Video Article

Contextual and Cued Fear Conditioning Test Using a Video Analyzing System in Mice

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Abstract

The contextual and cued fear conditioning test is one of the behavioral tests that assesses the ability of mice to learn and remember an association between environmental cues and aversive experiences. In this test, mice are placed into a conditioning chamber and are given parings of a conditioned stimulus (an auditory cue) and an aversive unconditioned stimulus (an electric footshock). After a delay time, the mice are exposed to the same conditioning chamber and a differently shaped chamber with presentation of the auditory cue. Freezing behavior during the test is measured as an index of fear memory. To analyze the behavior automatically, we have developed a video analyzing system using the ImageFZ application software program, which is available as a free download at http://www.mouse-phenotype.org/. Here, to show the details of our protocol, we demonstrate our procedure for the contextual and cued fear conditioning test in C57BL/6J mice using the ImageFZ system. In addition, we validated our protocol and the video analyzing system performance by comparing freezing time measured by the ImageFZ system or a photobeam-based computer measurement system with that scored by a human observer. As shown in our representative results, the data obtained by ImageFZ were similar to those analyzed by a human observer, indicating that the behavioral analysis using the ImageFZ system is highly reliable. The present movie article provides detailed information regarding the test procedures and will promote understanding of the experimental situation.

Video Link

The video component of this article can be found at https://www.jove.com/video/50871/

Introduction

The contextual and cued fear conditioning test is the behavioral paradigm used to assess associative fear learning and memory in rodents[1-3]. This test has been widely used to understand the neurobiological mechanisms of fear learning and memory in transgenic and knockout mice[1,4-16]. Freezing behavior, which is defined as complete immobility with the exception of breathing, is a common response to fearful situations. In this behavioral paradigm, after animals are exposed to a pairing of an auditory cue with an electric footshock, they respond to the fear-producing stimulus by displaying freezing behavior, which is measured as an index of associative fear learning and memory. This test requires less elaborate equipment, less physical exertion by the investigator, and much less training time for mice than other learning and memory tasks; it generally requires approximately 5-10 min/day per mouse for 2 days. Although the testing procedure is simple and requires little time to perform, the investigator must carefully observe and measure mouse behavior; therefore, several automated measurement systems have been developed to conduct the behavioral analysis[17-20]. Our video-analyzing system, which we developed with the ImageFZ software program, allows us to easily analyze freezing behavior and produce highly reliable results. This article provides detailed information on our testing procedure and describes how to use the ImageFZ software program.

Protocol

All of the experiments should be performed according to the guidance and protocols established by local Animal Care and Use Committees.

1. Apparatus Setting

1. The apparatus for the conditioning and context test is a square chamber with an electrifiable grid floor, a sound source, and a calibrated shock generator. Various chamber sizes are used, with dimensions varying from 54 cm x 27 cm x 30 cm [21] to 25 cm x 35 cm x 30 cm [22]. In this protocol, the apparatus consists of an acrylic square chamber (33 cm x 25 cm x 28 cm; transparent in the front and rear walls; white in the side walls) with metal grids (0.2 cm diameter, spaced 0.5 cm apart) covered by a transparent acrylic lid (**Figure 1A**). It is necessary to place the chamber on a white acrylic floor (**Figure 1B**) to analyze the behavior of black, agouti, or dilute brown mice because an image

analyzing system with the ImageFZ software program (available for free download, see Table of materials/reagents) distinguishes a dark subject from a white background in each captured video image. Albino mice also can be tested using black metal grids and a black acrylic floor (**Figure 1B**). Light-emitting diode (LED) lights are attached to the ceiling above the apparatus. The grid floor is illuminated at 100 lux by the LED lights. A speaker connected to a white noise/tone generator (**Figure 1C**) is mounted on a ceiling 5 cm above the lid to present an auditory cue (a white noise, 55 dB) as a conditioned stimulus (CS). The grids are wired to a shock generator (**Figure 1C**) to deliver an electric footshock as the unconditioned stimulus (US). The test chamber is placed in a soundproof room (170 cm x 210 cm x 200 cm) (**Figure 1D**) to minimize external noise during the tests. This condition also prevents the mice that are not currently being subjected to a test from hearing an auditory cue or the vocalization of the test mice.

2. The apparatus for the cued test is composed of a chamber that has different properties from the conditioning chamber, providing a new context. It is imperative to change the sensory cues as much as possible so that the mouse perceives the novel context as being unrelated to the conditioning chamber. Generally, a differently shaped box or a triangular chamber is used. In addition, different lighting and/or olfactory cues are also provided to the mouse. In this protocol, the apparatus is an acrylic triangular chamber (33 cm x 29 cm x 32 cm; white in each side wall) with a flat, white floor for black, agouti, or dilute brown mice or a flat, black floor for albino mice, covered with a transparent acrylic lid (**Figure 1E**). LED lights are attached to the ceiling above the apparatus. The illumination level of the floor is set at 30 lux. A speaker is mounted on the ceiling 5 cm above the lid to present an auditory cue that is the same as that provided to the mice at the time of conditioning. The triangular chamber is located in a different soundproof room from the room in which the conditioning and context test are performed.
3. Each chamber is equipped with a ceiling-mounted Charge Coupled Device (CCD) camera connecting to a Windows computer via quad video splitter and USB image capture device to monitor the mouse's behavior, and images of the apparatus and the mouse are captured and analyzed by the application software program ImageFZ (see Protocol 6). The white noise and footshock generators are automatically controlled by the ImageFZ software program; the start time and duration of the white noise and footshock must be written into a text file (see a sample text file 'simple-cond' shown in the video for the details on how the parameters are written in the file), which is read into the application.
4. Before each test begins, the acrylic walls and floors are wiped with a towel soaked in super hypochlorous water (pH 6-7), and the grids are cleaned with 70% ethanol to prevent a bias based on olfactory cues. The grids are wiped with ethanol instead of super hypochlorous water to ensure that the grids do not lessen their electrical conductivity due to rust.

2. Animal Preparation

1. Generally, two to four mice are housed per cage in a temperature-controlled holding room (23±2 °C) with a 12 hr light/dark cycle (*e.g.* lights on at 7:00 AM).
2. In this protocol, to reduce possible influences of cage transportation on behavior and to adapt the mice to the experimental environment, the cages containing the mice are transferred from the animal holding room into a soundproof waiting room adjacent to a soundproof testing room at least 30 min before each test begins.
3. All of the experiments (**Figure 2A**) should be performed during the same time period in the light or dark phase each day to minimize the behavioral variations produced by testing at different times[23,24]. In this protocol, all of the experiments are conducted between 1 hr after the onset of the light phase and 1 hr before the onset of the dark phase (8:00 AM to 6:00 PM in the light phase). If only one apparatus is available, mice of each genotype should be tested in a counterbalanced order to reduce potential effects of the experimental time and the testing order of subjects on behavioral performance. ImageFZ can control a maximum of 4 apparatuses. Testing 4 mice simultaneously by using 4 apparatuses in a counterbalanced order allows the researcher to save time and reduces the possible effects of the experimental parameters on mouse behavior.

