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EthoVision: A versatile video tracking system for automation of behavioral experiments

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The need for automating behavioral observations and the evolution of systems developed for that purpose is outlined. Video tracking systems enable researchers to study behavior in a reliable and consistent way and over longer time periods than if they were using manual recording. To overcome limitations of currently available systems, we have designed EthoVision, an integrated system for automatic recording of activity, movement, and interactions of animals. The EthoVision software is presented, highlighting some key features that separate EthoVision from other systems: easy file management, independent variable definition, flexible arena and zone design, several methods of data acquisition allowing identification and tracking of multiple animals in multiple arenas, and tools for visualization of the tracks and calculation of a range of analysis parameters. A review of studies using EthoVision is presented, demonstrating the system's use in a wide variety of applications. Possible future directions for development are discussed.

Manual Versus Automated Behavioral Observation

The behavior of animals is commonly recorded in either a manual or a semiautomated way. Traditionally, a researcher observes the animal; if the researcher considers that a certain behavior pattern is displayed, he or she notes the behavior, either by writing it down or by entering the data into an event-recording program (Noldus, 1991; Noldus, Trienes, Hendriksen, Jansen, & Jansen, 2000). Manual recording of behavior can be implemented with a relatively low investment, and, for some behaviors, it may be the only way to detect and record their occurrence; however, automated observation can provide significant advantages. Behaviors are recorded more reliably because the computer algorithm always works in the same way, and the system does not suffer from observer fatigue or drift. For instance, in contrast to manual observation, video tracking carries out pattern analysis on a video image of the observed animals to extract quantitative measurements of the animals' behavior (for more details about how this works, see below). Automated observation using video tracking is particularly suitable for measuring locomotor behavior, expressed as spatial measurements (distance, speed, turning, etc.) that the human observer is unable to accurately estimate (Burešová, Bolhuis, & Bureš, 1986; Spruijt, Buma, van Lochem, & Rousseau, 1998; Spruijt, Pitsikas, Algeri, & Gispen, 1990). Automated observation systems also allow the study of behaviors that occur briefly and are then interspersed with long periods of inaction (Martin, Prescott, & Zhu, 1992) and behaviors that occur over many hours, such as diurnal variation in behavior (Olivo & Thompson, 1988; Spruijt & Gispen, 1983).

Historical Development of Automated Observation

Technology for automated recording of animal behavior and movement has evolved dramatically in the past decade. Early systems, using hard-wired electronics, were able to track only a single animal in highly artificial environments (i.e., test arenas devoid of any substrate, bedding, or objects besides the animal). For example, an open field can be sampled with a grid of infrared beams either as the sole detectors (Clarke, Smith, & Justesen, 1985; Kirkpatrick, Schneider, & Pavloski, 1991; Robles, 1990) or in combination with other methods, such as placing a series of strain gauge transducers under the arena to estimate the animal's position (Gapenne, Simon, & Lannou, 1990). Various ways have been used to measure the magnitude of an animal's motion with types of touch-sensitive sensors. A crude estimate of movement can be gained by placing the animal on a bass loudspeaker and monitoring the loudspeaker's electrical output when its cone is moved

Many colleagues at Noldus Information Technology have contributed toward the development of EthoVision over the years; their hard work and dedication are acknowledged here. Special thanks to Berry Spruijt (Utrecht University), who has been an ongoing source of inspiration. We are also grateful to those users who provided us with their research design and data for use in the review section of this paper. We also thank Niels Cadee, Martine Janzen, Coen van Kaam, Tiffany Mayton-TheCrow, and Rudie Trienes for reviewing an earlier version of the manuscript and providing useful comments. Correspondence should be addressed to L. P. J. J. Noldus, Noldus Information Technology b.v., P.O. Box 268, 6700 AG Wageningen, The Netherlands (e-mail: l.noldus@noldus.nl; URL: http://www.noldus.com).

Note: The authors have a commercial interest in the product described in this paper.

by the rat (Silverman, Chang, & Russell, 1988). Sensors can also measure the position of the animal (and, hence, locomotion), for instance, by changes in the capacitance of a plate when the animal is in proximity to it (Clarke, Smith, & Justesen, 1992) or changes in body resistance (Tarpy & Murcek, 1984). Other comparable detection methods have included use of ultrasound (Akaka & Houck, 1980) and microwave Doppler radar (Martin & Unwin, 1980). A modern radar-based "actometer" is able to detect very small movements (which is particularly important in studies on insect behavior) and has the advantage of working in complete darkness (Knoppien, van der Pers, & van Delden, 2000). The position of a rat in an open field has been recorded by attaching the rat to a computer joystick via a series of rods attached by a collar to the rat's neck (Brodkin & Nash, 1995). Animal behavior can also be measured using a computerized version of a Skinner box (Skinner, 1938), in which the subject has to tap a touch-sensitive monitor (Morrison & Brown, 1990; Sahgal & Steckler, 1994). The motion of individual limbs can be monitored automatically using actigraph sensors, which detect movement by means of a piezoelectric accelerometer (May et al., 1996). Another technique for automatic classification of behavioral patterns uses sensors to detect the mechanical vibration of a platform on which a cage with a mouse is placed (Schlingmann, van de Weerd, Baumans, Remie, & van Zutphen, 1998).

Video Tracking

Video tracking systems were introduced in the early 1990s, offering clear advantages of flexibility, spatial precision, and accuracy over the various hardware devices listed above. However, with early systems the actual path of the animal still had to be entered manually by the experimenter, by following the track of the animal with a computer mouse (Pereira & Oliveira, 1994), a joystick (Morrel-Samuels & Krauss, 1990), or a digitizing tablet or similar device (Santucci, 1995). Another early method, still used in some commercially available systems, is to feed the analog video signal to a dedicated video tracking unit, which detects peaks in the voltage of the video signal (indicating a region of high contrast between the tracked animal and the background), and use this to produce the x,y coordinates of the tracked animals. This output is then fed to the serial port of a computer (Klapdor, Dulfer, & van der Staay, 1996; Vorhees, Acuff-Smith, Minck, & Butcher, 1992). These analog systems have the disadvantage of being relatively inflexible (dedicated to particular experimental setups) and can normally track only one animal in rather restricted lighting and background conditions.

Greater flexibility is achieved by the use of a video digitizer. The first video digitizers were of the column scan type. These had no internal memory of their own and were able to sample the video signal only at rather low rates (Olivo & Thompson, 1988). In contrast, a modern frame grabber uses a high-speed analog-to-digital converter to enable real-time conversion of the entire video image to a high-resolution grid of pixels. It is also possible to acquire digitized video images by first converting the video input to a digital video format, such as AVI, and then using the AVI file as the input for object detection (Derry & Elliot, 1997). However, this method has two disadvantages: (1) The AVI file quickly gets very large (and so only trials of a limited duration can be carried out), and (2) the method does not allow for real-time live data acquisition. Alternatively, a digital overlay board can also be used to obtain positional data of tracked animals without the need for a frame grabber (Hartmann, Assad, Rasnow, & Bower, 2000; Rasnow, Assad, Hartmann, & Bower, 1997).

A number of modern video tracking systems use frame grabbers to digitize analog video signals. This enables high-speed data acquisition and, therefore, tracking of animals that are moving relatively fast. However, most of these systems have severe limitations. They can track only one animal in one arena. If they can track multiple objects, they cannot identify them individually. They tend to require simple backgrounds (in terms of their gray scale values) and can deal with only a limited range of experimental setups. These systems also cannot handle color video.

THE ETHOVISION SYSTEM

Development History

The EthoVision video tracking system, which we present here, was developed to overcome the limitations of the techniques and systems mentioned above. EthoVision has been designed as a general-purpose video tracking, movement analysis, and behavior recognition system. On the basis of a high-resolution color video frame grabber and flexible software, it is a versatile image processing system designed to automate behavioral observation and movement tracking on multiple animals simultaneously against a variety of complex backgrounds.

Our group has been involved with the development, deployment, and support of EthoVision for almost a decade. A predecessor of the current EthoVision video tracking system was first developed in the 1980s at the Rudolf Magnus Institute for Neurosciences (RMI) of Utrecht University (Spruijt, Hol, & Rousseau, 1992). In 1992, Noldus Information Technology and the RMI joined forces in order to develop this prototype into a mature video tracking system. Over the years, development proceeded in close partnership with two universities (Utrecht University and Wageningen University) and three pharmaceutical companies (Bayer AG, Germany; H. Lundbeck A/S, Denmark; Solvay Pharmaceuticals b.v., The Netherlands). The first DOS-based implementation was released in 1993. The system has undergone numerous updates over the years, on the basis of feedback from users around the world, which has resulted in a comprehensive package for studies of movement and behavior. The software has recently been redesigned from the

ground up for 32-bit Windows platforms, with a greater range of features and a highly interactive graphical user interface for display of experimental design, experimental arena, and tracks of movement. The first Windows version of EthoVision was released at the end of 1999.

