FreezeFrame

ActiMetrics SOFTWARE

Freeze Frame

FreezeFrame is a computerized system for the **Conditioned Fear Test** that combines the sensitivity of the human observer with the objectivity and convenience of automation.

While infrared beams or video tracking systems can detect gross movements of an animal from one part of the cage to another, FreezeFrame can detect the minute movements of grooming, sniffing, turning and rearing.

By recording from 4, 8 or even 12 animals at once, FreezeFrame is ideal for high-throughput testing of large numbers of animals.

- **Not a tracking system.** FreezeFrame uses a proprietary motion detection algorithm that filters out shadows, light flicker and camera noise.
- **Detects movements as small as 1 mm.**
- **Fully validated.** >90% concordance between FreezeFrame and a trained human observer.
- **Multiple stimulus protocols** can be stored for training and testing phases of the experiments.
- **Display images on the computer screen.** No external monitor necessary.
- **Store images for later review.**
- **Export data and analyses in spreadsheet format.**
- **Easy setup.**
  - Sounds (white noise or tone) are delivered through the computer's speakers.
  - The shocker-scrambler is controlled directly from the computer.
  - 2 additional external stimuli may be controlled as well.

4-Hz sampling frequency.

4, 8 and 12 channel systems

- Record 4 animals at once using a single computer and camera with the FreezeFrame 4-channel system.
- Use 1 camera to record from 4 chambers or 1 camera for each chamber.
- Record 8 or 12 animals with 2 or 3 linked computers, each running the FreezeFrame 4-channel system.

System Requirements

Computer

FreezeFrame runs on Windows 9X/NT/2000/XP computers. We recommend:

- Pentium IV with a 1.2 GHz or faster processor
- 1 Gigabyte of memory
- Windows XP
- 15" monitor or larger (1280 x 1024 resolution)
- Sound card and speakers
- If you plan to store the acquired images, a CD or DVD writer for backing up data files.

Hardware

Camera. A black-and-white CCD camera with NTSC format output (supplied)

Image Acquisition board. National Instruments PCI-1405, PCI-1410 depending on configuration (supplied).

Shocker-scrambler, cage and cables. FreezeFrame will connect easily to most available fear conditioning hardware.

Hardware Installation

FreezeFrame can be used with National Instruments video acquisition boards, the PCI-1405, 1407, 1408, 1409 or 1410.

Before installing the video board, load the NI-IMAQ software from the CD provided with the video board.
Shut down the computer and plug the PCI-140X board into the open PCI-slot that is closest to the processor.
Restart the computer.

Camera connections

Single camera

- Connect the camera output connector to the "Video" input BNC on the PCI-14XX using the BNC cable provided.
- Connect the camera to a 24VAC power supply using the two-stranded wire provided. Polarity is unimportant.
- Plug the power supply into any 120V- 60Hz or outlet.

- **Two cameras** (See diagram below)

- Set up camera one as described above.
- Connect the multi-pin cable assembly provided to the PCI board.
- Connect the "video out" BNC connector on cameras 2 V0 and V1.
- Connect the CSYNCOUT BNC on the video board to the T connector on camera 2.
- Connect cameras to 24VAC power supply as above. One power supply can be used to both cameras.
- Plug the power supplies into any 120V-60Hz outlet.

- **Four cameras** (See diagram below)

- Connect the multi-pin cable assembly and breakout box (A6822) to the PCI board.
- Connect the "video out" BNC connector on cameras 1 2, 3 and 4 to V0, V1, V2 and V3.
- Put one BNC T-connector on the GEN LOCK (or SYNC IN) plug on cameras 2 and 3.
- Connect the CSYNCOUT BNC on the video board to the T connector on camera 2.
- Connect the other side of the T-connector on camera 2 to the T-connector on camera 3.

- Connect the other side of the T-connector on camera 3 to the GEN LOCK/SYNC IN connector on camera 4.
- Connect each camera to a 24VAC power supply as above.**Each power supply can be used to power 2 cameras.**
- Plug the power supplies into any 120V-60Hz outlet.
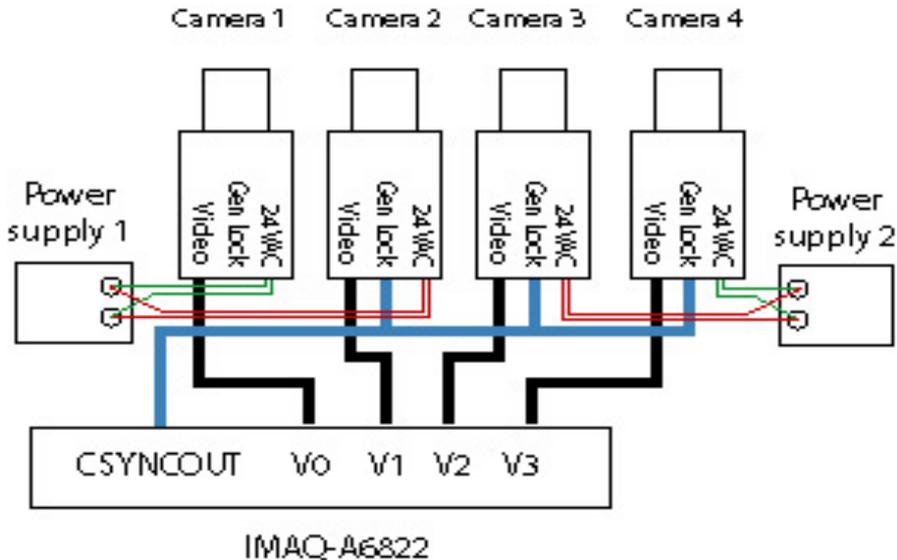
Configure the PCI-14XX

- Double-click the Measurement & Automation icon on the desktop, or from Windows's START menu, select Programs->National Instruments IMAQ -> Measurement and Automation. A Window entitled "MAX" should appear.
- In the list to the left of the window, open My System\Devices and Interfaces\IMAQ PCI-14XX \img0\Channel 0: rs 170
- From the Toolbar just underneath the menu bar, select IMAQ->Grab. You should now see a real-time image from camera 1. If no image appears, check the power and signal connections from the camera and video board. If you still have trouble, call National Instruments at 800-433-3488 and ask for help with NI-IMAQ.
- Close the MAX window.
- **For all systems:** Select "Channel 0: RS-170" in the configuration list. and click the Properties tool in the toolbar. Set the Bit Depth to "8 bit" in the dialog box that appears and then click OK.
- **For multiple camera systems only:** Select IMAQ PCI-14XX: img0 in the Configuration list and click on the Properties tool in the toolbar. Select the Operating Mode tab in the dialog box that

appears. Set Slave Acquisition Mode to "CSYNC External". CSYNC Source will be set to "Channel 0". Click OK.

