

Automated Analysis of Behavior: A Computer-Controlled System for Drug Screening and the Investigation of Learning

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ABSTRACT: Efforts to understand cognition will be greatly facilitated by computerized systems that enable the automated analysis of animal behavior. A number of controversies in the invertebrate learning field have resulted from difficulties inherent in manual experiments. Driven by the necessity to overcome these problems during investigation of neural function in planarian flatworms and frog larvae, we designed and developed a prototype for an inexpensive, flexible system that enables automated control and analysis of behavior and learning. Applicable to a variety of small animals such as flatworms and zebrafish, this system allows automated analysis of

innate behavior, as well as of learning and memory in a plethora of conditioning paradigms. We present here the schematics of a basic prototype, which overcomes experimenter effects and operator tedium, enabling a large number of animals to be analyzed with transparent on-line access to primary data. A scaled-up version of this technology represents an efficient methodology to screen pharmacological and genetic libraries for novel neuroactive reagents of basic and biomedical relevance. © 2006 Wiley Periodicals, Inc. *J Neurobiol* 66: 977–990, 2006
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INTRODUCTION

The current challenge before modern cognitive science is to understand and integrate the processes that span from the molecular genetics establishing the structure of the nervous system to the information

processing mechanisms that give rise to behavior and thought. The biomedical aspect of this program includes the search for useful neuromodulatory drugs and the understanding of the effects of various influences on normal cognition. Fundamental advances on these tasks require analysis of behavior in a variety of genetically and pharmacologically modified organisms. However, assessing behavior manually places significant limitations on experimental progress. These restrictions include the limited number of animals that may be analyzed by hand, the experimenter effects inherent in manual handling and observation by different individuals, and the difficulty in allowing other groups to analyze all of the primary data and potentially uncover trends missed by the experimenters.

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Indeed, these problems have been instrumental in a number of controversies in neurobiology. For example, the lack of consensus on the learning ability of planarian flatworms, and in particular on the ability of memories to persist during the regeneration of the central nervous system, was due in large part to small sample sizes necessitated by the tedium of training worms by hand, the inevitable but often important small differences in handling by different experimenters (observer bias, oversensitization of subject animals from handling), inconsistencies in exact protocols and controls, and the difficulties in making all of the primary data available to other groups in the field (Thompson, 1955; Corning, 1961; Cornwell, 1961; Humphries, 1961; Best, 1963; Lee, 1963; Murphy, 1963; Roe, 1963; Stephen, 1963; Wells, 1963; Hartry et al., 1964; McConnell, 1965; Jacobson, 1966; Chapoutier, 1967; Ungar, 1974; Sarnat, 1985). Driven by our lab's desire to mechanistically investigate memory during regeneration and tissue remodeling (McConnell, 1959, 1965; Corning, 1961; Best, 1963; Sheiman and Tiras, 1996), and to understand behavioral function in *Xenopus* larvae and flatworms whose large-scale CNS structure was altered by molecular or epigenetic manipulations during embryogenesis and regeneration (Nogi and Levin, 2005), we pursued the development of an automated system for the analysis of behavior.

A useful paradigm for such analysis necessitates the following properties: (a) it must be applicable to powerful genetic model systems such as zebrafish, *Xenopus*, and flatworms (as current efforts for automation of behavioral analysis are almost exclusively focused on rodents); (b) it must allow analysis of multiple animals simultaneously for larger sample sizes; (c) it must be fully automated to exclude experimenter effects and subjective scoring, thus allowing consistent reproducibility of experimental paradigms across labs; (d) it must allow convenient usage of essential control conditions (e.g., yoked controls); and (e) it must record all primary data so that it can be easily made available on-line for analysis by reviewers and other labs.

A number of previous efforts have been made in attempts to automate behavioral experiments. While the majority focused on rodents (Torello et al., 1983; Sanberg et al., 1985; Hulsey and Martin, 1991; Madrid et al., 1995; Valentinuzzi et al., 1998; Boisvert and Sherry, 2000; Nielsen and Crnic, 2002), some have addressed smaller organisms (McConnell et al., 1960; Lee, 1963; Corning and Freed, 1968; Tiras and Aslanidi, 1981; Sadauskas and Shuranova Zh, 1982; Fernandez de Miguel et al., 1989). We sought to improve these systems using more modern computer

and optical technology, to design a system ideally suited for molecularly tractable model systems, and to incorporate a crucial additional property: scalability.

A number of academic and commercial pharmaceutical projects have generated large genetic, proteomic, or small-molecule (drug) libraries that must be screened to identify compounds of interest to both biomedicine and basic biology (Bensing et al., 2001; Goodnow, 2001; Katayama et al., 2001; Koide et al., 2001; MacNeil et al., 2001; Nuttall, 2001; Cheung et al., 2002). Cell culture or simple organisms like yeast (Kirsch, 1993; Chen and Zhao, 2003) are obviously insufficient for identifying compounds that exert effects on complex multicellular systems or alter nervous system function in desired ways. Screens on multicellular model systems such as zebrafish (Zon and Peterson, 2005) have been successful in cell-biological assays, but the current necessity of manual analysis precludes effectiveness in high-throughput neurological screens. Thus, we pursued a system that could be scaled to provide an extremely powerful tool to biomedicine and neuropharmacology by allowing automated screens in small animal model systems for new compounds that, for example, increase learning and cognitive ability, expand memory, are sedatives or stimulants, counteract effects of neurotoxins, suppress pain, modulate the activity of other psychoactive compounds, or serve as antidotes to drug addiction.

Here, we present details on the design and construction of a prototype system for the automated analysis of behavior that meets all of the above criteria. We also provide sample proof-of-principle data obtained in a number of paradigms in planaria, to illustrate the operation and applications of this system to a model system that offers the advantage of a large database of existing behavioral data. Past controversies can be resolved using the proposed apparatus, and it offers significant promise for exciting molecular investigations of memory and regeneration in the same organism (Pietsch and Schneider, 1969, 1985, 1991).