3. Conditioning

1. Mice are placed in the conditioning chamber, and the mice are typically allowed to freely explore the chamber for 120 sec. Thereafter, the auditory cue, such as a white noise, tone, and auditory clicker, is presented as a CS for 30 sec, and a 0.1-0.8 mA footshock is given to the mice as a US during the last 2 sec of the sound. The presentation of the CS-US paring is repeated to strengthen the association. The mice are left in the chamber for a length of time after the last presentation to further establish the association between the context of the chamber and the aversive experience. In this protocol, after 120 sec of free exploration, an auditory cue (white noise, 55 dB) is presented for 30 sec, and a 0.3 mA footshock is delivered continuously during the last 2 sec of the white noise. After 90 sec, the pairing of the auditory cue with the footshock is given to the subjects again. The presentation of CS-US repeats three times per session (120, 240, and 360 sec after the beginning of the conditioning) (**Figure 2B**). Following the final footshock, the mice are left undisturbed in the chambers for 90 sec.
2. Before the conditioning session begins, run the ImageFZ application software program, select the plug-in menu 'FZ Conditioning and FZ Online (4 chamber)', and set the parameter values step-by-step as follows.
    1. Step 1: Project ID. Specify a folder where you want to store your data files.
    2. Step 2: Session Name. Type any words, *e.g.* the experimental date, in the 'Session' box, and select a reference text file in which the start time and duration of the white noise and footshock are written, in the 'Reference' box. A sample text file is shown in the video.
    3. Step 3: Parameter Settings. Enter parameter values in each box as follows.
        1. Rate (frame/sec): frame rate of image acquisition, *e.g.* 1 frame/sec.
        2. Duration (sec): in the case of conditioning, the total duration is 480 sec.
        3. Bin duration (sec): *e.g.* 60 sec; the data are analyzed in each block of 60 sec.
        4. Subject size - min (pixels): ImageFZ detects a mouse and noise as black particles (some mass of pixels) in a white background in each image. When the area of the black particle (pixels) is less than the 'Subject size – min (pixels)' value (*e.g.* 100 pixels), the particles are regarded as noise and are excluded from the image analysis.
        5. Subject size - max (pixels): when the sizes of the black particles are more than the size of the 'Subject size – max (pixels)' value, the particles are excluded from the analysis.
        6. Frame size - width/height (cm): chamber dimension, *i.e.* 33 cm wide and 25 cm high.
        7. Freezing criterion (pixels): *e.g.* 30 pixels, see the details in Protocol 6.

8. Freezing duration - min (sec): *e.g.* 2 sec; when no mouse movement is detected for only less than 2 sec, its behavior is not counted as 'freezing'.
9. Shock rate (frame/sec): see the details in Protocol 6.

4. Step 4: Subject ID. Enter the subject identification.
5. Step 5: Camera Settings. Control the brightness and contrast of the captured image.
6. Step 6: Threshold Settings. Adjust the threshold values to detect a black mouse as black pixels in a white background in each image and to judge mouse behavior as 'freezing' or 'non-freezing' (see the details in Protocol 6). To analyze an albino mouse, click on the checkbox 'invert mode', and adjust the threshold values appropriately.
7. Step 7: Set Cage Field. Specify the field of each chamber that you want to capture. After clicking the rectangle button in the toolbox, draw a rectangle around the floor of the chamber on the live image window. Next, select the chamber number and click the 'Set' button. Finally, click the 'Complete' button.

3. After the parameter settings are set, a preparatory test should be given using practice mice (mice not used as subjects) before the first test of the day to determine whether the image analyzing system and white noise/shock generators work without problems.
4. Move a home cage containing practice mice to the soundproof testing room from the adjacent waiting room, and place each mouse in the conditioning chamber. Immediately after placing the mice in the chamber, click the start button of ImageFZ. The application software will present auditory cues and/or electric footshocks to the mice in the order that you specify in a reference file.
5. After 480 sec has elapsed, return the mice to their home cage and return the cage to the shelf in the holding room.
6. Clean the chambers carefully. Then, click 'Next Analysis' button, and repeat steps 3.2.4-3.6 for the test mice.
7. ImageFZ stores live and trace images in the TIFF format. The program allows us to perform an offline analysis to reanalyze the images using modified parameter values. If you conduct an offline analysis, select the plug-in menu 'Fear Conditioning and FZ Offline' and select the data folder that you want to reanalyze. Thereafter, input the parameter values again, and click the 'Complete' button.

4. Context Test

1. After the conditioning session has been completed, the mice are returned to the same conditioning chamber and scored for freezing behavior to measure contextually conditioned fear (context test). A delay interval between the conditioning and the context test has been generally set at 24 hr. In this protocol, to assess recent memory and remote memory (measured by the test 1 day and more than 28 days after conditioning, respectively)[25], the mice are subjected to the context test approximately 24 hr and 30 days after the conditioning session. The mice are placed in the conditioning chamber and are allowed to freely explore the chamber for 300 sec without CS and US presentations (**Figure 2C**).
2. Run the ImageFZ software program and set the application software's parameter values in the same manner as in the conditioning (see section 3.2.3); however, modify the duration time of this test to 300 sec and select a reference text file for the context test. After changing the setting, a preparatory test should be given using practice mice to check the ImageFZ system.
3. Place each mouse into the conditioning chamber and click the start button. After 300 sec has elapsed, return the mice into their home cage, and leave the cage undisturbed until the cued test begins.
4. Clean the chambers. Then, click 'Next Analysis' button, and repeat steps 4.3-4.4 in the test mice.

5. Cued Test

1. Cued test is conducted on the same day of the context test or on the next day. In this test, mice are placed into another testing chamber with very different properties, providing a new context that is unrelated to the conditioning chamber for 3 min. At the end of the first 3 min, the auditory cue that is presented at the time of conditioning is given to mice for 3 min in the novel context environment. In this protocol, cued test is performed a few hours after the context test. Mice are allowed to explore the triangular chamber for 360 sec. In the first 3 min, neither a CS nor US is presented, and thereafter, a CS (a 55 dB white noise) is presented for the last 3 min.
2. Run the ImageFZ software program and set the parameter values in the same way as in conditioning, except modify the duration time of the test to 360 sec and select a reference text file for the cued test. After adjusting the setting, a preparatory test should be given using practice mice to check the ImageFZ system.
3. Place each mouse into the triangular chamber and click the start button. After 360 sec has elapsed, return the mice to their home cage and return the cage to the shelf of the holding room.
4. Clean the chambers. Then, click the 'Next Analysis' button and repeat steps 5.3-5.4 in the test mice.
5. To further test remote memory, repeat the Protocols 4-5 about 30 days after the conditioning session (**Figure 2A**).

