc. For variables which differ between subjects, select **Subject** as a **Scope**.

If you want you can pre-define all your trials here (click the **Add Trials** button), or you can enter the independent variable values as you carry out the trials. You can also prepare the trials in Excel, randomize them and then paste the values into your Trial List.

Furthermore, you can define a list of trials for batch acquisition. See **Acquire a series of trials** in the EthoVision Help.

Trial List				
<a href="#">Add Trials...</a> <a href="#">Add Variable</a> <a href="#">Add Videos...</a> <a href="#">Import External Data...</a>				
	System	User-defined	User-defined	User-defined
Label	Acquisition status	Treatment	Session	Animal ID
Description	The current status of acquisition per arena			
Type		Text	Text	Text
Format				
Predefined Values		Drug: Saline...	Pretest; Test	
Scope		Subject	Subject	Subject
Trial	Arena	Subject	No.	
Trial 1	Arena 1	Subject 1	1	Planned
	Arena 2	Subject 1	2	Planned
Trial 2	Arena 1	Subject 1	3	Planned
	Arena 2	Subject 1	4	Planned
Trial 3	Arena 1	Subject 1	5	Planned
	Arena 2	Subject 1	6	Planned
Trial 4	Arena 1	Subject 1	7	Planned
	Arena 2	Subject 1	8	Planned

**Figure 9.5** A trial list with four planned trials. Each trial includes two arenas, and therefore two subjects.

# Acquiring data

## PROTOCOL

### *Basic protocol (rats)*

- **Pre-test** trial (day 1, before drug administration). The animal is placed in the cylinder for 15 minutes. No scoring of immobility is performed during the first session. This session is needed to acclimatize the rats to the experimental situation and to induce a stable, high level of immobility during the test session.
- **Test** (day 2). After drug administration, the animal is placed in the cylinder for 5 minutes. Score the duration of immobility by summing the time spent immobile; score as immobile minor movements strictly necessary to maintain the animal's head above water.

### *Alternate protocol (mice)*

The mouse is placed in the cylinder. The trial lasts 6 minutes. Measure the latency to immobility from the start of the trial and the duration of immobility for the last 4 minutes.

See Castagné et al. (2011). *Current Protocols in Neuroscience* 8.10A.1-8.10A.14.

See **ACQUIRING TRACKS** on page 21.

## SCORE BEHAVIORS MANUALLY

If you want to score the subject's behavior manually, define them first under **Manual Scoring Settings** (page 164). After you have started the trial, watch the subjects in the video and in the **Manual Scoring** tab at the bottom of the screen, click the button to the category that applies. As soon as the behavior of the subject changes, click the button for the corresponding category.

## Notes

- **IMPORTANT** If you do tracking from video, make sure you de-select **DDS** in the Playback Control window.
- You can also score behaviors *after* tracking. See **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

## DETERMINING MOBILITY THRESHOLDS

To find the optimal **Highly mobile** and **Immobile** thresholds, you can run a few test trials and check in the **Analysis Results and Scoring** pane (**Dependent Variables** tab) the values for **Mobility state** in relation to the behavior the animal displays. If the **Mobility states** do not correspond to the behavior of the animal, in the **Analysis Results and Scoring** pane click **Dependent Variables** (see figure below), click the button under **Mobility state** and adjust the **Highly mobile** threshold and **Immobile** threshold.

Trial Status		Independent Variables	Dependent Variables	Manual Scoring		
Is and Scoring	Trial	Arena	Mobility	Mobility state		
			***	***	***	***
			Body fill	Highly mobile	Mobile	Immobile
			Mean	Current	Current	Current
	Trial 5	Arena 1	13.569290	true	false	false
		Arena 2	8.659130	false	false	true

**Figure 9.6** During acquisition, you can check the values of any dependent variable in real-time. In this example, **Mobility** and **Mobility state** have been chosen. **Mobility state** has three possible states, **Immobile**, **Mobile** and **Highly mobile**, depending on where the value of **Mobility** lies relative to some user-defined thresholds. In Arena 1, the animal is struggling and the current value of **Mobility** is about 14%. In order to score struggling as “Strongly mobile”, set the **Highly mobile** threshold to, for example, 12%.

For example, if the animal is struggling, its corresponding **Mobility state** is **Highly mobile**. If the current **Mobility** score is **Mobile** or **Immobile**, you probably need to reduce the **Highly mobile** threshold.

Note that you can also adjust the **Mobility** thresholds after acquisition. in the Analysis profile, add the **Mobility state** variable (see page 175), and visualize the data.

If you do not see the **Mobility state** variable on your screen, click **Show/Hide** on the toolbar and select **Show Dependent variables**, then **Mobility state** and **Mobility**.

## **DETERMINING ACTIVITY THRESHOLDS**

In the same way as you defined thresholds for **Mobility state**, you can define thresholds for **Activity state**. Use Activity state if Mobility state does not give satisfying results, for instance when detecting thrashing of the animal's limbs which may not be detected properly by **Mobility**.

Note that when you work with multiple cylinders in the same video image, Activity state gives good results when the size of the arena is the same for all cylinders. In the Arena Settings, adjust the contour of the arenas to make them similar.



# Data analysis

## DATA PREPARATION

### *Track editing*

Choose **Acquisition > Edit Tracks**.

You can fix tracking errors but normally you will not need to edit your data.

### *Track smoothing*

Choose **Acquisition > Track Smoothing Profile** > open **MDM 0.2 cm**. In this profile, the **Minimal Distance Moved** filter is used (option set to **Direct**, with a threshold of 2 mm). This filter filters out small movements which result from system noise.

Note that smoothing influences the track, that is, the coordinates of the center point of the subject, not the subject's detected area, and therefore does not influence the calculations of Mobility. It also has no effect on Activity analysis.

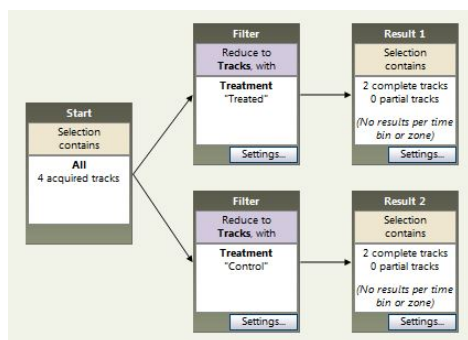
### *Review video and behaviors*

If you have scored behaviors manually, you can review the video and if necessary edit the data. Choose **Acquisition > Score behaviors manually**. For details, see **Acquire Data > Score behaviors manually** in the EthoVision XT Help.

### *Selecting data*

Choose **Analysis > Data Profile**.

You can select your tracks according to your independent variable values and also select parts of tracks (nesting). You can also create groups of tracks to obtain group statistics for each group. Figure 9.7 shows an example of a Data Profile to compare the animals that were treated with a drug, with those that were treated with saline.



**Figure 9.7** An example of data selection to compare two data sets in your experiment.

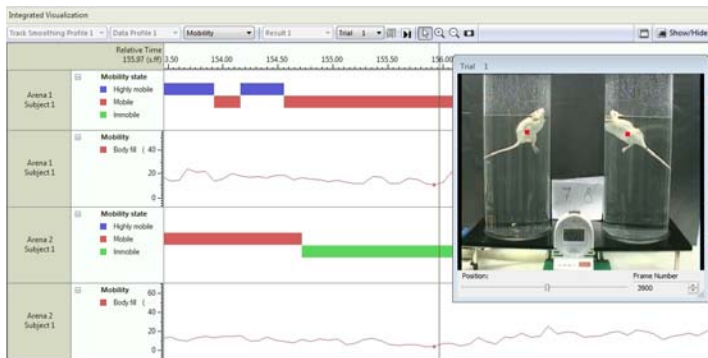
## INTEGRATED VISUALIZATION

Choose **Analysis > Results > Plot Integrated Data**.

Select a trial from the list on the tool bar. The video file and the Time Event Plot are displayed.

In the Time Event Plot tool bar you can choose which profiles to use for display of the variables. Figure 9.8 shows how variables are visualized in the sample experiment **Porsolt Forced Swim Test XT180** with the Analysis Profile **Mobility\_10** containing the variable **Mobility**.

Play the video and check whether the states scored under Mobility state correspond to the behavior of the animal. If they don't, fine tune the Mobility (or Activity) thresholds in the Analysis profile (see page 175). Then, check the results of your changes again in the Integrated Visualization.



**Figure 9.8** The *Plot Integrated Data* screen for the Analysis profile 'Mobility\_10' in sample experiment Porsolt Forced Swim Test XT180. The plot shows the variables **Mobility** (line plot) and the duration of **Mobility state** (colored bars) for both animals (here indicated as Arena 1 and Arena 2). The vertical line shows the current position in the Time Event Plot and the video window displays the corresponding frame. You can use this function to fine tune the **Mobility state** thresholds and detect the behaviors you are interested in.

## ANALYSIS PROFILE

### *Mobility analysis*

In an Analysis Profile, you select the dependent variables and statistics for analysis. For the experiment made with the template for the Porsolt Test, a dependent variable **Mobility** is already present.

Choose **Analysis > Analysis Profile > Open > Mobility**.

1. Double-click the row for the **Mobility state** variable.
2. Under **Outlier filter**, the **Averaging interval** is the number of samples on which the running average mobility is based. With an **Averaging interval** of 1 sample, sudden changes in surface area caused by reflections are not filtered out.

3. Under **Threshold**, set the **Highly mobile** and **Immobile** thresholds to the values you have previously determined (see **DETERMINING MOBILITY THRESHOLDS** on page 171).
4. Under **Calculate statistics for**, select the states you want analyze.
5. Click the **Trial Statistics** tab and select **Duration** and **Frequency**.  
Optionally select additional statistics in the **Group statistics** tab.
6. Click **OK**.

### ***Comparing EthoVision Mobility data with your ratings***

If you have scored the animal's behavior manually, you can easily compare your data with those scored by EthoVision (Mobility). Make sure that your analysis profile includes the three **Mobility states** and the behaviors scored manually, then visualize the data.

If the EthoVision data do not match your ratings at an acceptable level, adjust the Mobility threshold values in acquisition (page 171) and in analysis (see above).

### ***An alternative to Mobility analysis***

You can use **Activity state** if the **Mobility state** variable does not give good results, for instance when detecting thrashing of the animal's limbs which may not be detected properly by **Mobility state**.

**Activity analysis** is based on the temporal change in pixel value in the whole arena. Because it is not based on the detected animal's surface area, it is independent of tracking.

1. In the **Experiment Settings**, under **Analysis options** select **Activity analysis**. Click the video button next to it and watch the video tutorial to learn about **Activity analysis**.
2. In the **Detection Settings**, click **Activity**. Enter the **Activity threshold** for detecting a change in the pixels in the arena. Start with the default value and check the purple-colored pixels in the video image. Adjust the threshold in such a way that when the animal is immobile, those pixels are almost completely absent.
3. Run the trial.
4. In the **Analysis profile** add **Activity** and **Activity state**.

5. Visualize the data (Plot Integrated Data). There you see the scored **Activity states** and the subject in the video (see Figure 9.8 for an example with Mobility states). Adjust the *Activity state* settings in such a way that the states scored by EthoVision XT match the behavior of the subject at that time.



## Chapter 10

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# The Fear Conditioning Test

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Analyze the data .....	232
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# Introduction

## PRINCIPLE OF OPERATION

### *Fear Conditioning*

In a typical Fear Conditioning paradigm, single-frequency sounds are delivered as a neutral or conditioned stimulus (CS). Experimental procedures where the CS is administered are usually named Cued Fear Conditioning. Electric current from the grid floor is delivered as the naturally aversive, or unconditioned stimulus (US).

This chapter describes how you can use EthoVision XT with the Ugo Basile Fear Conditioning System NG-series 46000, to carry out fear conditioning experiments.

If you use a version of EthoVision XT earlier than 17.5, please refer to the EthoVision XT Application Manual for that version.

For an overview of Fear Conditioning, and how fear is measured in EthoVision XT, see the white paper which you can download from our web site:

[www.noldus.com/animal-behavior-research/fear-conditioning](http://www.noldus.com/animal-behavior-research/fear-conditioning)

### *Controlling the FCS stimuli from EthoVision XT*

1. Connect the EthoVision XT computer to the Ugo Basile Fear Conditioning System (FCS). For details, see **Physical setup** on page 184.
2. In EthoVision XT, create your experiment and declare the devices that you are going to use. Next, program the activation of the devices in the Trial Control Settings. For details, see the **The Fear Conditioning experiment in EthoVision XT** on page 207.

If you have multiple FCS cubicles, you can control the stimuli in each cubicle independently.

3. Carry out data acquisition. See **Acquire the data** on page 230.



### ***Experiment devices and parameters***

You can specify the devices and their parameters directly in EthoVision XT. This also makes it easier to test a device.

- Shock, with adjustable current intensity.
- Tone, with adjustable frequency and volume.
- White noise, with adjustable volume.
- Visible (white) light, with adjustable light intensity.
- IR light, with adjustable light intensity.
- Fan, which can be switched on and off.
- You can also have EthoVision XT check if the door of the FCS cubicle is open or closed.

**NOTE** If you work with EthoVision XT 16 or earlier, you must connect the EthoVision XT computer to the Ugo Basile Touchscreen Controller 40500-001. Please refer to the EthoVision XT - Application Manual for that version.

### ***Analysis of freezing***

The detection of freezing can be automated and based on analysis of the variable **Activity** in EthoVision XT. For details, see **Activity** and **Activity State** in the EthoVision XT Help, under **Dependent Variables in detail**.

## **CHANGES RELATIVE TO PREVIOUS ETHOVISION XT VERSIONS**

With EthoVision XT 17.5 and later versions you can control the Ugo Basile FCS without connecting the touchscreen controller. For new systems, the cabling is largely reduced and the system is easier to troubleshoot.

- Cable connections are way simpler - from the PC directly to the FCS cubicles through USB.

- When you create an EthoVision XT experiment, the devices of the Ugo Basile FCS are automatically added to the experiment.
- In the Trial Control Settings, you can easily recognize the names of the devices (e.g. Tone) instead of for example Line 1, as it occurred in the previous EthoVision XT versions.
- You can test the devices directly in the Trial Control Settings, before carrying out the actual tests.
- With FCS **Model 46110-NL** and **Serial Number higher than 0676U24**, you can control the IR light inside the cubicle directly from the software. You no longer need additional hardware. If you encounter problems, contact Noldus Support. See also **UPDATE THE CUBICLE FIRMWARE** on page 204.

See **Physical setup** on page 184.

## IF YOU HAVE THE “OLD” FCS SETUP

### *FCS controlled through the Noldus USB-IO box*

Your FCS setup is connected to EthoVision XT through the **Noldus USB-IO box**, and you have upgraded EthoVision XT to version 17.5 or later. In this case you can keep using the “old” connections. For the instructions, see the EthoVision XT 17.0 - Application Manual, which you can find on the downloads page of

[my.noldus.com](https://my.noldus.com)

If you purchased an upgrade kit for your setup, see **UPGRADE YOUR FCS SYSTEM** on page 200.

### *FCS with manual IR light controller*

Your FCS setup is equipped with a IR-light box placed on top of the conditioning cage and with the **IR light box manual controller**. You can keep using this configuration. However, you cannot control the IR light intensity from the software.

## FOR GENERAL INFORMATION

### *EthoVision XT Help*

For more details, choose **Help > Help Topics** or press **F1** in the software.

### *Other documentation*

On the Windows Apps screen, choose **Noldus > EthoVision XT 18 Other Documentation**. See in particular:

- For information on programming trial control, conditioning routines, actions and conditions, and the USB-IO box: the EthoVision XT 18 - Trial and Hardware Control - Reference Manual.
- For information on conditioning tests and examples: see other chapters in this manual.

You can find these and other manuals on the Noldus website at [my.noldus.com](https://my.noldus.com). Note that you need to log in first to access the downloads page.

### *About the Ugo Basile Fear Conditioning System*

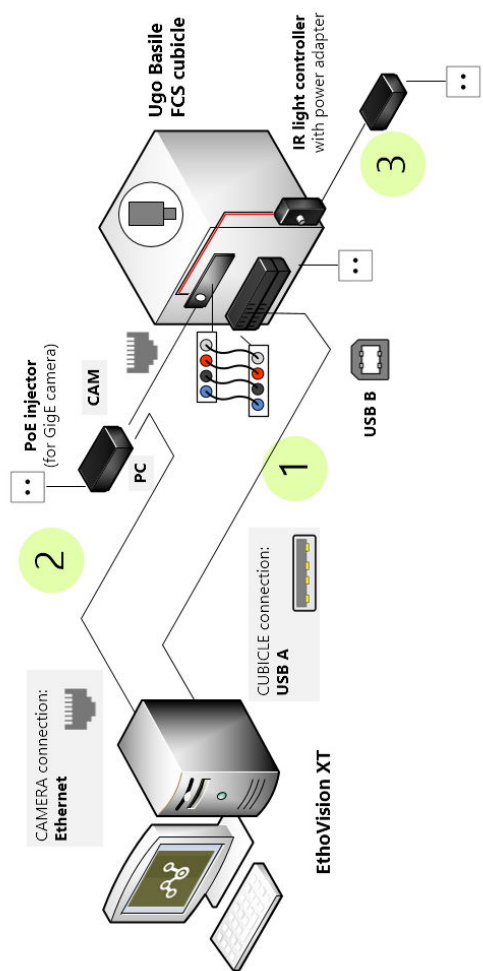
This system comes with the instruction manual Fear Conditioning Systems-NG Series 46000, in PDF format.

### *Technical Support*

If you encounter problems, browse to [my.noldus.com](https://my.noldus.com), and contact the help desk.

# Physical setup

## ONE-CUBICLE CONFIGURATION



**NOTE** The diagrams only include the connections essential for communication between EthoVision XT and the FCS. See also the Ugo Basile Fear Conditioning Systems NG Series instruction manual.

### ***Step by step instructions***

1. Connect the EthoVision XT computer to the Ugo Basile FCS using the appropriate USB cable. You need a USB cable with a type-A connector at one end and a type-B connector at the other end.



2. Connect the camera to the PC.

If your computer has an Ethernet board with one or two ports, you need a Power over Ethernet (PoE) injector (see also the figure on the previous page).



- Connect one of the ports of the Ethernet board of the computer to the **PC port (IN)** on the PoE injector.
- Connect the camera port on the back of the cubicle (see the figure below) to the **CAM port (OUT)** of the PoE injector.



- Next, connect the PoE injector to a power outlet.

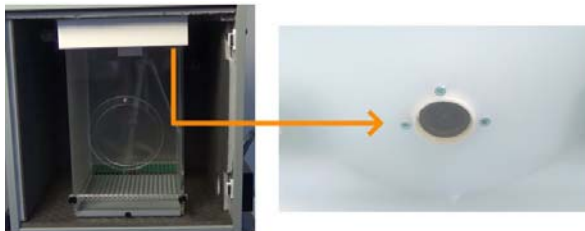
If your computer has a 4-port Ethernet board installed, and if this board receives power from the PC internal power supply, you do not need a PoE injector. Connect the camera directly to one of the board's Ethernet ports. See the EthoVision XT Help for details.

3. Connect the Ugo Basile USB Hub to the power outlet using the appropriate power adapter (see step 1).

With the current version of the FCS you can control the IR light from EthoVision XT. If you have an older version, you must use the **IR manual controller** (see page 188) or upgrade the FCS (Please contact Noldus for information).

### ***The video camera***

The camera is embedded in the IR light box at the top of the cubicle.



If you have not done yet so, you must configure the IP address of the camera and of the Ethernet port on your PC. You can find the IP address of the camera on the front panel of the cubicle's light box.



### ***Install and configure the cameras***

For details on how to install and configure the EthoVision XT cameras, and adjust the IP addresses, see **Camera Installation** in the EthoVision XT Help.

For more details on camera settings, see **CAMERA SETTINGS** on page 195.

### ***IR light box with software control***

FC systems of **Model 46110-NL** and **Serial Number higher than 0676U24** are equipped with a IR light box that is switched on automatically when the cubicle is powered up. The default intensity is 100%. You can control the IR light intensity directly from the software (Trial Control Settings > **Action** > **UB FCS IR Light**; see an example below). You no longer need additional hardware.

Hardware Action

Name

Action name: Hardware act (1)

Action to perform

UB FCS IR Light: Device E

Action to perform: Light On

Intensity Level: 100 %

Test Reset

Comment

OK Cancel

It could happen that your system is recent but is not yet updated for this option. Please contact Noldus for information. You can also update the firmware of the cubicles with the procedure on page 204.

### ***IR light box with manual control***

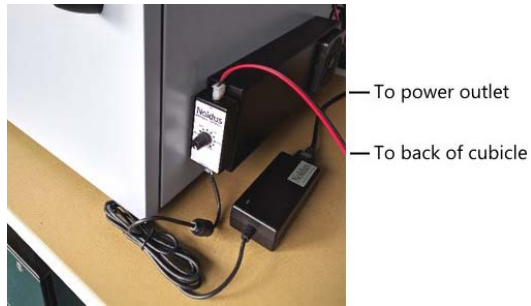
In older FCS models adapted to use with EthoVision XT, the cubicle is equipped with an IR light box above the conditioning cage, and a IR light controller to adjust the IR light intensity.

1. Connect the red and black cable that comes from the cubicle to the IR light controller.



2. Connect the IR light controller to a power outlet through its power adaptor. Attach the IR light controller as indicated on the labels.

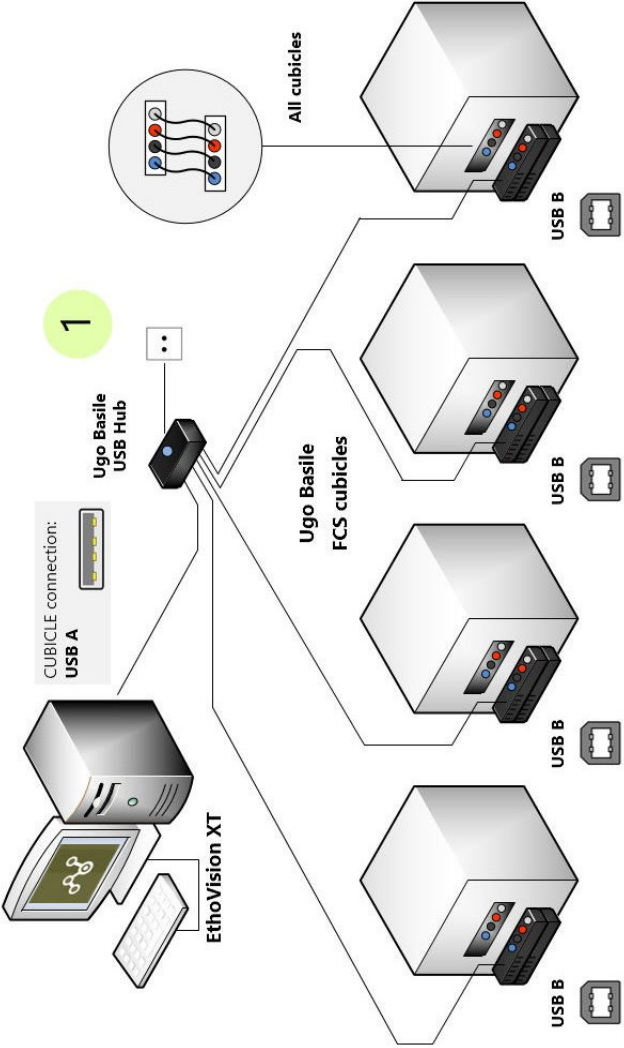




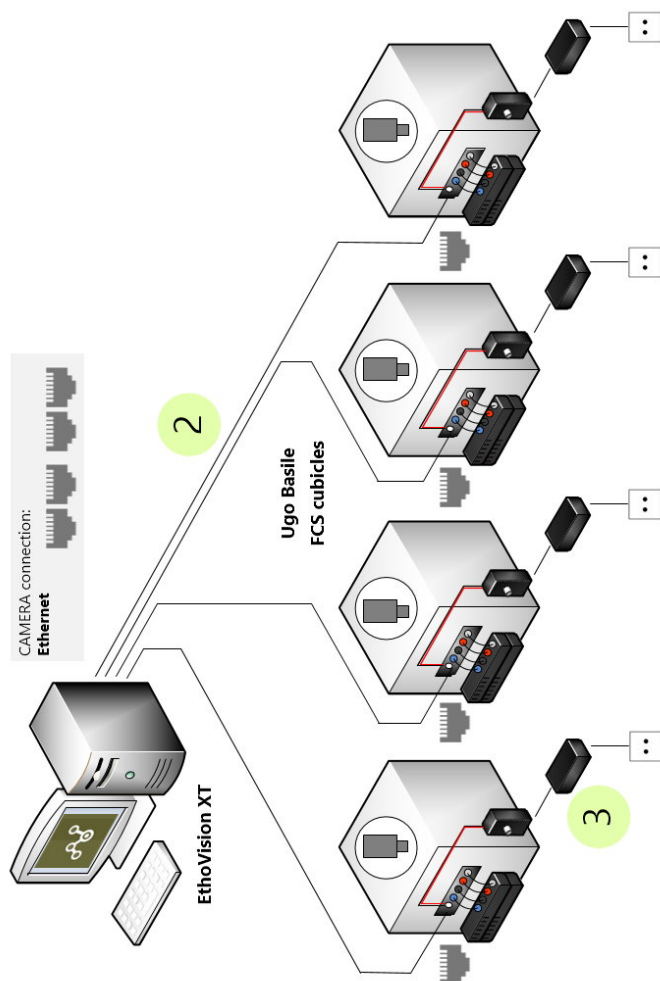
If you work with multiple cubicles, connect the IR controller as explained above for all the cubicles.

FOUR-CUBICLE CONFIGURATION

1 - Cubicles



## 2 and 3 - Video cameras and IR light controllers



Element 3 is for manual control of IR light, no longer necessary in the current version of FCS. See also page 181 and page 182.

### ***Step by step instructions***

See the schemes on the previous pages.

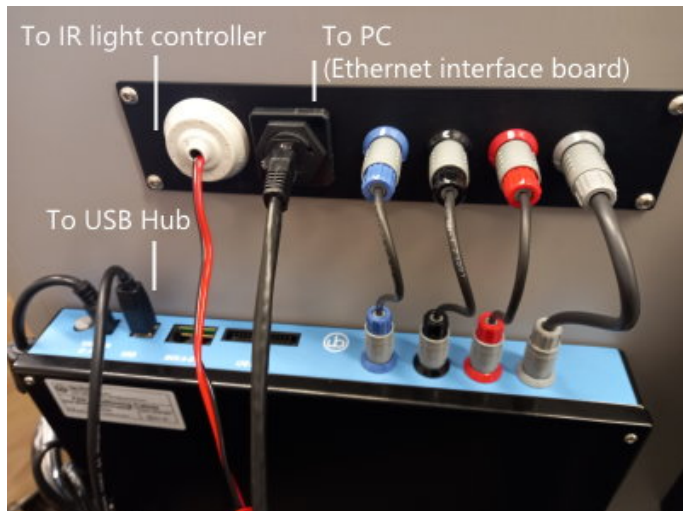
1. Connect each cubicle to the EthoVision XT computer through the Ugo Basile USB Hub.

For this you need 5x USB cables with a Type-A connector at one end and a Type-B connector at the other end.



2. Connect the Ugo Basile USB Hub to the power outlet using the appropriate power adaptor (see the top figure above).

3. Connect each camera to one of the ports of the Ethernet Interface board in the EthoVision XT computer (see the figure below). For this you need 4x Ethernet cables with RJ45 connectors. See also 2 in the figure on page 191.
4. Connect the Top Light Units to the power outlet through the appropriate power adaptors.



For more information on how to install and configure the Ethernet Interface board, see **Install the GigE cameras** in the EthoVision XT Help.

5. Connect one IR controller to each cubicle, as described in the procedure on page 188. See also 3 in the figure on page 191.

**NOTE** The external IR light controller is no longer necessary if you updated the cubicle. See **UPDATE THE CUBICLE FIRMWARE** on page 204.

### **Connect multiple cameras**

If your EthoVision computer is provided with a 4-port Ethernet interface board for connecting multiple GigE cameras, the cameras can

get the power from the PC itself. If you purchased the computer and Ethernet board from Noldus, the board is already powered by the PC.

Otherwise, open the computer casing and connect the Ethernet interface board with the internal power supply of the computer.

Once power is provided through the PC, connect each camera to one of the ports of Ethernet interface board. Label the ports and the cables so you always know which port is connected to which camera (and which cubicle).



### ***IP addresses***

**NOTE** If you have the Ugo Basile FCS equipped with USB cameras, connect the cameras directly to the EthoVision XT computer. Please skip this section.

If your FCS is equipped with Basler GigE cameras through Ethernet connections, each camera must have a IP address that matches the IP address of the Ethernet interface board on your PC.

The basic rules are:

- The first two numbers are the same for all cameras: **192.168**.
- The third number should be the same and unique for each camera-Ethernet port combination (see the examples below).
- The fourth number can be any, but must differ between camera and Ethernet interface board, and should suggest which cubicle (arena) it refers to.
- For example:
  - 192.168.1.**101** for camera 1 (cubicle 1) and 192.168.1.**51** for the Ethernet port 1 of the Ethernet interface board.
  - 192.168.2.**102** for camera 1 (cubicle 1) and 192.168.2.**52** for the Ethernet port 1 of the Ethernet interface board.
  - etc.

The IP address of each camera can be found on the front panel of the cubicle's light box. You need those addresses to configure the cameras.



For details about assigning IP addresses:

- In the HTML help: choose **Help > EthoVision XT Help > Camera installation > Install GigE cameras**.
- In the Video Tutorial: choose **Help > Video Tutorial > Set Up the Cameras**.

## CAMERA SETTINGS

### *Camera type and lens*

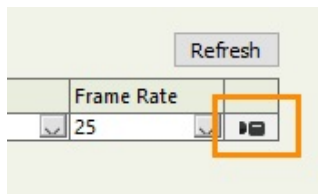
The camera used in Noldus systems for Fear Conditioning is a Basler camera acA1300-60gm with a 2.95 mm fixed focal lens, F2.0, 1/1.8".

### *How to view the live camera image*

In order to view the camera image, you must activate the IR light in the cubicle. The camera has a IR-pass filter that blocks visible light.

1. Make sure that the camera has been configured and connected to the PC (see above).
2. Start EthoVision XT and create a new experiment (**File > New**) or open an existing experiment without trials acquired.
3. Choose **Setup > Experiment Settings**.
4. Under **Video Source** select **Live tracking**.
5. Select the camera from the list under **Source**.

6. Click the camera button for the live view.



### ***Camera orientation***

The camera has been optimized at Noldus before shipping, therefore in most cases you do not need to adjust its orientation. If, for any reason, the video image is not optimized, follow the instructions below.

### ***Camera alignment***

This section applies for previous FCS versions which had Noldus IR light box on top.

Turn the three screws indicated to alter the camera orientation.



If you tighten one of the screws, the camera points more to the other side. Loosen the same screw to have the opposite effect. For example, tighten the screw **b** to have the camera pointing more to the back of the cubicle. Check the resulting image in EthoVision XT; make sure that the bottom of the cage is aligned at the center of the camera view.



### Camera software settings

The camera frame rate, exposure time and other settings can be adjusted in EthoVision XT, or in the camera software Pylon Viewer.

In EthoVision XT, choose **Setup > Experiment Settings**. Click the camera button and locate the settings you want to change. See below for the values recommended.

For multiple cameras, you need to adjust the settings for the first camera. Then, adjust the resolution of the **Merged** view.