MATERIALS AND METHODS

The species of planaria used in this study was *Dugesia tigrinia*, obtained from Ward's Natural Science. Planaria colonies were stored in rectangular plastic containers measuring 22 × 22 × 7 cm and filled with Poland Springs natural spring water. The containers were stored in an incubator at 22.5°C, and kept on a strict cycle of light (9:00 am to 6:00 pm) and dark (6:00 pm to 9:00 am) in order to facili-

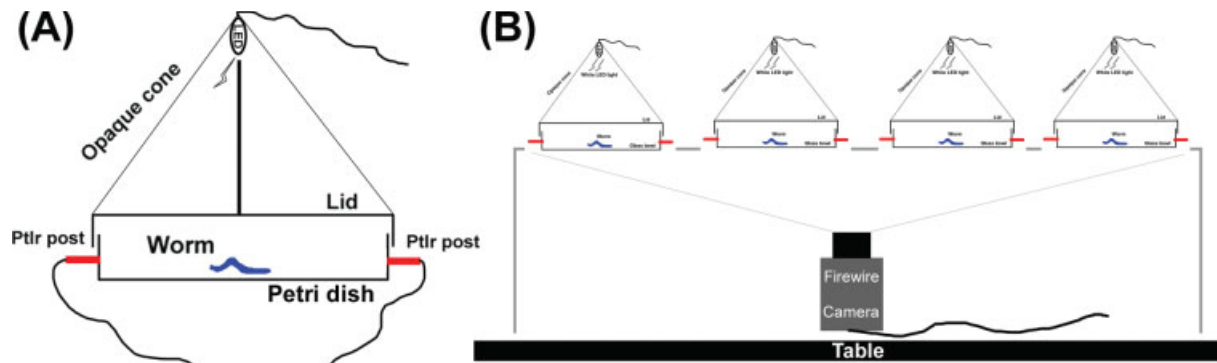


Figure 1 Schematic and arrangement of cells in learning apparatus. The entire training apparatus consists of an array of 12 cells (for the small-scale prototype) arranged in a grid. Each cell (A) contains a disposable 6 cm plastic Petri dish, and a specially constructed top fits snugly into it. Underneath the array of dishes is a camera and wide-angle lens such that all of the dishes are in its view (B). The lid contains a system of light-emitting diodes controlled by the computer, including weak red LEDs (long-wavelength light is known to be out of the range of planarian vision; Brown et al., 1968) to enable the camera to see the animals at all times, and a set of four bright white LEDs, each of which can be set to illuminate a single quadrant of the dish. Light diffusers and barriers are set up so that the light provided in a single quadrant is homogenous but does not spread to adjacent quadrants. Each cell's platinum-iridium (PtIr) electrodes and LEDs are connected to a specially designed addressable digital-analog converter, which is connected to the PC via a serial port. The computer is thus able to manipulate aspects of the worms' environment as desired by the algorithm.

tate colony health and expansion. Three planaria colonies were kept at any one time. The colonies are fed once per week with organic beef liver. The liver was purchased biannually from Valley Livestock Marketing Co-op, liquefied in a blender, and then frozen in teaspoon-sized aliquots. Prior to feeding, an aliquot was thawed at room temperature and then poured into the containers of planaria. The colonies were allowed to feed for 3 h, after which the remaining liver pieces are removed via pipette and discarded, and the water was changed twice to remove any remaining debris. The water was changed once again 3 days following feeding in order to remove excess slime build-up and to replenish the oxygen supply. On any given week only two of the three planaria colonies were fed, because planaria that were starved for a week were better for training, as they were less likely to spontaneously fission. The health of the planaria colonies was maintained through careful observation; they were viewed regularly under a dissecting microscope to monitor for any lesions or discoloration. If a colony became sick, it was transferred to a new container filled with spring water that was treated with 1 mL/L gentomycin (Invitrogen). The treatment continued daily for 1–3 weeks, until the symptoms disappeared.

For the duration of an experiment, individual worms were placed into a cell [Fig. 1(A)] and not fed during the experiment. Each cell consisted of a plastic Petri dish; electrodes for the delivery of current were made of a 90/10% platinum/iridium alloy (a biologically compatible, inert material that gives off no electrolysis products) and were constructed flush with the edge of the dish (so as not to create any corners in which worms could get stuck). Other details are given in the figure legends. For the drug experiments,

compounds made from stock solutions were dissolved in Poland Spring water and worms were kept in the compound throughout the experiment.

The camera used was a PixeLink PI-A661 (monochrome, 1.3 Megapixel resolution), with a Kowa HR F1.4/12 mm flatfield lens. The PC controlling the prototype device was a Dell Dimension 4600 with Windows 2000 Pro and a FireWire PCI card. The software controlling the device was written and run within Matlab v7.1 with image acquisition toolbox. The red LEDs ranged from 0–3.75 V; white LEDs ranged from 0.5–3.75 V (providing 7 Lux from the red LEDs and 315 Lux from the four white LEDs). The shocks ranged from 0–10.5 V DC; this range was chosen empirically based on shocks that result in a visible reaction in the worms (contraction and avoidance movements) but do not impair the health of the worms when applied repeatedly.