This paper constitutes the first comprehensive publication about EthoVision (version 2.2, the latest version at the time of writing) in the scientific literature. The specifications and functionality of the DOS program have never been published, which is why many of the features already present in that version are given in some detail here. In the sections below, we will outline the functionality of the EthoVision software and present an overview of studies using EthoVision, to demonstrate the system's use in a wide variety of applications.

How EthoVision Works

EthoVision is an integrated system, comprising various software and hardware components (Figure 1). A CCD video camera records the area in which the subject animals are (the scene). The analog video signal is digitized by a frame grabber and passed on to the computer's memory. The frame grabber can either digitize the camera's signal directly or take input from a video cassette recorder. The software then analyzes each frame in order to distinguish the tracked objects from the background, on the basis of either their gray scale (brightness) or their hue and saturation (color) values. Having detected the objects, the software extracts the required features—in the case of EthoVision 2.2, this includes the position of the mathematical center of each object (center of gravity) and its surface area. These values are written to a track file on disk. Calculations are carried out on the features to produce quantified measurements of the animals' behavior. For instance, if the position of an animal is known for each video frame, and the whole series of frames is analyzed, the average speed of locomotion of an animal during an experiment or the distance between several individually identified animals can be calculated. In addition, if certain regions are identified as being of

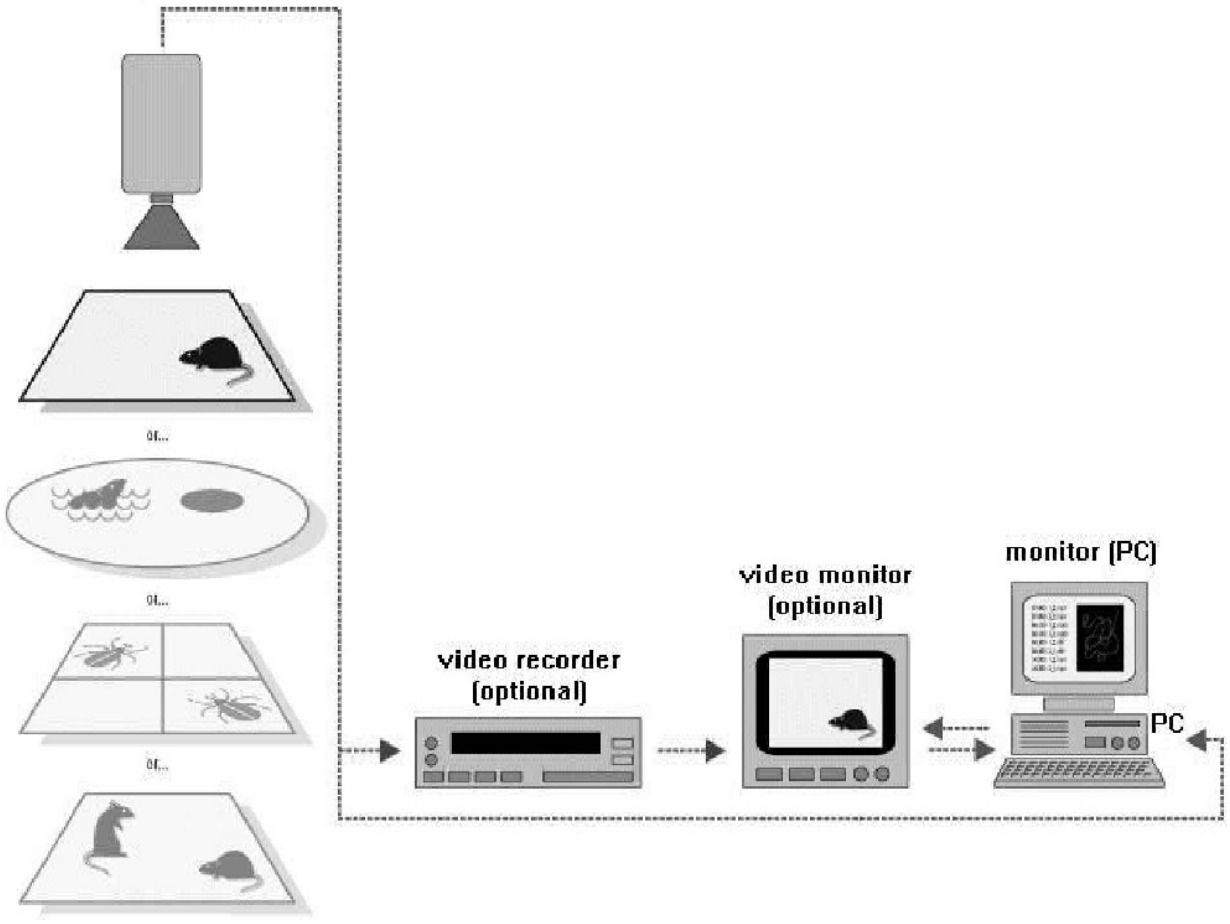


Figure 1. Diagram of an EthoVision setup. The analog signal from a CCD camera is fed into a frame grabber inside the computer, which digitizes the image. EthoVision then analyzes the signal to produce quantitative descriptions of the tracked animal's behavior. A video cassette recorder and monitor are optional components.

interest (e.g., the center and edges of an open field), the proportion of time spent by the animals in those regions can also be calculated.

ETHOVISION SOFTWARE SPECIFICATIONS

EthoVision may be thought of as consisting of a series of modules that function as an integrated whole. The functions of each of the modules are outlined below. The software has been written in Microsoft Visual C++ (using Visual Studio 6.0 with STL and MFC). The 32-bit code has been optimized for Windows 98, NT, and 2000.

File Management

Information in EthoVision is organized at several levels. The highest level is called a *workspace* (i.e., a container for one or more experiments). A workspace can be used to keep together experiments that have a specific relation (a similar setup, performed by the same experimenter, etc.). An *experiment* embodies a series of *trials* carried out with a particular experimental setup. The data from one animal collected during a trial (the *x*,*y* coordinates and body surface) is referred to as a *track*. In addition to the data files generated by EthoVision, the user can create *profiles*, which contain all the settings made for a particular function. The EthoVision Workspace Explorer enables the user to manage all these files through a simple interface (similar to the Windows Explorer), so that, for instance, settings defining the acquisition method (the tracking profile) can be dragged and dropped from one experiment to another (see Figure 2). An entire experiment or workspace can be backed up to a single file, to facilitate both safe keeping of the data and transfer between different computers. Tracks (and their associated independent variable values) can be imported into the current experiment from another experiment, enabling analysis of data across multiple experiments.

Experiment Design

Independent variables. As well as tracking objects, EthoVision also allows the researcher to define a complete experimental protocol, in terms of the independent variables of an experiment and their values. Up to 99 independent variables (e.g., treatment, room temperature, genetic strain, etc.) can be defined. EthoVision also automatically records a series of system variables, such as the profile (settings file) used, time and date of the trial, trial duration, and so on. These independent variables can be used to select, sort, and group data, both when plotting tracks and when analyzing the data. For instance, one can select to plot all the tracks from mice of a particular genotype or calculate the mean time taken to reach the target of a water maze by control rats relative to treated rats. When the trial is carried out, the user can, for each user-defined independent variable, select whether to accept the values predefined in the experimental protocol or enter new values. In addition, the possible range or a series of exact possible values can be defined for each independent variable (see Figure 3).

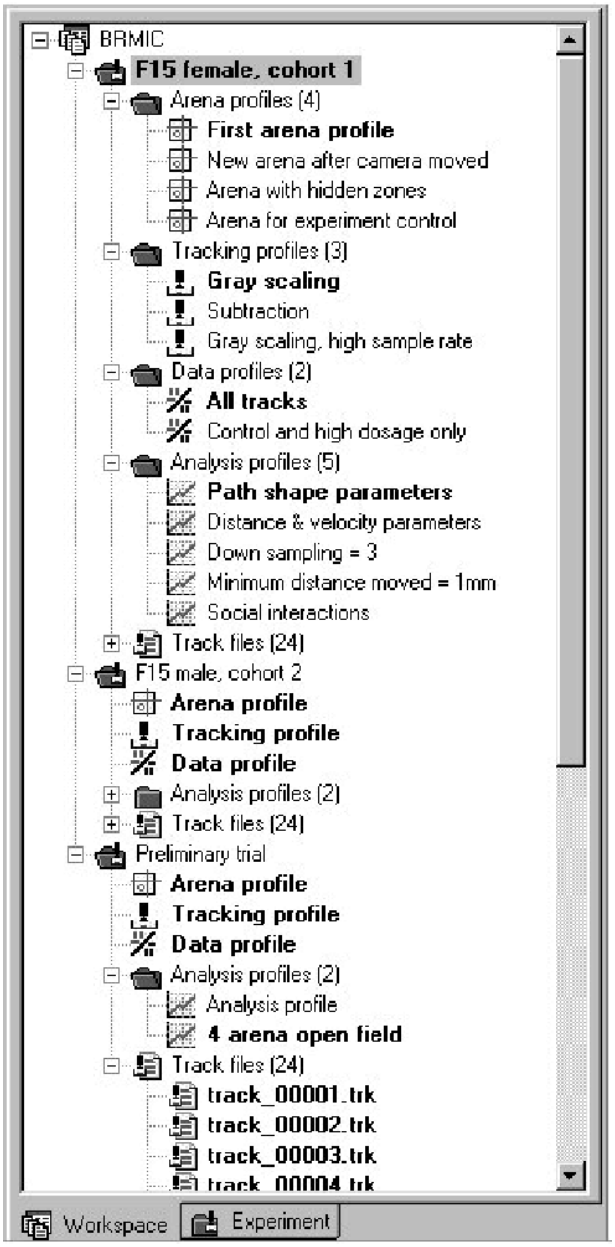


Figure 2. The EthoVision Workspace Explorer. The workspace "BRMIC" contains three experiments. Experiment "F15, female, cohort 1" is active (shown in bold), and it contains a series of different profiles, illustrating the different uses to which these stored settings may be put.