6. Image Analysis

1. Perform data acquisition and analysis automatically using ImageFZ. This application software program is based on the public domain ImageJ program (developed by Wayne Rasband at the National Institutes of Health and available at http://rsb.info.nih.gov/ij/), modified by Tsuyoshi Miyakawa (ImageFZ application software, available for free download, see Table of materials/reagents).
2. For all experiments, capture images at a given frame rate (*e.g.* 1 fps) with ImageFZ using a USB video capture device, including a video camera. To measure the distance traveled from the consecutive images, adjust the 'threshold min' value of the program (*e.g.* 80 pixels), which is set to segment the images into a black particle (a mouse) and a white background. The distance traveled is calculated from the distance between each set of the x-y coordinates for the center of gravity of the particle in the consecutive images.
3. To measure the freezing behavior from the consecutive images, adjust the 'threshold min (xor)' value of the program (*e.g.* 160 pixels), which is set to segment the images into a black particle (mouse) and background, and then calculate the amount of area (pixels) of nonoverlapping regions between particles of each pair of consecutive images. Adjust the value using the slider of the threshold tool until the black particle in each image matches the shape of the entire body of the mouse excluding the tail. If the area of the nonoverlapping region is below the 'freezing criterion' value (*e.g.* 30 pixels), the behavior is considered to be 'freezing' (**Figure 3**), which is generally defined as the complete

absence of any movement except for respiration and heartbeat. When the area exceeds this value, the behavior is considered to be 'non-freezing' (**Figure 3**). The judgment should be made based on the definition of freezing. Mice sometimes exhibit a subtle movement and a momentary immobility, which might not be considered as a freezing behavior that reflects fear. The immobility that lasts for a short time (*e.g.* less than 2 sec), which is likely different from the manifestation of fear, can be excluded from the analysis by setting the time threshold of freezing. To set the time threshold, input 'Freezing duration – min (sec)' value (*e.g.* 2 sec).

4. The ImageFZ program automatically calculates the distance traveled (cm) and the percentage of freezing. The results are saved in text files, and live and trace images are stored in a TIFF format. To measure the distance traveled (cm) as an index of electric footshock sensitivity, the ImageFZ program also acquires images at a high frame rate (*e.g.* 4 fps) for 6 sec, measured from 2 sec before the delivery of a 2 sec footshock until 2 sec after footshock during online analysis. To set the frame rate for image capture before, during, and after footshock, input a value in the 'Shock rate (frame/sec)' box. After the online analysis, perform offline analysis by selecting a plug-in menu 'FZ Shock Offline' to obtain the data for the distance traveled.
5. The parameter values of the ImageFZ program should be optimized to generate results similar to those obtained by human observers in preparatory tests. For manual scoring, the freezing behavior is continuously measured using a stopwatch and an event-recording program or an instantaneous time-sampling procedure every 3-10 sec, during analysis using ImageFZ software. Two observers typically conduct the behavioral observation. To adjust the parameter values of the ImageFZ program to ensure that the outcomes of the image analysis are consistent with those of human observers, perform an offline analysis of the ImageFZ program, modifying the 'threshold min (xor)' and 'freezing criterion' values. To perform the offline analysis, select the plug-in menu 'FZ Offline' and input any parameter values.

7. Troubleshooting

1. How can the ImageFZ program be obtained and installed?
   The ImageFZ program is available for free download from our website (see Table of materials/reagents), and runs on a Windows computer. Download the zip folder for ImageFZ and install the software on your computer. See the 'readme.txt' file for the installation details and follow the step-by-step instructions.
2. Why is the error message 'Error setting capture device' displayed?
   Check the connection of the camera cable and your driver installation of the USB image capture device. If there is no problem with the settings, then the ImageFZ software might not work with your image capture device. See the 'readme.txt' file concerning the appropriate device to use with ImageFZ software.
3. ImageFZ cannot detect the entire body of the mouse as a particle.
   Set the value of 'threshold min' and/or 'threshold min (xor)' lower than the current value. If ImageFZ cannot detect the mouse in a specific place, *e.g.* the corner of a test chamber, then insufficient testing conditions, such as a uniformly illuminated floor or a slightly contrasting difference between the mouse and the background, might exist. To solve this problem, adjust the parameter values (*e.g.* brightness and contrast of the live image and threshold values) of ImageFZ, control the number and position of lights, or use a white background for a black mouse.
4. Image capturing at a high frame rate slows down the computer during online analysis.
   Set the frame rate to a value lower than the current rate, and perform online analysis. The ImageFZ analysis, through image acquisition at 1 fps, is sufficient for accurately measuring freezing, as shown in the representative results section.
5. The results of the ImageFZ analysis do not agree with those of human scoring.
   Examine the stored image and judgment result files. If ImageFZ overestimates freezing, set the 'freezing criterion' to a value lower than the current value, and perform offline analysis. If ImageFZ underestimates freezing, set the 'freezing criterion' to a value higher than the current value.
6. In optogenetical and *in vivo* electrophysiological experiments, the fiber cable attached to the head of the mouse interferes with the judgment of freezing.
   Coat the cables in white for a black mouse, and change the position and angle of the camera until the cables are not detected.
7. What is needed for the offline analysis?
   Create a folder named 'Image_FZ' in the root directory of the ImageFZ program. In this folder, create the subfolders 'Images' and 'Sessions'. Move an 8 bit gray-scale image to the 'Images' folder, and create a text file in which the image file name is written in the 'Sessions' folder. Thereafter, run the ImageFZ offline analysis, and follow the instructions of the program.

Representative Results

In the fear conditioning test, human experimenters used to quantify the freezing behavior through labor-intensive direct observation[26-29], but recently photobeam-based computer measurement (*e.g.* the 'Freeze Monitor' system) and image-analyzing systems have been used to automatically measure the freezing behavior[26,30-32]. ImageFZ is an automated image-analyzing system, which produces results comparable to those obtained through human observation, as described below. Here, we compared the outcomes of human observation with those of ImageFZ analysis under varying parameters: 'Rate (frame/sec)' and 'Freezing criterion (pixels).' In this experiment, five male C57BL/6J mice (mean body weight ± SD (g), 31.4±3.55; mean body size ± SD (pixels), 351.6±62.2) were used at 15-27 weeks of age. The human observation was made using an event-recording program (a Macintosh OS9 software program); a key-pressing event that continued for 2 sec or more when a mouse displayed a bout of no movement was considered 'freezing'. The percentage of freezing was calculated every 60 sec in each test and used for correlation analyses. The percent of freezing scored by the 2 observers (interobserver reliability, for conditioning, r=0.879; for context test, r=0.957; for cued test, r=0.866, for all cases, r=0.888) was averaged to generate a human score. Correlations between the freezing percentages measured through ImageFZ with each frame rate (*i.e.* 1, 2, and 4 fps) and those obtained through human observations were examined. As illustrated in **Figure 4**, the freezing percentages calculated through ImageFZ (1, 2, and 4 fps) were highly correlated with the average value obtained from the measurements of the 2 observers. Notably, capturing images at a higher frame rate does not always produce the best correlation. Image analysis at 1 fps generated results similar to those obtained from human observers in each test. Correlations between the freezing percentages measured through human observations and using ImageFZ under each condition of the 'Freezing criterion (pixels)' (*i.e.* 20, 30, and 40 pixels) were examined. The freezing percentages calculated using ImageFZ at the 'Freezing criterion (pixels)' of 20, 30, and 40 pixels were, in all cases, highly correlated with those obtained through human observations (**Figure 5**). As shown in **Figure 5D**, when the