RESULTS

Basic Schematic of Device

The basic concept of the device is a series of Skinner cells monitored by a digital camera (Figs. 1 and 2). Each cell has one animal. The main point is that the environment in each cell is individually controlled by the software depending on the behavior of the animal within. The animals are shielded from external stimuli. A weak light out of the band of spectral sensitivity of the worms (far red) allows observation of ani-

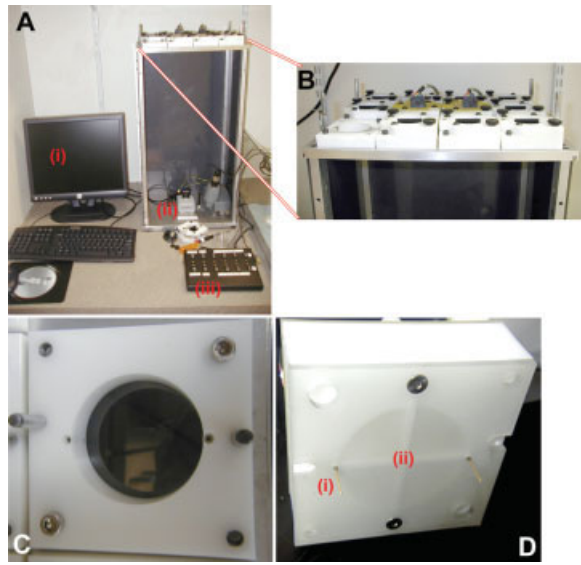


Figure 2 Actual photographs of the device. (A) The workstation consists of a PC (i), a vertical enclosure [(ii), shown here with the front panel removed] containing a high-speed camera below the cells, and a separate component containing a control panel and the circuitry mediating between the computer and the cells (iii). (B) The tray of cells, which consists of a number of nested components held down by screws for convenient disassembly, is on the top of the enclosure. (C) The base of each cell fits over the Petri dish. (D) The top fits over the enclosure, containing electrodes (i) and the quadrant barriers (ii).

mal position at all times. The current prototype (optimized for work with planaria) contains the ability to expose experimental animals to stimuli including one to four dish quadrants illuminated by bright light and a set of electrodes allowing electric shocks to be delivered to the water inside the cell. The mechanical design enables standard disposable plastic Petri dishes to be utilized inside each cell. The existing device is ideal for a plethora of experiments in any aqueous model species; modifications to adjust the device to a specific application are discussed below.

A number of specific construction details are important. The shock must be kept at constant current; thus, if the overall chamber resistance changes with time due to mucus deposition, the strength of the shock will remain level throughout the training period. Each cell lid is covered with an opaque material to allow different lighting conditions among the cells. LEDs are used to provide light but no heat to the quadrants individually. A rectangular array of 3×4 such cells are located within the view of a digital camera, which represents the main input to the software. The output, sent as text strings over the computer's serial port, contains instructions controlling

the state of a custom processing unit that exists as a separate printed circuit. This is a state machine that interprets the incoming control message and sets the lights, electric shock, and other parameters in each dish according to their respective bits (established by the software, depending on the particular behavioral paradigm being used).

The basic algorithm, implemented in the Matlab software platform, consists of the following steps (schematized in Fig. 3), performed in an iterative loop throughout the length of the experiment. An image is obtained of the animals in their dishes and parsed as follows to identify the centroid of each worm. Each animal's position and orientation is calculated by image-processing code containing the following transformations in order: background subtraction, morphological top-hat filtering using a diamond-structured element, conversion to binary (pixel intensity threshold of 0.9), binary area opening operation (removes small islands of white that are smaller than 10 pixels), dilation of remaining white pixels to connect any pixels in close proximity and to smooth out edges of white islands, majority elimination of remaining pixel spots (sets a pixel to 1 if five or more pixels in its 3-by-3 neighborhood are 1's; otherwise, it sets the pixel to 0).

The software then makes a decision (based on the details of the experiment as defined by the user before the start of the run) for each animal, as to whether it is to be rewarded or punished (via a brief electric shock to the water and/or an appropriate change in light conditions), or to receive no action. The position of each animal along with the action taken is recorded as a time-stamped record in an Excel spreadsheet, and the image taken of the dish is saved as a frame in an MPEG movie file. This process continues until the experiment is terminated by the experimenter or a predefined condition is reached.

A graphical user-interface to the software allows the user to define the experimental paradigm. This system can be used in two main modes: simple behavioral observation and analysis, and instrumental/classical conditioning paradigms. The former can be used to study actograms or assay light responses. The latter can be used to create a consistent environment where specific behaviors are encouraged. For example, the software might punish prolonged inactivity, location in a particular part of the dish, or more complicated relationships (sample tasks include: "when your quadrant is lit up, move to the next one to the left or be shocked"; or, "when the light comes on, you have 5 s to move to a spot within 5 mm from a wall"; or, "stay on a small agarose pad placed in the dish"). The possibilities are practically unlimited.