- For multi-camera setups, select and then try the grab for each Channel.
- Close the MAX window.

Configure the computer's speakers and sound card

FreezeFrame relies on the computer's sound card and speakers for generating conditioning stimuli. Connect the computer's speakers as in your computer manual and test the sound system. You can test the system by playing a CD (from the START menu, choose Accessories->Multimedia->CD Player), or by opening the Sounds control panel, selecting a sound and playing it using the rightward pointing arrow ("Preview:").

If the sounds do not play, check the speaker connections, whether they are turned on and whether the volume is set high enough. Next check whether the drivers for the sound card that came with the computer are properly installed and functional. If they are not, consult with the computer manufacturer.

If you are able to play a CD or a sound in the Sounds control panel, FreezeFrame should be able to play conditioning stimuli as well.

Connect the Shocker to the computer

PCI-1405 or 1407. Connect the shocker control input to the BNC connector labeled "TRIG" on the PCI-1407.The TRIG connector provides a 5V (TTL-compatible) signal. Check with the shocker manufacturer to make sure that a TTL signal is sufficient for triggering their device.

PCI-1408, 1409 or 14010. Connect the interface cable to the multipin connector on the PCI-1408/1409. Stimuli 2-4 (shock and 2 auxilliary outputs) correspond to the BNC connectors labelled T0, T1 and T2, in order.

Connect the Shocker to the cage

Details will vary depending on the shocker and cage you have. Consult the manufacturer's instructions.

Camera mounting

The camera can be mounted on any standard camera tripod or mounting bracket using a 1/4-20 screw. For a single channel system, the exact placement of the camera is not critical. The camera can be aimed from directly above the cage, or in front of and slightly above the cage, looking down from a 45 degree angle.

Alternatively, if the rear walls of the chambers are clear, the cameras can be mounted behind the chambers. This leaves the front of the chambers clear for easier loading of animals. One difficulty the rear-mounted cameras introduces is possible artefacts from the user walking in front of the chambers during a trial. Be sure to make the front door of the chambers opaque and to block off the space between the chambers so that the camera cannot see the space in front of the chambers.

Adjusting Image Quality **(Important)**

There are 5 critical adjustments that must be made to the image in order to insure proper operation of FreezeFrame. If any one of these conditions is not met, the system's sensitivity will suffer, as will the accuracy of your results. An example of a good camera setup is shown in the image below.

1) Camera position. Position the camera and adjust the zoom of the lens so that the **cage fills the image** as much as possible**.** For 1-camera setups, position the 4 cages as close together as possible.

2) Camera angle. Position the camera in front of the cage so that **all of the cage floor is visible**. With a 1-camera setup, it might be necessary to angle the right-hand cages to the left and vice versa so that the camera sees straight in to each cage.

3) Focus. Take extra care to **focus the image** properly**.** Incorrect focus will degrade the program's ability to detect fine movement.

4) Lighting. The absolute lighting level is not critical, as long as there is **enough light** in the chamber to give a clear image on the screen. If you do not have a auto-iris lens (one with a short wire that plugs into the camera), adjust the aperture manually so that a clear image is visible on the screen.

- With the aperture set to be too large, areas of the image will become uniformly white or washed out.
- With the aperture set to be too small, the image will become dark and grayed out.
- With the aperture set in the right range, small lighting changes will make little difference in the appearance of the image since they are compensated for by the automatic gain control of the camera

5) Reflections. It is critical that there be **no glare from highly reflective objects** on the screen. If you see light glinting off any shiny surfaces (that is, saturated parts of the image that are comletely white), adjust the lighting or camera angle, or as a last resort put tape over the reflective surface. Similarly, there should be no completely black areas of the image. An important check for too bright or too dark regions of the image is to run the FreezeFrame data collection program and view the images in Difference (Diff) Mode (see the FreezeFrame main window description). Most of the image should appear as grey snow that changes from frame to frame. Saturated parts of the image will appear a flat grey with no speckling and no change from frame to frame. It is important to minimize these areas by changing the lighting or the objects in the field of view.

Once the lighting is set, it should not be changed from trial to trial.

1 camera - 4 chamber systems

The multichannel system cuts the image up into 4 quadrants of equal size and performs the motion calculation independently on each image. The simplest arrangement is to cluster the 4 cages into a 2x2 array and point the camera at the entire array from above. Alternative arrangements are possible as well. For example, 2 upper cages can be set above and slightly set back from the 2 lower ones, and the camera pointed from in front of slightly above the array.

Make sure that the image of each cage is confined to its own image quadrant. This is most easily checked in the program, using the controls that allow viewing of each quadrant independently.

Multiple-Computer Systems (8 or 12 channels)

Install FreezeFrame hardware and software

Install the software and hardware on each computer as described in the [Installation section](#).

Identify the Master and Remote Computers

Select one computer (typically, the one with the fastest processor) to be the Master computer, and the second (and possibly third) computer to be Remote 1 (and 2).

Shock and Sound control.

You may connect all speakers and shockers to the Master computer. Alternatively, you may connect some speakers and shockers to the Remote computers as well. The program runs in near synchrony on the different computers and each puts out similar control signals.

If you will have each computer control various stimuli, be sure to set the Stimulus Polarity (Normal/Invert) controls independently on each computer using the [Output Configuration window](#).

All stimulus timing and Sound stimulus properties on the Remote computers are inherited from the Master and so do not need to be set on the Remote.

