



Fear Conditioning Test

(protocol, Dr. Juan Pina-Crespo)

Fear conditioning (FC) is a classical Pavlovian paradigm used to study learning, memory, and anxiety-related behaviors. This protocol provides comprehensive instructions for conducting fear conditioning experiments in mice using the San Diego Instruments (SDI) Freeze Monitor System (FMS). The FMS uses an array of infrared photobeams to detect animal movement. Freezing behavior—defined as the absence of all movement except respiration—is automatically quantified as the primary measure of conditioned fear.

Before starting your experiment, make sure to go over the following materials checklist:

Materials	Check
Morris Water Maze	
Spray bottle with 70% ethanol	
Disposable paper towels	
Camera & video tracking system (ANY-maze)	
Clean bedding	
Remote-controlled white noise generator	
Bright overhead lights	
Plastic beaker	
Timer	
Visual cues	

Shock calibration

Note: To be performed before every experimental session and whenever changing experimental parameters.

1. Set digital ammeter to mA range (0–2 mA)
2. Connect ammeter leads to two adjacent grid bars
3. Open SDI software and navigate to manual control/diagnostics
4. Select the chamber to calibrate
5. Set shock intensity to 0.50 mA
6. Set shock duration to 2 seconds
7. Deliver test shock
8. Record ammeter reading
9. Repeat 3 times and calculate mean

Tone calibration

1. Set meter to dB(A) and place it at center of chamber.
2. Measure tone output.
3. Go to SDI software and navigate to manual control.
4. Set tone frequency (e.g., 2800 Hz)
5. Activate tone
6. Record dB reading.
7. Adjust tone amplitude to achieve target (typically 80–85 dB).



Photobeam calibration

Check Beam Alignment

1. In SDI software, open Photobeam Diagnostics
2. Observe real-time beam status display
3. All beams should show "intact" (green) when chamber is empty

Test Sensitivity

4. Move a small object (pen) through the chamber
5. Verify beams break and recover appropriately

Clean beams

6. If beams show intermittent breaks, clean emitters/detectors with compressed air
7. Use lens paper with isopropanol for stubborn debris

Software configuration

Creating a new experiment

1. Double-click Freeze Monitor icon
2. Enter user credentials if required
3. Select New Experiment from File menu

Setting experiment parameters

1. Enter experiment name (e.g., "FC_Study1_Conditioning")
2. Select number of active chambers
3. Set session duration
4. Define sampling interval (typically 0.25–1.0 seconds)

Programming a stimulus protocol

Create a conditioning protocol

Navigate to protocol editor and program the following sequence:

Protocol for Standard Cued Fear Conditioning

Step 1: Baseline

- Start Time: 0:00
- Duration: 120 sec
- Stimuli: None (house light ON)

Step 2: CS-US Pairing 1

- Start Time: 2:00
- CS: Tone ON (2800 Hz, 80 dB)
- CS Duration: 30 sec
- US: Shock ON at 2:28 (0.5 mA, 2 sec)
- US co-terminates with CS at 2:30

Step 3: Inter-Trial Interval 1

- Start Time: 2:30
- Duration: 90 sec
- Stimuli: None

Step 4: CS-US Pairing 2

- Start Time: 4:00



- CS: Tone ON (2800 Hz, 80 dB)
- CS Duration: 30 sec
- US: Shock ON at 4:28 (0.5 mA, 2 sec)

Step 5: Inter-Trial Interval 2

- Start Time: 4:30
- Duration: 60 sec
- Stimuli: None

Step 6: CS-US Pairing 3

- Start Time: 5:30
- CS: Tone ON (2800 Hz, 80 dB)
- CS Duration: 30 sec
- US: Shock ON at 5:58 (0.5 mA, 2 sec)

Step 7: Post-Conditioning

- Start Time: 6:00
- Duration: 60 sec
- Stimuli: None

Stop: 7:00

Context Preparation

Context A (Conditioning Context)

Feature	Specification
Floor	Standard grid floor (stainless steel)
Walls	Clear Plexiglas (unmodified)
Lighting	House light ON (bright)
Odor	70% ethanol cleaning
Background	White noise or fan (constant)

Context B (Novel Context for Cued Test)

Feature	Specification
Floor	Smooth plastic insert (white or black)
Walls	Patterned inserts (stripes, checkerboard)
Lighting	Dim red light or house light OFF
Odor	1% acetic acid cleaning
Background	Different or no background noise

Day 1: Fear Conditioning

Step-by-step procedure

Preparation (15 minutes before first animal):

1. Power on all equipment
2. Launch Freeze Monitor software



3. Load conditioning protocol
4. Clean chamber with 70% ethanol
5. Allow to dry completely (3–5 min)
6. Verify all stimuli are functional (test tone, light)