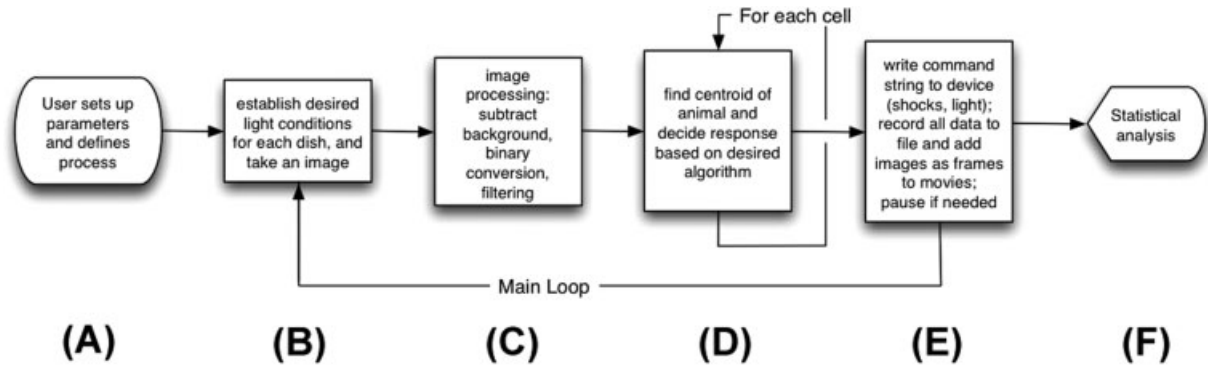


Figure 3 Schematic of learning algorithm. Each cell is essentially a kind of “Skinner box” that trains the worm on an instrumental learning task. The training algorithm consists of a reiterative loop performing the following steps. (A) First, the user sets up the parameters of the task for each dish—the behaviors that are to be punished, and the timing of the experiment, rest periods, and so forth. The control versus experimental conditions are set here. Then the main loop begins (B–E). (B) An image is obtained of the worms in their cells. (C,D) The image is parsed (using image analysis tools present in the Matlab software package), and each worm’s position is estimated. Note that some worms will be sitting on the vertical walls of the dish, and thus not visible (they are very flat). This is an important feature because it allows training of animals to be on the dish bottom versus dish side. (E) The software then makes a decision (based on the details of the experiment as defined by the user before the start of the run) for each worm, as to whether that worm is to be rewarded (by lowering the level of LEDs that are active—the worms don’t like bright light), punished (via a brief electric shock to the water or activation of LEDs), or receive no action (a control condition). The position of each worm along with the action taken is recorded as a time-stamped record in a database, and the image of each dish is appended to a QuickTime movie stack for each dish. (F) The process will continue until the experiment is terminated (weeks or days), at which point statistical analysis using the Microsoft Excel package is performed.

Controls are set up by having a given animal’s cell receive random stimuli (or those linked to the behavior of a master animal in a yoked control). Sensitization controls are easily handled (by specifying regular intervals of either light, shock, or both stimuli), and innate preferences are controlled by training half of the animals on a task opposite to the others (to avoid light vs. darkness for example), or by prescreening using 12 h observation periods and assigning alternate tasks to train against the preference, or by eliminating animals not meeting the behavioral criteria of a given paradigm. The main concept is that having set up such an environment, the computer automatically keeps track of the behavior of each animal. At any point during the trial, the experimenter can observe the progress of the experiment: the data, stored in Microsoft Excel format, allows easy plots of performance versus time for each animal, variability among animals, time spent in light versus darkness, total distance traveled by each animal, activity level throughout the experiment, percentage of time present in any given area of the dish, and so forth. Also important are the QuickTime format movies of each dish, allowing time-lapse or real-time observation of exactly what each animal was doing at each point in

the experiment. The frames of the movie are cross-referenced to the Excel processed data, so that any given period can be examined (and verified to ensure accurate data collection and interpretation by a given algorithm). The movie frames are also annotated with marks so that invisible events (such as electric shocks) are seen in the movie.

This system provides a number of immediate advantages. First and foremost, this provides complete consistency among labs replicating experiments, and removes experimenter bias in scoring behaviors. As long as a set of starting parameters is published with the results, any group will be able to independently run the same experiment. Second, because the procedure is fully automated, no user involvement is needed during the experiment—no blind (or double-blind) protocols are required, and the experiment can go on for weeks, involving millions of observations and training iterations/reinforcement (resulting in a training level exponentially beyond what can realistically be accomplished by hand). The removal of operator tedium results not only in extremely robust data with significant sample sizes, but allows much stronger learning in a consistent environment than usually results from a small number of training trials

that can be achieved manually in a given day. Moreover, because animals do not have to be handled during the experiment, this avoids sensitization to handling and confounding stimuli (animals may not like new environments and may act differently when they are moved from their “home” colony to a testing paradigm).

Third, any interested independent observer can view the progress of the experiment; if allowed by access controls, this can be done in real-time over the web. This means that many different groups can analyze data, and potentially uncover trends and relationships unforeseen by the original experimenter. The extensive data logging also allows other scientists to analyze all of the primary data, increasing the likelihood that others will discover novel trends via data-mining the original dataset. This can be particularly crucial when a set of rare (e.g., mutant) animals is being studied that cannot easily be duplicated outside a given lab. It also has important implications for low-budget high-school or undergraduate educational institutions because hundreds of students could have access to behavioral data being generated by such a device in a primary research institution.

Potential uses of this system are numerous. In its simplest mode, it can be used to provide detailed analyses of animals’ innate behavior and light preferences. By focusing on actogram measurements and conducting experiments at longer time-scales, experiments on circadian cycles can be greatly facilitated (Brown and Park, 1964). In the training paradigm, properties of memory and learning can be studied. One of the key features of this system is that the paradigms can be made incentive-based, which in many systems can produce very robust learning because the animals have the opportunity to avoid punishment by noticing patterns in their environment. Once a robust memory is formed, pharmacological, surgical, or molecular-biological interventions can be performed, and the recall can be automatically tested, enabling powerful analyses of the mechanisms of memory storage.

Sample Data: Proof of Principle

Our lab focuses on understanding biophysical control mechanisms during morphogenesis; one of the model systems we use is the free-living flatworm *Planaria* (*P. Platyhelminthes*, *C. Turbellaria*), which offers the unique ability to study regeneration and memory in the same organism. We have chosen to illustrate the function of this behavioral analysis system with proof-of-principle data in the planaria model system