Connect the Computers

Using the cables provided (with 9-pin connectors on each end), connect the COM1 (serial) port on the Master computer to the COM1 port on Remote 1.Some cables come with both 9-pin serial port connectors and 25-pin parallel port connectors on each end. Only the 9-pin connectors need be plugged in.

Connect the COM2 port on the Master computer to the COM1 port on Remote 2.

Configure FreezeFrame

Run FreezeFrame and use the File->To Master menu item to configure the Remote computers as Remotes.

On the Master Computer, activate the connections to the Remotes using the Settings->Remote 1 and Settings->Remote 2 menu items.

The FreezeFrame Recorder Menus and Controls

FreezeFrame starts running when it opens. With the camera and image acquisition board properly configured, you should see the camera image(s) in the window updating about 4 times per second. An asterisk (*****) on the controls below indicates that the setting of the control is telegraphed automatically to the Remote computers, if present.

File Menu

Data File. Use this menu item to select the file into which all data will be written. The currently selected file is displayed in the indicator underneath the image window. If the directory chosen for some reason does not exist, an warning indicator will appear.

To Remote/To Master. If you are using FreezeFrame on multiple computers that are connected to one another, use this menu item to configure the program as either a Master or Remote. In Master mode, the program controls the Remote computers, starting and stopping trials, and determining stimulus sequences. The Remotes in turn report their data back to the Master.

Quit. Exits FreezeFrame.

Operate Menu

Names. Opens a window that allows you to enter all the names for a trial in a single one-dimensional list. The TAB key moves the cursor from one trial to the next.

Avid IDs. Applicable only if you have Avid ID chips in your animals and an Avid chip reader connected to your computer. Contact Actimetrics for information.

Reference*. Collects a reference frame for later tracking of the animal. Same as clicking the **Reference** button underneath the image window. Collect a reference frame before placing the animal in the chamber.

Start*. Starts a trial.Same as the **Start** button. Commands the Remote computers to start at the same time.

Stop*. Aborts a trial without saving data. Same as the **Stop** button. Commands the

Remote computers to stop as well.

Settings Menu

Protocols. Opens the [Protocol window](#) for designing the stimulus sequence of the protocols.

Stimulus Configuration. Opens the [Stimulus Configuration](#) window, for setting the polarity of the shock and auxilliary control signals, and the sound stimulus characteristics.

Camera Setup. Opens the [Camera Setup](#) window, for setting the frame rate and the number of cameras.

Save Images*. Tells FreezeFrame to create an image file for each recorded animal. During the trial, each recorded image is stored in a file called <animal>.ffii, where <animal> is the name of the animal specified in the Animal Names control (below). The ffii file is located in the same directory as the data file.

Invert Vertical. When checked, inverts the image in the image window.

Invert Horizontal. When checked, flips the image left-to-right.

Remotes.

> **Remote 1.** When checked on the Master computer, tells FreezeFrame to control and read data from Remote computer 1.
> **Remote 2**. When checked on the Master computer, tells FreezeFrame to control and read data from Remote computer 2.
> **Remote 2**. When checked on the Master computer, tells FreezeFrame to control and read data from Remote computer 3.

Avid Setup. Applicable only if you have Avid ID chips in your animals and an Avid chip reader connected to your computer. Contact Actimetrics for information.

Large View. The program can be setup for two screen sizes. With Large View checked and multiple animals being monitored, the program will show all animals at once. Requires a display with 1280 x 1024 resolution. With Large View unchecked,

only one animal at once is shown in a smaller format. Appropriate for displays with 1024 x 768 resolution.

Help Menu

Help. Open this help file.

About FreezeFrame. Displays the Version number and Serial number.

Controls and Indicators

Animal Names Control

Local. An array of text boxes is located to the upper left corner of the FreezeFrame window. Prior to a trial, enter the names of the animals to be run into the controls. For 1-camera setups, the four controls correspond to the four quadrants of the image. For 4-camera setups, the 4 controls correspond to the 4 cameras, in the order upper left, upper right, lower left, lower right. These names are used to identify each trial in the freeze data file, and are used as the names of the image files if images are to be saved. As you enter each name, FreezeFrame checks the current data directory for an image file of that name and warns you if one exists by showing an "Overwrite" indicator in the space below the Names controls and disables the start button. Use the TAB key to move from one text entry box to the next.

Remote*. With FreezeFrame in the Master mode, and the Remote 1 or Remote 2 menu items checked, one or two more sets of **Animal Names** controls appear below the **Local** control. Enter names for the animals to be run on the remote computers here. The names are sent to the Remote computers and will appear on their screens.

Motion Index Indicators

Local. To the right of the **Name** controls is an array of indicators. The numerical indicator shows the motion index for each camera or image quadrant. The light below the indicator indicates freezing whenever the motion index falls below 10. This is only a rough guideline. The real threshold for freezing will be set in the FreezeView Analysis program.

Remote. With FreezeFrame in the Master mode, and the Remote 1, 2 or 3 menu items checked, additional sets of Motion Index indicators will appear below the first, one for

each Remote computer. Data are sent automatically from the Remotes and appear in these indicators. If the Remotes are not operating properly or the cable is not connected correctly, a No Data warning will appear. Similarly, an error indicator will appear if the serial port is not responding properly.

View Control

Click on the different buttons in the **View** control to view the image from each of the 4 cameras or from each of the 4 quadrants of the image. On 1-camera systems, click the **View All** toggle switch to view all the quadrants of the image at once.

TV/Diff Control

In **TV** mode, the image from the camera is displayed directly. In **Diff** mode, the image display shows the difference between the current and previous images.

Reference/Start/Stop*

These buttons control the start and end of a trial. See the corresponding menu items above.

Data File and Free Indicators

The Data File indicator shows the currently selected data file. Change the file using the FILE->OPEN menu item. The **Free** indicator shows how much space is available on the disk containing the current directory. The File Size indicator appears during a trial and shows the size of each of the image files being saved.