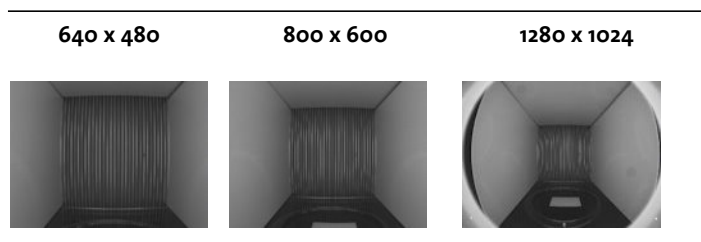
---

<b>Analog gain</b>	1
<b>Color space</b>	Mono8 (Y800)
<b>Video resolution single camera (H x V)</b>	640 x 480
<b>Video resolution merged view (H x V)</b>	1280 x 960
<b>Acquisition Frame rate</b>	25 or 30
<b>Exposure</b>	16000 $\mu$ s

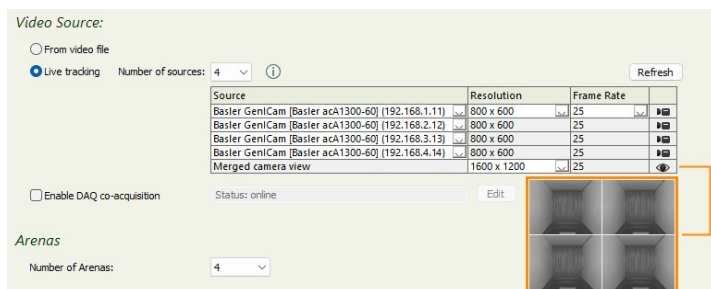
---

### Video resolution and camera zoom level

The camera lens of the FCS is a fixed focal lens. To adjust the zoom level, adjust the video resolution (in pixels) so that a portion of the maximal camera view is recorded. The following example may help you choose the video resolution. Note how the resolution setting crops the image.



In this example, EthoVision XT makes a video of 1600 x 1200 from four cameras set to 800 x 600.



**IMPORTANT** Whenever you change the resolution, open the Arena Settings and update the background image for your arenas before acquiring the data.

### *The Binning option*

If you need to reduce the video resolution without cropping the image, you can use the **Binning** option in the Video Settings of EthoVision XT.

In EthoVision XT, choose **Setup > Experiment Settings**. Click the camera button and in the Video Settings window click the **General** tab. Select the **Binning** option and leave the value **2**. The Binning option reduces the number of pixels of the single camera image by half but does not crop the image.

If you choose to select Binning, mind that the brightness of the image increases. Make sure that this does not have negative effects on detection. If the image is too bright, reduce the Exposure time or the Gain (see above).

The Binning option must be the same across cameras! Remember to select (or de-select) the Binning option for all cameras, before setting the resolution of the merged view.

### *Image brightness*

If the image from the camera is too dark, do one of the following based on what FCS version you have:

- If you have the IR light controller attached to the cubicle, turn the knob of the IR light controller all the way to the right (see page 188). In most cases this is enough to make detection optimal.
- Increase the IR light intensity up to 100% with the Trial Control Settings in EthoVision XT.
- Increase the IR light intensity using the software on the tablet PC.
- Place white plates at the sides and at the bottom of the FCS cage to increase contrast with dark-fur animals. Place dark plates in case of white animals.

To increase the brightness of the image further, you can adjust camera settings in EthoVision XT or Pylon Viewer:

- Increase the camera **Exposure time**. Be aware that a too high Exposure time may conflict with the camera frame rate. EthoVision XT shows a message in the Video Settings window if that is the case.
- Increase the **Gain** to 1 or 2. Mind that a higher Gain also creates more image noise.
- Another option to increase the brightness of the image without increasing noise is to set the image **Binning Horizontal** and **Binning Vertical** to 2. Make sure that the **Binning Mode** is **Summing** for both. However, with binning the video resolution (that is, the width and the height of the camera image, in pixels), is half of the original. See **The Binning option** on page 198.

Older versions of the Ugo Basile FCS have the IR light source placed at one of the top corners of the cubicle. You do not need to operate this light if you have the IR light box above the conditioning cage.

## UPGRADE YOUR FCS SYSTEM

### *Aim*

To adapt the Fear Conditioning System to use in combination with EthoVision XT 17.5 and later.

- The touchscreen controller is no longer needed.
- You can adjust the FCS settings (e.g. tone frequency, shock current) directly in EthoVision XT.
- The time needed for installation and setting adjustment is about one hour.

### *Prerequisites*

- You have received the following from Ugo Basile:
  - 1x upgrade box for each cubicle.
  - 1x power cable, 1x USB cable, 4x color-coded cables for each cubicle.
  - Two hexagonal Allen keys.



- Ugo Basile needs first the touch screen controller back, so that they can copy the settings of the touch screen controller / Fear

Conditioning box to the upgrade box. That way you can work with the same settings as before.

***Procedure***

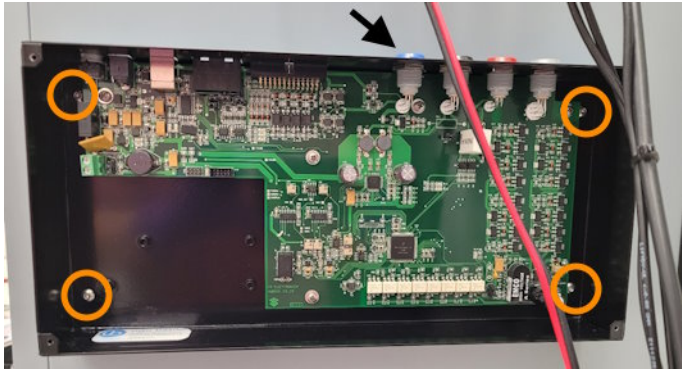
1. Remove the four screws at the corners of the upgrade box and remove the cover plate.



2. Locate the holes on the back panel of the cubicle.



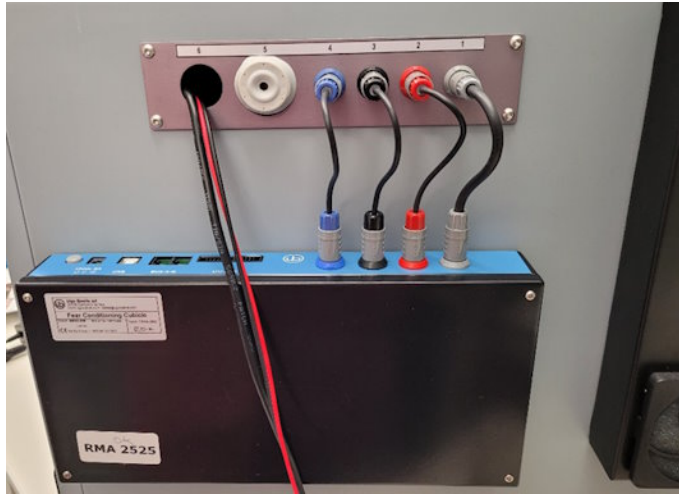
3. Attach the box to the back panel. Make sure that the cable connections are on top.



4. Put the lid of the box back in place and tighten the screws.



5. Connect the color-coded cables as shown in the next figure.



6. Finally, connect:
- The upgrade box to the PC (or USB hub, in case of 2 or more cubicles) using the USB cable that came with the upgrade box.
  - The upgrade box to a power outlet.
  - The camera to the PC's Ethernet board.

See also **Physical setup** on page 184.

7. Repeat the steps above for the remaining cubicles.
8. Next, follow the steps in the next section **The Fear Conditioning experiment in EthoVision XT** on page 207.

## UPDATE THE CUBICLE FIRMWARE

### *Aim*

To make the FCS cubicles compatible with the new IR illumination system that is fully controllable from EthoVision XT.

**IMPORTANT** Follow this procedure only when requested by Noldus Support.

### *What you need*

- The firmware updater installation files: **FW\_Updater Setup.msi** and **setup.exe**.
- The new firmware file **Cubicle.elf\_7.34.S19**.

See below for how to get those files.

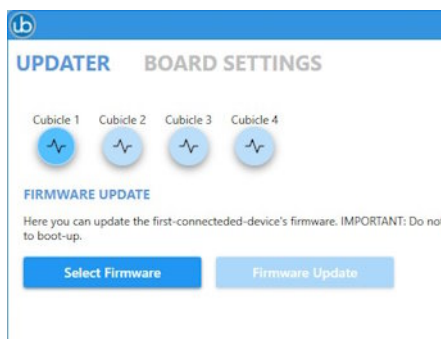
### *Procedure*

1. On the EthoVision XT full installation package, open **Drivers and Tools > Utilities > Ugo Basile FCS**.  
  
You can download the full installation package from [my.noldus.com](http://my.noldus.com).
2. Copy the file **Ugo Basile FCS FirmWare Updater [version number].zip** to your EthoVision XT PC and extract its contents.
3. Double-click the file **setup.exe**. Complete the installation procedure.
4. Power up the cubicle and connect it to the EthoVision XT PC through the USB cable.
5. Start the application.

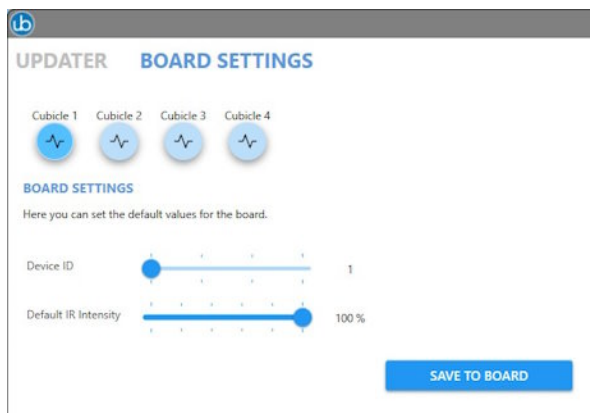


6. Choose **Select Firmware**.





7. In the window that opens, choose the file **Cubicle.elf\_7.34.S19**.
8. Click **Firmware Update**.
9. If the current version is 2.27 or newer, the update process should start automatically. Otherwise, you need to:
  - Disconnect both the power supply and the USB cable from the cubicle.
  - Reconnect both the power supply and the USB cable, approximately at the same time.
10. Wait until the **Erase** and the **Programming** processes are completed.
11. After the device has rebooted, you can change the tab to **BOARD SETTINGS**.



12. Leave or set the **Device ID** to 1 and set the **Default IR intensity** to the desired value (recommended: **100%**). This will be the default intensity at startup.
13. Click **Save to Board**.

### *Check the Ugo Basile Interface for EthoVision XT*

1. Open the **Control Panel > Programs and Features**.
2. Check that the software **Noldus - HardwareInterface UgoBasile -x64 package** version **1.0.14** or later is installed.

If a previous version is installed, uninstall it. Next, copy the file **HardwareInterfaceUgoBasile - X64 Package - [version number].msi** from the EthoVision XT 18 full installation package, under **Drivers and Tools > Prerequisites > HardwareInterfaceUgoBasile** to your PC, then run this file.

### *Test the IR light*

To test the IR light, in the Trial Control Settings create an **Action** based on the IR Light. Set the intensity to 100% and click **Test**. Click **OK** then check in the Detection Settings whether the IR light is still on.

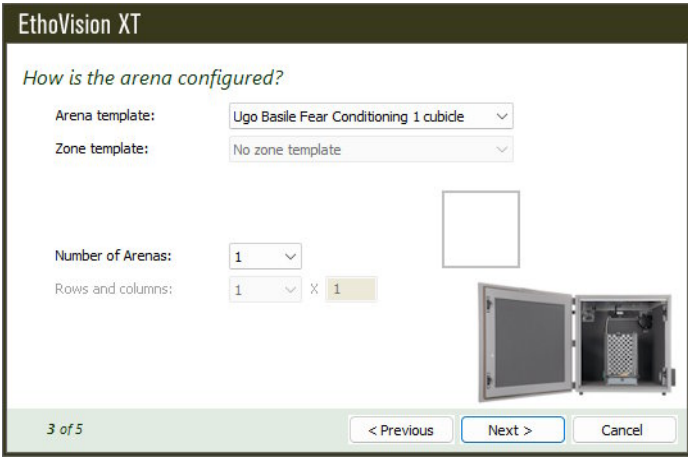
# The Fear Conditioning experiment in EthoVision XT

## PREREQUISITES

Make sure that all devices are connected and powered up.

## CREATE AN ETHOVISION XT EXPERIMENT

1. Start EthoVision XT and choose **File > New From Template**, then click **Apply a pre-defined template**.
2. On the **How is the arena configured** page, select:
  - If you work with one or two cubicles, **Fear Conditioning System 1 cubicle**. Next to **Number of arenas**, select 1 or 2.
  - If you work with four cubicles, **Fear Conditioning System 4 cubicles**. Next to **Number of arenas**, select 4.



The screenshot shows the 'How is the arena configured?' dialog box in EthoVision XT. The 'Arena template' is set to 'Ugo Basile Fear Conditioning 1 cubicle' and the 'Zone template' is set to 'No zone template'. The 'Number of Arenas' is set to 1, and the 'Rows and columns' are set to 1 x 1. A diagram of a single cubicle is shown. At the bottom, there is a '3 of 5' indicator and buttons for '< Previous', 'Next >', and 'Cancel'.

**NOTE** This template does not include pre-defined zones as they are not used often in Fear Conditioning.

3. When you are asked **How many subjects per arena will you track**, select **1** subject.
4. Complete the guided procedure and give the experiment a name. Next, click **OK**.

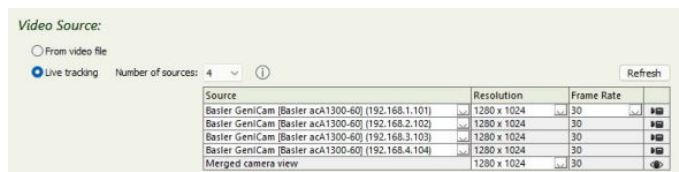
## EXPERIMENT SETTINGS

### *Cameras*

1. Choose **Setup > Experiment Settings**.
2. Under **Video Source**, next to **Number of sources**, select how many cameras (one per cubicle) you are working with. In the list under **Source**, select the name of each camera.

The IP address should tell which camera belongs to which cubicle, provided that you have assigned IP addresses

3. Next to Merged camera view, select **800 x 600** (cropped view of the cage) or **1280 x 1024** (full view of the cage). See **Video resolution and camera zoom level** on page 197.



### *Number of Arenas and Tracked Features*

**IMPORTANT** Do this before selecting the Ugo Basile FCS in the Trial Control Hardware Settings.

1. In the Experiment Settings, under **Arenas**, select the **Number of Arenas** you use, that is, the number of cubicles (1 to 4).
2. Under **Tracked Features**, in most cases **Center-point detection** will suffice for fear conditioning experiments.

### ***Activity analysis for freezing detection***

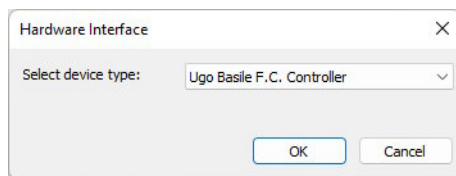
1. In the Experiment Settings, under **Analysis Options**, select **Activity analysis** if you want to analyze freezing behavior.\

**TIP** Click the **Video Tutorial** button next to that option for an overview of Activity analysis.

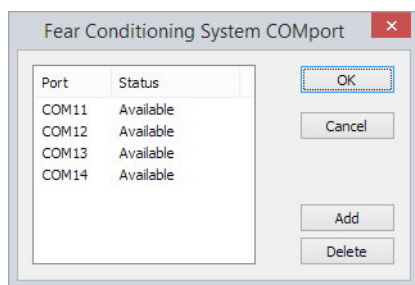
### ***Specify the Ugo Basile Fear Conditioning System***

**IMPORTANT** Have you already selected the number of arenas (see above)? Make sure that **Number of Arenas** selected corresponds to the number of FCS cages used in that experiment. Only after that, follow the instructions below.

1. In the Experiment Settings, under **Trial Control Hardware**, select **Use of Trial Control Hardware**, and click **Settings**.
2. In the **Hardware Interface** window, select **Ugo Basile F.C. Controller**.



3. In the **Fear Conditioning System COM Port Settings** window, click the **Add** button.
  - Select a COM port and click **OK**. Repeat this step to add the COM ports connected to all the FCS cubicles.
  - For a setup with the Ugo Basile USB Hub and four cubicles, add **COM11**, then **COM12**, **COM13** and **COM14**.



- For a setup with one cubicle connected directly to the PC, select the COM port associated with that cubicle. To find out which COM port is associated with a cubicle, see **COM PORT COMMUNICATION** on page 210. If you have created the experiment from a template, there may be one or more COM ports already selected in the **Fear Conditioning System COM Port Settings** window. If they do not match those associated with the cubicles of your setup, delete them and add the correct ones.
4. When finished, click **OK** and proceed with **ARENA SETTINGS: DEFINE THE ARENAS** on page 212.

### Notes

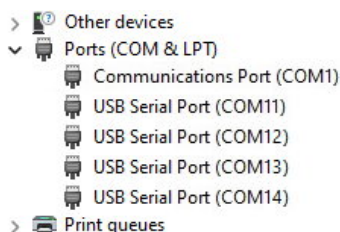
- **IMPORTANT** Take note of which cubicle is connected to which COM port. Make sure that only the necessary COM ports are added. If you added accidentally a wrong COM port, select that and click **Delete**.
- When adding more COM ports in the **Fear Conditioning System COM Port Settings** window, start with the port for cubicle 1. Disconnect cubicle 1 and see which COM port disappears from the Windows Device Manager (see **COM PORT COMMUNICATION** below). Next, reconnect the cubicle and add that COM port. Repeat those steps for cubicle 2, then 3, then 4.

## COM PORT COMMUNICATION

In order to know which COM port is associated with a specific cubicle, do the following:

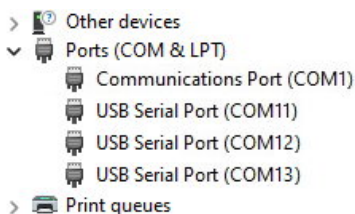
1. In Windows, open the **Device Manager**. Choose **View > Devices by type**.
2. Open the item named **Ports (COM and LPT)**.

If the cubicles are connected physically to the PC through the Ugo Basile USB Hub (see page 190) and powered up, you should see one up to four items **USB Serial Port (COMx): COM11 to COM14**.



**TIP** If you do not see the **Ports** item, from the **View** menu of the Device Manager select **Devices by type**.

3. Disconnect the USB cable from one of the FCS cubicles.
4. Look at the **Ports (COM and LPT)** list. One of the items has disappeared. That is the COM port currently associated with that cubicle. For example, after disconnecting cubicle 4 (**COM14**):



**TIP** Number the FCS cages and cubicles, and take note which COM port is linked with each cubicle. That makes troubleshooting easier.

If, after disconnecting a cubicle, a COM port other than the expected one disappears from the list, it may be that cables are swapped between the ports in the USB Hub and the corresponding cubicles.

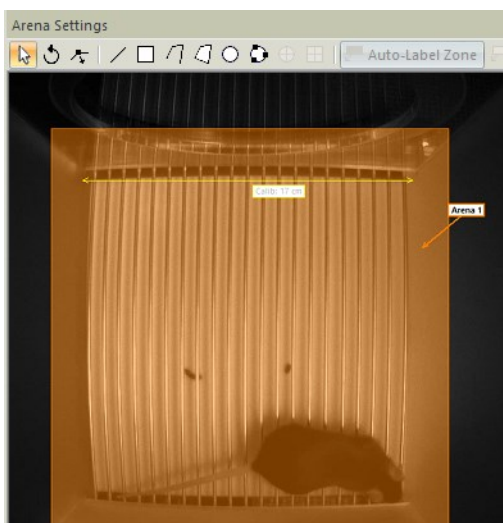
## ARENA SETTINGS: DEFINE THE ARENAS

### *Create and calibrate the arenas*

1. Choose **Setup > Arena Settings**.
2. Grab a background image from the cameras, calibrate and draw the arenas. For details, see **Arena Settings** in the EthoVision XT Help.

**TIP** To quickly calibrate, draw lines between opposite walls of the cage, next to the grid, and enter 17 cm (the cage width).

See page 197 for tips how to get a bright image of the cage.



3. If the image of a cage is not centered, see **Camera orientation** (page 196).
4. Proceed with declaring the FCS devices for each arena (next section).

## ARENA SETTINGS: DECLARE THE FCS DEVICES

Follow this procedure to specify the FCS devices that you want to use for each cage (arena).

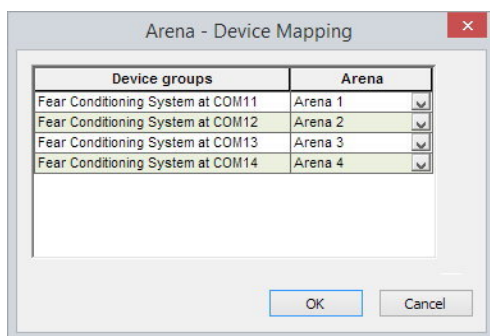


1. In the Arena Settings, click the **Arena – Hardware Mapping** button at the bottom-right corner of the EthoVision XT screen.

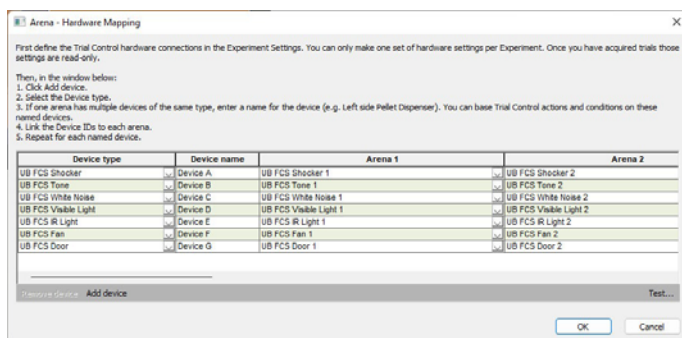
2. The **Arena - Device Mapping** window opens.

This window lists under **Device groups** the FC systems connected to the COM ports currently declared (page 208). The second column, **Arena**, lists the arenas currently associated with each system.

3. Check carefully that the name of each arena matches the physical FC system (and the COM port associated with it). If the two do not match, select the correct arena name under **Arena**.



4. Once you are ready, click OK. The **Arena-Hardware Mapping** window opens.



This window lists the names of the actual devices that EthoVision XT can control. By default, each device is uniquely associated

automatically to one arena based on the scheme in the window that opened in the previous step.

For example, all the devices of **Fear Conditioning System 1 at COM 1** are listed under **Arena 1**.

- The column **Device type** shows the types of device currently selected. If the type you want to use does not appear, add a new device (see page 214).
- In the column **Device name** you find names like *Device A*, *Device B*, etc. These are generic names for a specific type of device, which you find back when you program the activation of the devices in the Trial Control Settings. If you like, you can change them to Shock, Tone, etc.
- In the columns **Arena 1**, **Arena 2**, etc. you find the list with the names of unique physical devices. These are automatically assigned based on which FCS system connected to a specific COM port is associated with which arena in EthoVision XT (see through the COM ports (see above). Your task now is to assign each physical device to a specific arena.

**5. IMPORTANT** Test the devices! See page 218. You can also test the devices in the Trial Control Settings.

**NOTE** The item **UB FCS IR Light** listed in the Arena - Hardware Mapping window is for controlling the IR lamp located at the top-left corner of the cubicle, not the IR light given by the light box. You can control the IR light of the light box by turning the knob of the IR controller (page 191).

### ***Add a new device***

If the FCS devices are not listed in the Arena-Hardware Mapping window, do the following:

1. Click the **Add device** button.
2. Under **Device type**, select a device (e.g. **UB FCS Tone**).
3. Under **Device name**, either accept the default name or enter a new, meaningful name, for example *Tone*.
  - If you plan to activate a device type in all the cages at the same time, a generic name like *Shock* or *Tone* is fine.

- If you plan to activate a device type in different cages at different times, it is a good idea to specify a unique **Device name**, for example *Tone for Cage 1*, and assign that device only to the arena associated with that cage.
4. Locate the column for the arena you want to assign the device to, and select the physical device from the drop-down list. For example, under **Arena 1**, select **UB FCS Shock 1**; under **Arena 2**, select **Ugo Basile FCS Shock 2**, etc.

How many devices are available in a list depends on how many COM ports you have declared (see page 209). Make sure you do not select the same unique device under two or more arenas.

## EXAMPLES OF ARENA-HARDWARE MAPPING

### *EthoVision XT controlling one cage*

First define the Trial Control hardware connections in the Experiment Settings. You can only make one set of hardware settings per Experiment. Once you have acquired trials those settings are read-only.

Then, in the window below:

1. Click Add device.
2. Select the Device type.
3. If one arena has multiple devices of the same type, enter a name for the device (e.g. Left side Pellet Dispenser). You can base Trial Control actions and conditions on these named devices.
4. Link the Device IDs to each arena.
5. Repeat for each named device.

Device type	Device name	Arena 1
UB FCS Shocker	Device A	UB FCS Shocker 1
UB FCS Tone	Device B	UB FCS Tone 1
UB FCS White Noise	Device C	UB FCS White Noise 1
UB FCS Visible Light	Device D	UB FCS Visible Light 1
UB FCS IR Light	Device E	UB FCS IR Light 1
UB FCS Fan	Device F	UB FCS Fan 1
UB FCS Door	Device G	UB FCS Door 1

Remove device Add device Test...

OK Cancel

**NOTE** The item **UB FCS IR Light** is for controlling the IR lamp at the top-left corner of the cubicle, not the IR light box.

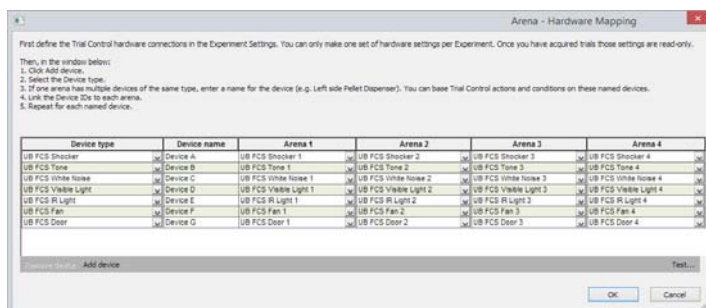
### **EthoVision XT controlling four cages - 1**

Use this configuration whenever you plan to administer a stimulus in all the FCS cages at the same time, for example, to activate the tone in all the cages exactly one minute after the start of the trial.

Define a **Device name** that is valid for all the cages (arenas). Under **Arena 1, Arena 2, etc.**, select the physical devices for each of those arenas.

In the following example, **Device A** represents the foot shock for all the cages. This means that when you use a hardware action **Device A - Shocker On** in the Trial Control Settings (see page 221), that action is carried out in all the cages.

- Make sure not to select the same physical device twice for two arenas.
- Repeat the steps for the remaining devices.



**NOTE** You can also use this configuration when tracking starts at different times in different arenas, for example when you release the subject at different times and EthoVision starts tracking after the subject has been detected.

### **EthoVision XT controlling four cages - 2**

Sometimes you need to apply different protocols to different cages (arenas). For example, to provide a stimulus after the subject has been immobile for one minute. Or, give a shock in cage 1 after 1 minute and

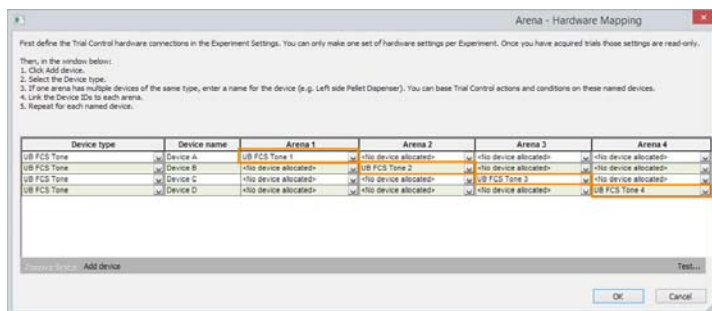
give a shock in cage 2 after a random time, or the like. In such cases the activation of a stimulus device is cage-specific.

You can create this configuration by linking a specific device name (e.g. Device A) only to a specific physical device in one of the arenas (e.g. Arena 1). Device A should not be assigned to other arenas.

Therefore, you must add four new devices (see page 214) of the same type, and assign each of them to one arena.

For example, in the case of UB FCS Tone:

- Device A: **UB FCS Tone 1** under **Arena 1**.
- Device B: **UB FCS Tone 2** under **Arena 2**.
- Device C: **UB FCS Tone 3** under **Arena 3**.
- Device D: **UB FCS Tone 4** under **Arena 4**.



Furthermore:

- Remember to select **<No device allocated>** in the remaining cells of the table.
- Note that with this setup tone stimuli are presented at different times in different cages. If the cubicles are near each other, the animal in cubicle 1 may be affected by the tone presentation in the other cubicles, when it is supposed not to hear any tone. Make sure that the cubicles are far apart or in different rooms when using non-simultaneous tone presentation.
- Repeat the steps for the other device types that you want to use.

- For more information about applying different protocols to different cages, see **Applying different protocols to different arenas** in the EthoVision XT 18 - Trial and Hardware Control - Reference Manual.

## TEST THE FCS DEVICES

### *Prerequisites*

- All the connections are set as in the section **Physical setup**.
- You have created an experiment and have specified the FCS in the Experiment Settings (see page 208).
- You have drawn and calibrated the arenas (see page 212).
- You have specified the FCS devices for each arena (see page 212).

### *Procedure for actions*

This applies to Visible Light, Tone, Shocker, White Noise, Fan, IR Light.

1. In EthoVision XT, choose **Setup > Trial Control Settings** and open one of the existing profiles.
2. In the **Components** pane, under **Actions**, click the button next to the name of the device you want to test.
3. In the window that appears, select the device, the type of action, adjust its settings, and click **Test**.
4. Verify that the devices works as expected. Next, click **Reset** to reset all devices to their default values.

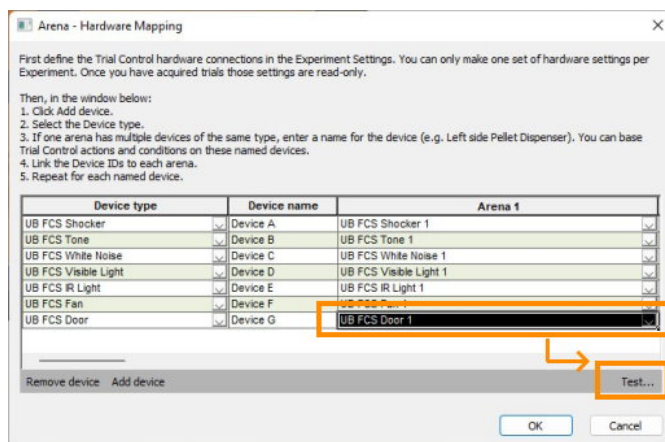
If the action is carried out in another cubicle, it means that the wrong device has been selected in step 3, or that the devices have not been mapped correctly in the Arena Settings (see page 212).

### *Procedure for the UB FCS door*

This applies to the device UB FCS Door.

1. In EthoVision XT, choose **Setup > Arena Settings** and click the **Arena-Hardware Mapping** button.

2. In the **Arena-Hardware Mapping** window, select the row for **UB FCS Door**. Next, click the **Test** button.



3. The **Test UB FCS Door** window opens on the screen. Open/close the door of the corresponding cubicle and check the message displayed in the window (**Door Open? True or false**).

### ***Verify that the shocker actually gives a shock***

Place a LED diode between two bars of the shocker grid, or touch a bar with a phase detector.



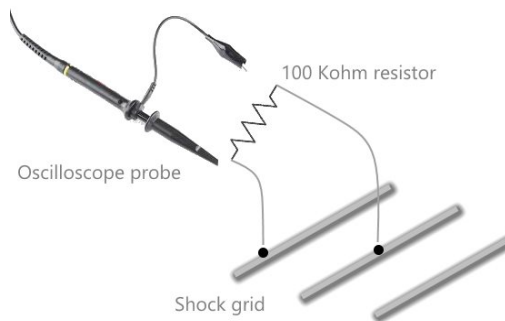
*A phase detector.*

If the shocker is connected correctly and working, the LED turns on. You can also touch the grid bars, however a current intensity around 1 mA is the threshold of feeling. A lower current may not be felt.

### ***Measure the amplitude of the shock***

**IMPORTANT** For this you need an oscilloscope and an oscilloscope probe with insulated channel and a 100 K $\Omega$  resistor.