for a number of reasons. *Planaria* exhibit much of the complexity of vertebrate systems: a well-differentiated nervous system, intestine, eyes, brain, three tissue layers, and bilateral symmetry (Bronsted, 1969; Sanchez Alvarado, 2004). *Planaria* possess a well-developed nervous system with true synaptic transmission and have what can be considered the first animal “brain” (Sarnat and Netsky, 1985). They have also developed sensory capabilities for the detection of light (Brown et al., 1968; Brown and Park, 1975) and other modalities (Brown and Park, 1964; Brown, 1966; Brown and Ogden, 1968; Fulgheri and Messeri, 1973; Brown and Chow, 1975; Mason, 1975; Miyamoto and Shimosawa, 1985). *Planaria* have been shown to exhibit learning and memory under classical conditioning paradigms as well as to perform more complex tasks requiring a surprising degree of intelligence (such as operant or instrumental conditioning); they also possess many of the neurotransmitters, receptors, and behaviors associated with higher cognition (McConnell et al., 1960; Jacobson, 1962; McConnell, 1965; Block and McConnell, 1967; Smith, 1985; Creti et al., 1992; Villar and Schaeffer, 1993; Eriksson and Panula, 1994; Sheiman and Tiras, 1996). They are an ideal organism for screening because they are small, and easy to raise and to subject to a multitude of reagents and manipulations. Moreover, evolutionarily, they are very similar to the ancestor of the *bilateria* clade, and have high relevance to human medicine and physiology both structurally and physiologically (Best and Morita, 1982; Sarnat and Netsky, 1985). *Planaria* offer an excellent combination of experimental tractability and sufficient complexity for asking a number of fascinating questions about basic functions of living systems (Eisenstein, 1997). Crucially, as a model system, planaria are quickly acquiring a powerful set of molecular biological reagents and techniques, enabling genetic and cell-biological investigations into their structure and function (Cebria et al., 2002; Sanchez Alvarado et al., 2002; Agata et al., 2003).

One of the most common protocols for demonstration of memory is to train an animal against its normal preference, because this can result in the greatest difference between controls and trained individuals. Therefore, to demonstrate a prescreening function as well as the use of this system for noninvasive behavioral observation and analysis, we began with a simple observe trial. A population of worms was monitored to ascertain the amount of time each animal spent in the light versus dark quadrants of a cell (Fig. 4). Interestingly, while a majority of the worms did indeed prefer darkness (consistent with the common wisdom about worm behavior; Walter, 1907;

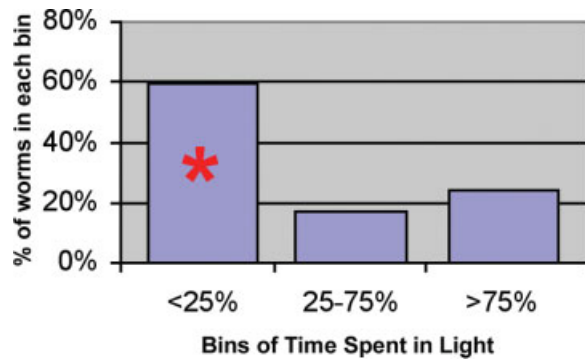


Figure 4 Sample prescreening data: innate light preferences. The histogram shows the use of this system in observation mode, to prescreen an experimental population for their light preference in the absence of any punishment protocol ($n = 59$). This is important for choosing individuals to use in training paradigms that teach a worm to behave against its innate preference (so that worms that aberrantly prefer light don't get inadvertently used in experiments where animals are trained to stay in light quadrants). When placed in a dish containing two light and two dark quadrants, 59.3% of the worms naturally spend less than 25% of their time in the light. Thus, cleaner data can be obtained by then utilizing only the worms from the group marked with an asterisk in subsequent learning experiments based on training against innate dark preference.

Reynierse, 1967), a significant percentage of our animals preferred light. This is important because if these individuals were used for a "learn to stay in the light" study, their native preference would reduce the difference between trained and untrained worms. Thus, this method allows the experimenter to rapidly select animals from a population for training; one might train the animals in the left-most photo-phobic bin for spending time in the light, while training the animals from the right-most photo-philic bin for spending time in the darkness.

The apparatus facilitates study of spatial relationships. It is easy to add agarose pads, mazes, miniature platforms, or other spatial cues for animals and reward spending time in a particular location within the dish. We chose the simple dichotomy provided by each worm's being horizontal, on the bottom surface of a dish, versus turned 90° , sitting on the vertical edge of a dish. We trained worms using a yoked control paradigm after prescreening for worms that, when left to their own devices, spent time on the dishes' walls. After 24 h of training (Fig. 5), where worms were punished with shock for being located on the edge, the worms were significantly more likely ($p < 0.001$) to spend time on the bottom of the dish—against their normal preference. Thus, the animals can sense their orientation and associate it with the

punishment. In a similar paradigm, we explored the ability to learn visual cues. An even more profound level of learning could be obtained in worms (Fig. 6) prescreened for photo-avoidance and trained to spend time in the light; the trained (but not the yoked control) worms changed from 10% light exposure to almost 100% light exposure within 3 days of training ($p < 6 \times 10^{-9}$). This learning paradigm can also be analyzed in terms of latency (time elapsed or number of trials before a series of correct responses); an example of such data is shown in Figure 7.

Finally, we studied long-term retention (asking whether the memory formed during these experiments is retained for significant time periods after the training ends; Fig. 8). Our data demonstrate that almost perfect performance is retained after 7 days of rest following a 3 day training period. This result supports our contention that a consistent environment that automatically rewards and punishes various behaviors avails the experimenter of an extremely robust memory for subsequent experiments. The classical planarian memory experiments done by hand

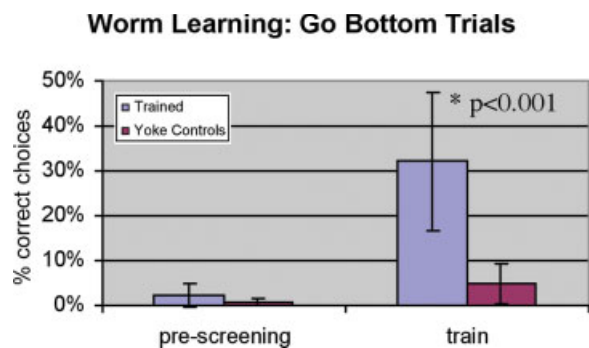


Figure 5 Example of learning for spatial orientation. This histogram shows sample data for a group of worms trained to stay on the bottom of the dish. The prescreening columns demonstrate that both the controls and the worms to be trained have a strong innate preference to sit on the vertical side of the Petri dish ($>97\%$ of the time). The difference in the percentage of correct choices made between a group of worms in training ($n = 6$) and a group of yoked controls ($n = 6$) during "Go Bottom" training trials was significant to $p < 0.001$ using a two-sample t test. In the training trial, the trained group of planarians was trained to move to the bottom of the dish using a combination of shock and light stimuli, and the yoked controls received stimulus corresponding to the stimulus received by their "master" (thus, their experiences paralleled those of another worm but there was no causal relationship between their behavior and the punishment they received). Training lasted 24 h, and feedback stimuli (DC shock and a flash of white LED lights) were applied for 1 s as punishment when the worm made the incorrect choice to stay on the edge of the dish.