Frame Rate Indicators

The **Frame Rate** indicator is most often not visible. It will appear only when FreezeFrame cannot collect and process images as fast as has been requested using the **Frame Rate** control in the Camera Setup window. When this condition occurs, the **Frame Rate** indicator will show the actual frame rate. A low frame rate does not affect the data collection seriously. See the Camera Setup window for details on how to set the frame rate.

Protocol Selector Control*

The protocol Selector is a popup menu to the lower left of the FreezeFrame window. The items in the menu are set by the user in the [Protocol Window](). The selected protocol is sent automatically to the Remote computers.

Protocol and Stimulus Display

The chart at the bottom of the screen gives a graphical indication of the protocol sequence. Horizontal bars in the four rows of the display show when each stimulus will come on during a trial. The time scale of the display also varies to indicate the duration of the trial. During a trial, a vertical yellow cursor scolls across the display to indicate the progress of the trial. As each stimulus is turned on and off, the four indicators to the right show the current status of the stimuli.

Setting up the protocols

Protocols in FreezeFrame are configured using the Protocol window. Open the Protocol window from the Main FreezeFrame window by selecting the Setting->Protocols menu item.

All changes you make in the protocols are saved automatically and immediately.

Protocol. FreezeFrame will store up to 20 different stimulus protocols. Select the protocol you wish to change using the Protocol pop-up menu.

Name. You can give each protocol a different name (though it is not necessary to do so). Simply type the name into the text box. The name will appear in the Protocol popupmenu as well.

Trial Duration. This is a text control specifying the total duration of the recording session in **minutes: seconds.** "5:00" is equivalent to "300", and specifies a trial duration of 300 seconds. The control will flash red when it is set to 0 as a reminder to change it to a more reasonable value. Trials of greater than 60 minutes are possible but not recommended.

Setting the stimuli

There are 4 different stimulus channels available when using a PCI-1408 or 1409: sound, shock, stimulus 3 and stimulus 4. Sound is delivered through the computer's speakers. Shock, stimulus 3 and stimulus 4 are delivered through the T0, T1 and T2 outputs of the 1408/9. These are TTL compatible outputs, delivering 0V when in the low state and 5V when in the high state. In the Output Configuration window, you may configure each input to be low (0V) when ON (Normal polarity) or high (5V) when ON (Inverted polarity).

The PCI-1407 provides only a single TTL ouput (Shock) through the BNC connector marked TRIG.

Onset/Duration array. You may specify up to 50 different stimulus epochs for each protocol. For each stimulus epoch, you must specify an onset time and a duration. Onset and duration may be entered as **minutes:seconds**, or as **seconds**. For example, "5:10" is equivalent to "310", and "1:45" is equivalent to "105".Perforce, a stimulus will not be delivered if its onset time exceeds the Trial Duration setting.

The values below will turn on the sound for 30 seconds at 3, 6 and 9 minutes into the trial. At the end of each sound, the shock will be delivered for 5 seconds.

1	3:00	30	6:00	30	9:00	30	
2	3:30	5	6:30	5	9:30	5	
3							
4							

Multiple Sound Stimuli. If you plan to use more than one type of sound stimulus (with different frequencies, pure tone or noise, different loudness, etc), then you must first set up the different sounds in the stimulus configuration window. It is possible to configure up to 10 different sounds. In the case that more than one is to be selected, you must specify which sound is to be used for each stimulus epoch in the protocol setup as shown below. Simply prefix the Onset time with the sound number and a "-". In the example below, the first epoch will present sound 2, the second sound 4 and the third sound 3. **If no prefix is given (as in the above example), sound 1 will be used by default.**

1	3-3:00	30	4-6:00	30	3-9:00	30	
2	3:30	5	6:30	5	9:30	5	
3							
4							

Epoch. Onset and duration times are visible only for 8 epochs at a time. To view epochs other than the first 8, use the **Epoch** numerical control or the **>>** and **<<** buttons on the left sode of the window. Also, with the NumLock control on your keyboard off, the <End> and <PageDn> keys will scroll the Onset/Duration array right or left.

Protocol Chart Display. As you enter times and durations for each epoch, a colored bar will appear in the chart at the bottom of the screen to give a picture of the entire protocol stimulus sequence.

Clear all. This button will delete all stimuli from the current protocol.

Exit. Closes the Protocol window.

Camera Setup Window

FreezeFrame can be used in 4 different configurations, depending on the hardware being used.

1 camera - 1 chamber. In the simplest configuration, FreezeFrame uses a single camera to view a single shock chamber.

2 cameras- 2 chambers. If a PCI-1408 or PCI-1409 is being used, you may connect 2 cameras to the board, each one viewing a different chamber.

4 cameras- 4 chambers. If a PCI-1408 or PCI-1409 is being used, you may connect 4 cameras to the board, each one viewing a different chamber.

1 camera- 4 chambers. With any of the boards, you may configure FreezeFrame to view 4 chambers with a single camera. In this case, the image is divided into 4 quadrants and the motion detection algorithm is performed independently on each quadrant. The chambers must be placed in a 2 x 2 array so as to fit one into each quadrant of the camera image. This can be a side-by-side array with the camera looking down from above, or a 2-up 2-down array with the camera looking from the front. In this case, the upper two chambers can be set back from the lower two slightly, so that the camera can look from in front, and slightly above the array (see Camera Setup).

Frame Rate. This control sets the rate at which FreezeFrame collects images and analyzes them. The maximum rate allowable will vary depending on how many cameras are being used, how many chambers are being analyzed, and how fast the computer clock processor is. A computer with a clock speed of 800 MHz or better can likely collect at 4 Hz with any configuration, and this is the default setting. With only 2 cameras and 2 chambers, a faster rate is possible, but not advisable for the reasons outlined below.

If the frame rate is set to be faster than the computer can handle, **Frame Rate** indicator will appear during a trial. This is not at all fatal to a trial. The data will be collected properly, but at a slower rate than is set in the Frame Rate control.

The 4 Hz rate is the fastest that is advisable. At faster rates, a trade-off begins to occur between temporal resolution in the data and motion detection threshold. The shorter the time between frames, the less the animal has a chance to move between frames and so the minimum detectable motion will decrease slightly. Frame rates lower than 4 Hz will not likely increase the motion detection threshold significantly, but will, of course, lower temporal resolution. Even a 1 Hz frame rate, however, is much greater resolution than most hand scoring and is sufficient to measure differences among animals with high reliability.