Scheduling trials. Prior to data acquisition, one can predefine the values of the independent variables for a series of individual trials one plans to run in an experiment. In this way, the design of the experiment and the testing can be done before the actual trials are performed. EthoVision can thus be used to schedule the trials (i.e., assist the experimenter in applying the correct treatment and select the correct animals). During data acquisition, the values of the independent variables are displayed on

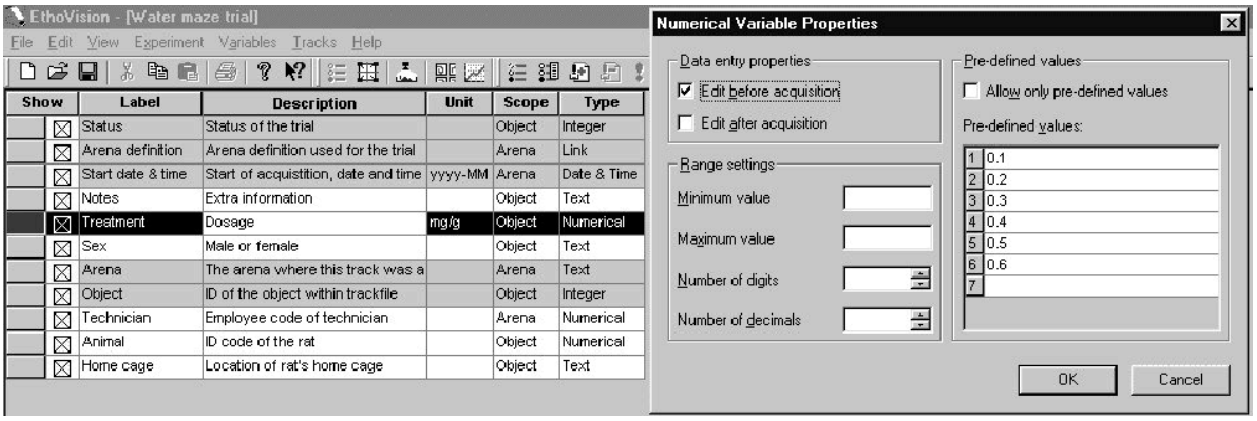


Figure 3. Defining independent variables with EthoVision. The system-independent variables are shaded, and the user-defined variables have a white background. The independent variable properties are also shown for the selected variable (treatment).

the computer monitor, providing immediate feedback on the characteristics of the trial (which animal is currently being tested, what the treatment is, etc.). Once the experiment design has been entered, it can be used in multiple experiments, thus reducing preparation time for repeated experiments.

Arena and Zone Definition

Arenas. Up to 16 enclosures can be placed under one camera, and each enclosure can be treated as a separate independent replicate (called an *arena*; see Figure 4). The animals in these arenas can be tracked simultaneously. The arena can be of any shape, which is easily defined using special drawing tools. Multiple arena profiles and zone definitions can be defined for each experiment. This is particularly handy when, for instance, a water maze experiment is prepared with multiple platform positions. When setting up the experiment, the user can assign the proper arena definition to each track file before the experiment is started. If one defines an arena in the video image in which object tracking takes place, the system ignores parts that do not belong to the defined arena during data acquisition. This reduces the chance that events outside of the experimental arena will interfere with the actual measurement. By using different drawing options (rectangle, circle, polygon, curve, line, and freehand), one can quickly draw any shape of experimental setup and enter it into the computer. This means that one can use the system for a water maze experiment, an open field test, or any other standard test. Because the signal from the video camera is directly displayed on the computer screen, the arena outlines can be traced with high accuracy.

Zones and points of interest. The user can define up to 99 regions of interest (called *zones*). These can be used in the analysis (e.g., to compute the time spent in different parts of a cage) and in automatic start and stop conditions. For instance, the trial can be stopped automatically when a mouse has visited all the arms of an eight-arm maze. Zones can be added together to make a cumulative zone (e.g., if a maze has more than one target). Zones can also be defined as *hidden zones*, for use with nest boxes, burrows, and so on. The system assumes that when an animal disappears from view and it was last seen adjacent to a hidden zone, it is inside the hidden zone. A hidden zone can also double as a normal zone: If the animal is visible within the boundaries of the zone, EthoVision assumes that it is on top of, for example, the nest box. All zones can be defined and altered either before or after data acquisition, allowing iterative exploratory data analysis of the effects of changing zone positions and shapes. Points of interest can also be defined (e.g., the center of an open field or a novel object that is placed in a stable).

Calibration. The arena can be calibrated so that parameters, such as velocity, are calculated in meaningful units, such as millimeters or inches, instead of pixels. There are two calibration methods. In *standard calibration*, the user measures a series of lines (e.g., a meter ruler placed on the floor of a cage). If the camera image is distorted, the standard calibration will not be accurate (the standard deviation of the calibration factors is given to enable the user to determine this), and the user can opt for *advanced calibration*. In advanced calibration, a grid of points is mapped onto the arena, and a series of coordinate points are entered, giving a calibration that is accurate for the entire image, even when it is distorted. This enables the distance-based parameters to be accurately calculated when the arena is in a room with a low ceiling, when an exceptionally large arena necessitates the use of a very wide angle or fish-eye lens, or when the lens is not directly overhead in relation to the arena.

Data Acquisition

Running a trial. After the user has defined the sample rate (up to 30 per second) and trial duration, EthoVision is ready for data acquisition. During tracking, the computer shows a combination of the recorded path, the detected object shape, the current arena/zone definition, and the original video image (see Figure 5). Elapsed time,