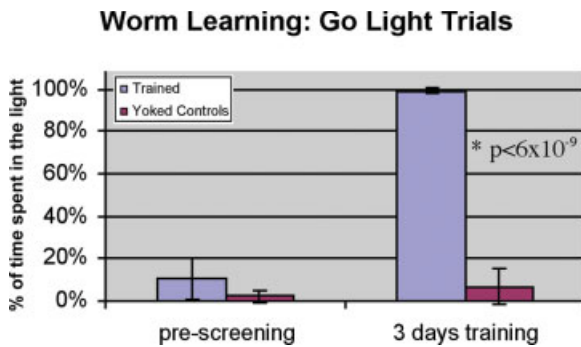


Figure 6 Example of learning for light level. This histogram shows the difference in the percentage of correct choices made between a group of worms in training ($n = 5$) and a group of yoked controls ($n = 5$) during training trials where the worms were punished for spending time in the dark quadrants. In the prescreening training section, worms were observed without stimulus to select those with an innate light preference of $<25\%$. Those worms who met this criteria were then trained in either the trained, or master, group or the yoke control group to move into two of four quadrants of a standard Petri dish that were lit with white LEDs. The trained group of planarians was trained to move into the lit quadrants of the dish using a combination of shock and light stimuli, and the yoked controls received stimulus corresponding to the stimulus received by their “master.” Training consisted of three 2 h trials spread over 3 days, in which the feedback stimulus was applied once every 30 s for 1 s (DC shock as punishment when the worm made the incorrect choice to stay in the dark, or a reprieve from all lights when the worm made the correct choice to stay in the light) after each image was analyzed. The trained group of worms showed a significant improvement in the averaged percentage of correct choices during training compared to the yoke controls ($p < 6 \times 10^{-9}$, two-sample t test assuming equal variance).

were very tedious (especially to obtain the large differences between trained and control worms) and memory recall was analyzed as “savings”—how fast a trained animal could relearn the task (Jacobson, 1962; McConnell, 1965). In this system, even after 7 days of rest in an environment where the target behavior was not reinforced, the worms remembered their task very well and did not require retraining. Moreover, the retention of the memory for 7 days is long enough for cut worms to regenerate, opening the possibility to study memory and brain regeneration in the same model system (McConnell et al., 1959).

As a final example, we sought to illustrate the use of this device to assay the effects of psychiatric drugs on worm behavior. A number of previous studies demonstrated that neuromodulatory reagents used in mammalian systems also have the expected effects on planarian behavior (Algeri et al., 1983; Sarnat and

Netsky, 1985; Kitamura et al., 2003; Raffa and Martley, 2005). We selected two compounds, p-chlorophenylalanine (PCPA) and reserpine—two modulators of serotonergic signaling that are known to have effects on activity levels (Garattini and Valzelli, 1958; Georgiev and Petkova, 1975; Hussey et al., 1983). Figure 9 illustrates a simple actogram analysis, where overall mobility of the worms was tracked (see Supplements 1–3 at http://server.drmichaellevin.org/worm_supplement.html for real-time movies of the worm behavior in each condition). While the data revealed significant variation among animals, the PCPA and reserpine, respectively, increased and decreased the locomotor activity level compared to controls. Thus, this system can be fruitfully used to analyze the effects of known and as-yet-uncharacterized compounds on behavior (as well as learning rate, retention, etc.).

DISCUSSION

Using the planaria model, we performed a number of different studies designed to illustrate the use of the automated behavioral analysis system. The automated nature of the process made it easy to test different paradigms and parameters (strength of shock, light levels, timing of training and rest periods, etc.). Moreover, by designating some of the dishes as controls and assigning yoke relationships, it was easy to compare animals undergoing training at the same time (thus minimizing the effects of environmental, circadian, and other confounding factors). While these data demonstrate multiple uses of this system for investigating memory and behavior in planaria, we believe this same paradigm can be used, with minimal modifications, with a wide variety of experimental model systems.

The experiments above surely raise many further questions about planarian ethology; there is much room for improvement and tantalizing directions for further studies of behavior and its genetic and epigenetic basis (the worms used in this study were wild-caught, but clonal isogenic animals can also be used to investigate the endogenous variability in cognitive function). Work currently in preparation will report primary data addressing the relationship between memory and regeneration in planaria and vertebrate model systems. Our goal here was not to derive conclusive answers about planarian learning, but rather to illustrate practical use of the system we have designed in the hopes that it will be utilized and extended by others. The planarian data demonstrate that this basic system provides automated analysis of