freezing criterion is set to a low value, the subtle movement of a mouse, considered to be 'freezing' by human observers, would be considered 'non-freezing' using ImageFZ. Conversely, if the criterion is set to a high value, the movement of a mouse, scored as 'non-freezing' by human observers, would be considered 'freezing' using ImageFZ (**Figures 5C, 5F**, and **5I**). Thus, to obtain the most reliable results, each parameter of the ImageFZ program should be calibrated using the data scored through human observations in each testing environment.

In addition, we compared the results generated by a human observer, using a photobeam-based computer measurement system (the Freeze Monitor system), to those obtained using ImageFZ (see **Figure 6**). The human observer was blinded to the treatment group and the results of ImageFZ scoring. For the parameter settings of the Freeze Monitor system, we used 3 measures of the percentage of freezing from a previously validated system[30]. Briefly, the number of 10-sec intervals in which the animals required more than 1 or 2 sec to cross the first new beam of the interval (1sec 10sec and 2sec 10sec, respectively) and the latency between the beginning of each 5 sec interval and the third new beam interruption within this interval (Latency3) were measured. The percentages of the intervals during which the mouse was freezing or the percentage of the total amount of time required to break the third photobeam were calculated.

The percentages of freezing measured in each system are illustrated in **Figure 6**. The groups were compared using two-way repeated measures ANOVA followed by t-tests (see **Table 1**). The freezing percentages measured using ImageFZ (**Figure 6B**) were more similar to those scored through human observation (**Figure 6A**) than the data obtained using a photobeam-based system (**Figures 6C-E**). The freezing percentages measured using the ImageFZ program in each test were highly correlated with those scored through human observation (conditioning, r=0.947; context test, r=0.970; cued test, r=0.934), whereas the correlations between the freezing percentages measured using the photobeam-based computer measurement system (1sec 10sec, 2sec 10sec, or Latency3) and the human observer were lower (conditioning, r=0.503, 0.593, and 0.761; context test, r=0.772, 0.819, and 0.912) compared to the correlations between the freezing percentages measured using ImageFZ and human observation (**Figures 7A** and **7B**). In addition, **Figure 7** reveals that the differences between the freezing percentages obtained through human observation and using ImageFZ in each mouse were the smallest differences. These results indicated that the freezing percentages measured using ImageFZ were similar to those obtained through human observation and that ImageFZ is highly accurate when measuring the amount of freezing.

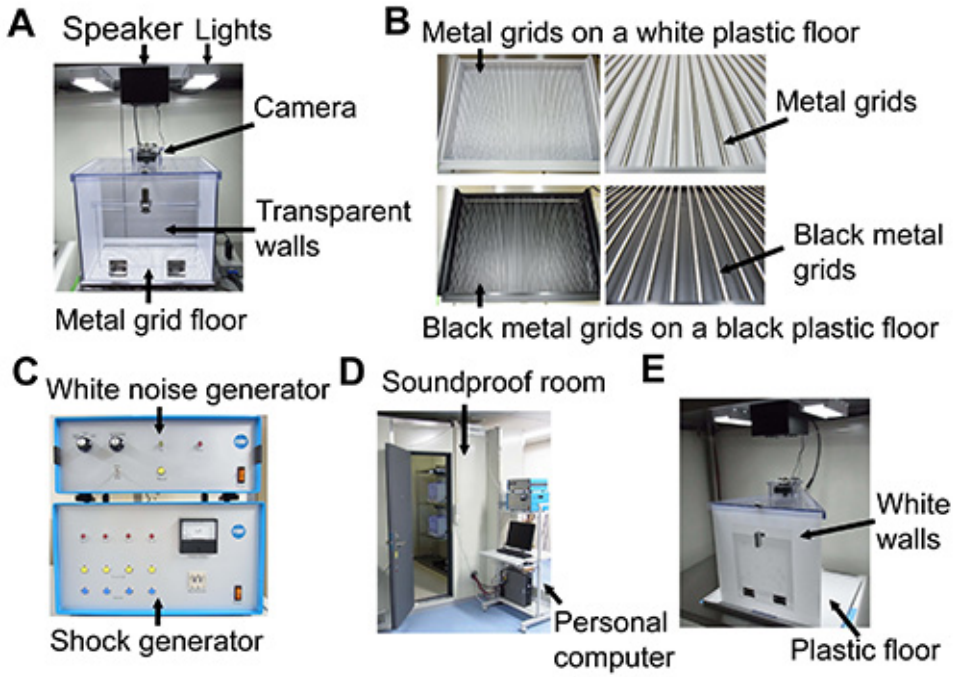

Figure 1. Apparatuses for the contextual and cued fear conditioning test. (**A**) An acrylic square chamber for the conditioning and context test, (**B**) metal grids on a white plastic floor for black, agouti, or dilute brown mice (top) and electrifiable black metal grids on a black plastic floor for white mice (bottom); enlarged images of the grids are shown in the right panel, (**C**) a white noise/tone generator and a shock generator, (**D**) a soundproof room, and (**E**) an acrylic triangular chamber with a flat floor for the cued test. Click here to view larger image.