1. Set the oscilloscope to 50V/DIV and time base 10 ms.
2. Connect the oscilloscope probe with a 100 K $\Omega$  resistor to the shock grid, as indicated in the following picture.



3. In EthoVision XT, in the **Arena - Hardware Mapping** window, select the shock device for a specific cubicle and click **Test**. Set the shock intensity to 1.5 mA.
4. Click the **Test** button to activate the shock. You should see a square wave on the display of the oscilloscope. Read the amplitude there; remember that with the current settings one vertical division corresponds to 50 V. Once you know the voltage, the current is equal to the voltage divided by the resistance (in this example 100000).



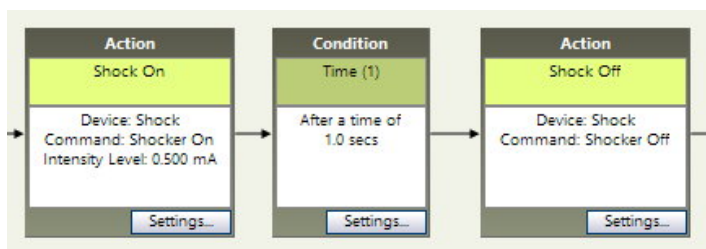
## TRIAL CONTROL SETTINGS

Follow this section to learn how to program EthoVision XT to send commands to the FCS devices at the right time during data acquisition.

### *Basic structure: Actions and Conditions*

To activate /deactivate a stimulus, use the **Actions** function. In general, two actions must be separate by some time. Here you find an example of activation of a stimulus for one second.

1. Choose **Setup > Trial Control Settings**.
2. In the Components pane, under **Actions** click the button next to the device name that you want to use, and specify the type of action.
3. In most cases you need to add a number of boxes in a sequence. For example, the sequence **Shock On (Action) > Time Condition > Shock Off (Action)** define the activation of the shock for a specific time set in the Time Condition.
4. Click and drag from the center of one box to the next to connect the boxes with the arrows.



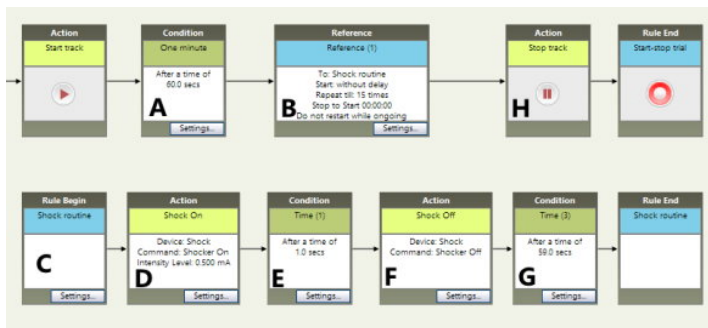
Depending on how you map the devices on the Arenas (see page 216), this action can be carried out in one or more cubicles simultaneously.

### *Recurring stimulation using Sub-rules*

In most cases you need to administer a stimulus at regular intervals, for example every minute. If you repeat the sequence depicted in the figure above, one for each round, you would end up with a large number of boxes to manage. Instead, create a **Sub-rule** that contains

the actions that you want to repeat. Specify how often and when the routine is repeated in the Sub-rule settings.

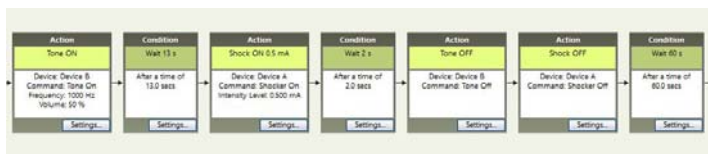
In the following example, a Sub-rule with the same instructions as above will be repeated 15 times. The shock is given every minute. After the start of the trial, the software waits one minute (box A), then the Sub-rule reference box (B) activates the Sub-rule (C). The stimulus is given as above (D-E-F). A time condition of 59 seconds (G) ensures that the next stimulus is given exactly one minute later (1 s of shock + 59 s = 60 s). The Sub-rule is repeated 15 times as specified in the Settings of box B. After that, the trial stops (box H).



### ***Cued conditioning: pairing tone and shock***

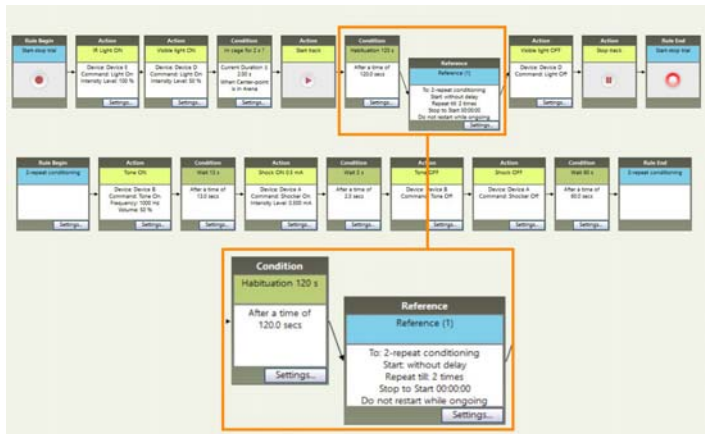
In the following example we follow the protocol by Curzon *et al.* (2009) [In Buccafusco (ed). *Methods of Behavior Analysis in Neuroscience*, Chapter 2]. During the conditioning session, a tone (Frequency 1000 kHz, Amplitude 50% of the maximum) is presented for 15 seconds. During the last 2 seconds of the tone presentation, the shock is administered (0.5 mA). Tone and shock end at the same time. After the shock, a period of 60 seconds with no stimuli follows.

This procedure is as follows:



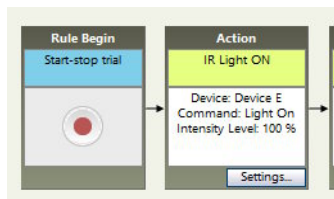
If you want to have this sequence repeated a number of times, place it within a **Sub-rule**.

In this example the Sub-rule is called by a Reference rule after 120 s of habituation.

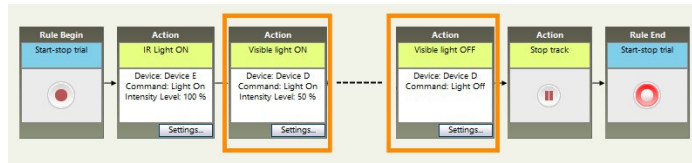


### Controlling lights

- **IR Light.** The FCS provided by Noldus has the IR light activated by default. If, for some reason, you notice that the IR light is off, you can activate the IR light at the start of the trial. To do so, add an action for the IR light just after the Start Trial box.



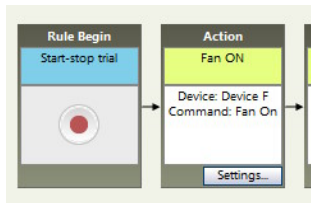
- **Visible Light.** In many FC protocols it is required that a house light illuminates the test chamber continuously during testing. Add an action for the UB FCS **Visible Light** and insert the **Light On** Action box after the **Start Trial** box. Add a second action box (this time with a **Light Off** action) immediately before the Stop Track box.



### Controlling the fan

The fan is by default activated when you start the cubicle.

To ensure that the fan is on at the start of a trial, also in the case that the fan was accidentally switched off during the testing phase (page 218), in the Trial Control Settings click the button next to **UB FCS Fan** and insert the **Fan On** Action box after the **Start Trial** box.



### NOTES

- If you add a **Fan On** Action at the start of the trial, the fan switches on at the start of the trial and switches off at the end of the trial.
- If you do not add any action about the fan in the Trial Control Settings:
  - If the fan was already on at the start of the trial (for example after you start up the cubicle), the fan stays on during the trial.
  - If the fan was off, for example when testing it in the Trial Control Settings, it stays off during the trial.

### Analyze data using Trial Control events

If you want to analyze the data in specific time segments, you can use the actions and conditions of Trial Control Settings to define specific intervals. See **ANALYZE INTERVALS** on page 233.

### ***For more information***

For more information and examples of actions, conditions and sub-rules, see the *EthoVision XT 18 - Trial and Hardware Control - Reference Manual*. To open this manual, choose **Apps > Noldus > EthoVision XT 18 Other Documentation**.

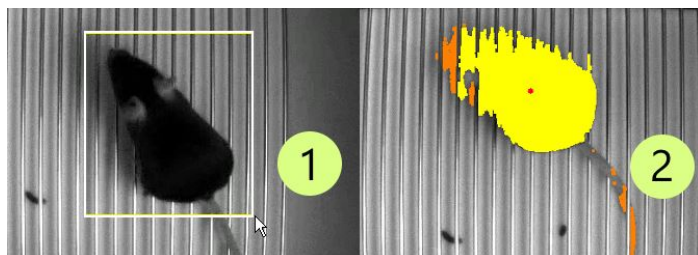
## **DETECTION SETTINGS**

Release the subjects in the FCS cages.

Choose **Setup > Detection Settings**.

### ***Detect the subject***

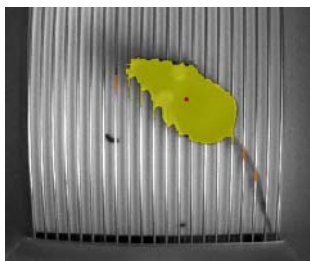
In the Detection Settings window, click **Automated Setup**. Draw a rectangle around each subject in the video image, and follow the instructions on the screen.



### ***Minimize the indentations caused by the grid***

In the Detection Settings, click **Advanced**. Under **Subject Contour**, choose what follows depending on the color of the subject and the grid bars:

- If the grid bars are the same color as the subject, use the first **Erosion** filter. Increase the value near **Erosion** until the grid is no longer detected. Normally 2-3 pixels are fine. Check that the whole body is detected, indicated by the yellow blob.

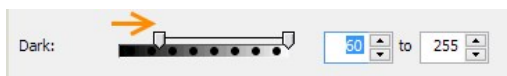


- If the grid bars are of a color other than the subject, and the detected subject appears to be broken in multiple parts, use the **Dilation** filter. Increase the value near **Dilation** until the yellow body is entirely over the subject's body. After that, reduce the size of the yellow blob by means of the second **Erosion** filter.

For details, see **Advanced detection settings: Subject contour** in the EthoVision XT Help.

### *Minimize the effect of the subject's shadow*

Make the contrast range smaller, to exclude the pixels in the shadow. For example, if the subject has dark fur, and the shadow is included in the yellow blob, then next to **Dark:** move the left slider to the right. This will exclude smaller values of contrast. You should see the yellow area over the shadow shrinking. EthoVision XT will not follow the shadow anymore.

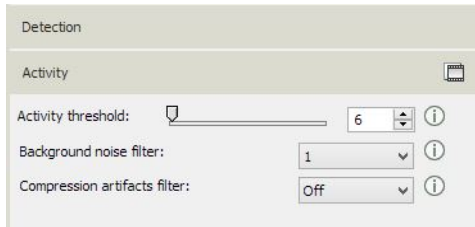


Do this in small steps, until the shadow is no longer detected. Make sure that the whole body is still detected in all parts of the cage.

**TIP** If the background looks too similar to the color of the subject, place a sheet of paper or plastic in the drawer that you find under the shock grid. Try to make as much contrast as possible between the subject and the background.

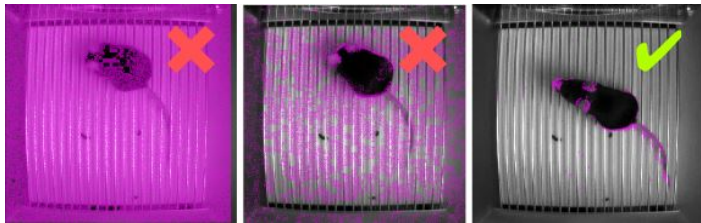
### *Set Activity analysis for freezing detection*

In the Detection Settings window, click **Activity**.



Adjust the settings in such a way that a large number of purple pixels appear when the subject moves.

- If the **Activity threshold** is too low, purple pixels also appear in the background (see the next figure, left and middle).
- Increase the value of **Activity threshold** until you see the purple pixels appearing only when for example the mouse moves its head (see the next figure, right).
- If the **Activity threshold** is too high, purple pixels disappear completely. That's not good either, because you need to have a sufficiently wide range of the amount of purple pixels in order to detect immobility (freezing).



When the subject freezes, the number of purple pixels should be minimized.

#### ***For more information***

- See **Activity settings** in the EthoVision XT Help.
- Click the video button next to **Activity**, and watch the video tutorial.



- For details about the advanced detection settings, See **Configure Detection Settings** in the EthoVision XT Help.

## TROUBLESHOOTING

*All the FCS devices are listed for Arena 1 only. For the other arenas, all is set to <No device allocated>.*

This may have occurred when you selected the number of arenas (for example, 4) *after* defining the COM ports for the FCS cages. If, at the time of defining the COM ports, the number of arenas was still 1, EthoVision XT assumes that all the devices must be associated with one arena, that is, Arena 1.

Do the following:

1. In the **Arena-Hardware Mapping** window, select each row and click **Remove device**. Do so to remove all the devices.
2. Choose **Setup > Experiment Settings**.
3. Under **Trial Control Hardware**, click **Settings**, then choose **Ugo Basile F.C. Controller**. In the window that opens, make sure that the COM ports are listed and click OK.
4. Under **Number of Arenas**, select the number of arenas you work with.
5. Choose **Setup > Arena Settings** and click the **Arena-Hardware Mapping** button. At this point the devices should be correctly selected under one of the arenas.

*The IR light is switched off, or its intensity is not optimal. What can I do?*

1. Create a new Trial Control Settings profile (**Setup > Trial Control Settings > New**).
2. Click the button next to **Action - UB FCS IR light**.
3. Select the device from the **UB FCS IR light** list.
4. Select **Light ON** and choose the **Intensity Level** (recommended: 100%).



5. Click **Test**, then **OK**.
6. Open the Detection Settings and verify that the chamber is illuminated.
7. **TIP** Keep the new Trial Control Settings profile so you can always access it in case you want to restart the IR light. Do not use this profile for tracking.

Another way to restore the default IR light is to restart the cubicle (disconnect and then reconnect the power).

See also **UPDATE THE CUBICLE FIRMWARE** page 204 to make sure that the default state of the IR light is to set the default light intensity.

***The fan is switched off. What can I do?***

Restart the cubicle (disconnect and then reconnect the power).

# Acquire the data

## THINGS TO CHECK FIRST

Before starting one or more trials, it is useful to do some checks.

### *Hardware*

- Check that all devices are properly connected according to the schemes in this manual.
- Check that all devices are powered up.
- Insert the conditioning cage in the cubicle as in the figure below. The cage fits between the two clear support angles attached to the IR light box.

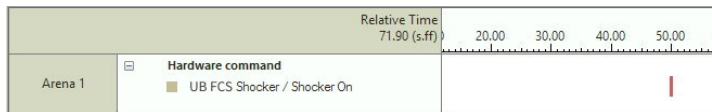


### *Test the devices In EthoVision XT*

- Choose **Setup > Arena Settings**. Open the Arena Settings you want to use. Click the **Arena – Hardware Mapping** button and test the devices (page 218).

### ***Run a test trial***

- To check that the system works correctly, run a test trial with a basic Trial Control settings profile that contains actions and conditions.
- After data acquisition, in the Analysis profile under **Hardware** choose **Hardware command** and define the event you want to view, for example Device A, Output 1 High (=Light on).
- Choose **Analysis > Results > Integrated Visualization** and check whether the event occurred at the expected time.



## **ACQUIRE DATA**

In EthoVision XT, choose **Acquisition > Open Acquisition**.

Follow the general procedure described in the EthoVision XT Help. Browse to **Acquire Data > Acquire one trial**.

# Analyze the data

## BASIC STEPS

1. Define the intervals you want to analyze. Choose **Analysis > Data Profile > New**. See **ANALYZE INTERVALS** below.

If you want to calculate statistics for the whole duration of your trials, you can skip this step.

2. Choose **Analysis > Analysis Profile > New**. Choose the variables you want to analyze. For example, velocity, time in zone, or activity. See *Dependent variables* below.
3. Choose **Analysis > Results > Statistics and Charts** to calculate statistics, or **Integrated Visualization** to view the video and the variable plotted against time.

## ANALYZE EVENTS

Add the following variables, if not already listed in the Analysis profile:

- **Activity state** to analyze freezing events. For more information about *Activity* and *Activity state*, and how to set the thresholds to detect freezing, see the EthoVision XT Help.
- **Distance moved**, and **Movement** to quantify locomotory behavior.
- **Hardware command** especially to visualize events like Light On, Shock On, etc. together with the video and the tracks (**Analysis > Results > Integrated Visualization**).
- **Trial Control Event** to visualize for example when a condition becomes true. Use this especially for testing purposes.

## ANALYZE INTERVALS

### *Define the intervals with the Nesting function*

By default, the whole trial timeline is selected for analysis. However, you may want to select part of the timeline, for example to analyze the Activity variable only when the white light was on. To do so, in the Data profile define an interval based on the event “White Light On”.

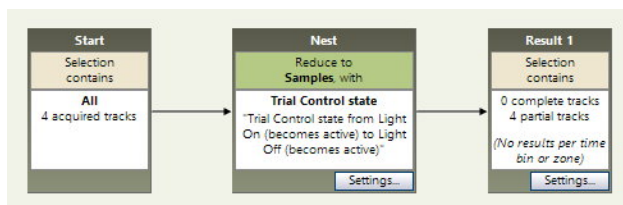
1. Choose **Analysis > Data Profile > New**.
2. Under **Nesting**, click the option corresponding to the type of interval you want.

Nesting	
Time	<input type="checkbox"/>
Movement	<input type="checkbox"/>
Acceleration state	<input type="checkbox"/>
Latency to zone	<input type="checkbox"/>
In zone	<input type="checkbox"/>
Mobility state	<input type="checkbox"/>
Trial Control state	<input type="checkbox"/>
Hardware state	<input type="checkbox"/>
Free interval	<input type="checkbox"/>

3. Specify the interval and place the **Nest** box between the **Start** and **Results** box.

### *Which options shall I choose?*

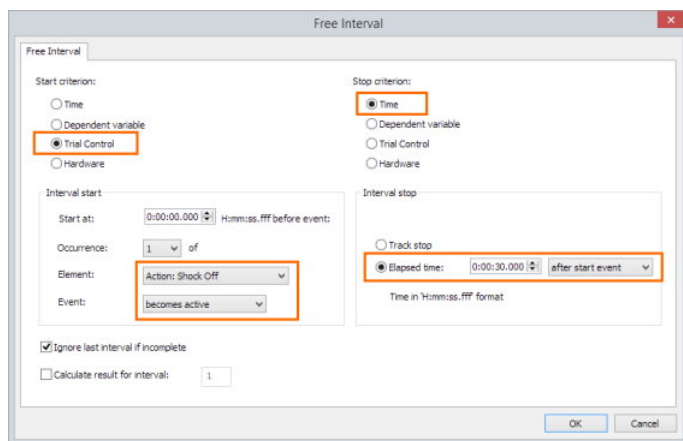
- To analyze the behavior of the subject when, for example, the white light was on, choose **Trial Control State**. In the window that appears, select the action that marks the start of the interval (e.g., Light On - becomes active), and the action that marks the end of the interval (Light Off - becomes active).



- If you want to analyze a complex interval, from A to B, where A and B are events of different type, for example, behavior and activation of a stimulus, choose **Free interval**.

For example, select the time from the end of the shock (**Shock Off - becomes active**) and the first instance of freezing (detected using Activity or Activity state).

- You can also combine events with time. In the following example, we select the 30 seconds interval immediately after the end of the Shock. From **Trial Control - Action: Shock Off - becomes active** to **Time - Elapsed time: 30 s after start event**. See the figure below.

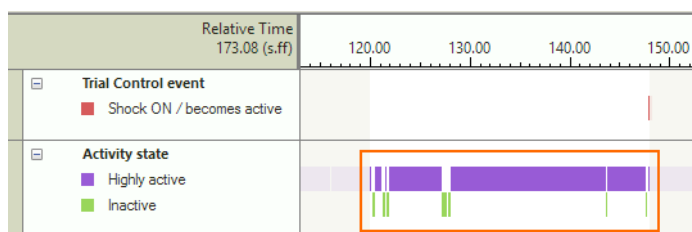


### **Visualize the intervals**

Once you have defined the intervals for analysis, it is always a good idea to plot the data and check that the intervals defined in the previous steps are correct.

1. Choose **Analysis > Results > Plot Integrated Data**.
2. The data selected within the intervals is shown in full colors while the data outside the intervals is showed in dimmed colors. Check that the intervals defined correspond to the time segments that you want to analyze.

In the example below, Activity is visualized within an interval defined as “from *Tone On* to *Shock On*”.



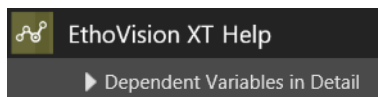
If the intervals are defined correctly, proceed with the statistics calculation.

### ***Calculate the statistics***

Choose **Analysis > Results > Calculate Statistics** and click the **Calculate** button. The results table shows the statistics of the dependent variables defined in the Analysis profile, within the intervals defined in the Data profile.

### ***For more information***

- For details about the analysis variables, see **Dependent Variables in Detail** in the EthoVision XT Help (F1).



- For more examples about analyzing intervals, see **Analyze Track Segments** in the EthoVision XT Help (F1).
- For more examples of control of hardware devices and PhenoTyper, see also the Chapter **Conditioning tests** in this manual.

# Technical specifications

## INFRARED TOP LIGHT AND CONTROLLER

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Noldus device numbers	Top light: UBIRT-001x Controller: UBIRC-001x
Voltage	24V (standard Noldus power supply)
Current consumption on max LED intensity	220mA
Light wavelength	940nm
Incorporated camera	1280 x 1024 pixels at max 60 fps (30 fps is recommended)
Dimensions (L x W x H)	Top light 28.6 x 29.3 x 8.5 cm (11 17/64" x 11 17/32" x 3 11/32") Controller 8.0 x 4.0 x 4.2 cm (3 5/32" x 1 37/64" x 1 21/32")
Weight	Top light: 860 g (30.3 oz) Controller 50 g (1.76 oz)
Power adapter	Type number TRH70A240-01E13VI Input 100-240V, 1.5 A Output 24V, 3.0 A CE marking: see the next page

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For the technical specifications of the Fear Conditioning system, see the **Instruction Manual FCS 46000.pdf** that comes with that system.



# DECLARATION OF CONFORMITY



Name of company: CINCON ELECTRONICS CO., LTD.  
 Address: No. 8-1 Fu Kung RD. Fu Hsing Park, Fu Hsing Hsiang,  
 Chang Hua Hsien, Taiwan, R.O.C.

Declares that the product

Adapter

TRH50A120; TRH50A150; TRH50A180; TRH50A190;  
 TRH50A240; TRH50A280; TRH50A360; TRH50A480;  
 TRH70A120; TRH70A150; TRH70A180; TRH70A190;  
 TRH70A240; TRH70A280; TRH70A360; TRH70A480;

referred to this declaration conforms with the standard(s) or directive(s) as far as applicable:

Product Safety Standard :	EN60950-1	2006+A11+A1+A12+A2
EMC Standards :	EN55022	2010/AC: 2011 Class B
	EN55032	2012 +AC:2013
	EN55024	2010
	EN61204-3	2000
	EN61000-6-1	2007
	EN61000-6-3	2007+A1: 2011+AC: 2012
	EN61000-3-2	2014
	EN61000-3-3	2013
Directives :	Low Voltage Directive	2014/35/EU
	EMC Directives	2014/30/EU
	ErP Directives	2009/125/EU
	RoHS Directive	2011/65/EU

This product must be used within other equipment and must not operated as a stand alone product.

The company named above will keep on file for review the following technical documentation:

- Technical drawings
- Other technical documentation

Manufacturer

Signature: Johnson Cheng

Date: Mar. 03 2017

Name: Johnson Cheng / President

## Chapter 11

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# Other Conditioning Tests

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# Introduction

EthoVision XT can be used in learning tests, such as the water maze test (see Chapter 3 **The Morris Water Maze Test**) and the conditioning tests (e.g., Pham et al., 2009).

The Trial and Hardware Control module of EthoVision XT can be used to operate hardware devices to be used in both classical and operant conditioning tests. In the latter, an action of the animal produces a result, for example, pressing a lever results in the food dispenser dropping a food pellet. This and other hardware actions (such as, turning on a light when the animal enters the shelter, or closing the door of a radial maze when the animal has exited that arm) can be operated through the Trial and Hardware Control in EthoVision XT. Learning can be demonstrated by a change in the frequency of the animal's action over time. For example, when the animal is presented with an aversive stimulus every time it performs a certain action, the performance of the action will likely decrease over time because the animal tries to avoid the negative stimulus.

To be able to operate hardware devices with EthoVision XT you need:

- **The Trial and Hardware Control module.** This module allows you to control hardware devices from within EthoVision XT.
- **The USB-IO Box.** This is the device that connects the EthoVision XT PC with external hardware devices such as the sound and light devices of PhenoTyper or a third-party hardware device.

This document describes how to set up EthoVision XT using Trial and Hardware Control in an operant conditioning test in PhenoTyper.

## ***PhenoTyper***

PhenoTyper is an instrumented cage to measure and test the behavior of laboratory rodents. See the following web page:

[www.noldus.com/animal-behavior-research/products/phenotyper](http://www.noldus.com/animal-behavior-research/products/phenotyper) for more information on PhenoTyper.

If you want to use EthoVision XT to operate a third-party hardware device, please contact Noldus Information Technology ([www.noldus.com](http://www.noldus.com)).

To see how a test with PhenoTyper is carried out in EthoVision XT, see the sample experiment **PhenoTyper hardware XT180** on the downloads section of the Noldus website ([my.noldus.com](http://my.noldus.com)). In EthoVision XT, choose **File > Restore Backup** and select this file. See also the document **Description of sample experiments of EthoVision XT.pdf** for more information.

### ***Fear Conditioning test***

In the fear conditioning test, you can use EthoVision XT to automatically detect freezing behavior as a measure of conditioned fear. For details, see page 179.

### ***References***

- Liu, W., Wang, X., Zhang, R., and Zhou, Y. (2009) Effects of postnatal exposure to methylmercury on spatial learning and memory and brain NMDA receptor mRNA expression in rats. *Toxicology Letters*, **188**, 230-235.
- Pham, J., Cabrera, S.M., Sanchis-Segura, C., and Wood, M.A. (2009) Automated scoring of fear-related behavior using EthoVision software. *Journal of Neuroscience Methods*, **178**, 323-326.

# Physical setup

The following suggestions are meant to optimize tracking:

- The camera should have a good view of the entire region the animals can be in (for zones like the shelter where the animal is not visible, you can define hidden zones).
- The lighting should be as even as possible throughout the arena and should be diffuse, so as not to cast strong shadows (which might be tracked instead of the animal).
- The background should contrast with the animal. If necessary use a different setup for light and dark colored animals.
- Either make sure the sides of the box are not reflective (e.g., sanding), or exclude the sides from the arena. If your objects are close to the sides then you will need to track the animal when it is against the side (so the camera sees the side instead of the base behind the animal).
- If you want to use Deep learning technique to track the subject's nose, keep in mind that there are additional requirements. Among other things, you need a powerful graphics card. See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.

## *For more information*

- About PhenoTyper: see the PhenoTyper - EthoVision XT 18 - Reference Manual.
- About using hidden zones: see the section **Shelters and other hidden zones** in the EthoVision XT Help.

# A conditioning test in EthoVision XT

Create an experiment. For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help. In the following sections we assume that you created an experiment using the PhenoTyper arena template.

## EXPERIMENT SETTINGS

1. Make sure that the PhenoTyper cages are connected to the EthoVision XT PC. See the PhenoTyper - EthoVision XT 18 - Reference Manual for how to connect PhenoTyper to EthoVision XT.

Connect a third-party hardware device, which can be controlled by TTL, to one of the TTL control ports on the USB-IO Box.

2. Open the experiment and choose **Setup > Experiment Settings**.

3. Check that the correct cameras are selected under **Video source**.

Select whether the resolution of the first camera and that of the mixed image. Note that depending on the number of cameras and the frame rate, you may need to reduce the resolution of the mixed image. For more information about installing and connecting multiple cameras, see **Camera installation** in the EthoVision XT Help and the PhenoTyper - EthoVision XT 18 - Reference Manual.

4. If you want to use Deep learning technique to track the subject's nose, under **Body point detection technique** choose **Deep learning**. Note that in order to use this technique there are additional requirements. Among other things, you need a powerful graphics card. See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.
5. Under **Analysis options**, select **Activity analysis** if you want to measure freezing behavior.

6. Next to **Hardware**, select **Use of Trial Control Hardware** and click **Settings**.
7. Select **Noldus USB-IO box**.
8. In this next step, you define the ports of the USB-IO box connected to PhenoTyper. Select **SDI port 13**, then **Top Unit Interface**. Select **Top Unit (Standard)** for all the rows below (depending on how many PhenoTyper are connected to a Top Unit Interface)

SDI Port 13	Top Unit Interface (PTTI-0010)	▼	
Interface Port 1	Top Unit (Standard)	▼	Top Unit (Standard) 1
Interface Port 2	Top Unit (Standard)	▼	Top Unit (Standard) 2
Interface Port 3	Top Unit (Standard)	▼	Top Unit (Standard) 3
Interface Port 4	Top Unit (Standard)	▼	Top Unit (Standard) 4

For PhenoTyper 2, and for more details and examples, see the **PhenoTyper - EthoVision XT 18 - Reference Manual**.

If you use third-party devices, also select the TTL ports that are connected to those devices.

9. Click **OK**.

### ***For more information***

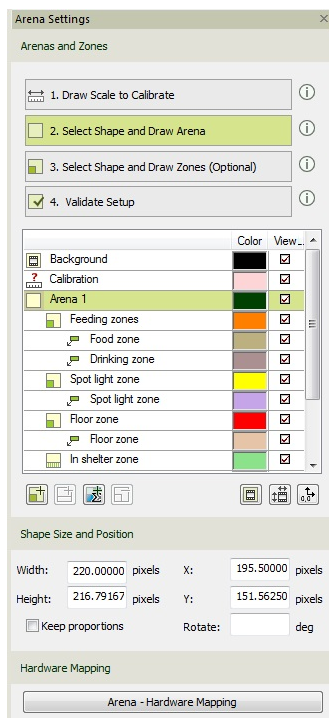
- About port mapping: the **EthoVision XT 18 - Trial and Hardware Control - Reference Manual**.
- About PhenoTyper connections and devices:
  - **PhenoTyper - EthoVision XT 18 - Reference Manual**.
  - **PhenoTyper - EthoVision XT 18 - Service Manual** for specific devices like the Pellet dispenser and the Lickometer.
- About general experiment settings: **Set Up an Experiment** in the **EthoVision XT Help**.

## **ARENA SETTINGS**

Choose **Setup > Arena Settings > open Arena Settings 1**.

We assume that you followed the general procedure on page 15.

Click the **Arena - hardware mapping** button at the bottom of the **Arena Settings** pane (see Figure 11.1).



**Figure 11.1** *The Arena Settings pane. In this example, Arena 1 contains four pre-defined Zone groups: Feeding zones, Spot light zone, Floor zone and a Hidden In shelter zone.*





If you only have one arena, the hardware will be assigned to this arena automatically. If that is the case, proceed with Trial Control Settings on page 245.

In the **Arena - Hardware Mapping** window, click **Add Device** for each device you want to add:

- **For PhenoTyper** – Under **Device Type**, select **Top Unit (Standard)**. Enter **Device Name** *PhenoTyper* and the corresponding Top Unit (Standard) # for each Arena (Figure 11.2).



- **For a third-party hardware device** – Under **Device Type**, select **Custom Hardware**. Enter a **Device name** and select a device from the list for each Arena.

Device type	Device name	Arena 1
Top Unit (Standard) 	PhenoTyper	Top Unit (Standard) 1 
Custom Hardware 	Buzzer	Buzzer 

**Figure 11.2** *Part of the Arena - Hardware Mapping window. In this example, two devices have been added: one PhenoTyper and a ‘custom’ Buzzer.*

Click **4. Validate Setup** to validate the Arena Settings.