Stimulus Configuration

Sounds

Selector. You may configure up to 10 different sound stimuli, each of which can be selected for any stimulus epoch in a protocol. Select one of the 10 sound stimuli to configure by clicking on the listbox to the left of the window. Sounds once configured are stored in a file and need not be updated each time the program is used.

Hiss/Tone. To the left, set whether the sound stimulus will be a white noise (hiss) or pure tone.

Frequency. If a tone, use the Frequency control to set the tone's pitch.

Amplitude. Adjusts the relative amplitue of the sound, from off (0) to the loudest your amplifier can produce (100).

Pulse. When on, the sound will be presented discontinuously during a stimulus epoch, 250 on - 250 off.

> **Rise Time**. At sound onset, the amplitude will increase linearly to the maximum over the number of milliseconds indicated. Similarly, at offset the amplitude diminishes linearly from the maximum to reach zero at the end of the sound.
> **Frequency**. Determines the frequency of pulsation.
> **Duty cycle**. Determines for what percentage of each cycle the pulsing sound is on.

Test. Momentarily turns on the sound as currently configured. If no sound can be heard, check the speaker connections, volume setting and sound card configuration in Windows.

Shocker and auxilliary stimulus outputs

Invert. 1 (PCI-1405/7) or 3 (PCI-1408/9/10) slide switches will appear that allow you to determine the polarity of the outputs for the shock (stimulus 2) and the auxilliary outputs (stimulus 3 and 4). When in the **Norm** position, the corresponding input will be low (0V) when off and high (5V) when on. In the **Invert** position, the stimulus will be inverted.

Test. While pressed, puts all three outputs in their ON state (high for **Norm** and low for **Invert**). Otherwise, the outputs are in their OFF state (low for **Norm** and high for **Invert**).

File Formats

FreezeFrame store several different types of files. All files are created automatically in the current Data directory, which is directory in which the currently selected Data file is located using the FILE->OPEN menu item in the main [FreezeFrame window](#).

FFDD data files. The frame-by-frame motion index is stored in a file with the extension "ffdd". When the data file is opened from the FreezeView program, the entire list of animals contained in that file is displayed.

For multicomputer systems, the Master computer's data file stores data from all animals on both the Master and Remote computers. On the Remote computers, only the data collected by that computer is stored in the data file.

Log file. FreezeFrame creates a text file that contains a list of all the animals run.

FFII Image Files. With the Save Images option selected (Settings->Save Images menu item in the main [FreezeFrame window](#)) FreezeFrame creates one image file for each animal in a trial. The name of the file is the name typed into the corresponding element of the **Name** control in the main window, and the extension is ".ffii". Each image is stored as a 2-D array of 8-bit unsigned integers, preceded by 2 4-byte integers that specify the size of the array. These bytes are stored in big-endian format (most significant byte first). Images can be converted to [QuickTime format](#) (with the extension .mov) using the FreezeView program.

Image files tend to be large. At 4 frames per second, the 320 x 240 pixel arrays accumulate at 18 MBytes per minute. If you are planning to save image files, a large hard drive and a mass storage backup device such as a fast CD or DVD writer are helpful.

FFTT threshold files. When you analyze the trials stored in a data file, the thresholds you choose for freezing are stored in a file with the same name as the data file, but with the fftt extension.

Data collection trouble shooting

No image

The most common trouble is the lack of an image in the image window. When this happens, the program gets very sluggish to respond.

Check to make sure the camera is connected to the computer properly and that the power is connected to the camera.

Once you check the connections for faults, stop and restart FreezeFrame to see if the image now appears.

Another convenient way to troubleshoot the camera input is using the Measurement and Automation Explorer that comes with the PCI-140X drivers. The icon for this program is on the desktop. Choose the Devices window, and then the 140X board. Use Snap or Grab to view the image from each camera.

Portions of the image (usually a horizontal strip) appear black.

Open the Measurement and Automation Explorer ("MAX", its icon is installed on the desktop by NI-IMAQ).

Open the Devices and Interfaces folder and select the image acquisition board (PCI-140X).

Select Channel 0 and click the Grab button in the toolbar. Expand the MAX window so that you are able to see the intire image.

If the black areas appear in the image, click Properties button in the toolbar and adjust the image limits (top, left, width and height) until the black areas disappear. Width should be set to 640, height to 480, and left to approximately 115.

The program seems not to be detecting small movements of the animal.

In order for FreezeFrame to be maximally sensitive to movement, the image of the shock cage should fill as much of the image as possible. In the 1-camera/4-chamber system, this can sometimes be difficult. Keep the 4 chambers as close together as possible by sliding them toward the center of the image, and raising the lower set of cages to be just underneath the upper set. In some cases, it might be necessary to convert to a 4-camera system so that each cage can be individually adjusted to fill it corresponding image.

The shocker does not seem to turn on at the appropriate times.

The usual cause for this problem is improper connection of the trigger output of the image acquisition board to the shocker. To make sure that the FreezeFrame program and its output is functioning properly, disconnect the trigger output from the shocker and measure voltage on the output with a voltmeter or oscilloscope. It should be within a few tenths of a volt of 0V when off, and within a few tenths of 5V when on (or vice versa if the **invert** control is on in the Stimulus Setup Window. If the voltages are correct, then contact the manufacturer of your shocker to help with the proper connections.

Trials seem to give inconsistent or highly variable results.

The most common cause of inconsistent results from trial to trial is problems in delivering the shock to the animals. The shock cage is a harsh environment for electrical connections: urine is very corrosive and the contacts between connectors and shock bars often fail. Check the video of trials in which animals are not freezing much to see that they are receiving the shock. Use an oscilloscope or voltmeter to check that each bar is receiving the shock signal. (It is good practice to do this on a regular basis, as often as once per week.) Clean the contacts in the electrical pathway between the shocker and the grid bars. On removable floor trays, check to see that the bars have not slid forward, especially the bars near the sides of the cages. If they have, they will no longer make good contact.