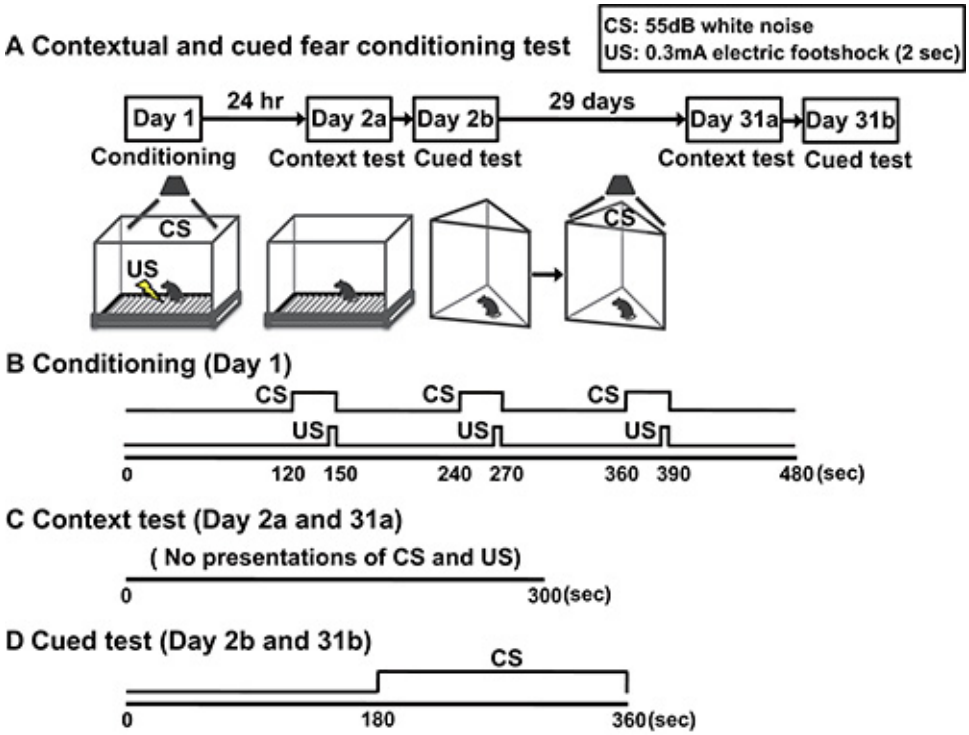

Figure 2. Schematic representation of the protocol. (**A**) Overview of the contextual and cued fear conditioning test, (**B**) conditioning, (**C**) context test, and (**D**) cued test. Click here to view larger image.

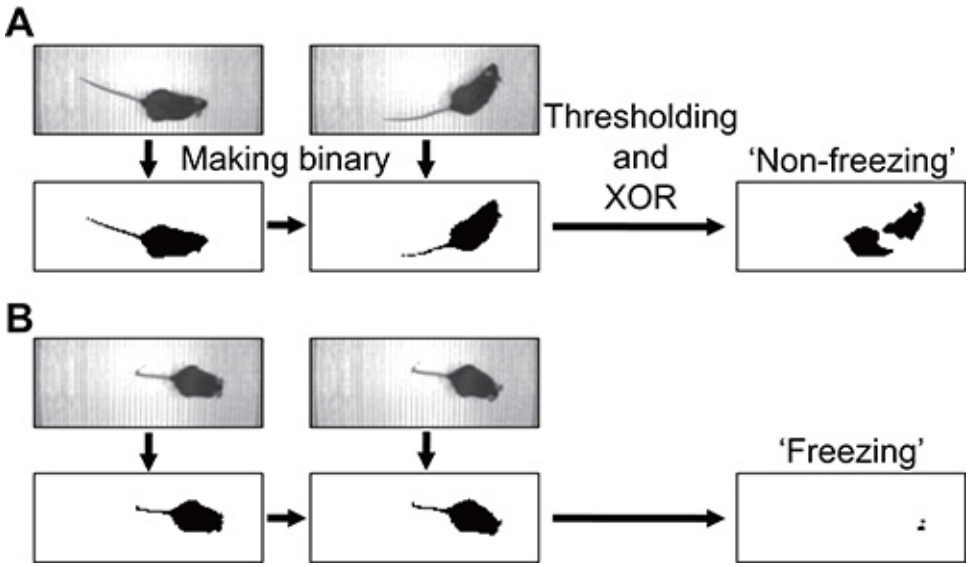

Figure 3. Image analysis by the ImageFZ software program. For each pair of successive images, the amount of area (pixels) through which the mouse moved is calculated by ImageFZ. When this area is below a certain threshold (*e.g.* 30 pixels), the behavior is judged to be 'freezing'. When the amount of area equals or exceeds the threshold, the behavior is considered to be 'non-freezing'. Click here to view larger image.