## TRIAL CONTROL SETTINGS

With the Trial and Hardware Control add-on module in EthoVision XT, you can control PhenoTyper and third-party devices in a variety of ways. For example:

- Start recording when the animal is detected on top of the shelter.
- Stop recording when the animal has performed a specific task 50 times.
- When the animal sits on the shelter, drop a food pellet. Repeat this task 25 times.

The template experiment contains two trial control profiles:

- **Default** – The default Trial Control Start-Stop rule.
- **Max. Trial duration 24 hrs** – In this profile, a trial stops after 24 hours.

### ***Maximum trial duration (tested)***

- Without video recording: 72 hours.
- With simultaneous video recording: 3 hours.

If you need to record video of the same subject for more than three hours, we recommend that you make a series of trials.

Below you find a few examples of Trial Control Settings. Modify the Trial Control Settings to suit your own application.

### ***Procedure overview***

1. The trial starts with a 30-second delay (See Start trial rule and **Condition ‘Start delay’** below).
2. Next, as soon as the mouse enters the LightSpot zone, with both the nose- and center-point, the spotlight of the PhenoTyper is turned on. When both the nose- and center-point are not in the LightSpot zone, the spotlight is turned off. This sequence is repeated three times (see **Sub-rule ‘Activation LightSpot’** on page 247).
3. After the Sub-rule is completed, the trial is stopped after a 5-second delay.

Choose **Setup > Trial Control Settings > New** and give this a new name.

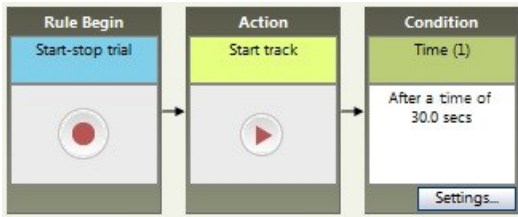
### ***Start trial rule***

1. To start recording as soon as the **Start trial** button is clicked:
  - Click the (default) Condition **In zone** box and press **Delete**.  
Keep this Condition if you want to start recording some time after starting the trial.

### ***Condition ‘Start delay’***

2. To insert a 30-second delay, after recording has started:
  - a In the **Components** pane, under **Conditions**, double-click the **Time Condition** or click the button next to it.
  - b In the **Time condition** window, type ‘Delay of 30 seconds’ in the **Condition name** field. In the **Condition is met:** group, select **After a time of 30 secs**. Optionally, enter a comment and click **OK**.

- c Insert the Condition box into the main sequence.

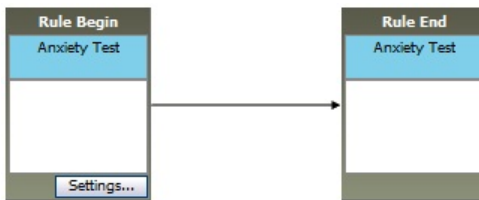


**Figure 11.3** Example of the Start trial rule and Condition ‘Start Delay’: with this sequence recording starts as soon as you click the **Start trial** button. Immediately after that, a 30-sec delay starts.

For more information about Conditions, see the EthoVision XT 18 - Trial and Hardware Control - Reference Manual.

#### **Sub-rule ‘Activation LightSpot’**

3. Because the sequence of turning on/off the spotlight depending on whether the animal is inside/outside the Spot Light Zone is repeated three times, you need to create a Sub-rule for this:
  - a In the **Components** pane, under **Structures**, double-click **Sub-rule** or click the button next to it.
  - b In the **Sub-rule** window, type ‘Anxiety Test’ in the **Sub-rule name** field. Optionally, enter a comment and click **OK**.



**Figure 11.4** The empty Sub-rule ‘Anxiety Test’ sequence.

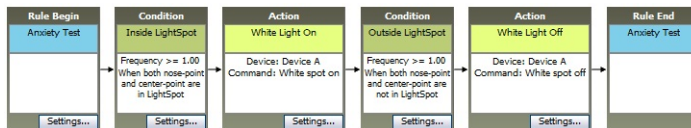
4. Create an **In zone** Condition for when the animal enters the Spot Light Zone:
  - a In the **Components** pane, double-click the **In zone** Condition or click the button next to it.

- b In the **In zone** Condition window, type 'Inside LightSpot' in the **Condition** name field.
  - c In the **Condition is met when:** group, from the **Statistic** list, select **Frequency**.  
Next, click the **Settings** button, select zone **Spot Light** from **In the following zones**, and select **When in any of the selected zones** from the list.  
In the **From the following body points** group, select both **Nose-point** and **Center-point**. Make sure to select **All selected points** from the list.
  - d Click **OK**.
  - e Insert the **Condition** box into the **Sub-rule** sequence.
5. Create a Hardware Action (White Light on) for when the previous Condition is met:
- a In the Components pane, under **Actions - Hardware** double-click **Top Unit (standard)** or click the button next to it.  
If a third-party device has been connected (see step 4 on page 243), under **Actions - Hardware** double-click **Custom Hardware**.
  - b Next to **Action** name, type in "White Light on".
  - c Next to **Action to perform**, select **White spot on** from the list and click **OK**.
  - d Insert the **Action** box into the **Sub-rule** sequence.  
If a third-party device has been connected, select an action from the **Custom Hardware**.
6. Create an **In Zone** Condition for when the animal leaves the **Zone LightSpot**:
- a In the **Components** pane, double-click the **In zone** Condition or click the button next to it.
  - b In the **In zone** Condition window, Type *Outside LightSpot* in the **Condition** name field.
  - c In the **Condition is met when:** group, from the **Statistic** list, select **Frequency**.  
Next, click the **Settings** button, select zone **Spot Light** from **In the following zones** and select **When not in any of the zones**.

In the **From the following body points** group, select both **Nose-point** and **Center-point**. Make sure to select **All selected points** from the list at the bottom.

- d Click **OK**.
  - e Insert the **Condition** box into the **Sub-rule** sequence.
7. Create a **Hardware Action** (White spotlight off) for when the previous Condition is met:
- a In the **Components** pane, under **Actions - Hardware** double-click **Top Unit (standard)** or click the button next to it.
  - b Next to **Action name**, type in *White Light off*.
  - c Next to **Action to perform**, select **White spot off** from the list and click **OK**.
  - d Insert the **Action** box into the Sub-rule sequence.
- If a third-party device has been connected, select an action from the **Custom Hardware**.

The Sub-rule *Anxiety Test* is now completed (see Figure 11.5).



**Figure 11.5** The Sub-rule 'LightSpot' with the sequence as describe above in steps 4-8.

### **Sub-rule Reference 'Start Anxiety Test'**

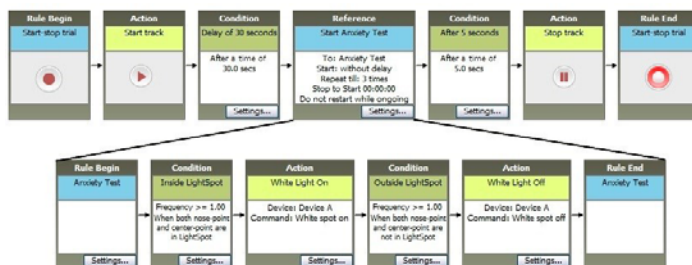
8. To create a **Reference** in the main sequence to the **Sub-rule** *LightSpot*:
  - a In the **Components** pane, under **Structures**, double-click **Sub-rule reference** or click the button next to it.
  - b Next to **Reference name**, type *Start Anxiety Test*.
  - c Next to **Reference to sub-rule**, select *Anxiety Test*.
  - d Next to **Stop Conditions**, select **Repeat per start condition**. Select **for a number of ... times** and set it to 3.
  - e Click **OK** and insert the **Reference** box into the main sequence.

### Condition 'Stop delay'

9. To insert a 5-second delay, before the trial is stopped:

- In the **Components** pane, under **Conditions**, double-click the **Time Condition** or click the button next to it.
- If the **Add a condition** window appears, it means that there is at least one condition of the same type in your experiment. You are asked to choose between creating a new condition, or re-use an existing one. Choose the option you require and click **OK**. If this window does not appear, skip this step.
- In the **Time condition** window, type 'After 5 seconds' in the **Condition name** field. In the **Condition is met:** group, select **After a time of 5 secs**. Optionally, enter a comment and click **OK**.
- Insert the **Condition** box into the main sequence.

Figure 11.6 shows the complete Trial Control Settings.



**Figure 11.6** *The Trial and Hardware Control. The main sequence is displayed at the top, the Sub-rule sequence at the bottom.*

### For more information and examples

- About PhenoTyper: PhenoTyper - EthoVision XT 18 - Reference Manual.
- About subrules, conditions and actions: EthoVision XT 18 - Trial and Hardware Control - Reference Manual.

## DETECTION SETTINGS

Choose **Setup** > **Detection Settings** > open **Detection Settings 1**.

We assume you followed the main procedure on page 17.

1. Check in the **Video** Section that the sample rate is set to:
  - **Rats**: 5 samples/second.
  - **Mice**: 12.5 samples/second.
  - **Nose-tail tracking**: 25-30 samples/second.
2. Under **Method**, choose your detection method and the contrast with the background.

If you selected to use the Deep learning technique to track the subject's nose, next to **Deep learning** click **Define** and select a box around the subject. Make sure that the box includes the subject's nose, and does not include objects of the same color as the subject. For details, see the EthoVision XT Help.

3. If you selected **Activity analysis** in the Experiment settings, open the **Activity** section in the **Detection Settings** pane. Enter the **Activity** threshold for detecting a change in the pixels in the arena. Start with the default value and check the purple-colored pixels in the video image. Adjust the threshold in such a way that when the animal is completely still, those pixels should be almost completely absent. Leave the **Compression artifacts filter** **Off** if you track live.

## TRIAL LIST

Enter your independent variables such as rat ID, first or second phase of the experiment, treatment (drug vs. control), dose, duration of treatment, name of the experimenter, etc. If you want you can pre-define all your trials here, or you can enter the independent variable values as you carry out the trials.

You can also prepare the trials in Excel, randomize them and then paste the values into your Trial List.

To carry out batch acquisition, plan your trials in the Trial list. Optionally select **Arena Settings**, **Trial Control Settings** and **Detection**

**Settings** for each trial. If you do not select these settings in the Trial list, the ones selected in the **Acquisition settings** pane (see next section) will be used for all trials.

Trial List				
Add Trials...		Add Variable		Show
Label	System		User-defined	User-defined
Description	Trial name		Acquisition status	Animal ID
	The name of the trial		The current status of acquisition per arena	Day
Type	Text		Text	Text
Format				
Predefined Values				
Scope	Trial		Arena	Subject
Trial	Arena	Subject	No.	
Trial 1	Arena 1	Subject 1	1	
	Arena 2	Subject 1	2	
	Arena 3	Subject 1	3	
	Arena 4	Subject 1	4	
Trial 2	Arena 1	Subject 1	5	
	Arena 2	Subject 1	6	
	Arena 3	Subject 1	7	
	Arena 4	Subject 1	8	
Trial 1	Planned	322	1	0.5
	Planned	333	1	0.5
	Planned	423	1	0.05
	Planned	335	1	0
Trial 2	Planned	324	1	0.5
	Planned	432	1	0
	Planned	222	1	0.05
	Planned	431	1	0

**Figure 11.7** A Trial List with two planned trials. Each trial includes four arenas (four PhenoTybers combined with a quad unit).

For data acquisition, see **ACQUIRING TRACKS** on page 21.



# Pre-Pulse Inhibition of the startle response (PPI)

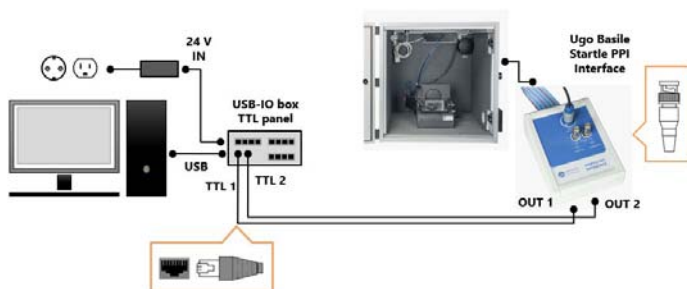
## INTRODUCTION

Startle Pre-Pulse Inhibition (PPI) is an experimental paradigm to study sensorimotor gating. With this term we intend the ability of the brain to filter out irrelevant sensory stimuli to suppress or decrease a motor response, in this case, a startle response.

The Startle PPI paradigm has been widely used in rodents, such as mice and rats, to study sensorimotor gating deficits in different animal models. In a typical PPI experiment, a startle baseline is measured as a response to a loud sound, or a light or air-puff. A pre-pulse stimulus is delivered shortly before the loud stimulus. That is supposed to decrease the startle reaction.

This section refers to the Ugo Basile system for the Pre-Pulse Inhibition of the Startle Reflex. You can have this system send TTL signals to EthoVision XT when a pulse is fired or terminated, and when an environmental output is activated or deactivated. This way the EthoVision XT tracking information is synchronized with the main events generated by the PPI system.

## CONNECTION SCHEME



For this you need the Ugo Basile PPI Startle Reflex Interface (see the figure above). This Interface box is connected to the PPI Startle Reflex system through a flat cable and has two additional output connectors, **OUT 1** and **OUT 2**. You can connect one or both outputs to TTL ports of the USB-IO box. Contact Noldus if you need the proper adapter cables.

- **OUT 1** can be enabled with each stimulus pulse (or pre-pulse). The status is ON while the pulse is fired and OFF when the pulse is terminated.
- **Out 2** can send out “environment” signals, like that for the visible light, the infrared light or the fan that can be turned ON or OFF at a specific time during the trial.
- See also the Ugo Basile Pre-Pulse Inhibition of Startle Reflex (PPI) User Guide.

### **EthoVision XT**

- In the Experiment Settings, define the TTL ports as **Custom Hardware**.

Ports	Device type	Device ID
TTL Port 1	Custom Hardware	Custom Hardware Stimulus
TTL Port 2	Custom Hardware	Custom Hardware Environment
TTL Port 3	<No device connected>	<No device type selected>

- In the Arena Settings, click the **Arena - Hardware Mapping** button and define the devices for each arena.

Device type	Device name	Arena 1
Custom Hardware	Stimulus	Custom Hardware Stimulus
Custom Hardware	Environment	Custom Hardware Environment

- In the Analysis profile, define a Hardware State variable for example to visualize the pulses together with the tracking data.

Device type:

Device:

Signal:

Value:

# Data analysis

In this section you find general information on how to analyze data in a conditioning test (see page 242).

## DATA PREPARATION

There are three optional steps you can take to prepare your data:

### *Edit the tracks*

Choose **Acquisition > Edit Tracks**.

You can fix tracking errors and swap back nose-points and tail-points that have been swapped in tracking. Normally you will not need to edit your data.

### *Smooth the tracks*

Choose **Acquisition > Track Smoothing Profile**.

You can use **Smoothing** and/or the **Minimal Distance Moved** filter to remove outliers from your data and correct the distance moved calculation as a result of body wobble.

### *Select tracks and intervals*

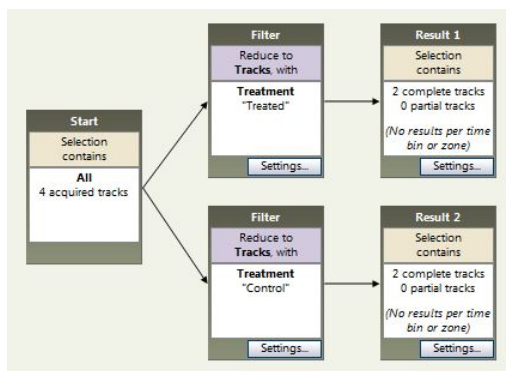
Choose **Analysis > Data Profile**.

Select your tracks according to your independent variable values and also select parts of tracks (nesting). You can also make groups of tracks to obtain group statistics. And you can define **Time bins** (see data selection below).

## DATA SELECTION

### *Groups of tracks*

Figure 11.8 shows a Data Profile to compare the animals that were treated with a drug, with those that were treated with saline. This way you create groups of tracks to obtain group statistics for each group.



**Figure 11.8** An example of data selection to compare two data sets in your experiment.

### *Time bins*

Using time bins means that you create time intervals of constant duration to analyze how variables change over time.

The default data selection does not specify time bins, so the behavior will be analyzed as a whole. If you define time bins and specify an interval length of, for instance, 1 minute, you can investigate whether a specific behavior changes over time.

To define time bins:

1. From the **Analysis** menu, select **Data Profile**.
2. Select **New** and type in *Time intervals*.
3. Click the **Settings** button on the **Result** box.

4. Select **Results per time bin** and enter the length of one interval, for example 30 s or 1 minute depending on the duration of your experiment. The data will be split in a number of intervals of the same length.

When the track duration is not an exact multiple of the time bin that you set, the last time bin is shorter than the others. To ignore this time bin, select **Ignore last time bin if incomplete**.

5. Click **OK**.

If the white spotlight is aversive to the animal, the time spent in the *Spot Light* zone should decrease in the course of the trial. In contrast, the time interval between consecutive entries into Zone LightSpot should show an increase. Via data analysis in EthoVision XT, this can be investigated.

## ANALYSIS PROFILES

Choose **Analysis > Analysis Profile**.

If you have multiple Data Profiles, create or open a Data profile without Results per time bin.

Below is described how to create two analysis variables:

- *In Zone*. This variable calculates how often and how long the mouse stayed in the illuminated zone.
- *Trial Control State* “Interval in Spot Light zone”. This variable measures the time elapsed until the mouse enters the light spot again. This is done for each repeat of the learning routine.
- *Trial Control State* “Duration Light on”. This variable measures the time that the mouse stays in the light spot in each repeat of the learning routine.
- *Trial Control Event* “Frequency light on”. This variable measures the number of times that the light is switched on.

### *In Zone*

1. Select **New** and type in **In Zone**.

- The new Analysis Profile contains the default dependent variables **Distance moved** and **Velocity**. Click on a dependent variable and press **Delete** to remove it.
2. Click the **Add** button next to **In Zone**.
    - a Under **In the following zones**, select **Spot Light**. Under **From following body points**, select both **Nose-point** and **Center-point** and select **All selected points** from the list at the bottom.
    - b In the **Trial Statistics** tab, select **Mean, Frequency** and **Duration**.
    - c Select additional statistics In the **Group Statistics** tab, if you created groups of tracks in your Data Profile.
    - d Click **OK**.

### ***Trial Control***

1. Click the **Add** button next to **Trial Control State**.
  - a From the **From Element** list, select **Rule: Anxiety Test**. From the **From Event** list, select **becomes active**.
  - b From the **to Element** list, select **Action: Light on**. From the **to Event** list, select **becomes active**.
  - c Select **Calculate statistics per interval**, for consecutive intervals: 1 to 3.
  - d In the **Trial Statistics** tab, select **Cumulative duration**.
  - e Select additional statistics in the **Group Statistics** tab, if you created groups of tracks in your Data Profile. Next, click **OK**.
  - f Right-click **Trial Control State**, select **Rename** and type "Interval in Spot Light zone".
2. Click the **Add** button next to **Trial Control State**.
  - a From the **From Element** list, select **Action: White Light On**. From the **From Event** list, select **becomes active**.
  - b From the **to Element** list, select **Action: White Light off**. From the **to Event** list, select **becomes active**.
  - c Select **Calculate statistics per interval**, for consecutive intervals: 1 to 3.
  - d In the **Trial Statistics** tab, select **Cumulative duration**.
  - e Select additional statistics in the **Group Statistics** tab, if you created groups of tracks in your Data Profile. Next, click **OK**.

- f Right-click **Trial Control State**, select **Rename** and type “Duration Light on”.
3. Click the **Add** button next to **Trial Control Event**.
    - a From the **From Element** list, select **Action: White Light On**.
    - b From the **From Event** list, select **becomes active**.
    - c In the **Trial Statistics** tab, select **Frequency**.
    - d Select additional statistics in the **Group Statistics** tab, if you created groups of tracks in your Data Profile. Next, click **OK**.
    - e Right-click **Trial Control Event**, select **Rename** and type “Frequency light on”.

### ***More options with the Free interval function***

The Free interval function in the Data profile and in the Analysis profile allows you to extend the possibilities to define the start and the end of intervals. For example, from the time a stimulus event occurs, to 10 seconds after that event.

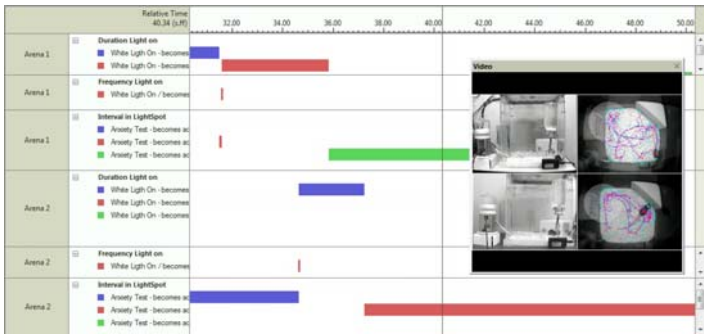
1. Choose **Analysis > Analysis Profile**.
2. Under **Miscellaneous**, click next to **Free Interval**.

For details, search the EthoVision XT Help for “Free interval”.

## **INTEGRATED VISUALIZATION**

When you Plot Integrated Data, the video file and the Time Event Plot are displayed. The Time Event Plot displays the variables of the active Analysis Profile.

Figure 11.9 shows the variables defined in the Analysis profile **Trial Control** described above, plotted against time.



**Figure 11.9** The Integrated Visualization for the an Analysis profile containing Trial Control variables. The Time Event Plot shows (from top to bottom) **Duration Light on**, **Frequency Light on** and **Interval in LightSpot**, all for each arena separately. The vertical line shows the current position in the Time Event Plot and the video window displays the corresponding frame in the video.



## **Chapter 12** ---

# The RotaRod Test

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The Rotarod test in EthoVision XT .....	265

# Introduction

## THE ROTAROD TEST

The rotarod test is widely used to evaluate drug effects on motor coordination in rodents. The principle of this test is that rats or mice are first trained to walk on a rod rotating at a certain speed. Once the animals have learned this, the effect of a test-compound on their motor performance is evaluated. Animals experiencing impaired motor coordination are unable to cope with the rotating rod and will drop off when the rotation speed exceeds their motor coordination capacity. The more disturbed the animals are, the sooner they fall off the rod.

The use of rotating rods to measure balance in rodents has been described since at least the 1950s (Dunham and Miya, 1957; Kinnard and Carr, 1957). Jones and Roberts (1968) introduced a significant modification of the basic design with an accelerating rod.

The rotarod is a horizontal cylinder that rotates along its long axis. It is situated above the cage floor, high enough to make sure rodents do not jump off the rod and low enough to avoid them being injured when they fall off.



**Figure 12.1** *Rotarod for mice.*

The speed of the rotarod is mechanically driven and can be constant or accelerated during the test.

The test consists of a *training* phase in which the rats or mice learn to walk on the rod at a certain speed. Then three *test trials* are carried out in which a drug is tested. A commonly measured parameter is the time until falling off.

With EthoVision XT the Rotarod test can be automated. A camera is placed above the Rotarod, which gives a top-view image of the setup. The rat or mouse that stays on the rod is present in the center of the camera image. When the animal falls off, or clings onto the rod and turns round on it, it moves out of the center of the image. This can be automatically quantified with various parameters in EthoVision XT.

## REFERENCES

- Dunham, N.W. and Miya, T.S. (1957). A note on a simple apparatus for detecting neurological deficit in rats and mice. *J. Am. Pharmac. Assoc. Sci. Ed.*, **46**, 208-209.
- Jones, B.J. and Roberts, D.J. (1968). The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod. *J. Pharm. Pharmacol.*, **20**, 302-304.
- Kinnard, W.J. and Carr, C.J. (1957). A preliminary procedure for the evaluation of central nervous system depressants. *J. Pharmacol. Exp. Ther.*, **121**, 354-361.
- Carter, R.J., Morton, A.J., and Dunnett, S.B. (2001). Motor Coordination and Balance in Rodents. In *Current Protocols in Neuroscience* (2001) 8.12.1-8.12.14.

### ***EthoVision XT and the Rotarod test***

Mahieu, M., Willems, R., Hoekstra, L., Ver Donck, L. (2012). Automated Detection of Aberrant Behaviour of Mice on the Rotarod: Use of EthoVision® XT. *Proceedings of Measuring Behavior 2012* (Utrecht, The Netherlands, August 28-31, 2012) 431-433.

## Physical setup

In most use cases, a video camera is positioned above the rod to record video. For systems with multiple rods, it is best to point one camera towards each rod. You can mix camera images directly with EthoVision XT or a video mixer.



**Figure 12.2** *Camera view.*

To detect the falling off the rod automatically, place the camera in front of the rotarod. You can have EthoVision XT detect the fall off event when the animal disappears from the arena, or a zone drawn around the rod.

# The Rotarod test in EthoVision XT

Create an experiment. For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help.

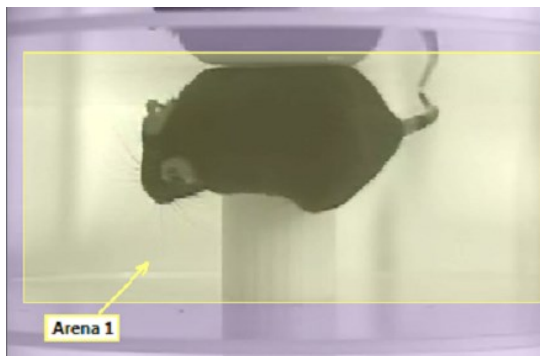
## EXPERIMENT SETTINGS

Select the number of cylinders as **Number of Arenas**.

## ARENA SETTINGS

Choose **Setup > Arena Settings > Open Arena Settings 1**.

After calibrating, click **2. Select Shape and Draw Arena**. The arena covers the region around the rod. make sure that the animal is always within the arena boundary.

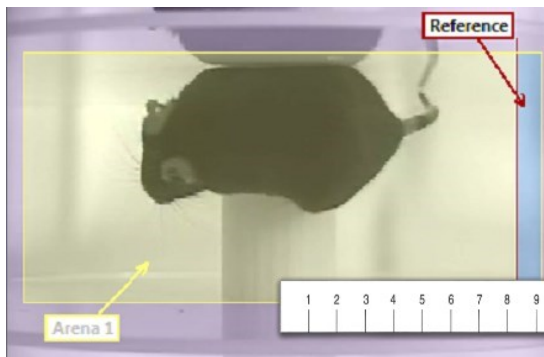


## Zones

Click **3. Select Shape and Draw Zones**.

To quantify the position of the subject's center point relative to the center of the rod, define a zone (Reference zone) at known distance

from the rotation axis of the rod. For example, draw a line (Reference line) at the right margin of the arena, and define a zone.



You can then analyze the distance of the subject's center point from the border of this zone. If you know that the border of the zone is for instance at 10 cm from the rod axis (Reference distance), then you can back-calculate the relative distance of the subject-center from the axis. The values of *Distance to zone* (border) diminished by the Reference distance will be either positive (when the subject moves forward on the rod) or negative (when it moves backward).

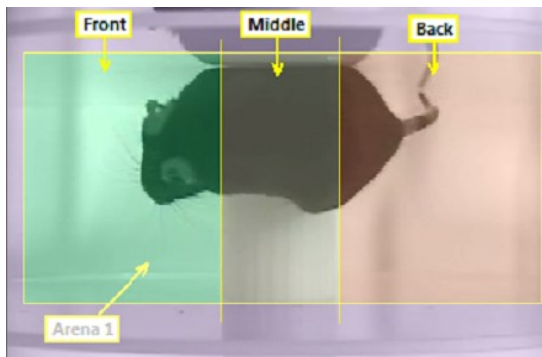
To measure the reference distance exactly, place a ruler on top of the rod, and refresh the background image. Then draw the Reference line at a specific distance from the rod.

Note: do not define the Reference zone in the middle of the rod. If you do that, distance from the zone border will always be positive, no matter what the position of the subject is.

### ***Zones for turnaround behavior detection***

To automate turnaround behavior (mice grip themselves to the rod and turn around without falling off), you can define zones in front and at the back of the rod.

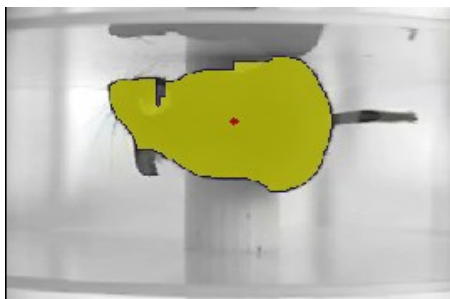
Divide the arena in three main zones: Middle (the rod area), the front and the back areas just outside of the rod.



It is then possible to detect turnaround behavior with the following sequence in the *Zone transition* variable: Middle (mouse in normal position) > Back > Front (The mouse reappears in view) > Middle (see **Data analysis** on page 269).

## DETECTION SETTINGS

Click **Advanced**. Under **Subject Contour**, select one or more pixels for the first **Erosion** and **Dilation** filter. Increase the first **Erosion** until the subject's tail is not detected anymore. Then, increase **Dilation** until the entire body is detected.



# Acquiring data

## *Training phase*

This phase lasts typically three days. Each subject is placed on the rotarod for a maximum of 1 minute. The trial is repeated a few times each day, with 5 to 10 minutes inter-trial interval.

## *Testing phase*

Each subject is placed on the rotarod for a maximum of 1 minute. The trial is repeated two times with 5 to 10 minutes inter-trial interval.

See also **ACQUIRING TRACKS** on page 21.

## *Scoring behaviors manually*

If you want to score when the animal falls off the rod, define the behavior *Falling off* under **Manual Scoring Settings**. After you have started the trial, watch the subjects in the video and in the **Manual Scoring** tab at the bottom of the screen, click the button for the arena (subject) that applies.



# Data analysis

Choose **Analysis > Analysis Profile > New**.

## ***Mouse position***

In the Analysis profile, choose **Distance to zone** and select the Reference zone (see page 265).

## ***Turnaround behavior***

In the Analysis profile, click the button next to **Zone transition**.

Select the following sequence: *Middle > Back > Front > Middle*.

The zones must be defined in the Arena Settings (page 266).

## ***Latency to fall***

If the camera is placed in front of the rotarod apparatus, you can measure the latency to fall by using the *In zone* variable. This calculated the time that the animal was detected in the arena (that is, until it fell off the rod).

See also **CALCULATE THE STATISTICS** on page 26.

## Chapter 13

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# The Y-maze Test

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# Introduction

## THE Y-MAZE

The Y-maze is constructed with three arms of equal length that extend from a central platform at a 120° angle. In the basic version, the animal is free to explore the maze arms. In a modified version, it is possible to close off some arms either to keep the animal in a specific arm for a fixed time once it has entered that arm, or to limit exploration to some arms, not others.



**Figure 13.1** A Y-maze setup with automatic doors in each arm.

The Y-maze is generally used to investigate *alternation behavior*, where animals are supposed to alternate choices of maze arms on successive opportunities. Irrespective of the precise function of alternation, the animal must remember which arm it had entered on a previous occasion to enable it to alternate its choice on a following trial. Therefore, the use of the Y-maze is based on the assumption that, following some pharmacological manipulation, it is primarily modified memory processes that are reflected in changed alternation rates.

## SPONTANEOUS ALTERNATION

### *Definition*

Spontaneous Alternation is a behavioral test for measuring the willingness of rodents to explore new environments. Rodents typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited. Many parts of the brain, including the hippocampus, septum, basal forebrain, and prefrontal cortex--are involved in this task (Conrad et al. 1996).

### *Classical procedure*

The animal is released in the center of the maze, and allowed to freely explore the three arms (A, B, C). Over the course of multiple arm entries, the subject is expected to show a tendency to enter a less recently visited arm ( $A > B > C > A > \dots$ ). The observer records the number of arm entries and the number of visits to the three arms without a re-visit in between; for example,  $A > B > C$ ,  $B > C > A$ , etc.) in order to calculate the percentage of alternation. An arm entry is scored when all four limbs are within the arm.