A second (though less common) problem that might cause inconsistent results can occur if the program is not detecting motion reliably. See the item "The program seems not to be detecting small movements of the animal" above.

No data from Remote computer (MultiComputer Systems only)

Check the cables that connect the Master computer to the Remote. COM1 on the Master computer should be connected to COM1 of Remote 1, and COM2 of the Master computer should be connected to COM1 of Remote 2.

Data analysis trouble shooting

The Analysis Program (FreezeView)

Opening a data file

Open File. Click on the FILE -> OPEN menu item to select a file for analysis (or double-click filename indicator). A standard Open File Dialog lets you navigate through the Windows file system to find a data file with the .ffdd extension.

Selecting an animal for analysis

Once a file is selected, the **Animals** list box at the right of the window shows the animals for which data were collected. Select an animal either by clicking on it or by using the **UP and DOWN arrow keys** to scroll through the list. As you scroll through the list, the displays are updated to show the data for the selected animal. Animals for which image files are available (in the same folder as the data file) are prefixed with *****.

Displays

- The motion index as a function of time. The data in the motion index graph can be written to a spreadsheet readable file by selecting the ANALYSIS -> EXPORT INDEX GRAPH menu item.
- Freezing as a function of time. Freezing is set to 0 or 1 depending on whether the motion index exceeds the freezing threshold.
- The stimuli as a function of time. The time during which each of the 4 possible different stimuli are on are shown as yellow, cyan, red and green fills.
- A histogram of the motion index (lower left graph). The data in the motion index histogram can be written to a spreadsheet readable file by selecting the ANALYSIS -> EXPORT HISTOGRAM menu item.
- Percent freezing. The numeric indicators show the percent time the animal spent frozen (see here for details)
  - while each of the stimuli was on (**stimulus 1, stimulus 2...**),
  - while each of the stimuli was off (**not stimulus 2, not stimulus 2...**),
  - for the whole trial (**Total**), and
  - while all stimuli were off (**no stimuli**).

Setting the Freeze Parameters

Freeze Threshold (Histogram cursor) A criterion level must be chosen for deciding when the animal has frozen. Set the freeze threshold by dragging the **vertical blue cursor** in the histogram display (lower left) or by using the digital **Threshold** indicator**.** The value is chosen by examining the motion index histogram. For most animals, there will be a large, narrow peak starting at 0 (freezing), and a second, broader peak between 50 and 100 (activity). There will be, in addition, a distinct trough between the two peaks in the range of 20-30. The threshold should be set to the value of the trough. To the right are shown examples of 5 histograms with the threshold set properly. The white text indicates the % freezing for the trial. Note the different scales for the different graphs.
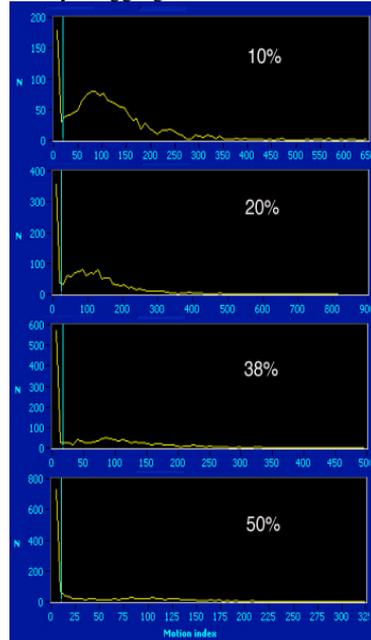

Some animals freeze very little (10% graph) and so have only a small peak or no peak at all at zero. Some animals freeze almost all the time (50% graph), and so the peak near zero will dominate with only scattered points at higher values.

You may set the threshold for each animal individually, or use the **Set all** button to set the threshold for all trials in the file to the current value. But if you use the **Set all** button, check each trial to make sure the thresholds are valid.

Thresholds are saved in a file with the same name as the data file, but with a different extension ".fftt". When moving data files to a different folder, bring the corresponding ".fftt" file along in order to maintain the previously set thresholds.

You can often see the appropriate threshold by looking at the Freeze Index vs. Time graph (upper right of the window) at a high vertical gain. To increase the vertical gain in the index graph in order to examine the periods of inactivity in more detail, double-click on the top number on the vertical scale and the desired range (e.g. 10), then hit <enter>. Below are images of the Freeze Index graph for a trial at normal vertical gain and then expanded to see the bouts of freezing in more detail. When the threshold is set properly, small changes in either direction should have only small effects on the Totoal % Freezing
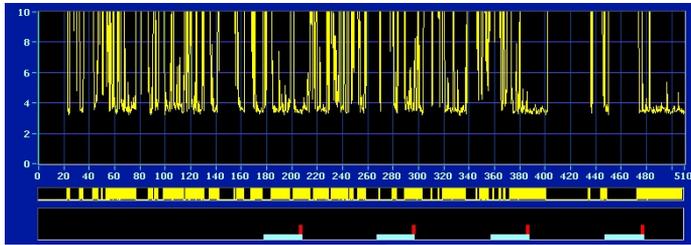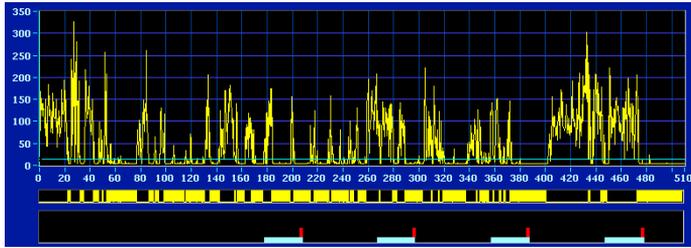
Bout (s)

Bout length is the duration that a single freezing episode must equal or exceed in order to be counted. With Bouts set to 1, for example, episodes of freezing (that is, episodes during which the motion index is continually below threshold) that last only 0.75 s or less will be treated as if the animal had not frozen at all during that period. As **Bout** and **Threshold** are changed, the freeze graph and % Freeze indicators will be updated to reflect the new parameters.