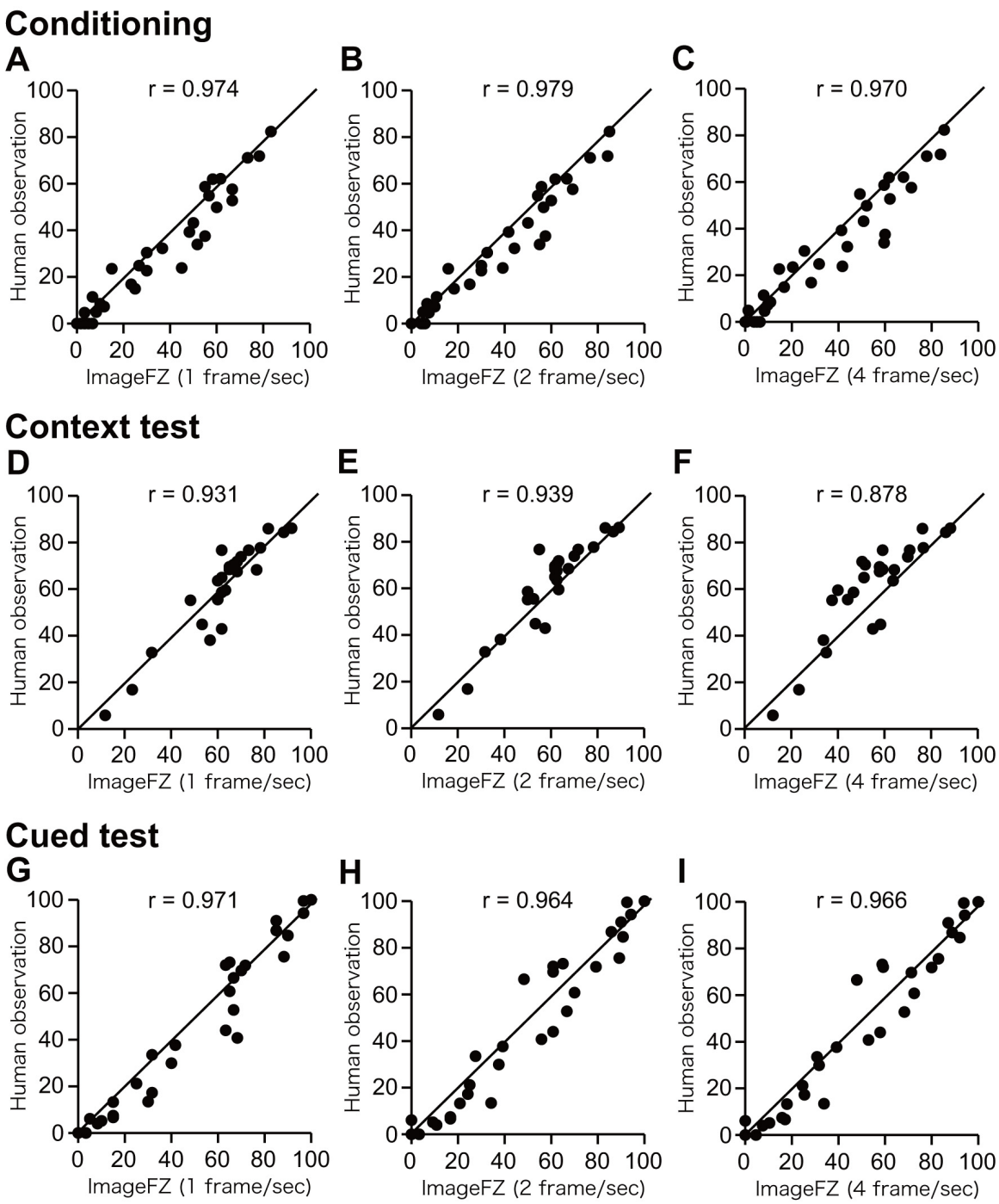

Figure 4. Comparisons of the freezing percentages calculated from images at different frame rates using ImageFZ with those measured through human observation. The fear conditioning tests were conducted using male C57BL/6J mice (n=5). During the tests, two observers scored the freezing behavior. Simultaneously, live images were captured at 4 fps using the ImageFZ program. The files captured at 4 fps were downsized after extracting the frames to correspond to images captured at 1 fps or 2 fps . The parameter values of 'Rate (frame/sec)' were set to 1, 2, or 4 fps , and freezing percentages in each 60-sec bin were calculated from image files using ImageFZ offline analysis. Each dot represents a freezing percentage of each 60-sec bin. Pearson's correlation coefficients between the data obtained from human observation and ImageFZ analysis were calculated. Click here to view larger image.

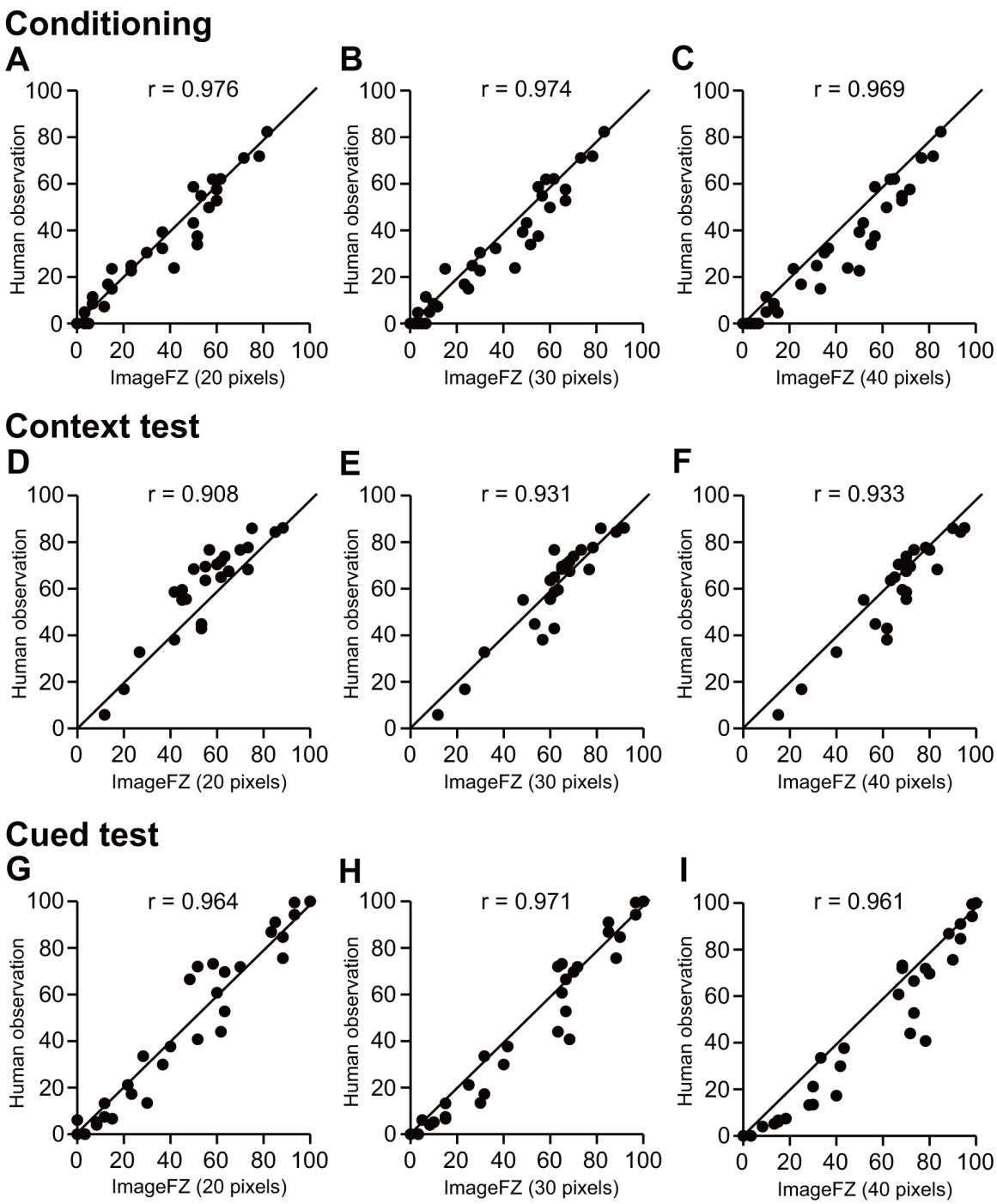

Figure 5. The freezing percentages calculated from the images at different freezing criterion values using ImageFZ and those measured through human observations were compared. The fear conditioning tests were conducted using male C57BL/6J mice (n=5). During the tests, two observers recorded the freezing behavior, and the live images were captured using the ImageFZ program. The freezing percentages in each 60 sec bin were calculated from the images (1 frame/sec) through ImageFZ offline analysis, setting the parameter values of 'Freezing criterion (pixels)' to 20, 30, or 40 pixels. Each dot represents a freezing percentage of each 60-sec bin. Pearson's correlation coefficients between the data obtained from human observation and the ImageFZ analysis were calculated in each test. Click here to view larger image.