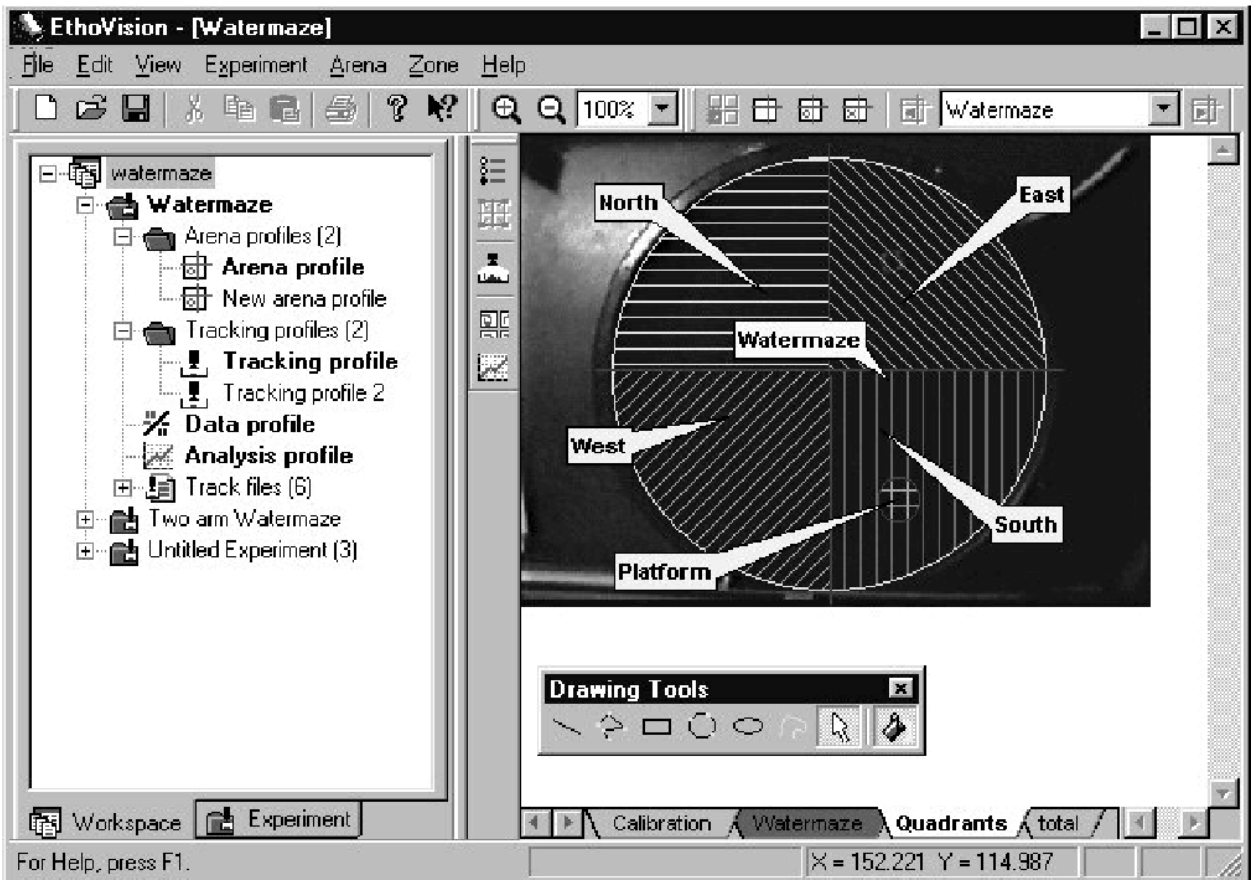


Figure 4. An EthoVision arena definition for a water maze experiment. The circular arena (region from which data are acquired; here labeled "Watermaze") is divided into four zones (labeled "North," "East," "South," and "West"). The small zone "Platform" defines the location of the platform. The area outside the arena is ignored, leaving data acquisition undisturbed by any movement that takes place in that part of the scene (e.g., an operator walking by).

number of samples, and various other statistics are also provided in real time. This allows immediate action if a tracking error occurs. An optional remote control can be used to start and stop trials at a distance from the computer (e.g., as you stand next to the experimental setup).

Object detection. To ensure optimal object detection in any experimental setup, EthoVision offers three different object detection methods. *Gray scaling* is the fastest. This method defines the animal as all connecting pixels that are both brighter than a low gray scale threshold and darker than a high threshold, and it defines the background as all other pixels. The thresholds can either be set manually by the user or be calculated automatically by the program. Gray scaling is a fast detection method (allowing the highest sample rate), but it cannot be used if the same gray scale values are present in both the animal's image and the background.

The second method, *subtraction*, first (before the start of the trial) makes a reference image, with no animals present, and then (during the trial) subtracts the gray scale value of each pixel of the reference image from the equivalent pixel of the live image. Any pixels that belong to objects larger than those defined as noise and that have a subtracted value other than zero constitute the difference between the reference and the live images, due to the presence of the animal. The user can define whether the animal has to be lighter or darker than the background or just different. This method tolerates more differences in light intensity across the scene. It thus becomes possible to separate an animal with light skin on a grayish background from its (black) shadow. The method is also suitable for animals with a heterogeneous fur pattern, such as hooded rats, spotted mice, or cows.

The third detection method, *color tracking*, uses the color of the animal (or a marker painted on it) to identify and track it. EthoVision uses the hue and saturation components of the hue–saturation-intensity (HSI) color space model to track objects (Spink, Buma, & Tegelenbosch, 2000; see Figure 6). By using both hue and saturation, EthoVision can better distinguish objects that are more similar in color to each other than if only using hue (e.g., objects with the same hue but differing saturation values; see Figure 7), which is why the system can track as many as 16 different colors in each arena at once (under

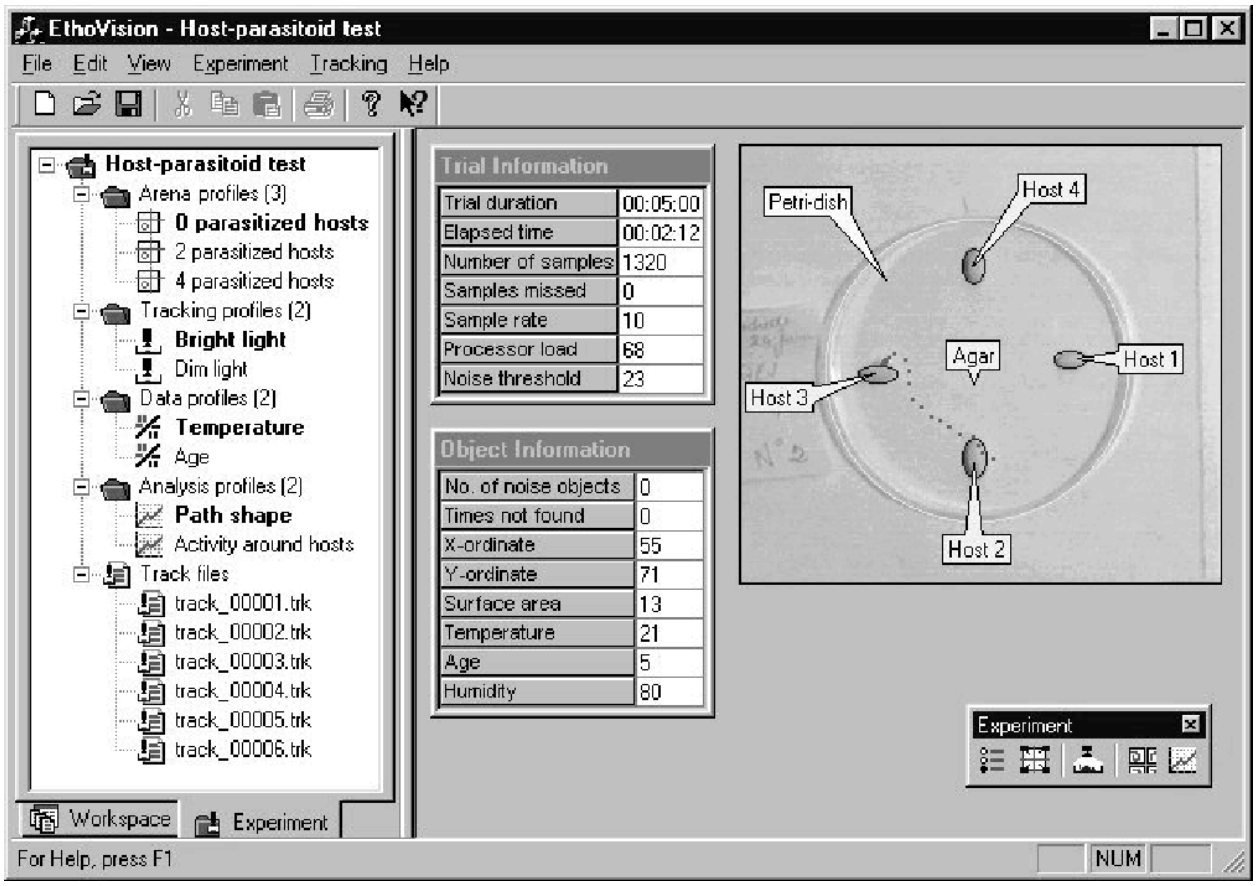


Figure 5. Data acquisition in progress during a test of a parasitic wasp searching for hosts in a petri dish. In addition to the live video image with overlaid zone borders and movement track, EthoVision shows tracking statistics (elapsed time, number of samples, processor load, etc.), measurement values of the object (*x*,*y* coordinates, surface area), and characteristics of the trial (independent variables). Image courtesy of J. P. Monge (University of Tours, Tours, France).

suitable conditions). In addition, the use of these two complementary detection descriptors makes the object identification more robust, so that, for instance, objects can be tracked more reliably if the light intensity (brightness) is uneven across the arena.

With all three methods, objects that are either smaller than the animal (e.g., droppings) or larger than the animal (e.g., reflections) can be excluded on the basis of their size.

Object identification. EthoVision distinguishes between two animals in the same arena on the basis of size. To distinguish two animals that are the same size, the user can partly color one animal to the same grayness as the background, thus reducing its apparent size (see Figure 8). Color tracking can be used to distinguish more than two animals (up to 16 per arena). If the animals being tracked are the same color, markers can be used to paint blobs on the animals, which the system will be able to track. For more details on color tracking, see the "Object Detection" section above.

Image resolution. The user can further fine-tune data acquisition by modifying the image resolution. EthoVision can track at low, medium, and high resolution. At high resolution (= 768 × 576 pixels), EthoVision can track an animal in an arena with a variable shape and up to 200 times the animal's size. By comparison, an infrared detector system typically operates in an arena of 0.5 × 0.5 m with 12–32 beams (though systems have been developed with a grid of 24 × 24 beams, in an arena 2 m in diameter; e.g., Robles, 1990). Other options include the possibility to manually adjust contrast, brightness, hue, and saturation, in order to optimize digitization of the video signal. This is useful when analyzing trials that were prerecorded on videotape. Furthermore, one can scan the complete arena or use a moving *scan window*. With the latter option, only a limited area around the object's last position is searched. As a result, tracking is not disturbed by occasional moving objects elsewhere in the arena (e.g., reflections). When an animal is temporarily out of view, EthoVision can be set to resume tracking by automatically repositioning the scan window.