Worm Learning Latencies: Go Light Training

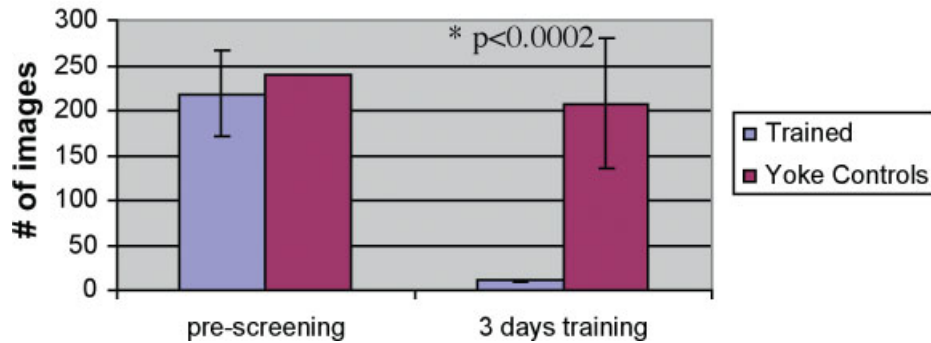


Figure 7 Measuring latency in learning paradigms. This histogram shows the difference in the latency (number of consecutive images taken during clock ticks of the main program cycle) required by the trained ($n = 5$) and yoke control ($n = 5$) groups of planarians before the worms stayed in the correct training location for 5 consecutive min. As in the previous graph, the training used in this paradigm was for prescreened worms (with innate light preference $<25\%$) to move into the two quadrants of a standard-sized Petri dish lit with white LEDs. The worms were trained for a total of 2 h, with images taken and feedback stimulus (DC shock as punishment and a reprieve from all light as reward) applied once every 30 s for 1 s. Latency is measured as the number of images that it takes before a worm remains in one of the “correct” quadrants for at least 5 consecutive min; as such, the minimum possible latency is 10 images, and the maximum possible latency is 240 images (the total number of images taken during the training trial). Once the worms achieved this criterion, they were given a 5 min rest period, during which time all LEDs were turned off, and no stimulus was applied. During prescreening, the trained and yoke control groups of worms both had average latencies greater than 210 images, but after training the trained group showed a significantly lower latency than the yoked controls ($p < 0.002$, two-sample t test assuming equal variances).

behavior in real time, can be used to train and test animals in memory experiments, and allows the evaluation of the behavioral effects of neuroactive compounds.

Crucially, this system is ideally suited to screening applications. When scaled up, in addition to facilitating inquiry into basic mechanisms of animal learning, this system is usable for screens of compounds that alter behavior. In this paradigm, each cell would receive one of the library’s compounds to be tested dissolved in the water. The software would then enable the search for reagents that increase activity (stimulants), decrease movement (paralytics or sedatives), alter normal behavior in other well-defined ways (e.g., render the animals more or less sensitive to environmental stimuli such as light or weak electric shock), increase learning rate, memory retention, or intelligence (ability to abstract more complex patterns from their environments), and so forth. Moreover, if each cell contains the same basic reagent, plus one element from a library, the system can be used to screen that library for compounds that augment or counteract the activity of that reagent. This can be used to search for new antitoxins, drugs that

prevent or ameliorate addiction, or reagents that modulate the activity of other psychoactive compounds. In addition to screening drug libraries for drugs to modulate endogenous processes, use of this system in genetically tractable model systems (such as those amenable to RNAi-based inhibition) will allow screens to identify endogenous gene products with roles in important aspects of cognitive function and nervous system activity. The ability to identify compounds and genes involved in these processes could significantly contribute to biomedical efforts addressing brain disorders, security threats from neurotoxin exposure, and treatment of drug abuse.

During the construction, testing, and use of this device a number of planned modifications have been formulated. We are currently developing a system with the following improvements. First, the basic system should have an individual CMOS camera underneath each cell. This would not only allow the whole system to be much smaller (height will be reduced by stacking trays of cells, because vertical height won’t be required to provide a complete field of view for a single camera), but would allow higher-resolution imaging. This will enable the use of this system for

Worm Learning and Memory Retention: Go Light Training

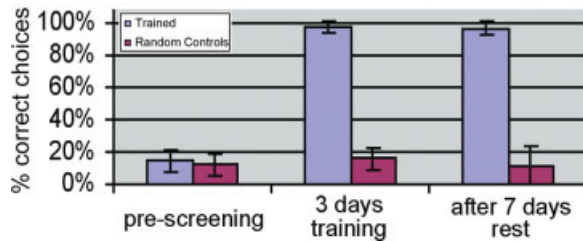


Figure 8 Example of analysis of retention of memory. This histogram shows the difference in the percentage of correct choices made between a group of worms in training ($n = 5$) and a group of random controls ($n = 5$) during “Go Light” training trails, as well as the memory retention of that task after 7 days of rest. In the prescreening training section, worms were observed without stimulus to pre-screen for an innate light preference of $<25\%$. Those worms who met this criteria were then trained in either the trained, or master, group, or the random shock control group to move into two of four quadrants of a standard Petri dish that was lit with white LEDs. The trained group of planarians was trained to move into the lit quadrants of the dish using a combination of shock and light stimuli, and the random controls received random stimulus established by the software. Training consisted of three 2 h trials spread over 3 days, in which the feedback stimulus was applied once every 30 s for 1 s (DC shock as punishment when the worm made the incorrect choice to stay in the dark, or a reprieve from all lights when the worm made the correct choice to stay in the light) after each image was analyzed. The trained group of worms showed a significant improvement in the percentage of correct choices during training compared to the controls ($p < 2.8 \times 10^{-8}$, two-sample t test assuming equal variance). Following training, the worms were removed for the ATA and allowed to rest for 7 days in a normal environment. After 7 days the worms were placed back into clean Petri dishes in the ATA and observed without feedback stimulus to determine whether they remembered to move to the two quadrants lit with white LEDs, or whether they reverted back to their innate photo-averse behavior. The trained group of worms performed significantly better than they did in the original prescreening trial ($p < 0.0009$, paired t test), whereas the random control group of worms did not ($p < 0.44$, paired t test).