### *Calculation of Alternation index*

Given a zone sequence:

$A > B > C > B > \dots$

An *alternation* is defined as multiple entries into the three different arms on overlapping triplet sets. In this example the first three visits are an alternation:

$A > B > C$   $> B > \dots$

The next overlapping triplet set,  $B > C > B$  is not an alternation.

The *Alternation index* is given by the number of alternations divided by the maximum number of possible alternations, that is, the number of alternations that would be obtained if the animal visited the zones in the sequence:  $A > B > C > A > B > C \dots$

For a 3-arm maze, like the Y-maze, The maximum number of alternations is equal to total number of visits minus 2.

Alternation index is expressed in percentage:

$$\text{Alternation index} = \frac{\text{Number of alternations}}{\text{Total nr. of zone visits} - 2} \times 100$$

### ***Spontaneous Alternation in EthoVision XT***

You can easily calculate the Alternation index using the outputs **Alternations** and **Max Alternations** in the **Zone alternation** dependent variable. See page 281.

For more information, see **Zone alternation** in the EthoVision XT Help.

## **DELAYED ALTERNATION**

The delayed alternation task allows assessing spatial working memory in a T- or Y-maze. The main difference with spontaneous alternation is that performance is evaluated between trials.

### ***Classical procedure***

In the first trial of the test, the animal is placed at the end of the start arm and has to choose between the two other arms that are baited. Once the choice made, the subject is removed and after a variable delay, is returned in the start arm. In this second trial, the one arm baited is now the opposite arm to which chosen during the first trial. The animal has to make a different choice than its first one (correct choice) to get the reward.

### ***Delayed Alternation in EthoVision XT***

For all tests where maze arms can be considered as targets of a choice, and others non-targets (errors), you can use outputs **Target first visits** and **Total errors** in the **Target visits and errors** dependent variable. See page 281.

For more information, see **Target visits and errors** in the EthoVision XT Help.

## REFERENCES

Conrad, C.D., Galea, L.A., Kuroda, Y., McEwen, B.S. (1996). Chronic stress impairs rat spatial memory on the Y maze, and this effect is blocked by tianeptine pretreatment. *Behav Neurosci* **110**: 1321–1334.

Conrad, C.D., Lupien, S.J., Thanasoulis, L.C., McEwen, B.S. (1997). The effects of type I and type II corticosteroid receptor agonists on exploratory behavior and spatial memory in the Y-maze. *Brain Res.* **759**, 76 – 83.

Hughes, R.N. (2004) The value of spontaneous alternation behavior (SAB) as a test of retention in pharmacological investigations of memory. *Neurosc. Biobehav. Rev.* **28**, 497–505.

### ***EthoVision XT and the Y-maze test***

Detrait, E., Brohez, C., Hanon, E., De Ryck, M. (2010). Automation of Continuous Spontaneous Alternation to Increase the Throughput for In Vivo Screening of Cognitive Enhancers. Optimization of the Ethovision System for the Y-maze Test in Mice. *Proceedings of Measuring Behavior 2010 (Eindhoven, The Netherlands, August 24-27, 2010)*, 141-144.

For a complete list of publications, see the results of [Google Scholar](#).

# The Y-maze test in EthoVision XT

Create an experiment. For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help.

For suggestions about optimize lighting, see page 87.

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

- **Number of Subjects per Arena: 1.**
- **Tracked Features: Center-point detection or Center point, nose-point and tail-base detection.**
- If you want to use Deep learning technique to track the subject's nose, under **Body point detection technique** choose **Deep learning**. Note that in order to use this technique there are additional requirements. Among other things, you need a powerful graphics card. See **Deep learning: Requirements and Limitations** in the EthoVision XT Help.

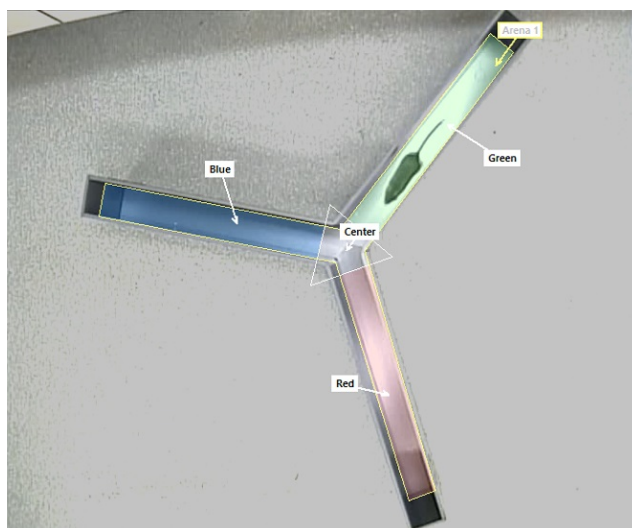
## ARENA SETTINGS

We assume that you followed the procedure in **ARENA SETTINGS** on page 15.

*For the Spontaneous alternation task*

1. Choose **Setup > Arena Settings > Open Arena Settings 1**.
2. Choose the video / camera image and click **Grab**.
3. Click **2. Select Shape and Draw Arena**. Resize and move the outline of the arena to make it overlap with the Y maze image.

For details on how to resize and move the outline the arena, see **ARENA SETTINGS** on page 15 and **Arena Settings** in the EthoVision XT Help.



**Figure 13.2** *Example of arena and zones in a Y-maze for spontaneous alternation. Zones are highlighted with different overlay colors.*

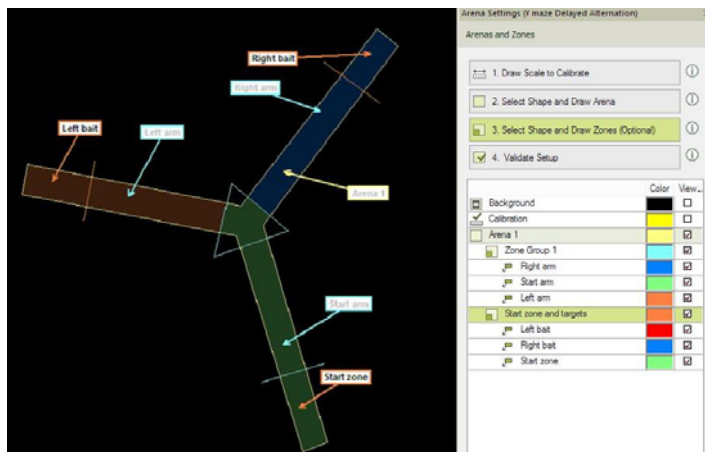
### ***For the Delayed alternation task***

Choose **Setup > Arena Settings > New**.

After calibrating and drawing the outline of the arena, click **3. Select shape and draw zones**. Draw the three arm zones. To draw the start box zone and the target zones (that is, the arm ends which will be baited with food), create an additional zone group (Figure 13.3).

For details on how to work with zone groups, and draw and edit shapes, see **Arena Settings** in the EthoVision XT Help.





**Figure 13.3** Example of arena and zones in a Y-maze for delayed alternation. On the right, the Arena Settings window shows two zone groups: **Zone Group 1** with the zones **Left arm**, **Right arm** and **Start arm** (these zones are like in the Arena Settings for spontaneous alternation), and **Start zone and targets** with the target zones (**Left bait** and **Right bait**) and the **Start zone**.

## TRIAL CONTROL SETTINGS

In the Trial Control Settings you can define conditions for the start and stop of the data acquisition.

Choose **Setup > Trial Control Settings > New**.

### Default Trial Control

The templates for the Y maze contain the following Trial Control Settings profiles:

- **Default.** Tracking starts when the subject's center point is detected for 1 second in the arena. Tracking stops at the end of the video or when you give the stop command.

- **Tracking Duration 5 min.** Tracking starts when the subject's center point moves from the start box to the center arm. Tracking stops after 5 minutes.

## DETECTION SETTINGS

Choose **Setup > Detection Settings**.

We assume that you followed the procedure in **DETECTION SETTINGS** on page 17.

Check in the **Video** Section that the sample rate is set to:

- For rats: 5 samples/second.
- For mice: 12.5-15 samples/second.

However, when tracking the nose-point, center-point and tail-base point of the rodents, always choose the maximum sample rate.

If you selected to use the Deep learning technique to track the subject's nose, under Method next to **Deep learning** click **Define** and select a box around the subject. Make sure that the box includes the subject's nose. Try not to include objects like walls, especially if they are the same color as the subject. For details, see the EthoVision XT Help.

## TRIAL LIST

### *Independent variables*

If you perform a Y-maze test where one of the arms is closed off or provided with food, it is always a good idea to create independent variables that mark the type of trial, and which you can use to select a subset of trials for answering a specific question. For example, create the independent variable *Correct choice* with possible values *Yes* and *No*, depending on whether the subject chose the arm not visited in the previous trial. At the end of each trial, enter the value for that subject. You can use this variable to filter the trials based on the value of *Correct choice*.

# Acquiring data

## PROTOCOLS

### *Continuous Spontaneous Alternation*

1. Release the subject in the center of the Y maze.
2. Start the trial.
3. Let the subject explore the maze for the time required (typically 5-6 minutes). Then, stop the trial.
4. Put the subject back in its home cage. Proceed with the next subject.

### *Forced-choice Alternation*

In this variant, the subject is forced to explore one of the two arms in the first trial.

1. Close one of the arms of the maze. Place the subject into the start arm, and allow it to explore the maze for 15 minutes.
2. After a fixed inter-trial interval, open the door in the blocked arm. Return the subject to the maze by placing it in the start arm. The subject is allowed to explore freely all three arms of the maze for 5 min. The Y-maze is often rotated between the two trials.

### *Two-trial Spontaneous Alternation*

In this task, the subject is confined for a fixed time in the T- or Y-maze arm chosen in the first trial. This allows to manipulate the time that the animal has experience with one of the arms.

1. In the first trial, let the subject access one of the arms after having left the start arm. Once an arm has been entered, close off that arm by lowering a guillotine door behind the subject. Leave the subject there for a fixed time (this is called *intra-trial interval*).
2. Remove the subject and, after an *inter-trial interval*, let it choose the arm in a second trial. This two-trial sequence is repeated a number of times.

### ***Delayed Alternation***

This task is usually designed with a T- or a Y- maze where one arm is the starting location, and the other arms are baited.

1. Release the subject in the start arm. Once the subject enters one of the other two arms, close the door for that arm (trial 1).
2. After a predetermined inter-trial interval, release the subject and let it freely choose one of the two arms (trial 2).

If the subject chooses the alternate arm, this is scored as alternation. This two-trial procedure is repeated several times.

See also **ACQUIRING TRACKS** on page 21.

# Data analysis

## ANALYSIS PROFILES

Choose **Analysis > Analysis Profile...**

The template experiment contains the analysis profiles:

- **Distance, Time & Movement.** To calculate the total *Distance moved* (a measure of activity of the animal), the mean *Velocity*, and the total time spent in the to states of *Movement* (moving and not moving). The group means with their standard errors are also calculated.
- **Zone alternation.** with the variables *In zone* (to calculate the frequency, total time and latency to first visit for each zone; and *Zone alternation*, to calculate the number of alternations and the maximum number of alternations for that set of zone visits (see below).

See the section below to create analysis profiles for specific tasks.

## ALTERNATION BEHAVIOR

### *Spontaneous alternation*

Choose **Analysis > Results > Statistics and Charts.**

Select the Analysis profile that contains *Zone Alternation* variable.

Locate the column **Zone Alternation** in the table.

To calculate the Alternation index, divide **Alternations** by **Max alternations** and multiply this by 100.

To change the Zone alternation definition, open the Analysis profile **Zone Alternation** and double-click the variable **Zone alternation**. Here you can specify which statistics you want to have. You can also enter a **Zone exit threshold**, to remove false re-entries in a zone from the

statistics, due to small movements of the body point near the zone borders.

For more information, see **Zone alternation** in the EthoVision XT Help.

### ***Delayed alternation***

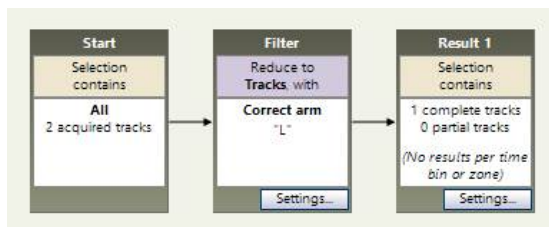
For delayed alternation tests, *Zone alternation* is not the correct dependent variable. To analyze correct and incorrect choices, use *Target visits and errors* to compare performance *between* trials. Because the target arm changes between trials (for trial 2, the target is the arm which was not visited in the previous trial), you must define multiple instances of *Target visits and errors*.

Whether an arm can be defined as “target” depends on the outcome of the previous trial. If the subject went to the Left arm in trial x, then for trial x+1 the target should be the Right arm, and a revisit to the Left arm should be scored as an error.

To do this, in the Trial List (**Setup > Trial List**) mark each trial with a variable which states which arm was the correct choice. By definition, the 1st trial of each subject has no “correct” choice (the values for subsequent trials depend on what the animal does in that trial).

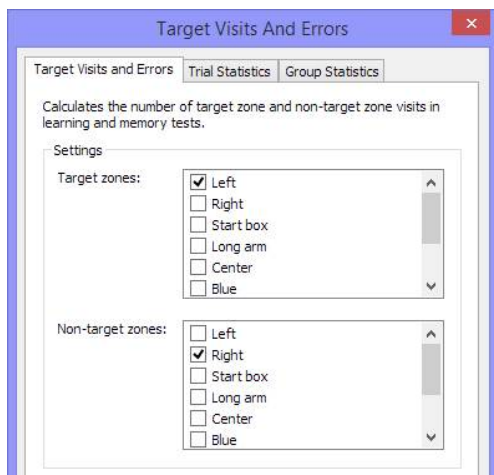
		User-defined
Label		Correct arm
Description		
Type		Text
Format		
Predefined Values		<input type="text" value=""/>
Scope		Subject
Trial	Arena	
Trial 1	Arena 1	
Trial 2	Arena 1	L
Trial 3	Arena 1	R
Trial 4	Arena 1	L
Trial 5	Arena 1	R
Trial 6	Arena 1	L

In the Data Profile, filter the trials based on this variable. Make two Data profiles, one for “Right arm correct” and one for “Left arm correct”.



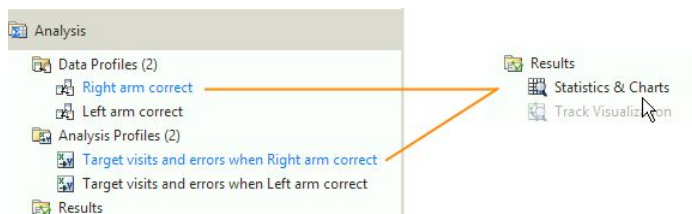
Next, create two analysis profiles. For example:

- Analysis profile *Target visits and errors* for when the target (correct choice) was the Left arm. The Right arm is a non-target; visits to that arm are scored as errors.



- Analysis profile *Target visits and errors* for when the target (correct choice) was the Right arm.

When you analyze the data, make sure to combine a Data profile with the correct Analysis profile.



## VISUALIZING DATA

Choose **Analysis > Results > Integrated Visualization**.

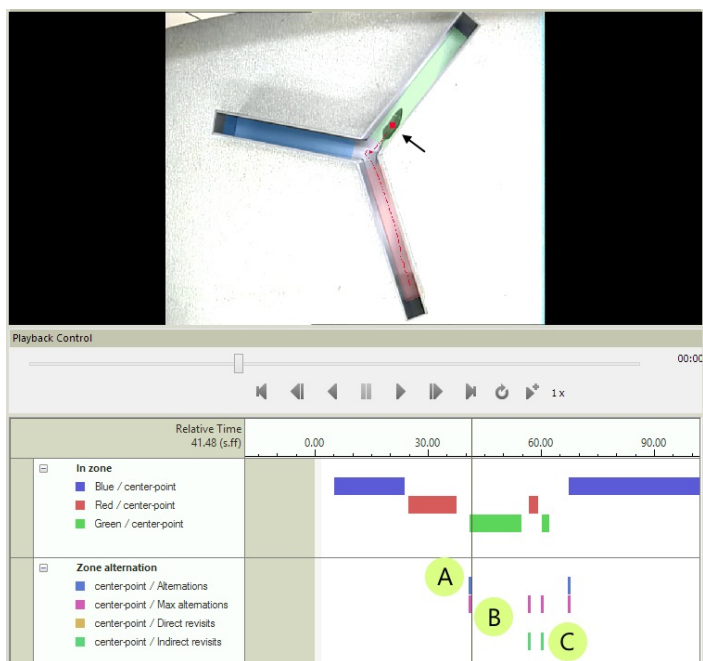
Figure 13.4 shows an example of visualization of spontaneous alternation behavior.

The first plot shows the variable *In zone* defined in the Analysis profile; there you can see which arms were visited during the trial.

The second plot is of *Zone alternation*. The subject visits first the zone marked in blue, then that in red, and when the subject enters the green -marked zone, an alternation is completed. This is scored as **Alternations** (blue vertical segment; A). At every visit except the last two in a trial, also **Max Alternations** is scored (purple vertical segment; B). This marks the moment a possible alternation could have occurred. This variable is useful to calculate the Alternation index (see page 281).

The plot also includes direct revisits (for example, green > green) or indirect revisits (green > red > green; C). The more revisits, the lower the Alternation index.





**Figure 13.4** Integrated visualization of the dependent variables *In zone* and *Zone alternation* for a Y-maze test. See explanations in the text.

## Chapter 14

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# Optogenetics Experiments

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# Introduction

## OPTOGENETICS AND ETHOVISION XT

In the last decade, a method was developed to specifically activate or even inhibit small groups of neurons with light. Today, scientists can insert light-sensitive receptor proteins (originally found in algae) into single mammalian neurons *in vivo*, making these neurons sensitive to activation by light of specific wavelengths. This allows scientists to control the activity of these neurons and study their downstream influence on a variety of biological processes [ref. 1, 2].

Optogenetics is an important development for behavioral research. Not only can the actual influence of specific neurons on behavior more specifically be determined, but also, with the help of an automatic video tracking system such as EthoVision XT, behavior can be manipulated via optogenetic methods in real time. With the Trial & Hardware Control module, EthoVision XT can control light pulses given by a third party device, based on the behavior of the animal (for instance, presence in a trigger zone).

In this manual we focus on the optogenetics solution for rodents that combines EthoVision XT, PhenoTyper (as test environment) and the Prizmatix optogenetics devices. For how to conduct optogenetics experiments with zebrafish, see the DanioVision DVOC-0041 - Reference Manual.

## EXAMPLES

### *Place preference*

The animal is placed in an arena with two distinct sides - one of which is paired with optogenetic stimulation. Depending on whether this stimulation is activating or deactivating, and which neurons are affected, this can be a rewarding or aversive stimulus for the animal. If in further sessions the animal spends more time on the stimulated sides, it is fair to conclude the stimulus had a rewarding effect. If the

animal avoids this side, the stimulus could then be considered aversive [ref. 3,4].

### ***Operant conditioning***

In operant conditioning tests, the animal learns to perform an action in order to get a reward or avoid an aversive stimulus. The behavioral response can be recorded with EthoVision XT, either by detecting the animal (or its nose point) in a certain zone, such as the feeder, or by analyzing the external signal initiated by the nose poke or lever press. Following this action, EthoVision XT sends out the command for optogenetic stimulation [ref. 3,4].

### ***Off-on-off stimulus test***

In an off-on-off stimulus test, you can program EthoVision XT to turn optogenetic stimulation on for a period of time during the test. For example, a 15 minute test during which continuous optogenetic stimulation takes place in the middle 5 minutes.

## **REFERENCES**

1. Guru, A., Post,R.J., Ho, Y., Warden, M. (2015). Making Sense of Optogenetics. *International Journal of Neuropsychopharmacology*, Aug 2015, 1–8.
2. Zalocusky, K., Deisseroth, K. (2013). Optogenetics in the behaving rat: integration of diverse new technologies in a vital animal model. *Optogenetics* 2013: 1-17.
3. Stamatakis, A.M., Stuber, G.D. (2012). Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. *Nature Neuroscience*, **15**(8), 1105-1107.
4. Kravitz, A.V., Tye, L.D., Kreitzer, A.C. (2012). Distinct roles for direct and indirect pathway striatal neurons in reinforcement. *Nature Neuroscience*, **15**, 816-818.

# Physical setup

## COMPONENTS (EXAMPLE)

### *Devices*

- PC with EthoVision XT installed.
- Noldus USB-IO Box.
- PhenoTyper with adapted Top Unit (optional).
- Prizmatix LED controller (for different light wavelengths).



**IMPORTANT** To control the LED through EthoVision, on the back panel of the LED controller, near/below the TTL connector, select **Ext** (but if you want to activate the LED manually using the button located on the front panel, you should select **Int**). The other switch on the LED controller, just near/above the **Ain 0-5V** connector, should be in the **Int** position.

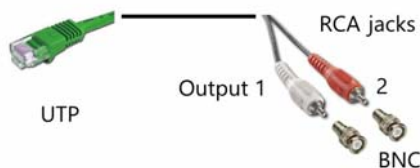
- Prizmatix Pulser (left) or PulserPlus (right) (with advanced setup).



### *Main cables and fiber cords*

(in bold, port names; power cords and video cables not listed)

- USB cable (USB-A to USB-B). Use this cable to connect the EthoVision XT PC to the USB-IO box [**USB**, type-B]. Also use this cable to connect the Pulser to the PC.
- UTP to BNC cable. To connect the USB-IO box [**TTL control**] to the LED Controller [**TTL**] or Pulser [**Trig. In**].



**NOTE 1** Each TTL port of the USB-IO box has two output lines. The white RCA jack corresponds to **Output 1** and the red RCA jack to **Output 2**. Take note of this when selecting the TTL commands in EthoVision XT page 299).

**NOTE 2** If you received UTP-to-BNC cables with four BNC connectors, only use those marked with **Output** to connect the LED controller or Pulser.

- BNC to BNC cable. From Pulser [**TTL Out**] to LED Controller [**TTL**].



- Fiber patch cord, core 1000  $\mu\text{m}$ . To connect the LED Controller [front panel] to the PhenoTyper Top Unit [Fiber Coupler adapter, see Figure 14.2B-C].
- Fiber patch cord, core 1000. This is located inside the PhenoTyper Top Unit. It connects the FC adaptor to the Rotary joint.

- Single fiber. To connect the Rotary joint fixed on the bottom plate of the PhenoTyper Top Unit to the implantable cannula. Available in various core diameters (e.g. 500  $\mu\text{m}$ ).

### ***Other components***

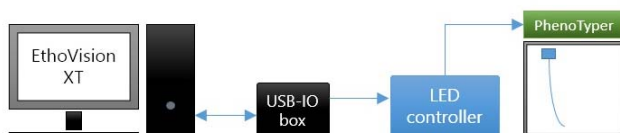
- Zirconia ferrules (sleeves) for 2.5 mm and 1.25 mm cannulae.
- Optogenetics implantable cannulae (2.5 mm, 1.25 mm).

## **BASIC SETUP**

### ***Aim***

With a basic setup, without the Pulser, EthoVision XT controls activation of the LED directly via the Noldus USB-IO box. With this setup you can, for example, activate the LED for one second.

### ***Functional scheme***



**Figure 14.1** *Activation of the optogenetics fiber directly from the USB-IO box.*

### ***Notes***

- Activation of the LED can be repeated during a trial using the Subrule function in the Trial Control Settings.
- For generating two pulses of different length, you must create separate Trial Control commands.

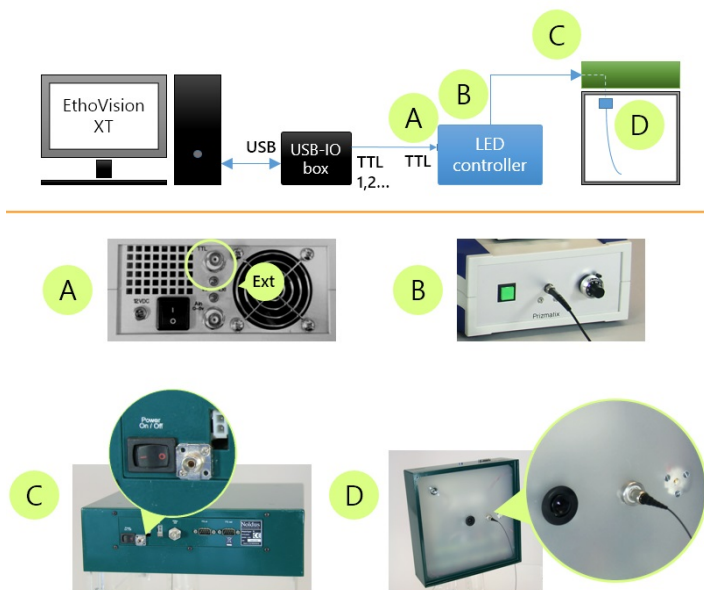
### ***Limitations***

- The pulse cannot be shorter than 0.1 s.

- Accuracy of onset, offset and duration of a pulse is approximately the time taken by three samples. When tracking at 30 fps, this time is 0.1 s. The higher the sample rate, the higher the accuracy.

For the advanced setup, see page 293.

### Connection scheme



**Figure 14.2** Cable connections for controlling the LED controller directly from EthoVision XT and the USB-IO box. **A.** Back panel of the LED controller. Under the TTL connector there is a switch; set it to **Ext**. This way you enable external control of the LED. Note that each TTL port of the USB-IO box has two output lines. Connect the LED controller to the USB-IO box using the white RCA jack if you use Output 1, or with the red jack if you use Output 2 (see page 290). The other switch on the LED controller, just near/above the **Ain 0-5V** connector, should be in the **Int** position so you can manually adjust the light intensity using the knob on the front panel. **B.** Front panel of the LED controller with patch fiber cord connected. **C.** PhenoTyper Top Unit adapted for connecting the LED controller. **D.** Bottom plate of the PhenoTyper Top Unit with Rotary joint and optical fiber.



**NOTE** If you received UTP-to-BNC cables with four BNC connectors, only use those marked with **Output** to connect the LED controller.

## ADVANCED SETUP (WITH PULSER)

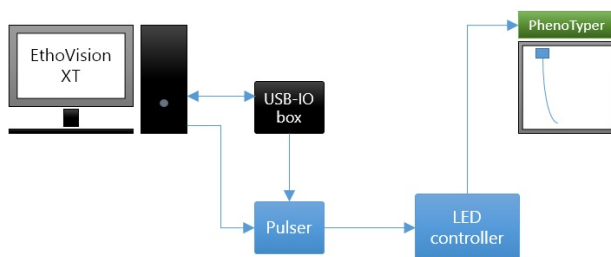
### *Aim*

With this setup, EthoVision XT controls activation of the LED via the Prizmatix Pulser and PulserPlus devices. With this setup you can send out a very specific sequences of pulses, at a frequency higher (max 0.5 kHz) than with the basic setup. The sequence is predefined in the Pulser/PulserPlus (see page 305).



**NOTE** PulserPlus is the device with three BNC connectors.

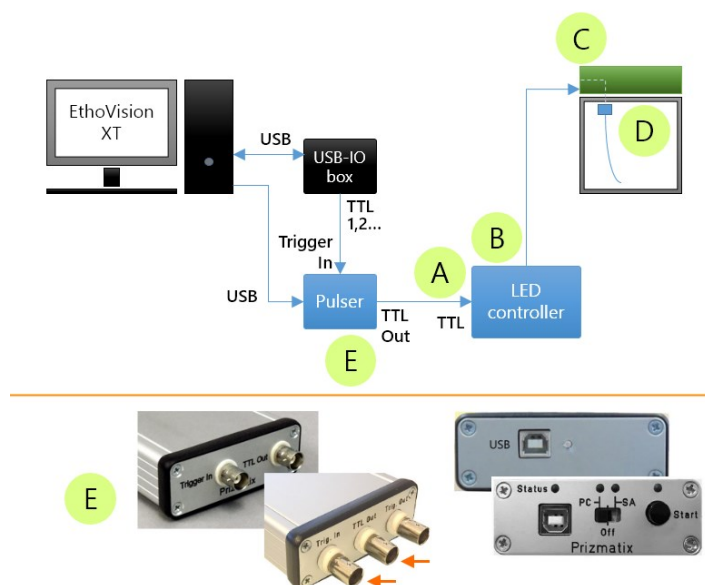
### *Functional scheme*



**Figure 14.3** Activation of the optogenetics fiber from EthoVision XT with a train of pulses programmed in the Prizmatix Pulser or PulserPlus.

**NOTE** For information about how to use the Pulser with its own software, see the Prizmatix Pulser/PulserPlus user manual which comes with the device.

## Connection scheme



**Figure 14.4** Cable connections for controlling the Pulser and the LED controller. For **A** to **D**, see Figure 14.2. On the LED controller, make sure that the switch near/below the TTL connector is in the **Ext** position. Also keep the switch near/above the **Ain 0-5V** connector in the **Int** position.

**E.** Front and back panel of the Pulser/PulserPlus. Note that each TTL port of the USB-IO box has two output lines. Connect the **Trigger in / Trig. In** port of the Pulser/PulserPlus to the USB-IO box with the white RCA jack if you use Output 1, or with the red jack if you use Output 2 (see page 290). Connect the Pulser/PulserPlus to the PC with a USB cable.

## Notes

- If you received UTP-to-BNC cables with four BNC connectors, only use those marked with **Output** to connect the **Trigger In/Trig. In** port on the Pulser/PulserPlus.

- **IMPORTANT** If you have the PulserPlus, set the Operation mode switch to **PC**. For more information on how to install the Pulser/PulserPlus, see the user manual that comes with the device.
- For more information and connection schemes, see also the section **The DanioVision Optogenetics add-on** in the DanioVision DVOC-0041 - EthoVision XT 18 - Reference Manual.

## MEASURE THE LED LIGHT OUTPUT

In some circumstances you may want to measure the LED light intensity at the tip of the cannulae.

For this you need a sensor and a power meter. Note that a standard power meter may not give accurate measurements. See this link for a few suggestions.

<https://www.prizmatix.com/blog/main/o/23/Optical-Power-Meters-for-Optogenetics>

An example is the Thorlabs S140C or Thorlabs S142C coupled with the Thorlabs PM100USB interface which shows the measurements on a computer screen.

See also [https://www.thorlabs.com/navigation.cfm?guide\\_id=37](https://www.thorlabs.com/navigation.cfm?guide_id=37)

# Optogenetics experiments in EthoVision XT

Create an experiment. For details, see Chapter 1 of this manual, or in EthoVision XT press **F1** and see **Setup an Experiment** in the EthoVision XT Help.

If you use PhenoTyper, you can create an experiment from a PhenoTyper template. Choose **File > New from Template** and in the guided wizard, from the **Arena template** list, choose **PhenoTyper** (for one cage) or **PhenoTyper quad** (for two to four cages).

## EXPERIMENT SETTINGS

Choose **Setup > Experiment Settings**.

### *General settings*

- Set **Video Source** to **Live Tracking** and select the camera image.
- Choose the **Tracked Features** that apply to your experiment.

### *Use of Trial Control Hardware*

1. Under **Trial Control Hardware**, Select **Use of Trial Control Hardware**.
2. Click **Settings** and choose **Noldus USB-IO box**.
3. In the window that appears, locate the **TTL port** of the USB-IO box that is connected to the LED controller, and from the **Device type** list choose **Custom Hardware**. Under **Device ID** give this device a unique name (for example *LED controller 1*, or *Pulser* if you use the Prizmatix Pulser/PulserPlus).
4. Repeat the previous step if you have more LED controllers or Pulsers.

**IMPORTANT** Make sure that **Custom Hardware** is selected in the Device Configuration window, not **TTL Tester** or others.

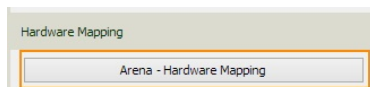
## ARENA SETTINGS

Choose **Setup > Arena Settings > Open Arena Settings 1**.

We assume that you followed the procedure in **ARENA SETTINGS** on page 15.