Image filtering. EthoVision calculates the mathematical point at the center of the digitized picture of the animal's body, including the tail (e.g., in a rat or mouse).

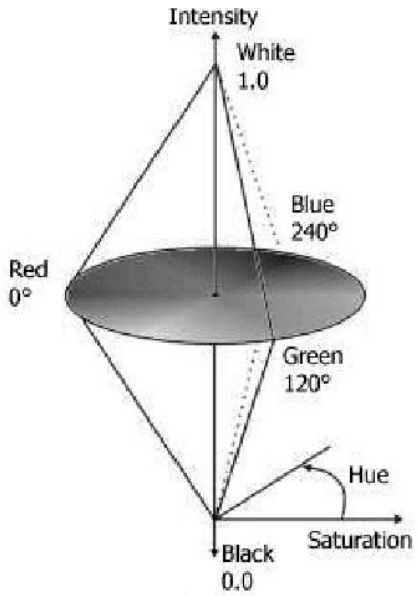


Figure 6. The HSI color space model. The horizontal distance from the center of a horizontal plane to its outside represents a color's saturation (fullness), the angle on that plane represents its hue (color), and the vertical distance (*z*-axis) represents its intensity (brightness).

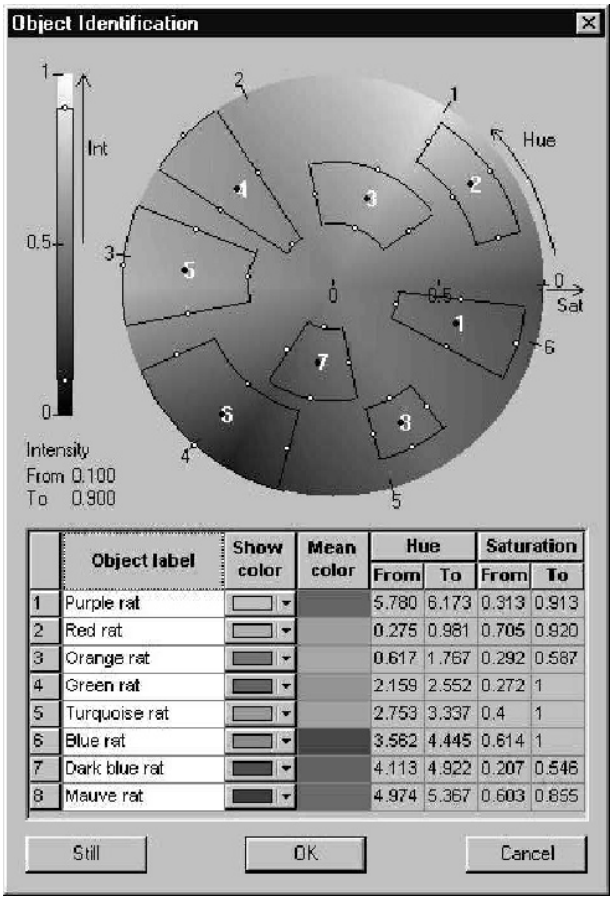
This means that this point is farther toward the animal's posterior than is correct. Furthermore, the system will still detect movement of the animal when only the tail is moving. In order to prevent this, the tail can be removed from the image using image filtering (erosion). The bars of a cage can also be filtered out using a similar technique (dilation). These filters also help to avoid accidental mix-up of the animal with background noise and to improve the computation of body-shape-related parameters.

Recording specific behaviors. In addition to automatically tracking the movement of the animal, EthoVision can automatically detect certain behaviors, such as rearing and moving (see the Appendix for details). To allow detection of rearing behavior of rodents using a single overhead camera, EthoVision stores the surface area of the animal's body for each sample taken. EthoVision also allows the researcher to manually record (by keystroke or mouse click) behaviors that cannot be detected automatically (e.g., sniffing).

Experiment control. EthoVision also has a facility for automatic experiment control (see Figure 9). This can be used to start or stop data acquisition depending on the location of the animal. For instance, the system can be set to start a trial when a rat enters a water maze and stop it when the rat reaches the platform or to stop the trial after the animal has been in a user-defined zone for more than a user-defined length of time. One can also let the system perform series of trials with user-defined number of trials and intertrial interval (e.g., 72 consecutive trials, each with 5-min duration and 55-min intervals in between), which is useful for studies of behavioral rhythms.

Selection and Visualization of Track Data

The user can specify data sets at several hierarchical levels for track analysis and visualization. The data can be selected, sorted, and grouped by independent variables. In addition, the track files can be split up (nested) by time. Thus, a selection of the tracks in an experiment can be plotted on a single screen—for instance, in a matrix of treatment level × genetic strain. Other variables, such as gender or day of the week, can be shown in different colors and line styles. Furthermore, time windows can be applied (e.g., just the first 60 sec of the track). The track plots can be displayed with or without the arena and zones or the background image of the experimental setup. The plots can be exported as graphic files in a variety of standard formats, ready for inclusion in presentations, reports, or research articles. Figure 10 shows a typical EthoVision visualization of tracks.



	Object label	Show color	Mean color	Hue From	Hue To	Saturation From	Saturation To
1	Purple rat			5.780	6.173	0.313	0.913
2	Red rat			0.275	0.981	0.705	0.920
3	Orange rat			0.617	1.767	0.292	0.587
4	Green rat			2.159	2.552	0.272	1
5	Turquoise rat			2.753	3.337	0.4	1
6	Blue rat			3.582	4.445	0.614	1
7	Dark blue rat			4.113	4.922	0.207	0.546
8	Mauve rat			4.974	5.367	0.603	0.855

Figure 7. Object identification based on color. Each shape on the disk (which is displayed in color on screen) represents a volume in HSI color space, and the user can drag the shape so that it includes the range of hues and saturations present in the animal or marker being tracked. When the color defined here matches the color of the animal or marker, the software displays those pixels in the color selected in the "Show color" column, thus giving visual feedback as to when the object is correctly identified.

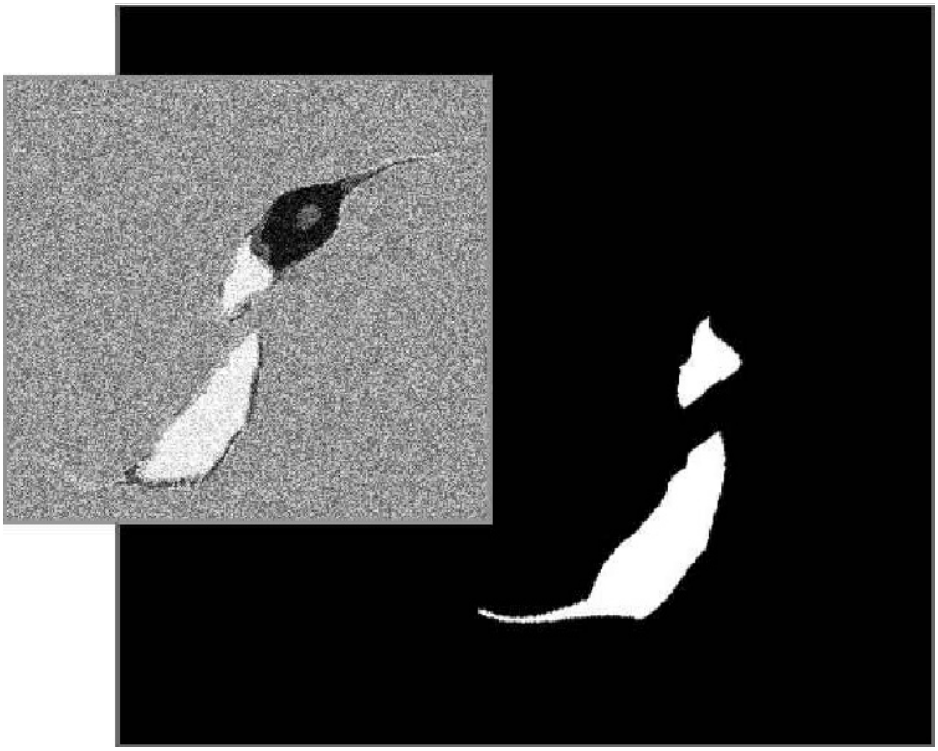


Figure 8. To distinguish two rats that are the same size, one has been partly colored black. After applying a gray level threshold, only the bright parts of the body remain, thus creating an apparent size difference.

EthoVision can replay the recorded tracks on the screen, allowing a detailed and interactive visual analysis of the data. One can choose for playback to be either at the original speed or at a user-defined speed and set the number of data points that are displayed simultaneously. One can also move through the tracks, showing each time only a selection of data points.