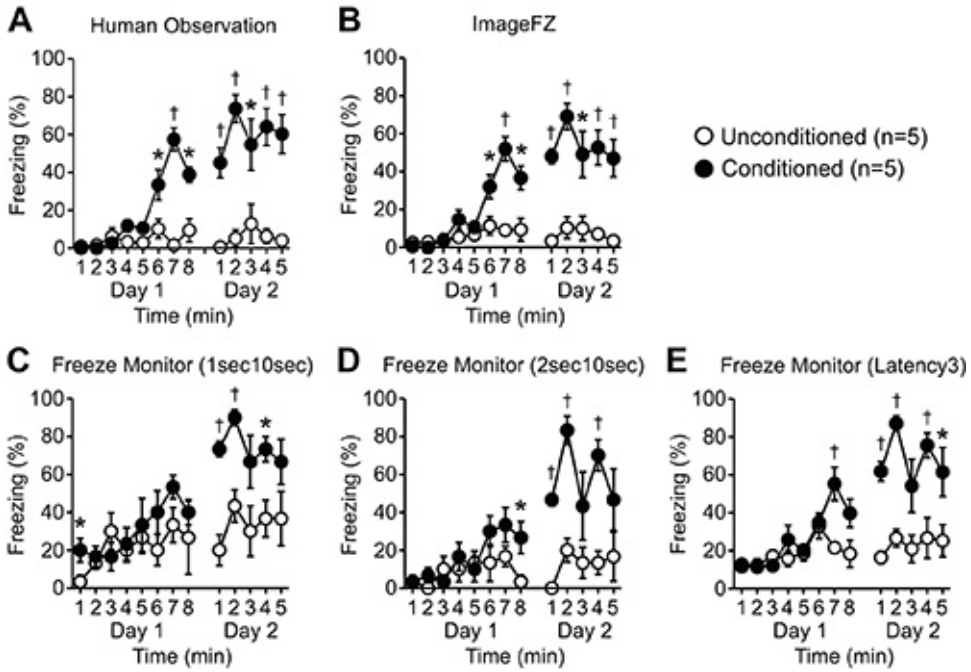

Figure 6. The percentages of freezing were measured using automated systems and human observation in unconditioned and conditioned groups of male C57BL/6J mice (n=5, each group). (**A**) Human observation, (**B**) ImageFZ, (**C**) Freeze Monitor system 1 (1sec 10sec), (**D**) Freeze Monitor system 2 (2sec 10sec), and (**E**) Freeze Monitor system 3 (Latency3). Group comparisons were performed using two-way repeated measures ANOVA followed by t-tests (unconditioned group vs. conditioned group, *, P<0.05; †, p<0.01). The data obtained using ImageFZ were similar to those scored through human observation. Click here to view larger image.

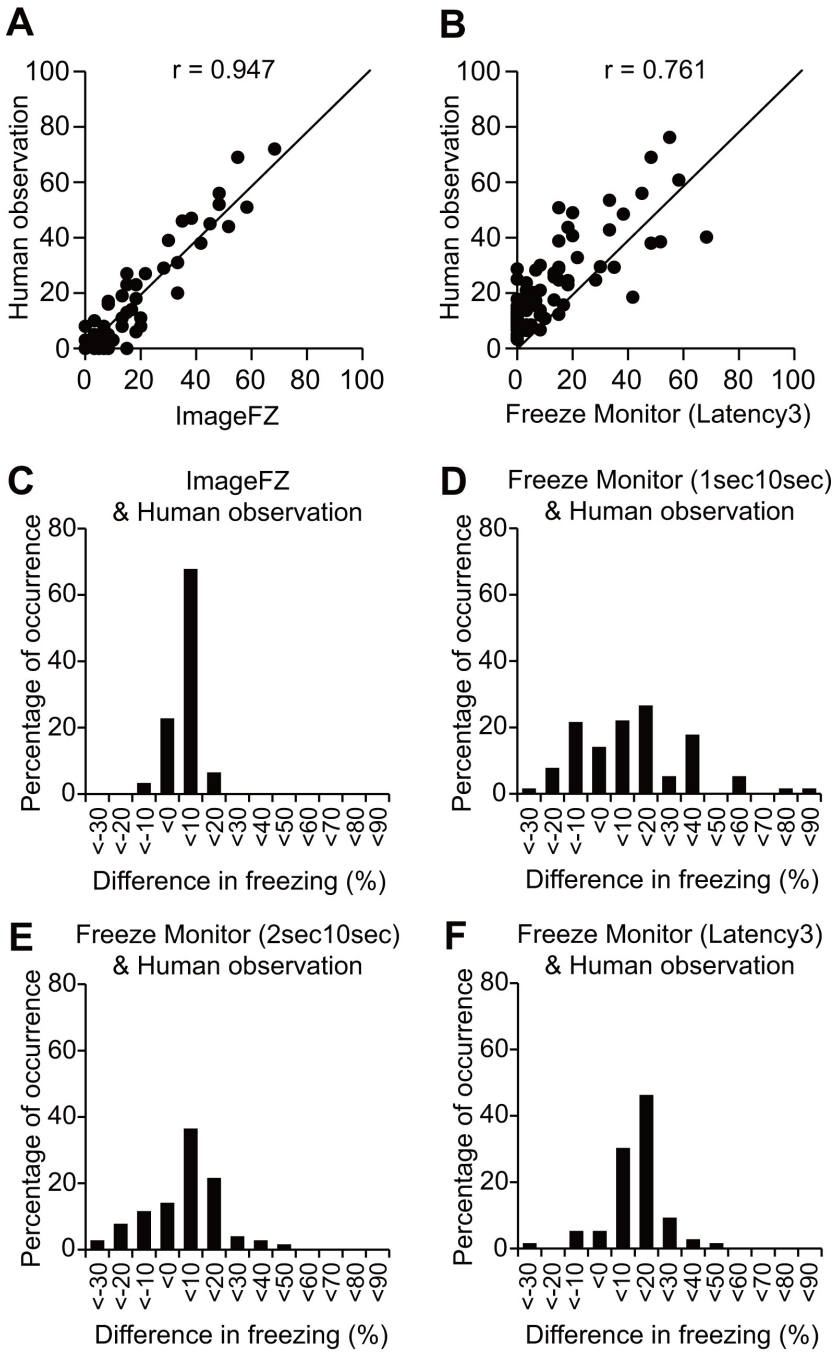

Figure 7. Correlation and frequency distribution of the differences between the freezing percentages, measured using automated systems and human observation. (**A-B**) Scatter plots and Pearson's correlation coefficients between freezing percentages scored through automated systems and human observation are shown. The freezing percentages, calculated using ImageFZ, were highly correlated with those obtained through human observation. (**C-F**) Occurrences of less than a 10% difference between the freezing percentages obtained from automated systems vs. human observation were highest when the data analyzed using ImageFZ were compared with those analyzed through human observation. Click here to view larger image.