Displaying images

If an image file for the selected animal is present in the same directory as the open data file, several buttons will appear over the image window (lower right) that can be used to play the image file as a movie.

Time cursor. The vertical blue cursor in the Freeze Index graph (top) is used to control the time at which the playback starts. For example, drag the cursor to time t = 10 sec, and press one of the playback controls to start the movie from that time. As the movie plays, the cursor moves to indicate the progress of the playback through the trial.

Playback controls.

> Plays the image at near real time, starting at the time in the trial set by the vertical blue cursor in the Freeze Index graph (top). Click the control again (or click the **||** control) to stop the playback.

>> Plays the image as fast as the computer will go. Playback starts at the time in the trial set by the vertical blue cursor in the Freeze Index graph (top). Click the control again (or click the **||** control) to stop the playback.

>| Advances one frame per click of the control.

<< Plays the image in reverse as fast as the hard drive and processor will go.

|| Stops playback..

TV/Difference. You may view the images as recorded, or as the pixel-by-pixel difference between the current image and the previous image.

The VIDEO -> INVERT VERTICAL and INVERT HORIZONTAL menu items flip the image vertically and horizontally.

Multicomputer Systems: When analyzing data files on the Master computer, index data are available for all animals recorded on all computers. Images are only available for animals whose image files are located in the data file directory. To view images for animals recorded on the Remote computers, you must transfer the image files for those animals to the Master computer (to the same directory as the data file). Or run the analysis program on the Remote computer.

Exporting QuickTime and AVI Movies/Compressing Image Files

It is possible to export QuickTime movies of all or part of a trial. These movies can be displayed in PowerPoint and other image presentation software. It is also possible to use QuickTime to compress the image files for viewing within FreezeView, greatly reducing the disk space required to store them.

Exporting Analyses

The program allows exporting of several statistical measures of freezing.

Exporting Analyses

To Export data, open the Analysis window by selecting the ANALYSIS -> EXPORT STATISTICS menu item in the main program window.

Interval Type

There are 3 different ways to specify the intervals for analysis.

- **Fixed Interval**. Divides the trial into periods of equal duration. The interval duration is set in the **Interval (sec)** that appears when Fixed Intervals are chosen. A graph of the %Freeze as a function of time is shown.
- **Custom Interval**. You may specify any number of intervals of arbitrary start time and duration. First specify the **number of intervals**. Then fill in the onset time and duration of each interval in the correspondign text edit boxes.
- **By Stimuli**. This analysis is similar to the indicators in the main window. The analysis is performed on:
  - The total duration of the trial
  - The time during which stimulus 1 (the sound) is on
  - The time during which stimulus 2 (the shock) is on
  - The time during which stimulus 3 is on
  - The time during which stimulus 4 is on
  - The time during which no stimulus is on
  - The time during which stimulus 1 (the sound) is not on
  - The time during which stimulus 2 (the shock) is not on
  - The time during which stimulus 3 is not on
  - The time during which stimulus 4 is not on

    Note that a stimulus may come on more than once during a trial. If, for example, stimulus 1 is on for the first and 3rd minutes of a 6 minute trial, Stimulus 1 on include both of those minutes, and Stimulus 1 not on includes minutes 2, 4, 5, and 6.

Analysis Parameter

- Once the intervals are chosen, the parameter for analysis must be chosen. The 4 choices are

- **Percent Freezing**. The percent of the time during which the animal was frozen.
- **Number of bouts.** The number of continuous bouts of freezing.
- **Mean bout interval.** The mean time between the start of consecutive bouts.

- **Mean bout duration.** The mean duration of bouts.

The selected parameter is calculated for each of the specified periods and displayed in the text box to the right of the window. Note that for all analyses, bouts of duration less than the **Bout duration** specified in the main window are excluded.

Note that when **By Stimuli** is the interval type chosen, the only analysis parameter available is Percent Freezing.

Exporting

Pressing the **Export** button performs the chosen analysis on each one of the trials (animals) in the open experiment file. The resulting data are written to a comma-delimited (csv) file, which can be opened in Excel or other statistical programs. If the button labelled "**Open Excel**" is set to the ON position, the file created will be opened automatically in Excel (assuming Excel is installed on the computer).

Trials in: Rows/Columns

This control determines the orientation of the data in the Excel spreadsheet.

Compressing and Exporting Image Files

FreezeFrame saves images in an uncompressed or raw format in the *.ffii files that are located in the same folder as the.fftt data file. In these files, the luminance value for each pixel of each frame is stored in a large 2-dimensional array. This scheme has the advantage of being fast, increasing the frame rate at which FreezeFrame can work. And the uncompressed format preserves all of the information in the images. The disadvantages of the raw ffii files are their large size and their incompatibility with other video presentation and editing programs. FreezeView contains 2 utlilities for converting the raw image files to ones that are in more standard video formats, compressing them at the same time.

In order to create QuickTime and AVI movies, the Windows version of Apple's QuickTime Player must be installed on your computer. Only the free QuickTime player is required. It is available from Apple at http://www.apple.com/quicktime.

Exporting images

From the Main FreezeView window, you may export all or part of the video stream for the currently selected animal. The resulting QuickTime (.MOV) or AVI files can be read into PowerPoint, placed on web sites or used in other presentation software. To export:

- Use the **Video controls** to move the Time Cursor (vertical light blue line in Motion Index graph) to the start of the portion of the trial you would like to export. Or drag the Time cursor directly.
- Select the ANALYSIS -> EXPORT MOVIE menu item. A Compression Settings window will appear.
- Select QuickTime or AVI
- Pick a Compression format using the **Format** pull-down menu. There are a daunting number of formats available with QuickTime. We recommend **MPEG 4** (MP4) for QuickTime or Indeo Video for AVI.
- Pick the level of compression using the **Compression** control. This setting will determine how compact the exported file will be. Low settings will give higher compression, lower image quality, and smaller files. Try exporting a short video clip with different settings to see which will suit your needs.
- Select the **Playback speed**. This will determine whether the video clip plays back in QuickTime at near real-time, twice real time, 3x or 4x real time.
- Click **OK** or **Cancel** and the Compression settings window will close.
- A file dialog will appear. Specify a **filename** for the exported image file. The appropriate extension (.MOV or .AVI) will be appended automatically.
- Once the file dialog closes, in the main FreezeView window, the video control buttons will have changed color. Play the desired portion of the video using the now-green video control buttons (**>| > >>**). While the controls are green, each displayed frame of the video will be stored to the QuickTime file of the selected filename. Note that using the different play buttons does not affect

the playback speed you will see when you play the file in the QuickTime player. They only affect how quickly the file is written and displayed in FreezeView. The **Playback Speed** control in the Compression Settings Window determines how fast the video will play in QuickTime.