Data Analysis

Data selection for analysis is carried out in the same way as for data visualization, and, in fact, the same data profile is used so that the data selection made for visualization is automatically available for analysis (and vice versa). The user can group tracks by the values of independent variables (e.g., the mean velocity can be calculated for all animals that received a particular treatment) and further fine-tune the data using filter settings (e.g., minimal distance moved) to eliminate slight "apparent" movements and using down-sampling steps to eliminate redundant data if a lower sample rate describes the path better. A wide range of quantitative measures of behavior is available in EthoVision, including parameters for location and time, path shape, individual behavioral states, and social interactions, and these can be described with a full range of descriptive statistics. The parameters, statistics, and raw data (i.e., series of x,y coordinates and other extracted image features for each individual as a function of time) can be exported in a variety of formats for further analysis and production of graphs in third-party programs, such as Microsoft Excel, SAS, SPSS, The Observer (Noldus et al., 2000), WinTrack (Lipp & Wolfer, 2000; Wolfer & Lipp, 1992), or SEE (Drai, Benjamini, & Golani, 2000). More detailed information about these parameters is given in the Appendix.

REVIEW OF STUDIES USING ETHOVISION

In contrast to some other video analysis systems (e.g., Mukhina, Bachurin, Lermontova, & Zefirov, 2001; Pan, Lee, & Lim, 1996; Spooner, Thomson, Hall, Morris, & Salter, 1994), EthoVision is a generic tool that can be used in a wide variety of setups and applications. There are, of course, practical limitations; however, in principle, EthoVision can be used to track animals in any situation where the animals are within sight of a camera and in a delimited and constant area that is no more than 200 times the size of the animal. The majority of studies using EthoVision are carried out using small rodents, but the system is also used in research on insects, fish, birds, primates, farm animals, and other mammals. The review below gives an overview of the main types of research using EthoVision; however, it is by no means an exhaustive list of all current uses of EthoVision.

Neuroscience and Pharmacology

The most common application of EthoVision is as a tool for measuring the behavior of rats and mice that have had their brain chemistry altered by application of

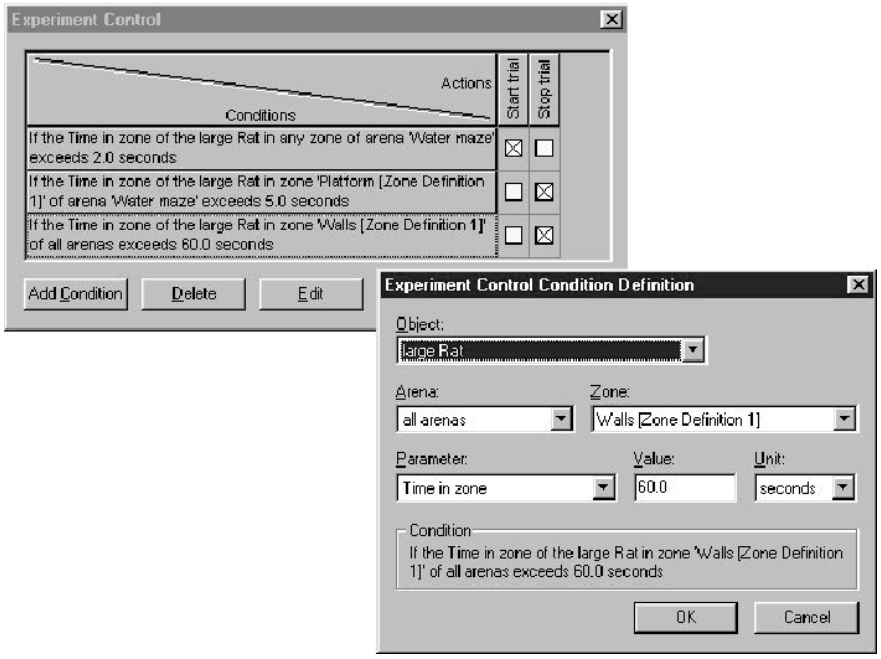


Figure 9. The Experiment Control dialog, with three conditions and two actions. The properties of the third condition are shown in detail.

drugs or neurochemicals (e.g., Ploeger, Spruijt, & Cools, 1994; Sams-Dodd, 1995), surgery (e.g., Eijkenboom & van der Staay, 1998; van Rijzingen, Gispen, & Spruijt, 1995), or the use of a particular genotype (e.g., Minichiello et al., 1999; Spink, Tegelenbosch, Buma, & Noldus, 2001). Research of this type often combines the above techniques (e.g., testing whether injecting a mineralocorticoid receptor antagonist can counter the effects of a lesion; Oitzl, Josephy, & Spruijt, 1993). The brain functioning and behavior of rats and mice are often used as models of human brain functioning and behavior in the study, for instance, of the effects of addiction (Chow, Tomkins, & Sellers, 1996; Miczek, Nikulina, Kream, Carter, & Espejo, 1999) or sleep deprivation (Meerlo, Overkamp, Benning, Koolhaas, & van den Hoofdakker, 1996). The majority of neuropharmacological studies use either the Morris water maze to test spatial learning (Morris, 1984; see, e.g., Dalm, Grootendorst, de Kloet, & Oitzl, 2000; Lipp & Wolfer, 2000; see Figures 4 and 10) or the open field test to study anxiety and/or social interaction (e.g., Spruijt et al., 1992; van den Berg, Spruijt, & van Ree, 1996). These tests are ideally suited to automatic data acquisition and analysis with EthoVision; they take place in controlled conditions in a simple defined area, and the resulting behavior can be readily quantified.

With the Morris water maze, the researcher tests spatial memory by measuring how long a rat or mouse takes to swim to a hidden platform. When it reaches the platform, it can stop swimming. The animal is first trained, and it normally relies on visual clues to find the platform again. The test thus usually assesses both visual processing and spatial memory. EthoVision can be used to define a zone where the platform is and measure the time taken for the rat or mouse to reach the zone. It can also be used to assess the reason an animal might "fail" the test. For instance, some strains of mice hug the walls when stressed (thigmotaxis) and, therefore, never find the platform. This can be an appropriate behavioral response in the animals' natural environment, and it is important not to confuse this with failing to learn where the platform is because of an error in its learning mechanism (Gass et al., 1998; Lipp & Wolfer, 2000). Figure 10 shows the tracks from a Morris water maze experiment.

With an open field test, the researcher simply places one or more animals in an open space (usually a square enclosure with the side about 5–10 times as long as the length of the animal's body). As an animal becomes less anxious, it spends less time by the walls and more time in the open (e.g., Meerlo et al., 1996). Novel objects can be introduced into the field, and the animal will investigate the object, spending a variable amount of time by the object, depending on the animal's state (Whishaw, Haun, & Kolb, 1999). One of the strengths of EthoVision is that the arena can be divided up into a large number of zones, of any size and shape, so that the positions of objects in the field and areas around the object can be accurately defined and the positions and movements of the animal can be measured in relation to those zones. In addition, the zones can be defined after data acquisition has occurred, so that if areas of interest become apparent only after an initial analysis, these can be investigated further. Moving novel objects can also be placed in the