### *Arena-Hardware Mapping*

1. Make sure that you have drawn all the arenas.
2. At the bottom of the **Arena Settings** window, click the **Arena-Hardware Mapping** button.



3. In the **Arena-Hardware Mapping** window, click **Add device** one or more times depending on how many physical devices (LED controllers or Pulsers) are connected.
4. Assign the devices to the arenas.

For setups with one cage (and one device), that is automatically assigned to Arena 1.

Device type	Hardware interface type	Device name	Arena 1
Custom Hardware	Noldus USB-IO Box	LED controller 1	LED controller 1

For setups with multiple cages (and devices), assign each device to the corresponding arena.

5. For each row, rename the **Device name** (for example, rename *Device A* to *LED controller 1* or *Pulser 1*).

**TIP** Give devices unique names. That is also handy when analyzing the data and events of specific devices.

### *To test the LEDs*

**NOTE** This procedure is only valid for the Basic setup (without Pulser).

1. In the **Arena-Hardware Mapping** window, Click the row that corresponds to the device you want to test and click the **Test** button.
2. Choose **Output 1 High** and click **Test**. Check that the LED is activated.
3. Choose **Output 1 Low** and click **Test**. Check that the LED is deactivated.

## TRIAL CONTROL SETTINGS

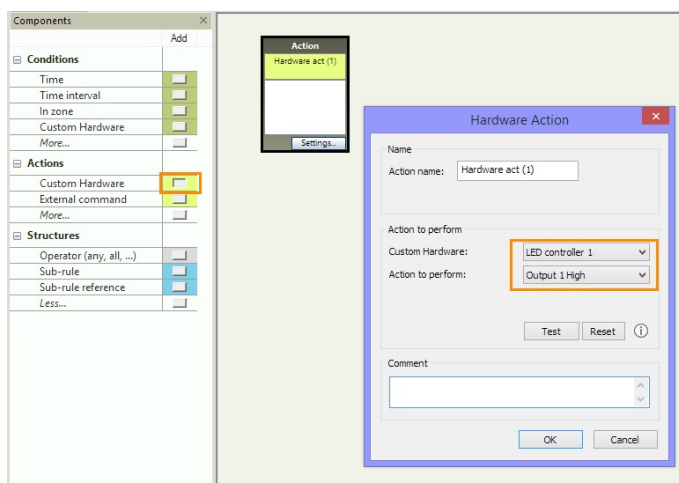
In the Trial Control Settings you can program activation and deactivation of the LED controller during the trial. If you use the Prizmatix Pulser, see page 305.

Choose **Setup > Trial Control Settings**.

Below you find a simple example. It shows how to switch on a LED one minute after the start of the trial, keep the LED active for one second, and then switch it off.

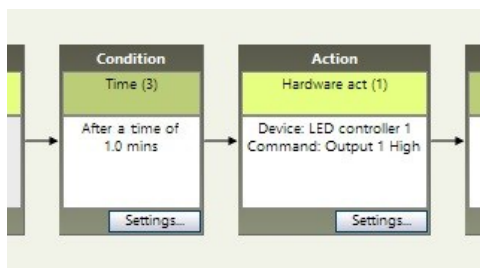
### *To switch on the LED*

1. Under **Actions**, click the button next to **Custom Hardware**.
2. From the **Custom Hardware** list select the LED controller device (this is the name given under **Device name** in the **Arena-Hardware Mapping** window; see page 298).
3. From the **Action to perform** list select **Output 1 High** (or **Output 2 High** if you connected the USB-IO box to the LED controller using the cable with the red RCA connector - see page 290).
4. **OPTIONAL** Rename the **Action name** (for example, rename *Hardware act (1)* to *Start Pulse*).



5. Click **OK** and insert the new **Action** box in the Trial Control sequence, usually after a condition.

In the example below, the stimulation is given one minute after the start. Therefore, a **Time Condition** box is inserted between the **Start track** box and the **Action** box just made.



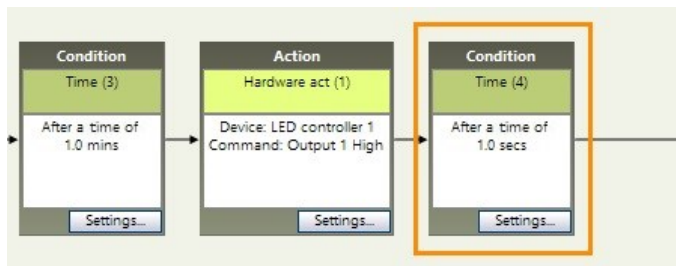
**NOTE** With this Trial Control procedure, the stimulus does not stop unless you insert another **Action** box that specifies **Output 1 Low** (see below).

### ***To switch off the LED after some time***

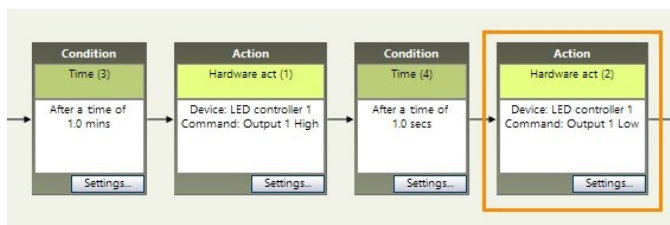
1. Follow the instructions on the previous page to program activation of the pulse.



2. Under **Conditions**, click the button next to **Time**. Select **After a time of** and enter the duration of the pulse (minimum possible: 0.1 s). Next, insert the **Condition** box immediately after the **Action** box.



3. Under **Actions**, click the button next to **Custom Hardware**.
4. From the **Custom Hardware** list select the LED controller device (this is the name given under **Device name** in the **Arena-Hardware Mapping** window; see page 298).
5. From the **Action to perform** list select **Output 1 Low** (or **Output 2 Low** if you connected the USB-IO box to the LED controller using the cable with the red connector).
6. **OPTIONAL** Rename the **Action name** (for example, from *Hardware act (2)* to *Pulse Off*).
7. Click **OK** and insert the new **Action** box after the second Time **Condition** box.



The sequence can be read as follows:

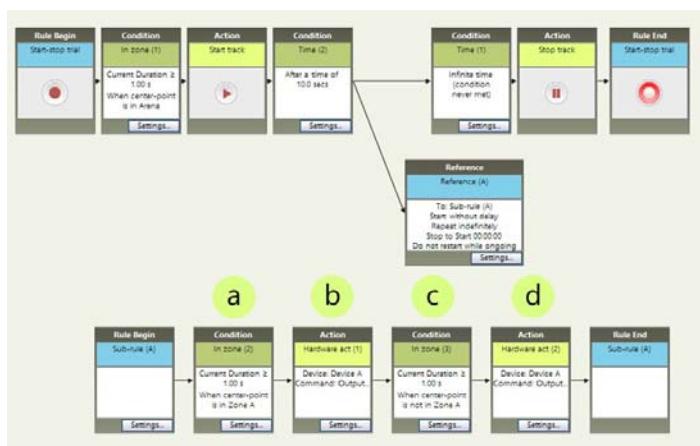
One minute after the start of tracking (first box), activate the LED controller (second box), then wait one second (third box) and deactivate the LED controller (fourth box).

### ***To activate the LED when the animal is in a zone***

It is often required to activate the LED multiple times, for example, every time the subject enters a trigger zone, or every time the subject presses a lever.

In such a situations you need to create a *Subrule* that can be repeated indefinitely. The Subrule contains the following instructions (see the next picture, from left to right):

- Check that the subject is in the zone (or the lever is pressed) (a). In this example the condition is based on “Current Duration”, that is, it becomes true when the subject is found in the zone for a specific time.
- When the previous condition is met, turn the LED on (b; for details, see page 299).



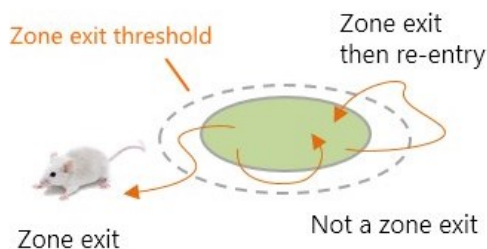
**Figure 14.5** Trial Control Settings for activating the LED when the subject is in a specific zone. **a.** Condition box that checks if the animal is in the zone. **b.** When the condition **a** becomes true, then the Action box activates the LED. **c.** Condition box that checks if the animal is out of the zone. **d.** When the condition **c** becomes true, then the Action box switches off the LED. Next, the Subrule starts again with condition **a** being evaluated. The Reference box (at the top) specifies that the Subrule is repeated indefinitely until the end of the trial.

- Check that the subject is out of the zone (or presses another level/ performs an action to be linked to the end of stimulation) (c).
- When the previous condition is met, turn off the LED (d).

**NOTE** You can also base the “In Zone” conditions on the statistic “Current”. However, in such case when the subject walks along the zone border its center point will jitter between somewhere outside the zone and inside the zone. For this reason the “Current” value of the In Zone variable will rapidly change from *true* to *false* and vice versa, therefore the conditions based on this variable become true and false very often. To prevent this from happening, in the “In Zone” Condition window click **Settings** and enter a **Zone exit threshold**. This way the subject is still considered in the zone (Current In Zone = *true*) if its body point is found within the threshold distance from the zone border. The subject is only considered out of the zone (Current In Zone = *false*) if it crosses the threshold.

Threshold

Zone exit threshold:
cm
*i*



**TIP** Reduce the zone by a distance half of the exit threshold; for example if the exit threshold is 4 cm, reduce the zone border by 2 cm. This way you do not overestimate the size of the zone when using the exit threshold. For details, in the EthoVision XT Help see **Dependent Variables in Detail > In zone**.

## DETECTION SETTINGS

Choose **Setup > Detection Settings**.

We assume that you followed the procedure in **DETECTION SETTINGS** on page 17.

### *Set the sample rate*

In the **Video** section, check that the sample rate is set to:

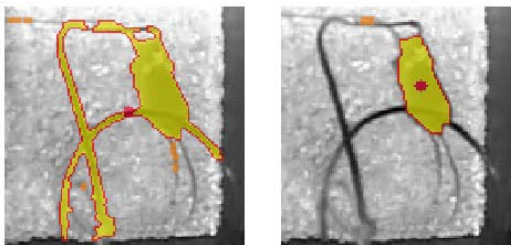
- **Rats.** 5 samples/second.
- **Mice.** 12.5 samples/second.

However, when tracking the nose-point, center-point and tail-base point of the rodents, always choose the highest possible sample rate, for example 25 or 30 samples/s, depending on your camera.

### *Remove the effect of cables and fibers*

When working with physiology and optogenetics setups, the cables and fibers often make detection of the subject impossible with the default settings (see the figure below, left).

To filter fibers out of the video image (right), use the **Subject Contour** options. If the fiber is the same color as the subject, like in the figure below (left), then increase the value in the first **Erosion** filter until the fiber is no longer detected (figure below, right). Increase **Dilation** to make the entire body fully detected. Leave the second **Erosion** filter to zero.



If the fiber has a different color and “cuts” the yellow blob in two, try first **Dilation**, and then increase the second **Erosion** filter.

# Using the Pulser

## PULSE SEQUENCES

### *Aim*

To specify a train of pulses of frequency up to 0.5 kHz.

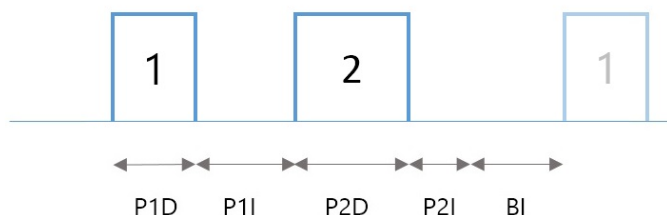
### *The Pulser software*

The Pulser/PulserPlus can send out a very specific modulated pulse sequence. You can program the Pulser by specifying a number of parameters in a command line (string command; see below). This command line is sent out from EthoVision XT to the Pulser.

### *Structure of the pulse sequence*

*Pulse train.* Two pulses (1 and 2) are organized in a Pulse train. This is described by (see Figure 14.6):

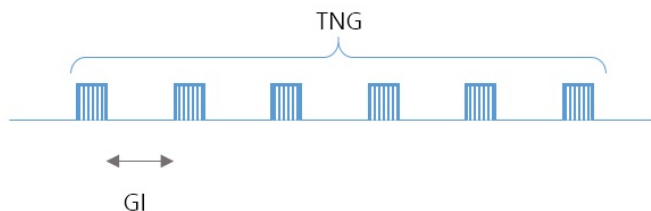
- The duration of pulse 1 (P1D).
- The interval between pulse 1 and pulse 2 (P1I).
- The duration of pulse 2 (P2D).
- The interval between pulse 2 and the end of the train (P2I).
- The interval between trains (BI).



**Figure 14.6** Parameters describing a train of pulses 1 and 2.

**Number of trains.** A pulse train can be repeated a number of times specified by the parameter TNT (see below). The total duration of the signal is given by TNT times the total duration of the other five parameters.

**Group of pulse trains.** A number of trains defined as above can be seen as a *group*. The duration of the group is defined automatically by the parameters above. Multiple groups of pulse trains are therefore defined by the interval between groups (GI) and the total number of groups (TNG).



**Figure 14.7** Parameters describing groups of pulse trains.

### Structure of the string command

echoMTNT,BI,P1D,P1I,P2D,P2I,TNG,GI,OM,NoT,ToDy,ToDu,ToIt,ToNu@>  
[COM port number]

Where

<b>echo</b>	DOS command that sends the string.
<b>M</b>	Command prefix. Do not put commas between this and the next parameter.
<b>TNT</b>	Total number of trains.
<b>BI</b>	Interval between trains (msec).
<b>P1D</b>	Duration of pulse 1 (msec).
<b>P1I</b>	Interval between pulse 1 and 2.
<b>P2D</b>	Duration of pulse 2 (msec).

<b>P2I</b>	Interval between pulse 2 and end of train (ms).
<b>TNG</b>	Total number of groups of pulse trains.
<b>GI</b>	Interval between groups (sec).
<b>OM</b>	Operational mode. For Pulser [0,1,2,3], for PulserPlus [100 to 103] where 0 (100) = Do not use the trigger input 1 (101) = Use the trigger for single sequence 2 (102) = Use the trigger. Perform the and when finished wait for the next trigger. 3 (103) = Use the trigger. Perform the sequence as long as the trigger is "high". This is the recommended option. See also the Pulser/PulserPlus User Manual.
<b>NoT</b>	Number of Triggers [1, 2,...].
<b>ToDy</b>	Delay of TrigOut from last train finish (not used by EthoVision XT).
<b>ToDu</b>	Duration of TrigOut (not used by EthoVision XT).
<b>ToIt</b>	TrigOut interval (not used by EthoVision XT).
<b>ToNu</b>	Total number of TrigOut (not used by EthoVision XT).
<b>@</b>	End of command (no comma before it)

### **Example**

In an optogenetics protocol we want to stimulate the animal with light bouts of 20 ms separated by 20 ms off. This would result in 25Hz stimulation. P1D = P2D = P1I = P2I = 20 ms.

The resulting string command we need to send to the Pulser would be (Note: OM = 3 for Pulser, 103 for PulserPlus):

```

MTNT BI P1D P1I P2D P2I TNG GI OM NoT
echo M1, 0, 20, 20, 20, 20, 1, 0, 3 1,
                                     (103),

```

ToDy ToDu TolT ToNu

o, o, o, o @

### Notes

- Although the total number of trains TNT is set to 1, the pulses are sent out continuously because the Operation mode is set to 3. In this case the pulse trains are generated as long as EthoVision XT sends the TTL signal “high”. When EthoVision XT sends the TTL signal “low”, the sequence stops.
- If one wants to stimulate the animal as long as the animal is in a specific zone, in the Trial Control Settings the TTL signal “high” must be associated with a condition based on the variable *In zone* (see an example on page 314).
- With Operation mode 3, during generation of the pulse sequence the LED is lit on continuously.
- If you have the Pulser and the older Pulser software (version 2.1) the command above would be: C echo 1,o,20,20,20,20,1,o,3,1@.

## CONFIGURE THE COM PORT FOR EACH PULSER

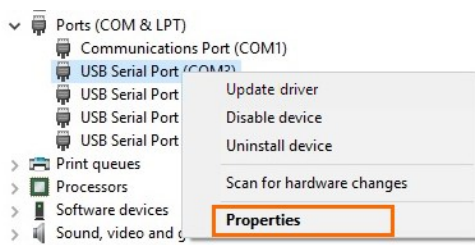
### Aim

To configure the COM port in such a way the Pulser/PulserPlus accepts commands from EthoVision XT.

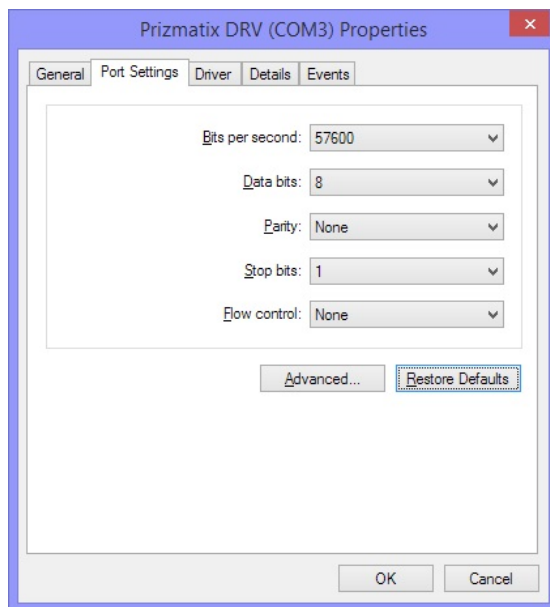
### Procedure

1. Connect the Pulser/PulserPlus to the PC using the USB cable (type-A to type-B connectors).
2. In the **Control Panel**, open the **Device Manager**.
3. Under **Ports (COM & LPT)**, right-click **USB Serial Port** and select **Properties**.





4. Click the **Port Settings** tab.
5. Make sure that the parameters are as below, then click **OK**.



6. Take note of the port number (in the example above it is COM3).
7. Repeat the procedure for each Pulser/PulserPlus.

### Notes

- The COM port number is retained in the Pulser, so if you disconnect the Pulsers and reconnect them, you do not have to assign the COM ports again.
- **TIP** To know which COM port is assigned to a Pulser/PulserPlus device, disconnect the USB cable from the Pulser/Pulser Plus. Then, re-connect it. The item that appears under **Ports (COM & LPT)** indicates the COM port.
- **TIP** To change the COM port for a specific Pulser, in the **Control Panel**, open the **Device Manager**. Under **Ports (COM & LPT)**, right-click the COM port, select **Properties**, **Port Settings**, then **Advanced**. From the **COM Port Number** list select the COM port among those available.

## PROGRAM THE PRIZMATIX PULSER/PULSERPLUS WITHIN ETHOVISION XT

### Aim

To program the pulse sequence.

### Prerequisites

- The Pulser driver is installed. If not, see page 335.
- The COM port is configured as described on page 308.
- **IMPORTANT** Make sure that **Custom Hardware** is selected in the EthoVision XT Device Configuration window, not TTL Tester or others. See page 297.

### Procedure 1 - Initialize the COM port

In EthoVision XT, open the experiment and choose **Setup > Trial Control Settings > New**. Follow all instructions below.

1. Under **Actions** choose **External command**.
2. Next to **Select program to run** browse to

C:\Windows\System32\cmd.exe

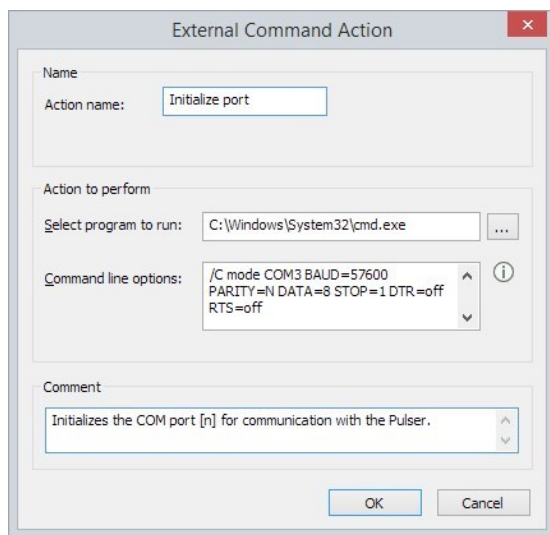
3. Add the **Command line options** as follows.

/C mode COMn BAUD=57600 PARITY=N DATA=8 STOP=1 DTR=off  
RTS=off

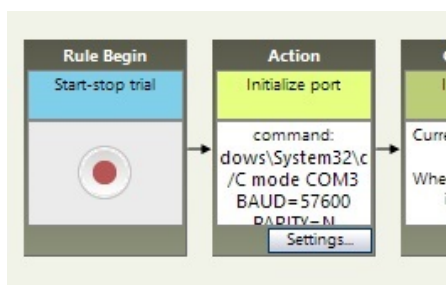
**IMPORTANT** In place of “n”, enter the number of the COM port for that Pulser.

**NOTE 1** The command /C closes the window after sending the command line.

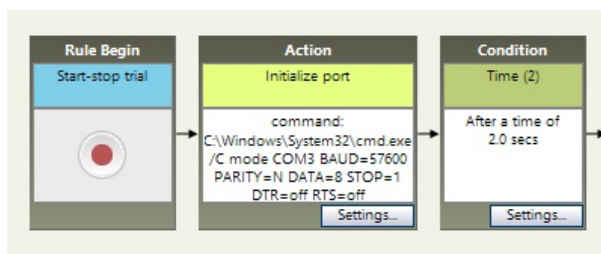
**NOTE 2** If you have the Pulser and the older Pulser software (version 2.1) the command above would be: /C mode COMn BAUD=57600 PARITY=N DATA=8 STOP=1 DTR=on.



4. Click **OK** and insert the resulting box after the **Start Trial** box.



5. Under **Conditions** choose **Time**.
6. In the **Time Condition** window, select **After a time of 2 secs**.
7. Insert the **Time Condition** box after the **Action** box.



#### *Why this Time condition?*

A Time condition is placed to allow the instructions in the external command to be processed, before the next external command (that is, the one defining the pulse sequence; see below) is received by the Pulser. Because the two external commands act on the same program **cmd.exe**, if this Time condition is absent the second command may not be executed by the Pulser which is still busy with the first command.

**IMPORTANT** If you use multiple Pulser, there may be more time needed to initialize the ports. If the Pulser does not respond it could be due that the 2 s waiting time is not enough. Set a longer time, like 5 seconds or the like.

### ***Procedure 2 - Define the pulse sequence***

The aim of this procedure is to have EthoVision XT send the command that specifies the sequence parameters.

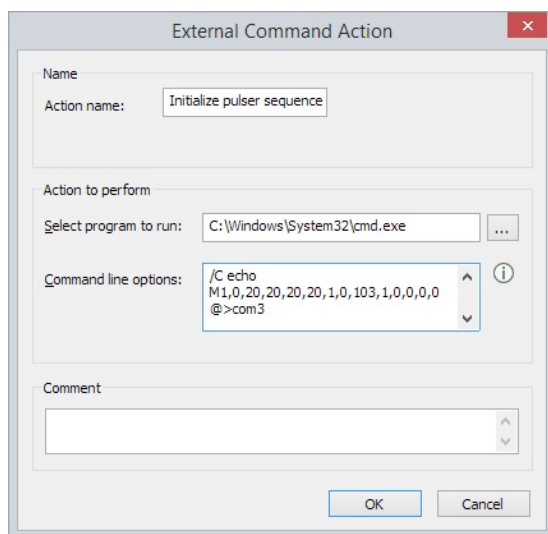
1. Under **Actions** choose **External command**.
2. Select the **Program to run** and the **Command line options** as follows (remember to enter the number of your port instead of [n]):

```
/C echo MTNT,BI,P1D,P1I,P2D,P2I,TNG,GI,OM,NoT,ToDy,ToDu,Tolt,  
ToNu@ > com[n]
```

(see page 306 for an explanation of the parameters).

For example, for a continuous pulse sequence 20 ms on and 20 ms off, and with COM port 3:

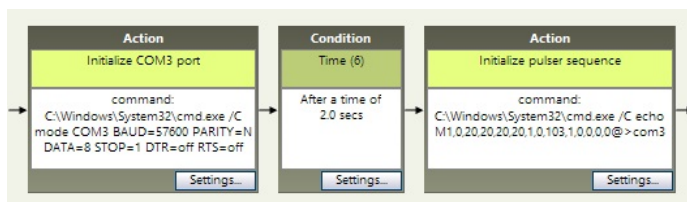
```
/C echo M1,0,20,20,20,20,1,0,103,1,0,0,0,0@ > com3
```



**NOTE 1** The Operation mode 103 (for PulserPlus, or 3 for Pulser) means that the pulse sequence is generated as long as EthoVision XT sends the TTL command to the Pulser.

**NOTE 2** If you have the Pulser and the older Pulser software (version 2.1) the command above would be: `/C echo 1,0,20,20,20,20,1,0,3,1@> com3`.

3. Click **OK** and insert the resulting box after the Time **Condition** box created in the previous procedure.



When this procedure is carried out, the Pulser “knows” what sequence to send out to the LED Controller when it is triggered. The last piece is to program one or more actions in the Trial Control procedure that activate the Pulser at the right time. See the next section.

### ***Procedure 3 - Program activation of the pulser***

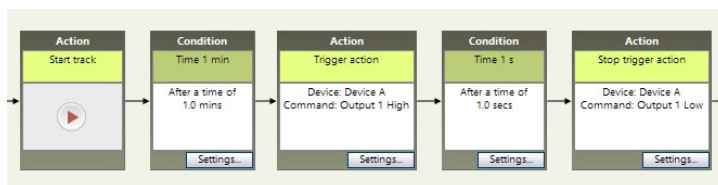
In general activating the Pulser requires four elements:

1. Trigger condition (wait that the condition is met).
2. Action that triggers the pulser.
3. Stop condition (wait that the condition is met).
4. Action that stops the pulser.

In this example, stimulation is set to start one minute after the start of tracking, and last 1 second. For more examples see page 316 and 318.

1. Under **Conditions** choose **Time** and set it to 1 minute.
2. Under **Actions** choose **Custom Hardware**. Choose the Pulser device. This device must be defined in **EXPERIMENT SETTINGS** (page 297) and **ARENA SETTINGS** (page 298). Under **Action to perform**, select **Output 1 High**.
3. Under **Conditions** choose **Time** and set it to 1 second.

4. Under **Actions** choose **Custom Hardware**. Choose the Pulser device as in step 2. However, under **Action to perform**, select **Output 1 Low** for deactivating the Pulser.
5. Place the four boxes one after the other.



**TIP** Add a condition after the last box, otherwise tracking stops immediately after stimulation stops.

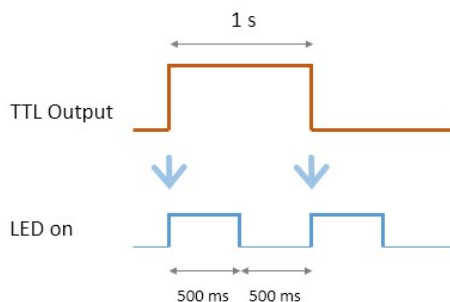
If you want these actions to be repeated, insert them in a Subrule. In the settings of this box, under Stop conditions specify **Repeat indefinitely** (or as long as required).

### Note

Consider the following sequence:

```
/C echo M1,0,500,500,0,0,1,0,103,1,0,0,0,0
```

This means that the LED is switched on for 0.5 s, then is switched off for the next 0.5 s. If the Time condition between the trigger actions is set to 1 second, at the end of that time the TTL output sent to the Pulser is still high; this triggers another pulse. As a result, the LED switches on two times (On, Off, On, Off), for a total of 2 seconds.



To have just one sequence, set the Time condition to a time slightly less than one second, for example 0.9. If, on the other hand, you want to have a sequence like in the figure above, we recommend to set 1.1 s.

### ***Procedure 3b - Program activation based on zone visit***

In this example stimulation is given every time the subject visits a zone named A. The Trial Control procedure is as follows

1. Trigger condition: Is the subject in zone A?
2. Action that triggers the pulser.
3. Stop condition: Is the subject **not** in the zone?
4. Action that stops the pulser.

The sequence must be placed in a Subrule, and the Subrule reference is set to **Repeat indefinitely**, so the Pulser is triggered again at the next zone visit. See a similar Subrule on page 302; set the Action boxes based on the Pulser, not the LED controller.

### ***For more information***

For more information on Subrules, see the EthoVision XT 18 - Trial and Hardware Control - Reference Manual. To open this manual, choose **Apps > Noldus > EthoVision XT 18 Other Documentation**. For more information on the Pulser, see the Prizmatix Pulser/PulserPlus User Manual.



## PROGRAM THE PRIZMATIX PULSER/PULSERPLUS WITH THE PRIZMATIX SOFTWARE

It is also possible to define a LED activation sequence with the software that comes with the Pulser/PulserPlus. EthoVision triggers the Pulser with a TTL signal, as shown above. The advantage is that the Prizmatix graphical interface makes the definition simple. However, you cannot change the sequence definition during a trial.

A few things to remember:

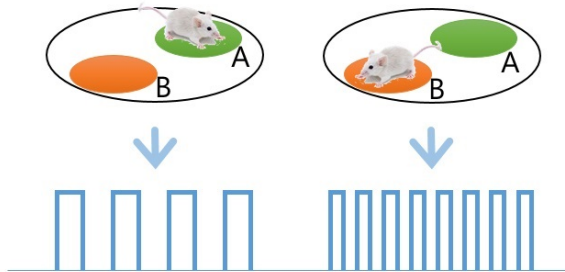
- Keep the Pulser software open during your trials. If you have multiple Pulsers, open one instance of Pulser software for each Pulser.
- Make sure that the pulse sequence is defined in the Pulser software.
- Check that the Pulser software indicates that a COM port is selected (**COM3**, **COM4**, etc.) and shows the message **Prizmatix Pulser is connected**. Do this for each instance of Pulser software.
- On the Pulser software, set the Operation Mode to **(3) Execute pulse sequence after trigger HIGH, then stop when LOW**.
- Before starting the trial, click the **Start sequence** button. If you work with multiple Pulsers, do so in each instance of the Pulser software. The Pulser software now waits for the trigger signal from EthoVision XT.
- For connection schemes and further details, see the chapter **The DanioVision Optogenetics add-on** in the DanioVision DVOC-0041 - EthoVision XT 18 - Reference Manual.

# Advanced applications

## DIFFERENTIAL STIMULATION DEPENDING ON ZONE

### *Aim*

To give a pulse sequence with frequency  $f_A$  when the subject is in zone A, and a pulse sequence with a frequency  $f_B$  when the subject is in zone B.

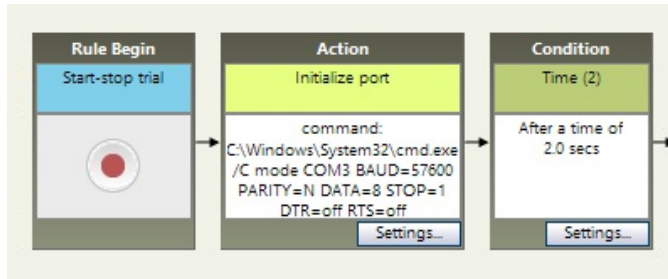


### *Prerequisites*

In the Arena Settings, you have defined two non-adjacent zones, A and B.

### *Procedure*

1. Create new Trial Control Settings.
2. In the main Trial Control procedure, right after the Start Trial box, define an External command box to initialize the port for the Pulser. For details, see page 310.
3. Add a Time condition and set it to 2 seconds.



4. Add one External command, to the program  
C:\Windows\System32\cmd.exe with the line options:

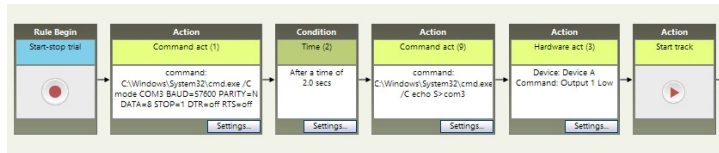
**/C echo S>com[port number]**

In this example **/C echo S>com3**.