- Click the red stop-playback button (||) to close the movie file.

To playback the newly created QuickTime or AVI video file, double-click its icon in Windows Explorer and it will open in the QuickTime Player or Windows Multimedia Player. The file can also be imported into PowerPoint, html files or other multimedia files.

Warning. Do not try to overwrite an existing QuickTime file while the file is open in the QuickTime Player. An error will result.

Compressing the image files for playback in FreezeView

It is also possible to compress all the FreezeView image files associated with a data file in one batch operation. That is, you can copy all the images from each raw image file to compressed, QuickTime-compatible *.MOV or *.AVI files. When you then play a video from within the FreezeView program, the images shown will come from the newly created QuickTime file rather than the original raw video file. The advantage is that the QuickTime files can be 10 or more times smaller than the ffii files, and so occupy less disk space. The disadvantage is that the compressed images are not quite as sharp as the originals, depending on the level of compression chosen. As with files written one at a time with the EXPORT MOVIE function, the image files created en masse can also be played in the QuickTime Viewer, PowerPoint etc. To compress all the image files in the folder containing the currently opened data file:

- Select the VIEW->COMPRESS IMAGE FILES menu item.
- The Compression Settings Window will appear.
- Pick a Compression format using the **Format** pull-down menu. We recommend **MPEG 4** (MP4).
- Pick the level of compression using the **Compression** control. The setting will determine how compact the exported file will be. Low settings will give higher compression, lower image quality, and smaller files. Try exporting a short video clip with different settings to see which will suit your needs. MP4 with a compression setting of about 350 gives 10-fold compression and yet gives a very nice image.
- Select the **Playback speed**. This will determine whether the video clip plays back in QuickTime at near real-time, twice real time, 3x or 4x real time. It will NOT effect the speed at which the files play in the FreezeView program itself.
- Click **OK** and the Compression settings window will close (or select **Cancel** to stop the compression process).
- A new window will open. The uncompressed images are displayed as they are being compressed and stored. Various indicators show the file currently being compressed, the progress through the list of files, the progress in the current compression, the compression format being used and the compression ratio acheived (that is, the size of the compressed file expressed as a percent of the size of the raw file).You may cancel the process at any time.
- When all the ffii files in the folder have been compressed, the window will close.

During the compression process each file named <animal>.ffii, is compressed and stored in a new file called <animal>.MOV or .AVI. Whenever a file called <animal>.MOV or .AVI is present in the data folder, FreezeView will automatically use it when displaying images, even if the corresponding <animal>.ffii file is also present. You may therefore discard the original ffii files once you have decided that you are satisfied with the level of compression and the quality of the images. We recommend that you archive the original files on CD, DVD or tape if possible. But this is not required.

Tail Suspension Data Collection

FreezeFrame can be used for collection of data from tail suspension experiments. Set up the video card, cameras and FreezeFrame program the same as you would for fear conditioning experiments. You may use one camera for 4 animals (placed in the 4 quadants of the image), or record 1 animal each with up to 4 cameras.

Set-up

Aim the camera horizontally toward the animal from one side

Put a uniform background background behind the animal for maximum contrast (white or light gray for dark animals; black or dark gray for white animals.

Adjust the field of view (zoom and camera distance) so that the animal is near or slightly below the center of the screen. Include all of the tail and some of the tail support in the image.

Adjust the lighting so that the shadow of the animal is not directly behind the animal. Light from above and slightly in front of the animal. Or for dark animals on the light background, the lights can be put above the animal, between the animal and the background.

Recording

Record trials using the FreezeFrame program as for fear conditioning. Set up the duration of each trial in the Protocol window. During the trial, the program will calculated freezing as normally does for fear conditioning. These numbers are not useful for Tail Suspension, but you will reanalyze the records for tail suspension using the Tail Suspension analysis program.

Tail Suspension Data Analysis

Install and run the Tail Suspension program.

Opening a data file

Open File. Click on the FILE -> OPEN menu item to select a file for analysis (or double-click filename indicator). A standard Open File Dialog lets you navigate through the Windows file system to find a data file with the .ffdd extension.

Selecting an animal for analysis

Once a file is selected, the **Animals** list box at the right of the window shows the animals for which data were collected.Select an animal either by clicking on it or by using the **UP and DOWN arrow keys** to scroll through the list. As you scroll through the list, the displays are updated to show the data for the selected animal.Animals for which image files are available (in the same folder as the data file) are prefixed with *****.

Re-tracking a trial for Tail Suspension

When FreezeFrame records a trail, it calculates a freeze index appropriate for fear conditioning. In order to measure motion for Tail Suspension, you will need to reanalyze each trial. This only needs to be done the first time you view a trial. **For safety, make and store a copy of the ffdd file before you modify with the Tail Suspension program.** The video (ffii) files are not affected, so there is not need to copy them other than for standard backups.

- Select a trial in the list to the right of the main analysis window.
- Select the **ANALYSIS ->TAIL SUSPENSION** menu item.
- Use the yellow cursors to select an area around the animal. Include the tail, but not the supporting apparatus. Include an area around the animal about twice its size.
- Select Realine or Fast using the toggle switch at the upper right to determine how the trial will be displayed during analysis.
- Set the tracker
    - Select the animal color. AUTO for auto detection, or white or black for light or dark animals.
    - Set Threshold method to AUTO
    - Set Area threshold to 25
    - Set Manual threshold to 100
- Click the Run button and let the trial complete.
- Select the SAVE (or CANCEL) menu item either to save (or ignore) the new analysis and return