	ANOVAs		
	Condition	Time	Condition x Time
Day 1 (conditioning)			
Human	F(1,8)=28.53, p=0.0007	F(7,56)=20.79, p<0.0001	F(7,56)=16.58, p<0.0001
ImageFZ	F(1,8)=13.97, p=0.0057	F(7,56)=21.40, p<0.0001	F(7,56)=11.69, p<0.0001
Freeze Monitor (1sec10sec)	F(1,8)=5.16, p=0.0528	F(7,56)=2.39, p=0.0329	F(7,56)=0.72, p=0.6572

Freeze Monitor (2sec10sec)	F(1,8)=4.07, p=0.0782	F(7,56)=3.44, p=0.0039	F(7,56)=1.52, p=0.1803
Freeze Monitor (Latency3)	F(1,8)=4.44, p=0.0682	F(7,56)=9.94, p<0.0001	F(7,56)=4.33, p=0.0007
Day 2 (context)			
Human	F(1,8)=42.94, p=0.0002	F(4,32)=1.91, p=0.1336	F(4,32)=1.48, p=0.2302
ImageFZ	F(1,8)=49.61, p=0.0001	F(4,32)=2.06, p=0.1087	F(4,32)=0.83, p=0.5174
Freeze Monitor (1sec10sec)	F(1,8)=20.28, p=0.002	F(4,32)=1.63, p=0.1918	F(4,32)=0.55, p=0.6997
Freeze Monitor (2sec10sec)	F(1,8)=40.20, p=0.0002	F(4,32)=2.66, p=0.0504	F(4,32)=1.20, p=0.3306
Freeze Monitor (Latency3)	F(1,8)=35.30, p=0.0003	F(4,32)=2.49, p=0.0626	F(4,32)=1.09, p=0.3793

Table 1. Comparisons of statistics.

Discussion

The contextual and cued fear conditioning test is one of the most widely used paradigms to assess learning and memory. This test is a form of Pavlovian conditioning in which an association is made between a context and/or a conditioned stimulus (auditory cue) and an aversive stimulus (electric footshock). After even a single pairing of the context/auditory cue and footshock, mice exhibit long-lasting freezing when faced with either the context or the cue. In this test, freezing behavior is used as an index of fear memory. Pharmacological and lesion studies have revealed that memory formation, consolidation, and retrieval are regulated by several brain regions, such as the amygdala, hippocampus, and prefrontal cortex[3,33-35]. In addition, molecular genetics studies have demonstrated the role of specific genes and molecules involved in learning and memory in these brain regions using genetically engineered mice[36]. Therefore, this test is simple and useful for exploring the neurobiological basis underlying fear learning and memory. In this movie article, we introduced our protocol to provide experimenters with detailed information to understand and easily perform the test.

Freezing behavior was quantified through direct observation by human experimenters. A well-trained experimenter is expected to produce reliable, stable results across observations. However, this method involves potential problems, such as differences in the observational method, observer biases, and simple quantification mistakes, making it difficult to directly compare the results from independent experimenters and different laboratories. An automated photobeam-based computer measurement system has also been used[26,30-32]. However, this system also presents potential problems in measuring freezing behaviors. Because of the sensor arrangement, this system might be unable to detect small head movements that would typically be scored as 'active' through human observation. In addition, trembling during freezing might be considered as nonfreezing because when an animal freezes, intermittent interruptions in the photobeam are observed as a consequence of trembling. As an alternative method, automated image- and video-analyzing systems have been developed[17-20,37,38]. Anagnostaras *et al.*[37] described a few systems with image analysis software programs that have good validity and score freezing well[17,20,37-38]. However, most of these systems and analysis programs have to be obtained from commercial suppliers and are typically costly. We developed the ImageFZ software program for the analysis of freezing behavior, and this program is distributed as a free software program. ImageFZ detects the mouse as a body of pixels (a particle) and discriminates subtle mouse movement as 'freezing' or 'non-freezing' depending on the amount of area of nonoverlapping regions between particles of each pair of consecutive images. As shown in the representative results, measurements using the ImageFZ program are consistent with or more accurate than obtained using other methods. Thus, the ImageFZ program automatically measures the behavior that human observers judge as freezing using defined criteria. In addition, the ImageFZ program calculates the distance traveled (cm) before, during, and after footshock exposure, facilitating an assessment of the shock sensitivity and analysis of the freezing behavior.

Methodological differences exist between laboratories. These differences may result in difficulty in comparing data among laboratories and in replicating results in different laboratories. To obtain more stable and comparable data, it is necessary to standardize the test protocol as much as possible. The analysis system with ImageFZ leads to the automation of test procedures, which can contribute to the standardization of protocols used across laboratories.

Several behavioral responses must be considered when analyzing freezing behavior. First, when animals face a fearful situation, they may flee instead of freezing[39]. Fleeing is one of the fear responses, and its occurrence will lead to underestimating fear memory. Second, freezing may depend on a general activity level, and the activity level in experimental and control mice needs to be examined. For example, although mice lacking the M1 muscarinic acetylcholine receptor showed reduced levels of freezing compared to wild-type mice, various behavioral tests indicated that the results may be attributed to their hyperactivity phenotype instead of their memory impairment[18]. ImageFZ calculates the distance (cm) traveled by the subjects. The data are available to examine whether or not differences exist in the general activity levels between subjects. If there is a group difference in the distance traveled, one possible approach to the problem is to consider the distance traveled during the first 2 min of training as the baseline activity and to use a suppression ratio (suppression ratio = (activity during testing)/(activity during baseline + activity during testing)) as a secondary index of fear[17,40]. Finally, a difference in pain sensitivity, inducing the changes in reactivity to an electric footshock, if any, may result in variations in freezing behavior. ImageFZ also calculates the distance traveled (cm) in detail from 2 sec before an exposure of a 2 sec footshock to 2 sec after its exposure (for 6 sec), which can be used as an index of footshock sensitivity.

Video-analyzing systems have been developed to measure the freezing behavior of albino, black, agouti, and dilute brown mice. ImageFZ uses a black floor tray and black grids to examine white mice (see **Figure 1B**). The black grids are made of specially processed metals with coated black paint and have an electrical conductivity similar to that of the noncoated metal grids, which are typically used for black mice. ImageFZ also analyzes the freezing behavior in rats and other rodents through adjustments of the program parameters. In the current version of ImageFZ, the behavior of the subject is recorded using a video camera from the top wall to analyze freezing. ImageFZ could also be used in a set-up where the images are captured from the side of the chamber. In addition, the ImageFZ controls a maximum of 4 apparatuses. This feature allows the researcher to simultaneously examine 4 mice, saving time and reducing the potential influences from differences in the execution time of each

subject and the testing order on behavior. Thus, ImageFZ simplifies the testing procedure and analysis of freezing behavior, and this program facilitates testing with less labor and without any training for the behavioral experiments.

In the Miyakawa lab, we have assessed more than 110 strains of genetically engineered mice and wild-type control mice in the contextual and cued fear conditioning test using the video analyzing system to elucidate the effects of a given gene on learning and memory[41-42]. We have obtained a large set of raw data for more than 5,000 mice. The raw data that was used for published research articles[4-16] are included in the 'Mouse Phenotype Database' as a public database (URL: http://www.mouse-phenotype.org/). The present movie article provides detailed information regarding the details of our experimental procedure and promotes the understanding of the testing situation.

Disclosures

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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