For each animal:

Step	Action	Time
1	Record animal ID in software	—
2	Gently remove mouse from home cage using cupped hands or tail base	—
3	Place mouse in center of conditioning chamber	0:00
4	Close chamber door and cubicle door	—
5	Start protocol immediately	0:00
6	Monitor session via software (do not disturb)	0:00–7:00
7	Protocol delivers stimuli automatically	—
8	At session end, remove mouse promptly	7:00
9	Return mouse to home cage	—
10	Save data file	—
11	Clean chamber with 70% ethanol	—
12	Allow 3–5 min for odor dissipation	—
13	Proceed to next animal	—

Time	Event	Expected behavior
0:00	Session start	Exploration
0:00-2:00	Baseline period	Active exploration, low freezing
2:00	Tone 1 ON	Orienting response
2:28-2:30	Shock 1	Jump, vocalization
2:30	Tone 1 OFF	Freezing, reduced activity
2:30-4:00	ITI 1	Gradual recovery, some freezing
4:00	Tone 2 ON	Freezing to tone
4:28-4:30	Shock 2	Jump, vocalization
4:30-5:30	ITI 2	Increased freezing
5:30	Tone 3 ON	Strong freezing to tone
5:58-6:00	Shock 3	Jump, vocalization
6:00-7:00	Post-shock period	High freezing
7:00	Session end	—

**Day 2: Contextual Fear Test****Timing:** 24 hours (± 1 hour) after conditioning**Preparation:**

1. Set up Context A (identical to conditioning)
2. Clean with 70% ethanol
3. Load contextual test protocol

For Each Animal:

Step	Action	Time
1	Record animal ID	—
2	Place mouse in conditioning chamber (Context A)	0:00
3	Start protocol	0:00
4	Record freezing for 5 minutes	0:00–5:00
5	No stimuli presented	—
6	Remove mouse at session end	5:00
7	Return to home cage	—
8	Save data	—
9	Clean chamber	—

Expected Results:

- Conditioned mice: 40–70% freezing
- Control mice (no shock): <10% freezing



Day 2: Cued Fear Test

Timing: 2–4 hours after contextual test (same day)

Preparation:

1. Configure Context B (novel context)
 - Insert plastic floor
 - Add patterned wall inserts
 - Change lighting (dim/red)
2. Clean with 1% acetic acid
3. Load cued test protocol

For Each Animal:

Step	Action	Time
1	Record animal ID	—
2	Place mouse in novel chamber (Context B)	0:00
3	Start protocol	0:00
4	Baseline period (no tone)	0:00–3:00
5	Tone presentation (no shock)	3:00–6:00
6	Remove mouse at session end	6:00
7	Return to home cage	—
8	Save data	—
9	Clean chamber with 1% acetic acid	—

Expected Results:

Period	Conditioned Mice	Control Mice
Pre-CS (0–3 min)	<20% freezing	<10% freezing
CS (3–6 min)	50–80% freezing	<15% freezing

Data Collection and Analysis

Step 1: Export Raw Data

1. In Freeze Monitor software, select File → Export Data
2. Choose format: CSV or Excel
3. Export options:
 - Time-binned data (recommended: 30-sec or 1-min bins)
 - Event markers (CS, US onset/offset)
 - Raw activity counts
 - Calculated freezing percentages

References

1. Curzon P, Rustay NR, Browman KE (2009). Cued and Contextual Fear Conditioning for Rodents. In: Buccafusco JJ, editor. *Methods of Behavior Analysis in Neuroscience*. 2nd edition. Boca Raton (FL): CRC Press/Taylor & Francis; 2009. Chapter 2. PMID: 21204331.



2. Shoji, H., Takao, K., Hattori, S., Miyakawa, T (2014). Contextual and cued fear conditioning test using a video analyzing system in mice. *J. Vis. Exp.* (85), e50871, doi:10.3791/50871 (2014).
3. Forner S, Kawauchi S, Balderrama-Gutierrez G, Kramár EA, Matheos DP, Phan J, Javonillo DI, Tran KM, Hingco E, da Cunha C, Rezaie N, Alcantara JA, Baglietto-Vargas D, Jansen C, Neumann J, Wood MA, MacGregor GR, Mortazavi A, Tenner AJ, LaFerla FM, Green KN (2021). Systematic phenotyping and characterization of the 5xFAD mouse model of Alzheimer's disease. *Sci Data*, 8(1):270. doi:10.1038/s41597-021-01054-y (2021).

Manuals:

SDI-Animal-Stimulator (<https://sandiegoinstruments.com/product/animal-stimulator/>)