much smaller organisms, such as *C. elegans*. The camera sensitivity and LEDs should be increased in spectral width so that fluorescently tagged organisms can be tracked. Fluorescent markers that highlight a particular group of cells, organs, or neuronal paths (Offield et al., 2000; Haycraft et al., 2001) will make it easy for the software to identify mutants or drugs that alter the normal pattern. Moreover, physiological screens using can be carried out by devices that measure the fluorescence of vital or reporter dyes, such as pH-

and voltage-sensitive dyes (Buckler and Vaughan-Jones, 1990; Seksek et al., 1991; van Erp et al., 1991; Epps et al., 1994), which can give specific information on *in vivo* processes in real time. For example, screens using voltage- and pH-sensitive fluorescent dyes are easily envisioned to identify drugs that change the pH of specific tissues, or mutants resistant to the membrane-depolarizing effects of common

Effect of Drug Treatments on Planarian Speed

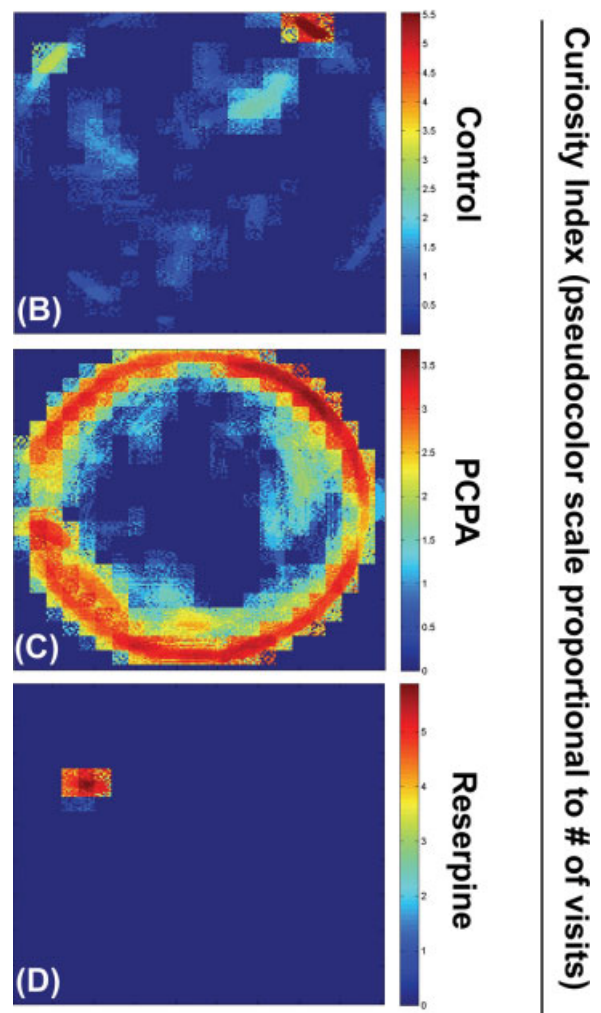
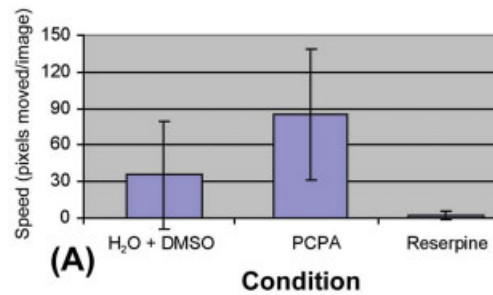


Figure 9

toxins such as polytoxin (Yamashita et al., 2000; Naitoh et al., 2001).

Aside from these generally useful enhancements, a number of changes can be made to modify the system for use with other systems and novel experimental paradigms. The individual cells can be fitted with sensors for specific chemicals (dissolved oxygen, waste products, pH sensors, toxins, etc.) to monitor or screen for any desired physiological reaction (Epstein and Walt, 2003; Mano et al., 2003; Thrush et al., 2003; Lee et al., 2004; Popovtzer et al., 2005; Tian et al., 2005; Zhang et al., 2005). The image analysis software could be augmented with morphometric analysis modules (Klingenberg et al., 1998; Klingenberg and Zaklan, 2000; Albertson and Kocher, 2001), allowing more sophisticated changes in neural morphology to be followed (especially when used in combination with high-resolution imaging and fluorescent tracing labeling of neural components). Depending on the species used (and its visual system), the spectral qualities of the LEDs may need to be changed. Vibration can easily be added to the environment of each cell (using piezoelectrics and dampening insulation between cells), as can Peltier thermocouples to provide temperature differences as stimuli or reward/punishment.

The use of this automated technology will not only reveal much about the cognitive properties of many different species, but will also allow the rapid derivation of high- and low-intelligence strains, the analysis of which in genetically tractable model systems will provide important insights into the basis of intelligence. Ultimately, massively scaled-up and miniaturized versions of this device will be used for high-throughput screens of novel neuroactive compounds (Kanigan et al., 2000; Crane et al., 2001; Anquetil et al., 2003; Martel and Hunter, 2006). Through dis-

covery of such reagents, and of endogenous genetic components regulating aspects of behavior, we envision new vistas in cognitive science.

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Figure 9 Example of analysis of drug effects on behavior. This histogram shows the average speed of planarian movement observed over 3 h after 24 h of drug treatment. The worms were monitored for their speed, position, and distance traveled in unlit dishes in the apparatus. For each drug, $n = 8$, and significance is calculated compared to the control ($H_2O + DMSO$ as carrier). Both PCPA and Reserpine had a significant and opposite effect on planarian movement rate ($p < 0.025$ and $p < 0.034$ respectively, two-sample t test assuming equal variance). Drug concentrations were as follows: PCPA = 625 $\mu g/mL$; Reserpine = 21 $\mu g/mL$. All three treatments were made up with 0.5% DMSO of final volume. Sample occupancy plots of each point in the dish for one worm in each group are shown in panels (B–D). Red end of pseudocolor scale indicates more visits to that region; blue colors indicate fewer visits.

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