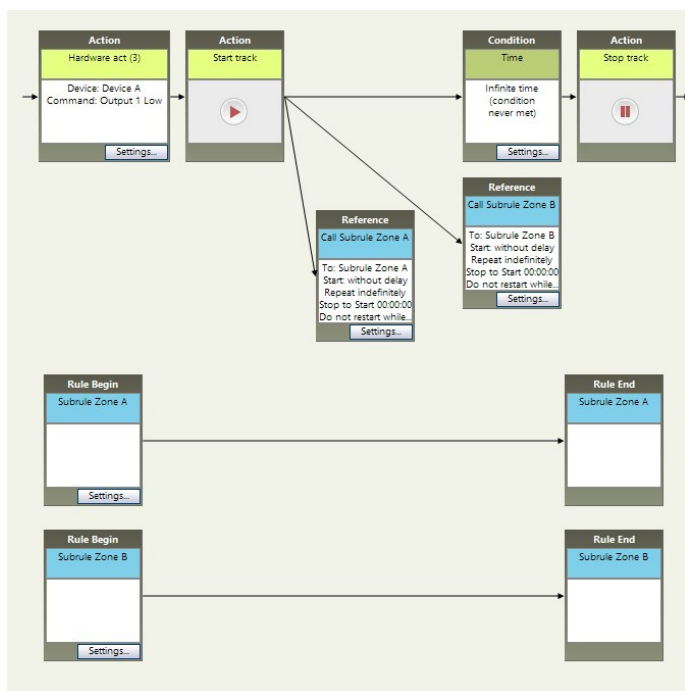
This instructions cancels the current pulse sequence definition stored in the Pulser in case you have used other definitions in a previous trial.

5. Add one Hardware command, to set the Output 1 to Low.

Result:



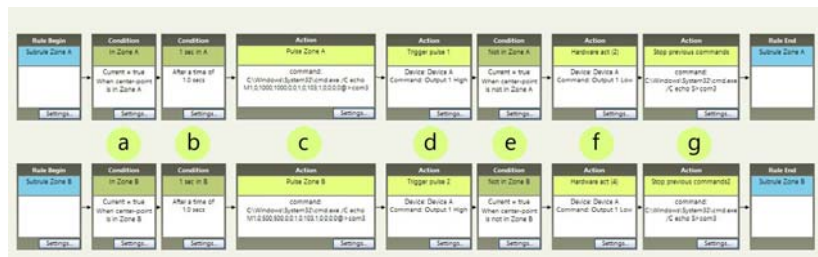
6. Create two Subrules, one for zone A and the other for zone B. In the main Trial Control sequence, after the **Start track** box, insert the two reference boxes. For both Reference boxes, select **Repeat indefinitely**.



7. In each Subrule, define the following instructions; see the next picture.

### Notes

- **IMPORTANT** Do not use zones that are adjacent; always make some space between the two.
- The 1-second **Time** condition (boxes **b**) has been inserted in both Subrules to ensure that there is enough time between the reset of the Pulser port at the end of one Subrule (box **g**) and the new pulse sequence definition in the other Subrule (box **c**) when the animal switches from one zone to the other.



**Figure 14.8** **a.** In zone Condition: Is the animal in the zone A (or B) (Current = true)? **b.** Time Condition: Wait 1 second. **c.** External command: Define the pulse sequence (for details, see page 313). **d.** Custom hardware Action: Output 1 High. **e.** In zone Condition: Is the animal NOT in zone (Current= true)? **f.** Custom hardware Action: Output 1 Low. **g.** External command: Stop the last sequence definition.

These instructions check that the animal is in a particular zone (**a**). If it is, after one second (**b**; see below for why this 1-s has been added) the corresponding pulse sequence is defined (**c**) and the Pulser is triggered (**d**). When the animal leaves the zone (**e**), stimulation stops (**f**) and the sequence definition is canceled (**g**).

- If the animal enters the other zone, the instructions in the other Subrule after (**b**) will be executed; the corresponding stimulation starts.

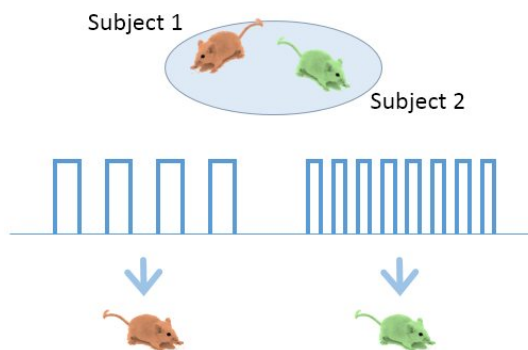
- Box **c**: See **Procedure 2 - Define the pulse sequence** on page 313.
- Boxes **d-e-f**: See **Procedure 3 - Program activation of the pulser** on page 314.
- Box **g** is an External command to C:\Windows\System32\cmd.exe with line option `\C echo S>com[port number]`. See also step 4. This command stops the pulse sequence definition and makes sure that the Pulser listens to the next definition in case the subject enters the other zone.
- You need as many Subrules as trigger zones.
- **NOTE** See a remark under **To activate the LED when the animal is in a zone** (page 302) for more options about the statistic to be used for the “In zone” condition.

## MULTIPLE SIMULTANEOUS STIMULATIONS IN ONE ARENA

### Aim

To generate different pulse sequences and send them simultaneously to different subjects in one arena.

**NOTE** Using multiple Pulsers is necessary when stimulation in two or more subjects can occur at the same time.

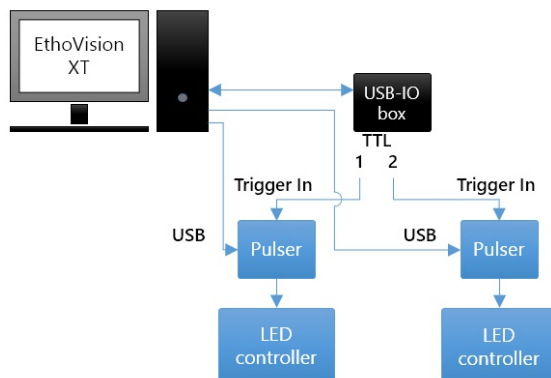


### Basic steps

1. Follow the instructions on page 335 to install the driver for the Pulser on the EthoVision XT computer.
2. Follow the instructions on page 308 to configure the driver for a specific Pulser (COM port). When connecting multiple Pulsers to the EthoVision XT computer, the Device Manager shows multiple instances of **Prizmatix DVR (COM[n])** or **USB Serial Port (COM[n])**. Repeat that procedure for each instance.



3. Connect the **Trig. In** port of each Pulser/PulserPlus devices to a TTL port of the USB-IO box.



4. In EthoVision XT (Experiment Settings; see page 297), assign a TTL port to Pulser 1, and another TTL port to Pulser 2, etc.

**TIP** Rename the **Device IDs** to something like Pulser 1 and Pulser 2.

Ports	Device type	Device ID
TTL Port 1	Custom Hardware	Pulser 1
TTL Port 2	Custom Hardware	Pulser 2

5. In EthoVision XT (Arena Settings), click the **Arena-Hardware Mapping** button. Add two devices and assign them to the arena.

Device type	Device name	Arena 1
Custom Hardware	Pulser 1	Pulser 1
Custom Hardware	Pulser 2	Pulser 2

6. In EthoVision XT (Trial Control Settings), for each pulser, initialize the COM port and define the pulse sequence. Follow the instructions from page 310 and repeat that procedure for each Pulser.

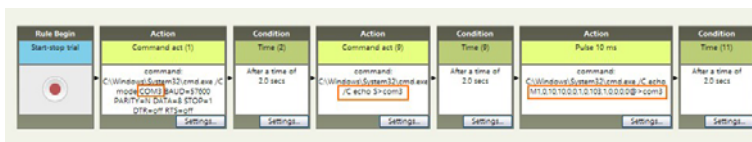
### Details

For each Pulser, define three external command **Action** boxes, and place them in a linear sequence before the **Start track** box: one for

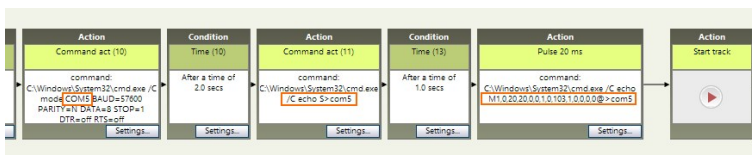
initializing the port (**Procedure 1 - Initialize the COM port**, page 310), one for stopping previous definitions (for example from a previous trial; see page 319), and one for defining the pulse sequence (**Procedure 2 - Define the pulse sequence**, page 313).

In this example, Pulser 1 is set to a pulse sequence of 20 ms on and 20 ms off (25 Hz stimulation; boxes A for initialization and C for pulse definition; see the following picture); Pulser 2 is set to a pulse sequence of 10 ms on and 10 ms off (50 Hz stimulation; boxes D,E).

First, for Pulser 1 (com 3):

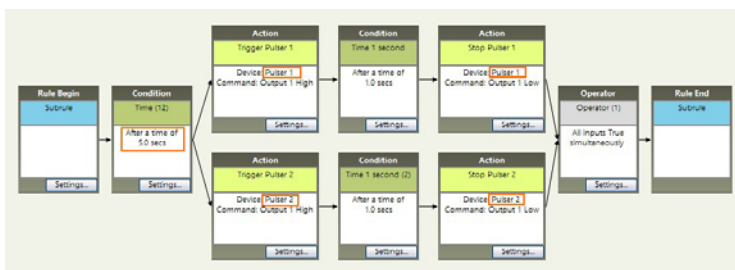


Then for Pulser 2 (com 5):



Note that actions are separated by Time conditions (1-2 s). For the reason why that is done, see page 312.

Complete your Trial Control procedure (to the right of the Start track box). In this example, the Subrule for triggering the Pulser for 1 second stimulation, and with an interval of 5 seconds, looks like this:





### Notes

- Different protocols must be defined with different Subrules. For example, create a **Subrule 1** for controlling Pulser 1, and **Subrule 2** for controlling Pulser 2.
- **TIP** When using multiple Pulsers, it is handy to use batch files. For example, the instructions for stopping sequence definitions for com3 and com5 would look like:

```
echo S>com3
```

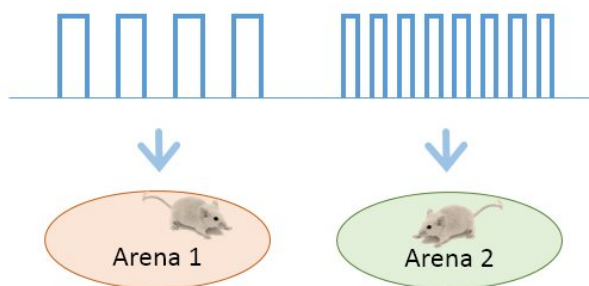
```
echo S>com5
```

Enter these instructions in a text file, and rename it with extension **.cmd**. Then instead of the single instructions, create an External command to this cmd file.

- **IMPORTANT** Test the system thoroughly before the real experiments. When using multiple Pulsers, there may be more time needed to initialize the ports. If the Pulser does not respond it could be due that the 2 s waiting time is not enough. Set a longer time, like 5 seconds or the like.

## SIMULTANEOUS STIMULATIONS IN TWO OR MORE ARENAS

When you run a trial with multiple arenas, EthoVision XT controls multiple pulsers, each assigned to a specific arena. This is only possible with the aid of batch files (see below).



### *Why are batch files needed?*

A batch file is necessary to send a commands for initializing the COM port and define the pulse sequence for a specific arena. The reason for this is that the Trial Control rules are applied to each arena independently, but the External commands specified in those boxes are not arena-specific. You can imagine two identical copies of the picture of the previous page, one for each arena. This means that an external command would be sent twice to the same COM port, which may result in unexpected behavior. To prevent this from happening, a batch file is needed that sends the command lines based on the name of the arena.

You need two batch files, one (“Initialize COM ports.cmd”) for initializing the COM port, and one for defining the pulse sequences (“Define sequences”). The two batch files are activated one after the other, with a time interval in between. A third batch file (“Stop sequences”) stops the definition of sequences previously used, and is handy when you use different sequences in the same trial or between subsequent trials.

**NOTE** If you do not have those batch files, please contact Noldus IT, or follow the instructions below.

***To create the batch file “Initialize COM ports”***

1. Open the Windows Notepad and enter the following (here we assume that four arenas are used, with COM ports 3, 4, 5 and 6):

```
@echo off

set arena=%1

set arena=%arena:~2,-2%

If "%arena%"=="Arena 1" C:\Windows\System32\cmd.exe /C mode
COM3 BAUD=57600 PARITY=N DATA=8 STOP=1 DTR=off RTS=off

If "%arena%"=="Arena 2" C:\Windows\System32\cmd.exe /C mode
COM4 BAUD=57600 PARITY=N DATA=8 STOP=1 DTR=off RTS=off

If "%arena%"=="Arena 3" C:\Windows\System32\cmd.exe /C mode
COM5 BAUD=57600 PARITY=N DATA=8 STOP=1 DTR=off RTS=off

If "%arena%"=="Arena 4" C:\Windows\System32\cmd.exe /C mode
COM6 BAUD=57600 PARITY=N DATA=8 STOP=1 DTR=off RTS=off
```

2. Save the file, and give it the extension **.cmd**.

**IMPORTANT** Make sure that “Arena 1”, “Arena 2” etc. are the exact name of the arenas in the Arena Settings. Make sure to avoid leading or trailing spaces in the names. Note that this differs from that in EthoVision XT 17 and earlier versions. Also, make sure to enter the correct COM port number of your Pulsers (see page 309).

***To create the batch file “Define sequences”***

1. Open the Windows Notepad and enter the following:

```
@echo off

set arena=%1

set arena=%arena:~2,-2%

If "%arena%"=="Arena 1" C:\Windows\System32\cmd.exe /C echo
M1,0,500,500,0,0,1,1,103,1,0,0,0,0@>com3
```

```
If "%arena%"=="Arena 2" C:\Windows\System32\cmd.exe /C echo
M1,0,500,500,0,0,1,1,103,1,0,0,0,0@>com4
```

```
If "%arena%"=="Arena 3" C:\Windows\System32\cmd.exe /C echo
M1,0,500,500,0,0,1,1,103,1,0,0,0,0@>com5
```

```
If "%arena%"=="Arena 4" C:\Windows\System32\cmd.exe /C echo
M1,0,500,500,0,0,1,1,103,1,0,0,0,0@>com6
```

The example above would produce a sequence of 500 ms pulses (frequency 1 Hz). Replace the “500” with the pulse durations and intervals you require.

2. Save the file, and give it the extension **.cmd**.

### *To create the batch file “Stop sequences”*

1. Open the Windows Notepad and enter the following:

```
echo S>com3
```

```
echo S>com4
```

```
echo S>com6
```

```
echo S>com7
```

Replace the port number with those assigned to your Pulsers.

2. Save the file, and give it the extension **.cmd**.

### *Basic steps*

1. Follow the steps 1 to 4 on page 322.

**TIP** Rename the **Device IDs** to for example Pulser 1 and Pulser 2.

2. **IMPORTANT** In EthoVision XT (Arena Settings), make sure that the name of the arenas is **Arena 1, Arena 2**, etc.
3. In EthoVision XT (Arena Settings), click the **Arena-Hardware Mapping** button. Add two devices, and assign each pulser to only one arena.

Device type	Device name	Arena 1	Arena 2
Custom Hardware	Pulser 1	Pulser 1	<No device allocated>
Custom Hardware	Pulser 2	<No device allocated>	Pulser 2

**IMPORTANT** Make sure that **<No device allocated>** is selected in the remaining cells under Arena 1, Arena 2, etc.

4. In the Trial Control Settings, define the following boxes, and insert them between the **Start trial** and the **Start track** box (see the following picture).

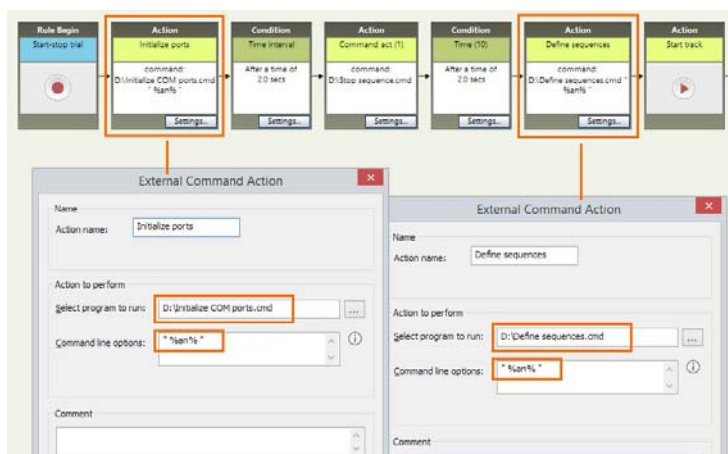
An External command for the first batch file, “Initialize.cmd”. Enter “%an%” in the **Command line options**.

A time condition, set to two seconds.

An External command for the first batch file, “Stop sequences.cmd”. Command line options are not necessary.

A time condition, set to two seconds.

An External command for the second batch file, “Sequence.cmd”. Enter “%an%” in the **Command line options**.



5. Complete your Trial Control procedure to the right of the **Start track** box. Different protocols must be defined with different Subrules. For example, create a **Subrule 1** for controlling Pulser 1, and **Subrule 2** for controlling Pulser 2.

# Data Analysis

## SIMPLE DATA SELECTION

Choose **Analysis > Data Profile > New**.

*To select the track segments when the LED was activated*

1. Under **Nesting** choose **Hardware state**.
2. From the **Device type** list, choose **Custom Hardware**.
3. Choose the **Device**, the **Signal** (**Is output 1 High**), **Value** (**true**).

Hardware State

Hardware State Variable

Calculates the statistics for a hardware state.

Device type: Custom Hardware

Device: LED controller 1

Signal: Is output 1 High

Value: true

OK Cancel

4. Click **OK** and insert the **Nest** box in the Data profile sequence.
5. Choose **Analysis > Analysis Profile > New** and define the variables you want to calculate for when the animal was stimulated (for example, the distance moved).

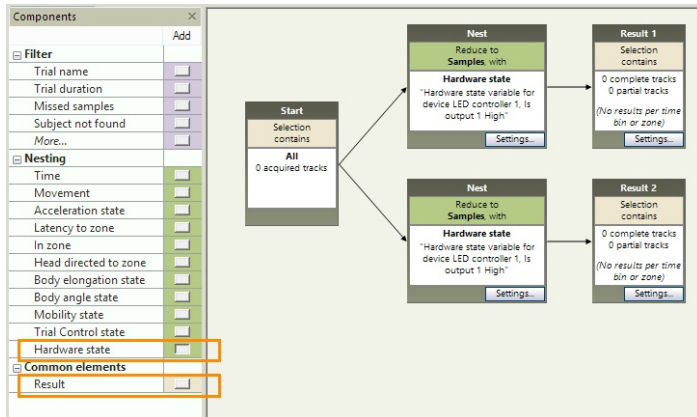
*To select the track segments when the LED was not activated*

1. Under **Nesting** choose **Hardware state**.
2. From the **Device type** list, choose **Custom Hardware**.
3. Choose the **Device**, the **Signal (Is output 1 High)**, **Value (false)**.
4. Click **OK** and insert the **Nest** box in the Data profile sequence.
5. Choose **Analysis > Analysis Profile > New** and define the variables you want to calculate for when the animal was not stimulated.

## ADVANCED DATA SELECTION

*To compare two sets of track segments (LED activated vs LED not activated)*

Create the two **Nest** boxes as described above. Place each box in a separate branch ending with a **Result** box. To create another **Result** box, under **Common Elements** choose **Result**.



Note that if you use the Pulser to generate high frequency optical pulses, the individual pulses are not “seen” by EthoVision XT. Because EthoVision XT controls the Pulser via the single TTL start/stop commands (“Output 1 High/Low”), it only “sees” the time that the

command was sent out (Output 1 High/Low). Therefore, the pulse sequence is seen as a one time segment.

For more information on data selection, see the EthoVision XT Help.

***To select the track segments when a particular frequency was generated (setup with pulser)***

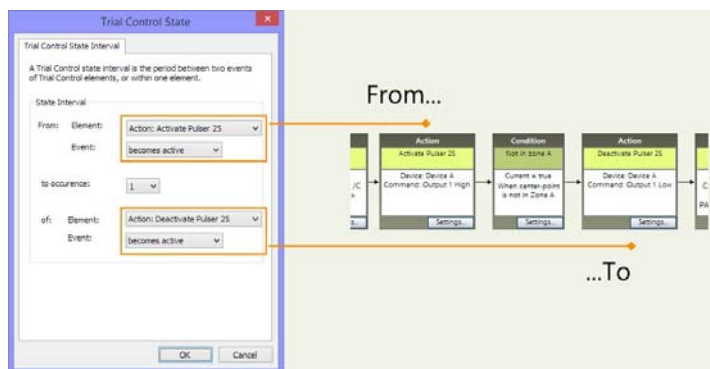
Prerequisite: your Trial Control procedure includes control of frequency of stimulation, like that described on page 318. Here, two Subrules were defined, one for zone A (stimulation at 25 Hz) and one for zone B (stimulation at 50 Hz). Within a Subrule, Hardware actions “Activate Pulser” and “Deactivate Pulser” can be used to mark the time that a particular frequency was used.

1. In the Data Profile, under **Nesting** choose **Trial Control State**.
2. Choose the name of the actions that refer to a particular frequency.

**From Element Action:** [action that triggers the Pulser]

**To Element Action:** [action that stops the trigger]

From the Event lists, select **becomes active** for both elements.



3. Click **OK** and insert the **Nest** box in the Data profile sequence.
4. Choose **Analysis > Analysis Profile > New** and define the variables you want to calculate.



## CALCULATING STATISTICS

Once you have selected the track segments you are interested in, choose the variables you want to calculate.

Choose **Analysis > Analysis Profile > New**.

- To calculate the number of times that the optical stimulation was activated, in the Analysis profile choose **Hardware command**. Choose the following:
  - **Device type** = **Custom Hardware**.
  - **Device** = the LED controller (or Pulser).
  - **Command** = **Output 1 High**.
  - Under **Trial Statistics** choose **Frequency**.
- To calculate the total time that stimulation was activated, in the Analysis profile choose **Hardware state**. Choose the following:
  - **Device type** = **Custom Hardware**.
  - **Device** = the LED controller (or Pulser).
  - **Signal** = **Is Output 1 High**.
  - **Value** = **true**.
  - Under **Trial Statistics** choose **Cumulative Duration**.

To calculate the statistics, choose **Analysis > Results > Statistics and Charts**.

## INTEGRATED VISUALIZATION

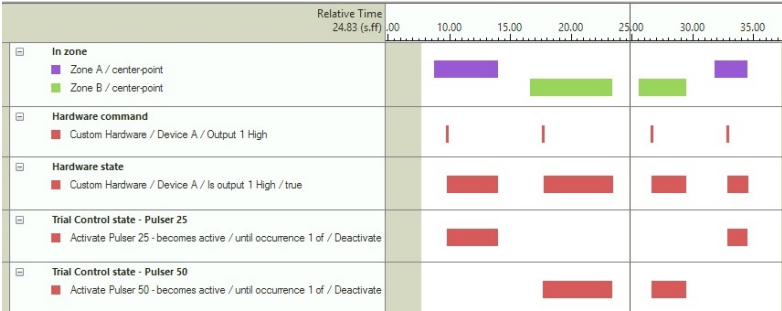
Choose **Analysis > Results > Integrated Visualization**.

The following picture shows the integrated visualization for a few dependent variables defined in the Analysis profile.

The Trial Control protocol is the same as that explained from page 318: a stimulation of 25 Hz is given when the animal is in zone A; a stimulation of 50 Hz is given when the animal is in zone B.

- **In zone**. Shows when the animal was in zones A and B. To show this variable, you must define it in the Analysis profile (choose **In zone** under **Location**).

- Hardware command.** Marks the time when the Output 1 High was sent out from EthoVision XT. This happened 1 s after the animal was detected in a zone (see page 321).
- Hardware state.** Marks the time interval when Output 1 stayed High.
- Trial Control state - Pulser 25 / Trial Control state - Pulser 50.** Marks the time that the Hardware command within one of the Subrules was High. That command triggered the pulser for a specific simulation (25 or 50 Hz). This way instances of **Hardware state** can be split according to the frequency of the stimulation.



# Installing the Prizmatix Pulser

Follow this section if the Prizmatix Pulser/PulserPlus is not properly installed on the EthoVision XT computer.

## SOFTWARE INSTALLATION

Insert the Prizmatix Pulser software CD in the EthoVision XT computer's CD/DVD ROM drive, or download the most recent version from [www.prizmatix.com/software.htm](http://www.prizmatix.com/software.htm) under **Pulser/PulserPlus Software**.

Double-click **setup.exe**. Follow the instructions on the screen and at the end of installation restart the computer. Follow the instructions below to update the driver.

**NOTE** The installation procedure differs depending on whether you have the Pulser or PulserPlus. For more information, see the Prizmatix Pulser/PulserPlus User Manual.

## DRIVER UPDATE

Do this for each Pulser you use, when they are not recognized.

1. Connect the Pulser to the computer with the supplied USB cable.
2. In the **Control Panel**, open the **Device Manager**.
3. Under **Other Devices**, right-click **Unknown device** and select **Update driver**.
4. Choose **Browse my computer for driver software**.
5. Click **Browse** and select the following folder  
C:\Program Files (x86)\Prizmatix Pulser [version number]\Drivers  
Then click **Next**.

**NOTE** This location may differ on your PC if you downloaded the drivers and saved them on another location.

6. In the **Windows Security** dialog click **Install**.
  7. At the end of the update process click **Close**.
  8. In the **Device Manager**, under **Ports**, you should see **Prizmatix DRV**.
- To configure the Pulser driver for EthoVision XT, see page 310.



## Chapter 15

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# Experiments with Calcium Imaging

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# Introduction

## ETHOVISION XT AND CALCIUM IMAGING

To understand the working of neural circuits in the brain, it is vital to be able to view neural activity simultaneously with behavioral data. Calcium Imaging is a microscopy technique to optically measure the calcium ( $\text{Ca}^{2+}$ ) status of a cell, a tissue or a medium. This allows to monitor the electrical activity in single neurons at a spatial scale that is not possible to achieve with other techniques.

The Inscopix nVista miniature microscope system enables researchers to conduct genetically targeted calcium imaging over time in awake, freely behaving rodents, and has provided insights from multiple brain areas, from the prefrontal cortex all the way to the hypothalamus. nVista's compact, sleek plug-and-play interface makes it easy to add powerful new neural circuit insights to any laboratory's behavioral research arsenal.

You can combine EthoVision XT with the **Inscopix nVista** system in such a way that EthoVision XT takes control over the recording activity of nVista. For this you need the Noldus USB-IO box interface connected between the two systems.

## ETHOVISION XT AND OPTOGENETIC STIMULATION

In vivo Calcium Imaging is often coupled with Optogenetic stimulation to causally link neural circuit activity and behavior. For this purpose, the **Inscopix nVoke** system is coupled with EthoVision XT, which can trigger both calcium imaging and the optogenetic LED stimulation. The two triggering systems can be set independent of one another. Similarly to what is done for the EthoVision XT and the nVista system, you need the Noldus USB-IO box interface connected between EthoVision XT and nVoke.

## NVISTA VS NVOKE

- **nVista** is Inscopix system for in vivo Calcium imaging in free behaving animals. It includes a head-mounted miniature microscope that enables one-photon epifluorescence imaging of calcium dynamics, a correlate of neural activity.
- **nVoke** is the Inscopix system that enables simultaneous in vivo Calcium imaging and optogenetic manipulations.

## ETHOVISION XT LICENSE NEEDED

To enable control of Inscopix devices by EthoVision XT, you need the Trial and Hardware Control module. If you are not sure whether this function is activated, in EthoVision XT select **Help > About EthoVision XT > License Info**.

If the **Trial and Hardware Control** checkbox is not selected, contact Noldus to purchase an upgrade with this module.

## APPLICATION EXAMPLES

### *Place preference in the PhenoTyper*

The nVista microscope is triggered every time the mouse enters a target region of the PhenoTyper, as defined in the EthoVision XT software.

In an experiment that combines Calcium Imaging and Optogenetics, the PhenoTyper was divided in two side zones, and in each session one side was assigned as stimulus (LED)-paired side. Each time the mouse crossed to the stimulation side, stimulation was delivered until the mouse crossed back into the non-stimulation side (Stamatakis *et al.* 2018).

### *Contextual fear conditioning*

Simultaneous Calcium Imaging and Optogenetic stimulation are performed during a fear conditioning experiment. Optogenetic



stimulation during the shock phase reduced the percent time freezing during context retrieval (Jimenez *et al.* 2020).

### **Motor behavior**

Simultaneous optogenetics and calcium  $\text{Ca}^{2+}$  imaging is performed in different populations of cells to look into the functioning of fast-spiking interneurons in the control of motor behavior in an open field (Owen *et al.* 2018).

## **REFERENCES**

- Jimenez *et al.* (2020). Contextual fear memory retrieval by correlated ensembles of ventral CA1 neurons. *Nature Communications* 11: 3492. doi:10.1038/s41467-020-17270-w
- Owen *et al.* (2018). Fast-spiking interneurons supply feedforward control of bursting, calcium, and plasticity for efficient learning. *Cell* 172(4): 683-695. doi: 10.1016/j.cell.2018.01.005
- Stamatakis *et al.* (2018). Simultaneous optogenetics and cellular resolution calcium imaging during active behavior using a miniaturized microscope. *Frontiers in Neuroscience* 12: 496. doi: 10.3389/fnins.2018.00496

# Physical setup

## HARDWARE COMPONENTS

*Noldus USB-IO box PTIO-002x*



*Noldus Optical Isolated TTL-IO interface PTISO-00x0*

Also known as opto-isolator.

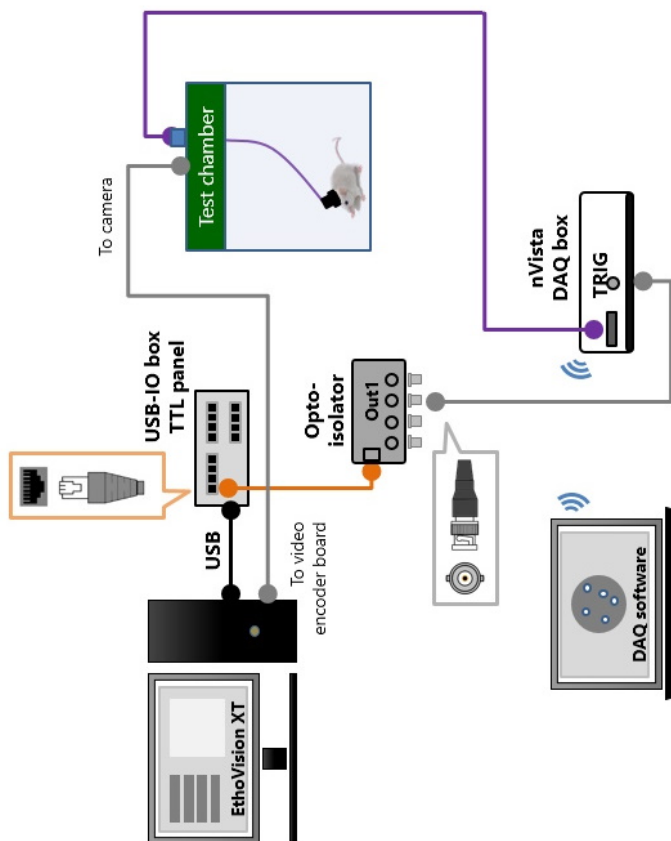


*Inscopix nVoke or nVista DAQ box*



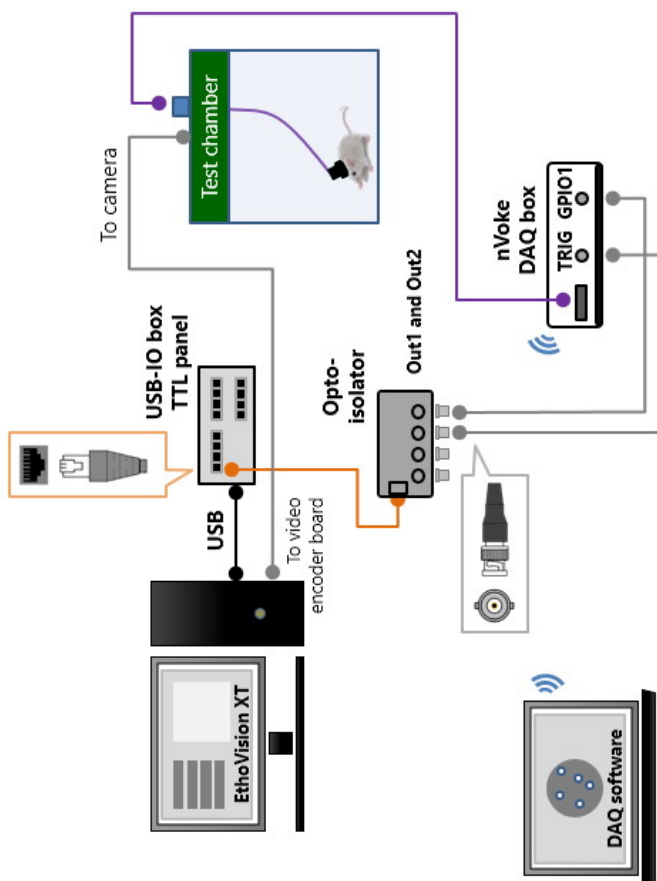
## CONNECTION SCHEMES

### *Calcium Imaging (nVista)*



**Figure 15.1** Basic connections for triggering video recording from EthoVision XT in nVista/nVoke systems. Connect one of the TTL ports of the USB-IO box to the Opto-isolator using a network cable (here in orange). Connect the Out-1 port of the Opto-isolator to the TRIG port of the Inscopix device using cables with BNC connector (in gray).