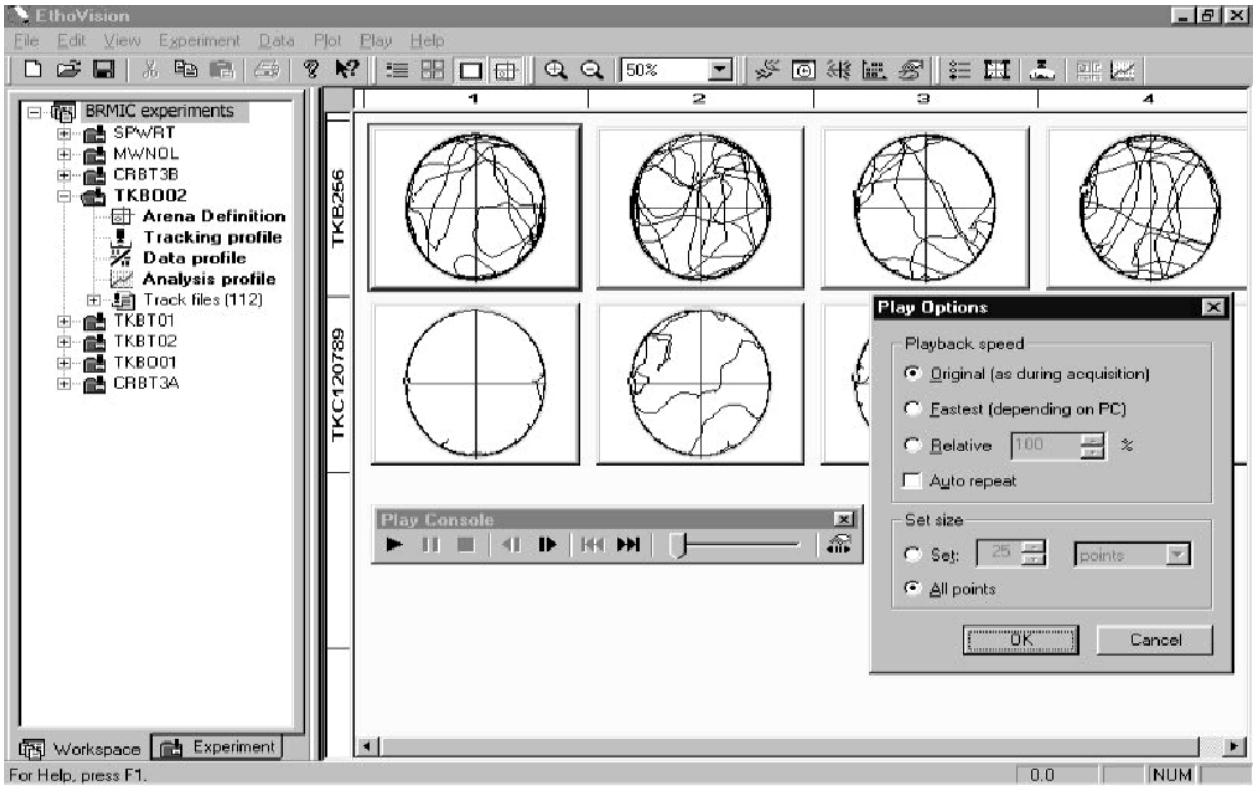


Figure 10. Visualization of an EthoVision water maze experiment (data from Minichiello et al., 1999). The Workspace Explorer can be seen on the left. In the main window, eight tracks are plotted, sorted by trial number (1–4) and genetic strain. The "Play Console" (with VCR-type controls) and "Play Options" dialog box are also shown.

open field, and these can be tracked in the same way that the animals are tracked (Spruijt et al., 1992). The way that the animals move in relation to the other objects can then be quantified by EthoVision, and this can be interpreted in terms of approach, avoidance, and so on.

Toxicology

A standard assessment for the effects (or lack of effects) of toxins and suspected toxins is the measurement of any changes in behavior under standard conditions (Moser et al., 1997). EthoVision is used by a number of groups for these measurements. For example, Hany et al. (1999) looked at the behavioral effects on rat pups of exposing their mothers to PCBs during the gestation period. The behavior of the rats in an open field was measured using EthoVision. The open field was divided into inner and outer zones, and it was shown that rats exposed to PCBs avoided the outer zone. Because there was no difference in the total distance moved between treatments, this was interpreted as a change in the emotional state of the rats, rather than an impairment of motor function. Weinand-Härer, Lilienthal, Winterhoff, and Winneke (1996) also used EthoVision to analyze the effects of PCBs on rat pups' performance in a Morris water maze. Another typical toxicological application is research on the behavioral effects of nicotine (Kim et al., 2000). Healing et al. (1997) concluded that EthoVision can be used to reliably assess changes in motor activity to a standard acceptable to regulatory authorities.

Entomology

Although EthoVision is most commonly used to track rats and mice, it can also be used successfully to track insects and arachnids. For example, Bouman, Simek, Zemek, Dusbabek, and Zahradnickova (1999) looked at the effect of female status on behavioral interactions between male and female *Ixodes ricinus* ticks, whose walking pattern was tracked and analyzed by EthoVision. The latency to contact between male and female ticks and speed and duration of movement of males toward females were interpreted as indicators of female attractiveness over short distances, and the mean distance between the sexes was interpreted as an index of the overall attractiveness of the female. Bouman et al. found that the males did not walk randomly but were attracted to the females and that the males were more attracted to the females that were engorged on guinea-pig blood.

Kröber and Guerin (1999) also studied ticks with EthoVision. They analyzed the path moved by *Boophilus microplus* and *Ixodes ricinus* in relation to a patch of water. In general, as soon as one of the tick's legs touched the water, the tick rotated and walked away from the

water. However, if the ticks were dehydrated, they did not avoid the water. It was hypothesized that ticks have to strike a balance between their need for water vapor to maintain water balance and the danger of freezing when coming into contact with ice crystals in the winter.

Parasitic wasps such as *Encarsia formosa* are of great commercial value in biological control of greenhouse pests. A video tracking system such as EthoVision can be a useful tool in selecting species suitable under given conditions for controlling certain pests. For example, Drost, Qiu, Posthuma-Doodeman, and van Lenteren (2000) measured the velocity and turning rate of parisitoids of *Bemisia argentifolia* whitefly, in order to characterize the searching pattern and to identify the best natural enemy. The wasps were tracked in a petri dish lit from below, which gave a good high-contrast image. The edge of the leaf disk was defined as a zone and was excluded from analysis by nesting (because the boundary causes unnatural 180º turns). The data will be used to create a simulation model of host-searching behavior for each of the species studied. Krips, Kleijn, Wilems, Gols, and Dicke (1999) carried out a similar study to determine the effects of leaf hair density on the searching efficiency of *Phytoseiulus persimilis* (a predatory mite used for biological control of spider mites); although a species may have been successfully used to control the pest on a plant with smooth leaves, it may be quite unable to cope with leaves with dense hairs. Movement parameters have the potential of becoming a standard measurement in the quality control of mass-reared insects (van Schelt, Buma, Moskal, Smit, & van Lenteren, 1995). EthoVision has also been used in studies of behavioral aging in *Drosophila* (Le Bourg & Minois, 1999).

Animal Welfare

EthoVision has been used to study stress effects on a variety of species, including captive animals such as farm animals (Bokkers & Koene, 2000; Šustr, Špinka, & Newberry, 2000), laboratory animals (Ruis et al., 1999), and zoo animals (Fuchs, Kramer, Hermes, Netter, & Hiemke, 1996; van Kampen, Schmitt, Hiemke, & Fuchs, 2000). For example, battery hens were tracked with EthoVision as they walked toward food. The latency to pecking the food and the speed of walking toward the food were measured and used to assess the animals' motivation and ability to walk (Bokkers & Koene, 2000).

Šustr et al. (2000) marked the front and rear of pigs with different colors and tracked these with EthoVision. They were then able to use the EthoVision data to analyze pig play and pig fights in terms of mutual spatial position and orientation. First, the data were corrected for samples missed when, for example, the head was dipped down and out of sight of the camera. Then, the actual positions of the heads and snouts were calculated, using the positions of the markers on the pigs and the known distances from the markers to the heads and snouts. These positions were then used to calculate how close the pigs were to each other and which pig was "active" (i.e., its snout was nearer to the body of the other one). Finally, the angle of the two bodies and point of contact were calculated.

Fish Behavior

One of the first uses of an early predecessor of the current EthoVision program was to study locomotor activity in Arctic char (Winberg, Nilsson, Spruijt, & Höglund, 1993). This species is still being studied with EthoVision. The char are placed in a fluvarium, the upstream end of which is divided into two halves. Each half receives water from a separate source. Every 90 min, the tanks are switched, and the fish are tracked for the latter half of each 90-min period. The tanks are backlit with light with a wavelength of less than 750 nm, which is detectable by a monochrome camera but not by the fish. The analysis is based on the frequency that the fish are in each half of the fluvarium (which is defined as an EthoVision zone), which is in effect a Y-maze. Bjerselius, Olsén, and Zheng (1995a, 1995b) found that male fish avoided the half of the fluvarium that had elevated levels of the female hormone indicating that the female was not spawning. If, instead of a semiochemical being added, the two halves of a fluvarium were attached to tanks containing other char, the char in the fluvarium had a different frequency of being in the two halves of the fluvarium, depending on whether the water came from fish that were siblings (Olsén & Winberg, 1996) and whether the siblings had an identical major histocompatability complex (Olsén, Grahn, Lohm, & Langefors, 1998).

Ylieff, Sanchez-Colero, Poncin, Voss, and Ruwet (2000) were able to use EthoVision to track damsel fish (*Chromis chromis*) marked with colored pearls to quantify variables that cannot be measured accurately by direct observation methods. Having determined that the colored marker did not affect velocity, distance moved, or time in various zones, they investigated the effects of water temperature and fish density on the behavior of the fish. They found that, at high temperatures, the fish swam faster when they were near the surface, which was hypothesized to be an adaptation to escape from predatory birds.

Van Ginneken et al. (1997) used EthoVision to combine calorimetry of tilapia with analysis of activity. In this way, the effects of various movements on the increased metabolic rate of the fish under various light and oxygen conditions could be analyzed.