## Calcium Imaging + LED stimulation (nVoke)



**Figure 15.2** Basic connections for triggering video recording and OG-LED stimulation from EthoVision XT in nVoke systems. Connect the Out1 and Out2 ports of the Opto-isolator to the TRIG port and the GPIO port of the Inscopix device using cables with BNC connector. Note that a TTL port on the USB-IO box can send out signals through two independent lines (Output 1 and Output 2). Make sure that the Trial Control procedure sends the correct commands to the Inscopix DAQ box through the two lines Out 1 and Out 2, one for TRIG and the other for GPIO. See also page 353.

## NOTES

### *Why use a Opto-isolator?*

If you do not have the Noldus Opto-isolator, you can connect the USB-IO box and the Inscopix directly. For this you need a cable with an Ethernet (RJ45) at one end and a BNC connector at the other end. However, we recommend to use a Noldus Opto-isolator also to prevent ground loops to occur and preserve the TTL signals. Use one opto-isolator per TTL port of the USB-IO box.

# Basic settings

## ETHOVISION XT: EXPERIMENT SETTINGS

After creating a new experiment, choose **Setup > Experiment Settings**. Define the number of arenas, the features that you want to track, and the camera properties.

To define the Inscopix devices:

1. Select **Use of Trial Control hardware**, and click the **Settings** button.
2. Depending on the type of USB-IO box you use, choose either **Noldus USB-IO box** or **Noldus Mini USB-IO box**.
3. Locate the TTL port that you use to connect the USB-IO box (see page 343), and from the **Device Type** list choose **Custom Hardware**.
4. Under **Device ID**, enter a name for the device. For example, *nVista Video*, or *nVoke Video*, *nVoke LED*.
5. Repeat the steps 3-4 above to declare more devices. If you want to trigger Calcium Imaging and LED stimulation, you must use two TTL ports from the USB-IO box, one for the TRIG input and one for the GPIO[n] input of the nVoke device. See Figure 15.2 and page 353.
6. Click **OK**. Next, open the Arena Settings (see below).

## ETHOVISION XT: ARENA SETTINGS

Choose **Setup > Arena Settings**. Calibrate and define the outline of the arena(s) and the zones.

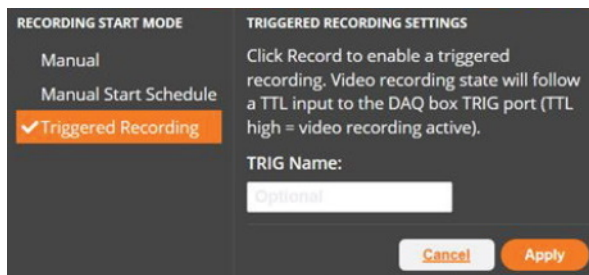
To map the Inscopix devices to the arena(s):

1. Click the **Arena - Hardware Mapping** button at the bottom-right corner of the screen.
2. Check that the device appears under the arena name. In the case of multiple devices, assign each of them to one arena and make sure that they do not occur in the cells for other arenas.

3. Optionally, edit the **Device name** (default: Device A, B, etc.). You will see this name back in the Trial Control Settings and in the Analysis profile.
4. Repeat the steps 2-3 to map the remaining devices.

## INSCOPIX DEVICES

1. Connect the microscope to the subject and pass the cord through the tracking chamber/PhenoTyper and connect it to the Inscopix device (see page 343).
2. On the Inscopix computer, launch the data acquisition software and adjust the imaging settings, like the frame rate and the gain.
3. In the data acquisition software, select to trigger from external hardware. If you do Calcium Imaging only, you can use the Basic Recording mode in the Inscopix software. Make sure that the Recording Start mode is set to Triggered Recording.



4. When using optogenetics stimulation, in most cases you must use the Advanced Recording mode. Specify the type of stimulation, power, number of repeats etc. See also the **nVista and nVoke User Manual** for examples.

## OG-LED Configuration Parameters

Click the OG-LED button to start the pulse protocol.

Type: ☒ Excitatory ☐ Inhibitory

Power (mW/mm<sup>2</sup>): 0.2

Range = 0 - 20 mW/mm<sup>2</sup>, Step = 0.1 mW/mm<sup>2</sup>

Repeats: ☒ # of repeats ☐ Indefinitely

Repeat Count: 1

Range = 1 - 1000, Step = 1

Pulse Frequency (Hz): 20

Range = 1 Hz - 100 Hz, Step = 1 Hz

Pulse Width (ms): 5

Range = 1 ms - 47 ms, Step = 1 ms

Pulse Train Duration (min : s : ms):

0 : 1 : 0

Range = 00 ms - 120 min, Step = 5 ms

OGT Pulse Duration: Pulse Train (min : s : ms):

0 : 0 : 0

Range = 0 ms - 120 min, Step = 5 ms

Delay from Trigger (min : s : ms):

0 : 0 : 0

Range = 0 ms - 60 min, Step = 5 ms

Cancel

Save



# Program control of devices in EthoVision XT

## TRIAL CONTROL SETTINGS

The Trial Control Settings in EthoVision XT allows you to program activation of calcium imaging and optogenetic stimulation by Inscopix devices at specific times or when specific events occur, for example when the subject enters a target zone of the arena.

In addition, in Trial Control Settings you can define the conditions for the start and stop of data acquisition.

In EthoVision XT, choose **Setup > Trial Control Settings > New**, and enter a name for the new settings profile.

### *For more information*

For more information on how to work with Conditions and Actions in Trial Control Settings, see the EthoVision XT Help (press **F1** in EthoVision XT) and the EthoVision XT 18 - Trial and Hardware Control - Reference Manual, which you can find in the Apps screen under **Noldus > EthoVision XT 18 Other Documentation**.

## EXAMPLE 1 - IMAGE RECORDING (NVISTA)

### *Use case*

EthoVision XT starts tracking after locating the subject's center point in the arena. Next, the EthoVision XT sends out a high TTL pulse to the TRIG port of nVista. After a time delay of 10 minutes, EthoVision XT stops tracking the subject.

### *Hardware connections*

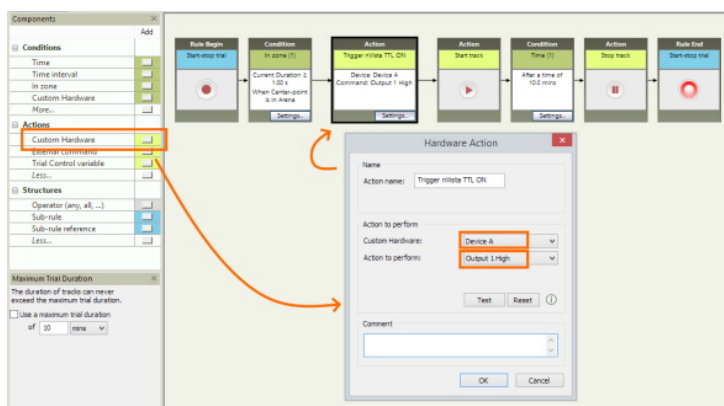
See the scheme on page 343.

### Prerequisites in EthoVision XT

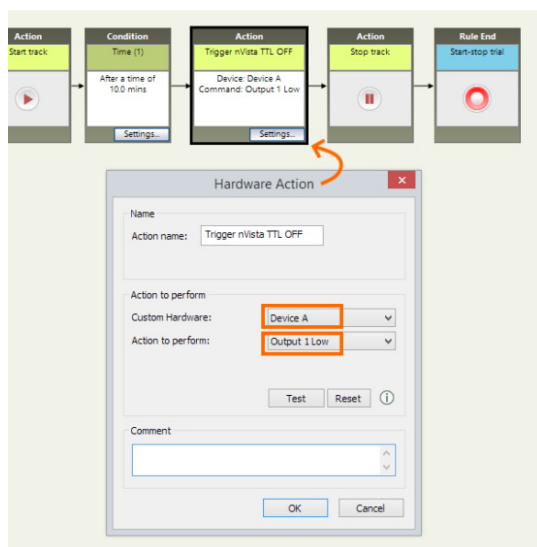
You specified the TTL port and output line (either 1 or 2) for TRIG in the Experiment Settings and mapped the port to the arena (see page 346).

### Procedure

1. In the Trial Control Settings, under **Actions**, click the button next to **Custom hardware**.
2. Specify the device (e.g. Device A) and the type of signal (**Output 1 High**).
3. Optionally, edit the name in the **Action name** field.
4. Click OK and insert the Action box between the **Condition - In zone** box and the **Action - Start track** box.



5. Click **Settings** in the **Condition - Time** box and select 10 minutes. This way tracking stops after 10 minutes.
6. If you want to stop recording in nVista when you stop behavior tracking, bring a new Action box in the flow line which instructs the system to send out a low TTL pulse to nVista. Make sure that you place the Action box at the right of the 10-minutes time box.



**TIP** In the Hardware Action box you can test whether Ethovision XT triggers the nVista. To do so, click the **Test** button.

**NOTE** Make sure to instruct nVista/nVoke to record video based on the TRIG signal.

## EXAMPLE 2 - MANUAL VIDEO RECORDING, TRIGGERED OG-LED STIMULATION

### Use case

EthoVision XT starts tracking after locating the subject's center point in the arena. You start video recording in nVoke manually by clicking the **Record** button. At the planned time, for example at 10 minutes after start, EthoVision XT sends out a high TTL pulse to the GPIO[n] port of nVoke.

- In the Inscopix DAQ software, under Video Recording configuration, make sure that Trigger is set to NONE and Control is set to Video Recording ON.

### **Hardware connections**

- See the scheme on page 344. You do not need to use the TRIG port of the nVoke device.
- Set the GPIO[n] to Input in the Inscopix DAQ software.

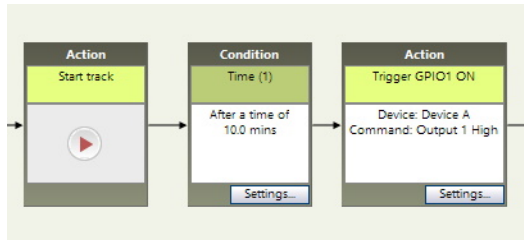
### **Prerequisites in EthoVision XT**

In the Experiment Settings, you defined the TTL port and output line (either 1 or 2) connected to the GPIO[n] of nVoke. In Arena Settings, you mapped that port to the arena (see page 346).

### **Procedure**

Because calcium imaging is started manually in this example, you only have to program the activation of the OG-LED. In the following example, the Time condition is set to wait 10 minutes before the triggering action.

1. In the Trial Control Settings, under **Conditions**, click the button next to **Time**.
2. Specify the time (e.g. 10 minutes). Optionally, edit the name in the **Condition name** field.
3. Click OK and insert the Condition box after the **Action - Start track** box (or wherever it applies in your protocol; for example in a sub-rule).
4. In the Trial Control Settings, under **Actions**, click the button next to **Custom hardware**.
5. Specify the device (e.g. Device A) and the type of signal (**Output 1 High**).
6. Optionally, edit the name in the **Action name** field.
7. Click OK and insert the Action box at the right of the **Condition** box just created.



8. When that applies, define a Condition and an Action (Output Low) for stopping the trigger action and insert them in the trial control flow line.

### Notes

- In the Inscopix DAQ software, make sure to set the **Trigger** mode for the GPIO[n] port to **Follow**.
- When the Trigger is set to low, LED stimulation is stopped. To set the Trigger to Low, add an Action to the same device/TTL port and output line as above, and select the signal **Output Low**.
- You can also let the DAQ software run its own pulse protocol while the Trigger is ON. In that case the Control for the GPIO[n] port is set to **Pulse protocol**.
- For more information, see the nVista and nVoke User Manual.

## EXAMPLE 3 - TRIGGERED VIDEO RECORDING AND LED STIMULATION

### Use case

EthoVision XT starts tracking after locating the subject's center point. in the arena. Both video recording and the OG-LED are triggered by EthoVision XT when the corresponding conditions (time or event) are met.

### **Hardware connections**

- See the scheme on page 344.
- Set the GPIO[n] to **Input** in the Inscopix DAQ software.

### **Prerequisites in EthoVision XT**

In the Experiment Settings, you defined two TTL lines from the USB-IO box: one connected to the TRIG and the other to the GPIO[n] port of nVoke. In Arena Settings, you mapped the ports/lines to the arena (see page 346).

### **Procedure**

Define actions and conditions as explained for the previous examples.

- Condition to trigger video recording.
- Action to trigger video recording through the TRIG port.
- Condition to trigger OG-LED stimulation.
- Action to trigger the OG-LED stimulation through the GPIO[n] port.

When applicable, define additional conditions and action to stop video recording and OG-LED stimulation.

### **Notes**

- In the Inscopix DAQ software, make sure to set the **Trigger** mode for the TRIG port to **Follow**, and the Control is set to **Video Recording ON**. In an alternative configuration, you can set Control to **Recording schedule** to record video for a specific time.
- In the Inscopix DAQ software, make sure to set the **Trigger** mode for the GPIO[n] port to **Follow**. Select the type of Control you require (e.g. Pulse protocol).
- See the Example Configuration #3 in the nVista and nVoke User Manual.

# Detection settings

## GENERAL

Ensure your subjects are detected well by Ethovision XT.

Choose **Setup > Detection Settings**.

See also **DETECTION SETTINGS** on page 17. For more details, see the EthoVision XT Help.

### *Sample rate*

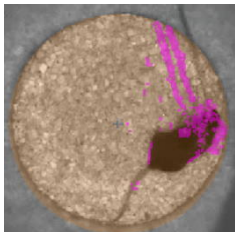
In the **Video** section, specify the sample rate. Some commonly-used values are:

- For tracking rats: 5 samples/second.
- For tracking mice: 12.5 samples/second.
- When tracking the nose- and tail-base points of rodents: 25-30 samples/second.

## DETECT IMMOBILITY

There are two ways to detect immobility of the subject during the trial.

- **Activity** measures the number of pixels that change their intensity, so whenever the subject sits still, the Activity value will be low. However, when the subject is connected to a microscope cable, the cable can move continuously and keep Activity high.



As a result, Activity measurements may not detect immobility with sufficient accuracy. Instead, use Mobility.

- **Mobility** is based on the change of the detected shape (i.e., the yellow blob). You can define Mobility in the Analysis profile, so you do not need to specify anything in the Detection Settings. However, make sure that the yellow blob covers the entire body of the subject and is not affected by the cable (see below).

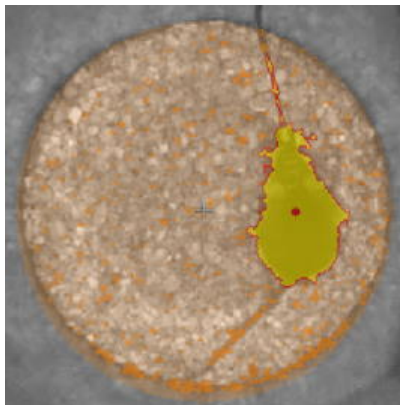
## REMOVE THE EFFECT OF THE MICROSCOPE CABLE

When the microscope cable is detected as the subject, it changes both the Mobility measure and the position of the center point. The latter affects distance moved and other readouts.

### *Step 1 - Define the contrast*

The first thing to do is to make sure that the entire body of the subject is well detected throughout the arena. Use for example Dynamic subtraction and choose the smallest range of contrast that enables detection of the subject's body.

In this example the cable is still detected. This will be removed in the next step. Make sure that the contour of the subject is not too much indented and noise (i.e. the orange areas) is limited.

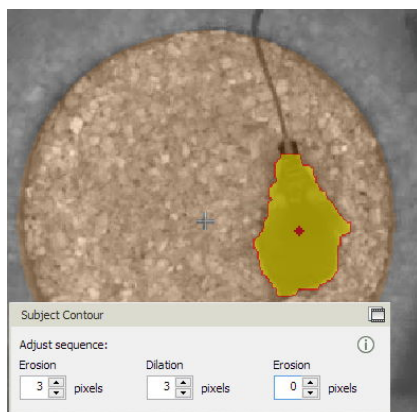




### ***Step 2 - Erode and then dilate the contour***

In the Detection Settings, locate the **Subject Contour** options. Select **1** or a higher value for the first **Erosion** and a similar value for **Dilation** until the cable is no longer highlighted in yellow.

The Erosion filter removes the pixels from the contour (and therefore the cable too); the Dilation filter restores the original size of the yellow blob but excludes the cable.



# Aligning data

## SYNC SIGNAL

To align the data streams from EthoVision XT and Inscopix nVista/nVoke, there are two solutions:

- Have EthoVision XT send a time code signal (TCAP).
- Have EthoVision XT send a sequence of TTL pulses.

Either way, the Inscopix system records the signal in one of its channels. The difference between the two is that with TCAP you need to export the TCAP signal that is stored in Inscopix back to EthoVision XT, together with the Inscopix data, so that EthoVision XT reads the time code and aligns the Inscopix data with the tracks.

### **Note**

The nVista/nVoke system can also send a sync pulse to EthoVision XT through the USB-IO box every time a video frame is recorded. However, the pulse generated from the SYNC port of nVista/nVoke has a duration of 10 ms. That is too little for EthoVision XT to detect and record the signal. Therefore, this solution is not optimal to sync the two data streams.

## USING THE TCAP SIGNAL

### **Prerequisites**

For this solution you need:

- The External Data add-on module for EthoVision XT. Contact Noldus if you need to purchase it.
- An interface cable between EthoVision XT and the BNC connector of the nVista/nVoke DAQ box. Contact Noldus to have one made for you.

- A way to convert the Inscopix data to a format compatible with EthoVision XT: fixed sample rate, without gaps.

### ***Procedure***

1. Set the TCAP signal in the Experiment Settings of EthoVision XT, and specify the sample rate of the Inscopix channel that will receive that signal.
2. Start acquisition in Inscopix and then start the trial in EthoVision XT. nVoke/nVista records the TCAP signal, which should be visualized on the system's screen.
3. Export the Inscopix data to text files. Note that here the sample rate must be fixed and all samples must be written in the file, otherwise import won't work.
4. After import EthoVision shows the TCAP signal and other Inscopix data together with the track data.

For more information, see **External Data** in the EthoVision XT Help.

## **USING A TTL SIGNAL**

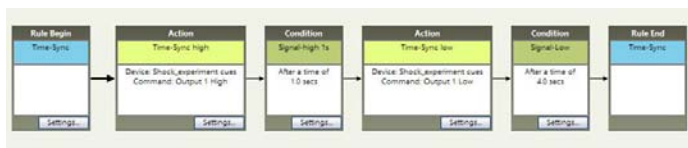
### ***Connections***

Connect a TTL port of the USB/IO box to one of the GPIO ports of the Inscopix DAQ box. For this you need a cable with a RJ45 connector at one end and a BNC connector at the other end.

### ***Procedure***

1. In the Experiment Settings in EthoVision XT, define a TTL port of the USB-IO box. Select **Custom Hardware** from the list. In the Arena Settings, map that TTL port to one of the arenas.
2. In the Trial Control Settings, add a subrule that activates the TTL port every few seconds. For example: **TTL Output 1 High** for 1 second, then **TTL Output 1 Low** for 4 seconds. Let the subrule repeat a number of times or when a Trial Control variable reaches a specific value. In general, make sure that the ON-OFF sequence runs through the duration of the trial.

Example:



**TIP** To stop the subrule at the end of the trial, add an Action box immediately before the Stop Trial box, where a Trial Control Variable, for example END, gets the value 1. In the Subrule reference, specify to end the subrule when END =1.

3. Run a test trial and make sure that Inscopix records the signal coming to the GPIO port.
4. Visualize the data in both systems. In EthoVision XT, you can visualize the TTL signal by selecting, for example, **Trial Control state** in the Analysis profile and defining the state from the action **Output 1 High** to the action **Output 1 Low** (here the numbering depends on which TTL line you use).



5. If the data are well aligned, the time between the first and the last pulse should be the same in EthoVision XT and Inscopix, or differ by a few milliseconds.

### **For more information**

- The nVista/nVoke User Manual.
- The EthoVision XT 18 - Trial and Hardware Control - Reference Manual.

## MARK ETHOVISION EVENTS ON THE CALCIUM IMAGING TIMELINE

You can also use the GPIO ports on the nVoke/nVista box to send specific events to the Calcium Imaging software.

In the following example, a setup that includes a fear conditioning system, all GPIO ports are set to **digital** and work as **inputs**.

- **GPIO-1** receives a signal from a TTL port of the USB-IO box that is set to high when tracking starts.
- **GPIO-2** receives a signal from a TTL port of the USB-IO box that is set to high when a 5-kHz tone is given.
- **GPIO-3** receives a signal from a TTL port of the USB-IO box that is set to high when a 15-kHz tone is given.
- **GPIO-4** receives a signal from a TTL port of the USB-IO box that is set to high or low when the optogenetic stimulation is given/stopped, respectively, or when a shock is given/stopped, respectively.

# Data analysis in EthoVision XT

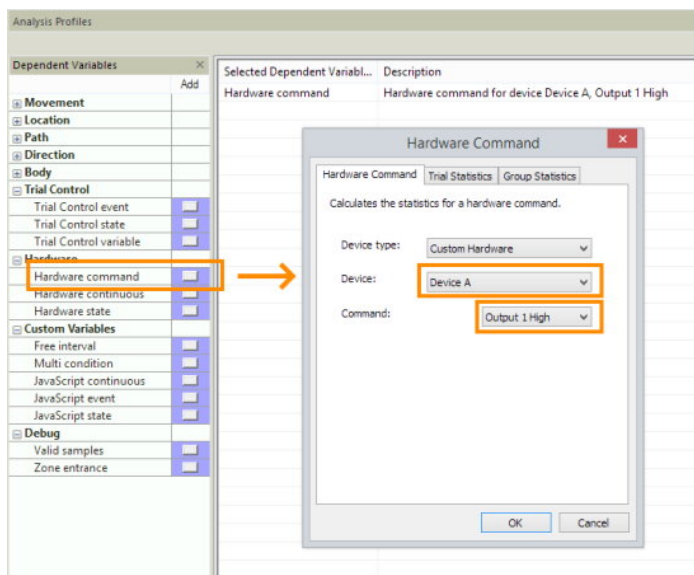
Once you acquire your trials, you want to pair the behavioral tracking with the calcium imaging/optogenetic stimulation events.

To view and analyze those events, you must first define them in the Analysis profile. Here we report a few examples.

Choose **Analysis > Analysis Profile > New**.

## DEFINE A SIMPLE EVENT

With the variable **Hardware command** you can visualize the time that the USB-IO box sends a trigger command to nVista/nVoke.

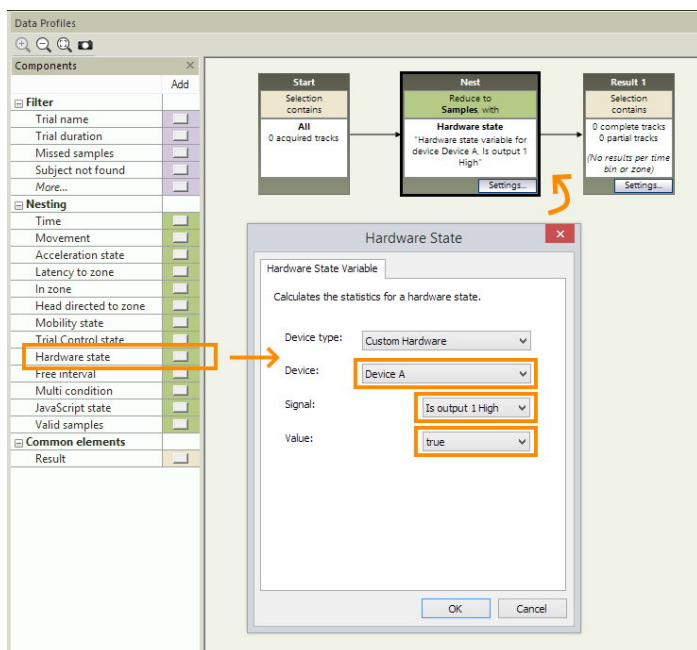


1. In the **Dependent Variables** pane, under **Hardware**, choose **Hardware command**.

2. Choose the device and the output state, for example **Output 1 High**.  
Note that Output represents one of the two communication lines present in each TTL port of the USB-IO box. Choose the line that you used in the Action box in the Trial Control Settings to trigger the device (see the examples on page 349).
3. Visualize the event (**Analysis > Results > Integrated Visualization**).

## ANALYZE INTERVALS (GLOBAL)

You can analyze the behavior of the subject within an interval based on events. For example, calculate the average velocity of the subject from the time that stimulation starts (i.e., when the TTL Output 1 is High) to the time that stimulation end (i.e., nVoKe is triggered with Output 1 Low). To analyze the behavior within intervals, you must use the Nesting function in the Data profile.

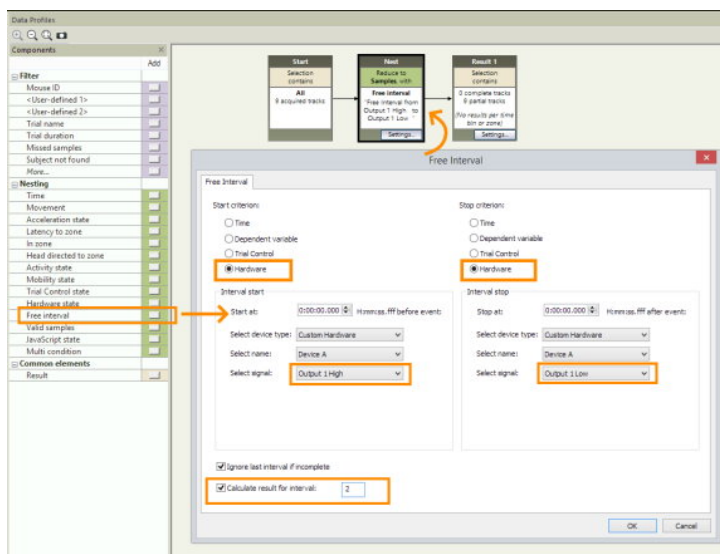


1. Choose **Analysis > Data Profile > New**.
2. In the Components pane, under **Nesting**, choose **Hardware state**.
3. Select the device and the status of the device. For example, **Device A - Is Output 1 High - true**. This selects the time that the TTL Output 1 signal remains high.
4. Insert the box in the flow line.
5. Visualize the interval (**Analysis > Results > Integrated Visualization**).

**NOTE** If Output 1 stays high multiple times during one trial, EthoVision XT considers the cumulative time that Output 1 stays High, and that is used as the analysis interval. When you want to analyze the time defined by a specific triggering event, see below for a different solution.

## ANALYZE SPECIFIC INTERVALS

You can use Free Intervals to specify a single interval based on one of the occurrences of a triggering event. Suppose that nVoke is triggered in ten intervals, and you want to analyze the second interval.





1. Choose **Analysis > Data Profile > New**.
2. In the Components pane, under **Nesting**, choose **Free interval**.
3. For both Start criterion and Stop criterion, select **Hardware**. Define the start and stop event. For example, Start criterion: **Output 1 High**; Stop criterion: **Output 1 Low**.
4. Select **Calculate Result for interval** and choose the occurrence. In this example, 2.
5. Insert the box in the flow line.
6. Visualize the interval (**Analysis > Results > Integrated Visualization**).
7. In the Analysis profile, define the variable that you want to calculate within the interval (e.g. velocity).

**NOTE** You can also use the commands of Trial Control to define intervals. For example, define an interval that starts when the tone cue is given (i.e. when the Action *Tone cue* becomes active). You can also use Conditions (e.g. the interval starts when the Condition *Wait 30 seconds* becomes active). If an Action follows immediately a Condition, then the time that the condition becomes true is essentially the same as the time that the following action becomes active, because both boxes are processed in the same sample time. In that case it does not matter which box you use for defining the interval.

## EXPORT THE RAW DATA

You can export the raw data (behavior and hardware events) with their time stamps, for example to sync EthoVision XT data with calcium imaging in a third-party application.

Choose **Analysis > Export > Raw data**.

- Note that **Raw data** also include the Trial Control events and states (e.g. a condition being true) once you include them in the Analysis profile.
- Select the **Hardware log** option if you want to export hardware events.

## ALIGN ETHOVISION XT AND INSCOPIX DATA

### *Computer clocks*

Ensure that the two computers used for data acquisition (EthoVision XT and Inscopix IDAS) have their clocks synchronized, for example using Network Time Protocol. Point all PCs at a time server or point one and set it up to act as a time server to the other.

### *Unix time*

Inscopix software often exports time stamps to Unix (Epoch) time. Unix time is also known as Epoch Time or Unix timestamp. It's a system that counts the number of seconds that have elapsed since the Unix Epoch, i.e. January 1st, 1970. To put in simple words, Unix time is the total number of seconds that have elapsed since 00:00:00 UTC Thursday, 1 January 1970.

### *Convert EthoVision XT time to Unix time*

You can use an Excel formula to convert the trial time in EthoVision XT to Unix time.

In general, the formula goes like this:

Unix time = (EthoVision Time - DATE(1970,1,1))\*86400

Because the trial (or recording) time in EthoVision XT is relative time (e.g. 0.200), you need to convert it to absolute time using the Reference time cell in the raw data (A in the next image).

Then use a second formula (B) that adds the trial (or recording) time to the reference time A.

	A	B	C	D	E	F
26	Reference duration		+ 00:00:07.967			
27	Reference time		3-10-2019 1:32:50.367 PM		A	1552224770.367
28	Sof file					
29	Missed samples	0.0 %				
30	Subject not found	0.0 %				
31	Interpolated samples	0.0 %				
32	Postprocessor (SIR) calculatio	Tr::eldrUndefined				
33	LED pattern					
34						
35	Trial time	Unix time	Recording time	X center	Y center	Area
36	s		s	cm	cm	cm <sup>2</sup>
37	1.067	1552224771.434		0	-1.54751	0.074889
38	1.1	=F\$27+A38		0.033	-1.54738	0.071847
39	1.133	1552224771.500		0.067	-1.5476	0.072705
40	1.167	1552224771.534		0.1	-1.54663	0.071229
41	1.2	1552224771.567		0.133	-1.54622	0.072911
42	1.233	1552224771.600		0.167	-1.54646	0.07324
43	1.267	1552224771.634		0.2	-1.5465	0.075142