DISCUSSION

As can be seen from the "Review" section of this paper, one of the strengths of EthoVision is that it is flexible enough to be used in a wide variety of experimental setups and applications and with a wide-ranging assortment of species. Some other systems have been specifically designed for a particular experiment, such as the Morris water maze (e.g., Mukhina et al., 2001; Spooner et al., 1994), whereas EthoVision can work with any shape of arena, with a large variety of backgrounds and lighting conditions. The ability of EthoVision to track multiple animals in an arena means not only that social interac-

tions can be studied (Rousseau, Spruijt, & Gispen, 1996; Sams-Dodd, 1995; Sgoifo, Kole, Buwalka, de Boer, & Koolhaas, 1998; Spruijt et al., 1992) but that separate parts of the animals can be marked and tracked independently (Šustr et al., 2000). The sophisticated range of data selection options and the wide array of variables that EthoVision can calculate to quantify the animals' behavior (parameters) mean that the user is able to use the system to produce accurate descriptors of complex behaviors in diverse situations and is able to gain an overview of the behavior from the visualization of the animals' tracks.

Future Developments

Of course, despite the power and flexibility of a system such as EthoVision, there is always room for further development and improvement. We are currently involved in, and in the past have carried out, a number of special projects developing customized versions of EthoVision. Which of these customizations are integrated into the standard software depends on both the technical feasibility and interest in the application. The following aspects are some indications of the way in which the EthoVision system might be developed in the future.

Support of digital video. At the moment, the EthoVision software takes its input from an analog video camera, the signal of which is digitized by a frame grabber. A logical development in the future is that EthoVision would be able to take direct input from either digital cameras or digital video files.

Analysis of thermographic images. The EthoVision system is designed to measure animals' behavior and not the physiological changes that may have influenced that behavior. Of course, it is used in many studies with a physiological aspect (as in many of the studies cited in this "Review" section), and physiological data gathered at the same time are commonly analyzed together with the behavioral data. A specialized application of EthoVision can use the system to directly measure a physiological parameter. It is well known that stress can cause changes in body temperature of various homeothermic species, such as rats and mice (Clement, Mills, & Brockway, 1989). However, the very act of inserting a rectal probe or implanting a biotelemetry device (both are commonly used techniques to measure an animal's body temperature) stresses the animal and raises its temperature (van der Heyden, Zethof, & Olivier, 1997). As an alternative, Houx, Buma, and Spruijt (2000) have developed a noninvasive method to measure body temperature. An infrared thermographic video camera is connected to a customized version of EthoVision, and the gray scale value (i.e., brightness) of each pixel in the image is proportional to the animal's temperature at that location. Thus, the animal's behavior and temperatures of different regions of the body can all be measured synchronously. Further research is necessary before this technique can become a standard tool.

Synchronization of behavioral data (video) and physiological signals. In order to carry out a full statistical analysis of variables derived from both EthoVision track files and various other data, it is necessary for the various data streams to be synchronized. At the time of this writing, we are carrying out a research project, together with several partners, to resolve this issue (Hoogeboom, 2000).

Automatic detection of complex behaviors. In its current implementation, EthoVision is able to detect whether an animal is moving or whether a rodent is rearing, but (in common with other commercial systems) it cannot automatically detect other body postures or behavioral patterns (unless, of course, it is possible to define them in terms of the existing parameters). Research on automatic classification of body postures and behavioral acts in digitized video sequences was already in progress more than two decades ago (Kernan et al., 1980) but has not, to our knowledge, found its way into a commercial product so far. Recently, however, considerable progress has been made with the use of model-based pattern recognition, statistical classification, and neural networks to automatically detect rodent behaviors, such as sitting, grooming, and stretched attend (Rousseau, van Lochem, Gispen, & Spruijt, 2000; van Lochem, Buma, Rousseau, & Noldus, 1998). Further refinement of these and other algorithms (e.g., Heeren & Cools, 2000; Twining, Taylor, & Courtney, 2001) is necessary before they can be incorporated into the EthoVision system. This development is likely to benefit from recent work done on automated coding of human facial expressions, using digital image processing, active shape modeling, and neural network techniques for pattern classification (Ekman & Friesen, 1978; Lanitis, Taylor, & Cootes, 1997; Tian, Kanade, & Cohn, 2001).

Tracking large and indeterminate numbers of animals. Tracking large and continually varying numbers of animals, especially if crowded close together, calls for techniques quite different from the object identification methods used by EthoVision. Some progress has been made using techniques such as track segmentation and reconstruction (Buma, Moskal, & Liang, 1998) and active modeling and prediction of the animals' shapes (Bulpitt, Boyle, & Forbes, 2000; Sergeant, Boyle, & Forbes, 1988).

Availability

EthoVision is commercially available from Noldus Information Technology and various international distributors. Readers can contact the first author for more information or visit the EthoVision homepage on the Web (http://www.noldus.com/products/ethovision/).

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APPENDIX
EthoVision's Analysis Parameters

Each selected parameter (e.g., velocity) is calculated for each individual sample. The user selects which statistic is required (e.g., mean), and the combination of the two is displayed (e.g., mean velocity) per track or per group (e.g., the mean of the total distance moved for all animals that received a given treatment). Input filters can be applied to the data to down-sample the points and/or exclude samples where there is just an apparent movement due to, for example, breathing. Most of the parameters (e.g., distance moved and velocity) are continuous variables, but some are state variables because they are either true or false (in zone, relative movement, moving, rearing, and manually recorded behaviors).

Location and Time

Distance moved. The length of the vector connecting two sample points (i.e., the distance of the center of gravity of the tracked animal between one sample and the next). This is calculated using Phythagoras' theorem ($a^2 + b^2 = c^2$).

Velocity. Distance moved per time unit (i.e., speed).

Distance to point. The distance between the center of gravity of the tracked animal and a location inside or outside the arena defined by the user.

Distance to zone center. The shortest distance between the center of gravity of the tracked animal and the center of a user-defined zone.

Distance to zone border. The shortest distance between the center of gravity of the tracked animal and the border of a user-defined zone.

In zone. Whether or not the animal is in a particular zone. Zones can be redrawn at any time. They can also be added together to form *cumulative zones* and can be *hidden zones* for use with burrows or nest boxes.

These basic parameters are used to calculate a series of derived parameters (see below).

Path Shape

This category describes the geometrical shape of the path traveled by an animal. Some of these parameters can be based on unsigned degrees (absolute) and signed degrees (relative).

Heading. The direction of movement in relation to a user-defined reference line.

Turn angle. Angle between the movement vectors of two consecutive sample intervals (absolute or relative).

APPENDIX (Continued)

Angular velocity. Speed of change in direction of movement (i.e., amount of turning per unit of time [absolute or relative]). The angular velocity of each sample is the turn angle for that sample, divided by the sample interval.

Meander. Change in direction of movement relative to the distance moved (i.e., amount of turning per unit distance [absolute or relative]). The mean of each sample is the turn angle for that sample, divided the distance moved from the last sample.

Individual Behavioral States

Activities of the animal are assigned to behavioral states.

Movement. Whether or not the tracked animal's velocity exceeds a user-defined level. The user can set thresholds for both "start velocity" and "stop velocity," and the parameter is calculated over a running average of a user-defined number of samples. The parameter has two states: "moving" and "not moving."

Rearing. The state in which a rodent's body is vertically erected. It is a useful parameter for assessing exploratory behavior. It is detected by measuring the decrease in surface area when the rat or mouse stands up. The user can define a running average and percentage decrease in area to customize the parameter for different species and conditions.

Manually recorded behaviors. While EthoVision is tracking the animals, the user can register any predefined behaviors by pressing a key (or with the mouse), and these can be analyzed in exactly the same way as any of the state variables automatically measured by EthoVision.

Social Interactions

On the basis of relative movement between pairs of simultaneously tracked animals, distances and movements are classified and assigned to particular parameters of social behavior.

Distance between objects. The distance between the center of gravity of two animals.

Proximity. Whether or not the tracked animal is closer than a defined distance from another animal. The user can define threshold values for both "in proximity" and "not in proximity" to ensure that the frequency of transitions is biologically meaningful.

Relative movement. Whether or not an animal shows a relative displacement toward ("moving to") or away from ("moving from") another tracked animal. EthoVision does not just measure whether two objects get closer to or farther away from each other, but the speed and direction of movement is taken into account so that it is possible to correctly assess situations where Object A is moving toward Object B, but B is moving away from A (Spruijt et al., 1992).

Speed of moving to and from. The distance-weighted speed at which an animal moves toward or away from another animal. Closer objects are given a heavier weighting, and movements between objects a long way from each other have a slower speed of movement. "Speed of moving to" is calculated only if the relative movement is "moving to."

Net relative movement. The signed, distance-weighted change in distance between two animals. Net relative movement combines the two parameters "Speed of moving from" and "Speed of moving to" and can be used to quantify avoidance and aggression.

The parameters of social interactions, based on the relative movement between pairs of objects, can also be calculated for the relative movement between animal(s) and zones/points of interest (i.e., a static position in the arena). This allows the user, for example, to calculate the speed with which an animal moves toward a novel object or when an animal is in proximity with the center of the open field.

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