Structured Tracking of Alcohol Reinforcement (STAR) SOP

Developed by the Siciliano Lab

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Contents

Overview

This is the SOP for the Structured Tracking of Alcohol Reinforcement (STAR) procedure developed by the Siciliano Lab. The purpose is to capture individual differences in drinking behavior that develop over time with exposure to alcohol. As originally performed, the experiment consists of a training phase followed by a baseline, Pre-test. Animals then go through an event or series of events (in our case, a period of binge drinking through a two-bottle choice task) and then finally re-run the same test as in baseline as a Post-test comparison. Individual changes in animals' consumption patterns from Pre- to Post-test are examined and used to determine how the test affects animals' disposition to compulsive drinking behavior.

Importantly, while this SOP describes the procedure most commonly implemented in our lab, virtually all of the STAR parameters are designed to be flexible and can be altered to address specific experimental questions. For example, the capped intake acquisition procedure is useful for measuring drinking behaviors during early exposure without learning confounds – this is critical for our questions which focus on preexisting AUD vulnerability, but can be removed to shorten the acquisition period if only later timepoints are of interest. Experimental timeline, reinforcement schedule, ethanol concentration, and many other variables are also flexible. As described in detail here, the *sine qua non* features of STAR are operant schedules of reinforcement and multivariate phenotyping analysis to classify animals based on alcohol intake and punishment resistance.

That said, longitudinal quantification of induvial differences in any behavior requires procedural consistency and careful experimentation. The general guidelines below can also be altered but changes should be implemented with caution and consistency must be retained.

General Experimental Guidelines

Some things will be marked in order of importance:

CRITICAL – Part or step that is of utmost importance to the experiment running correctly
Important – Part or step that should be paid close attention to or will provide benefits in the future if done correctly
Optional – Not critical to experiment, though might have additional benefits further down the line if done
Experimenter Note – Thing that was done that is not critical to the experiment, but from experience has been found to be useful

Consistency

CRITICAL: Consistency is the bedrock of the STAR. Animals should start behavior and be fed at the same time every day to the best extent possible. Since every animal will acquire at a different rate and multiple animals are typically run sequentially in the same operant box each day it is not possible to match every experimental factor across all subjects. However, since differences in individual animal's behavior is the focus, giving every animal the most consistent day-to-day experience will result in the most accurate and reproducible results.

Timeline

Animals should be run during their dark (active) cycle each day. Bring animals to experimental space to acclimate for at least 30 minutes prior to running the experiment. The experimental timeline used in our lab requires 28 consecutive days of behavior after acquisition criteria have been met (acquisition time varies by subject), though exact experimental timeline varies based on the speed at which animals acquire and the specific task and schedules being tested. For a visual timeline of the experiment, see here.

Solutions Making and Reporting Intake

Our experiments typically use 15% EtOH diluted in dH20, measured as volume/volume (v/v). Regardless of concentration used, dilutions should be made from 190 proof (95%) nondenatured ethanol and water should be from a consistent source.

- **CRITICAL:** *Nondenatured ethanol must be used* as denatured alcohol contains additives that are unsafe for consumption. 100% ethanol is unstable without additives and can develop impurities when exposed to air, and therefore should not be used even if it is nondenatured.
- **CRITICAL:** Ethanol's specific gravity (0.79 g/ml at room temperature) must be accounted for when calculating grams of ethanol intake from volume consumed
    - Conversion from volume of v/v solution to grams of ethanol consumed is calculated as $[g = mL\ x\ 0.79\ x\ \%v/v]$
        - Example: 1mL of a 15% ethanol v/v solution contains 0.1185g of ethanol. $[0.1185 = 0.79\ x\ 0.15]$
    - Animal weights vary substantial over the course of the day. Thus weight should be assessed at a consistent timepoint to limit artifactual variance in g/kg intake calculations. We always weigh animals immediately after completion of the session to obtain accurate measures without concerns of handling-induced effects on behavior.

Box Cleaning

Since ethanol is consumed during this experiment, boxes should be cleaned with non-ethanol based cleaning solutions. We use 0.03% (v/v) acetic acid diluted in dH20.

Materials

Operant Box Parts

All operant behavior was run in operant boxes from Med Associates (St. Albans, VT). Since a Med Associates behavioral setup is already needed to run the experiment, parts will be broken down into sections based on their importance to STAR directly, vs. other parts of the experiment. Feel free to contact the authors for further details of their specific experimental setup.

Custom chambers or setups from other vendors should readily amenable to STAR experiments. The most critical component is volumetric motorized sippers so ethanol access can be fully controlled and quantified by the experimenter. Accurate, time-resolved lickometers are also highly recommended for any experiment and are indispensable for experiments which require synchronization of behavior with *in vivo* recording equipment. Our exact setup is described below.

General Parts

Can be different based on setup specific to your lab/experimental environment. For Med Associates parts, a "W" at the end of the part number denotes that they fit the Extra Wide Modular Test Chambers opposed to the standard modular mouse chamber (ENV-307A). Always double check your setup to make sure parts are compatible.

- Standard Multi-Density Fiberboard (MDF) Sound Attenuating Chamber (SAC) – ENV-022SA
- Extra Wide Modular Test Chamber with Modified Top for Mouse – ENV-307W-CT
- Stainless Steel Grid Floor for Extra Wide Mouse Modular Mouse Chamber – ENV-307W-QD

CRITICAL Parts

- Retractable Sipper w/ Graduated Pipette for Mouse – ENV-352AW
- Graduated Pipette (only) – MSUB-ENV-352A1
    - **Experimenter Note:** we purchased two sipper tubes per box being run, one for each solution being used (in our case, ethanol and quinine). Since each retractable motor moves a slightly different amount, this allowed us to keep using the same tubes per box each day and avoid any changes different sipper tubes might have as well as avoid any cross contamination of solutions
- Triple Contact Lickometer Controller – ENV-250C
- Illuminated Nose Poke response for Wide Mouse Modular Chamber – ENV-313W (2x per box)

    Experimenter Note: The setup must include operanda but does need to be nose pokes – any standard type, such as levers, are suitable.

Optional Parts

- White Noise Amplifier with Cage Speaker for ENV-307W Wide Mouse Chamber – ENV-325SW
- Cage Speaker for ENV-307W Wide Mouse Chamber – ENV-324W
- Shock / Lickometer grid switch - ENV-250S (only needed if doing shock and lickometer readings concurrently)
- Sound level meter – Model 732A, BK Precision (for normalizing volume of white noise in operant chambers)

Video Recording Parts

Experimenter Note: An infrared camera was mounted to the ceiling of each sound attenuating chamber, providing a bird's eye view recording of each box. This is useful for a variety of reasons, mainly watching animals as they run the behavior to keep an eye out for any abnormalities and also a visual check to make sure the quantitative measurements (e.g. nose pokes and licks) are being recorded correctly. Additionally, videos can later be used for quantitative analyses to address specific experimental questions. Security camera setups are well-suited for this task as many cameras can be synchronized and organized through one system. We use Security Camera Warehouse (Asheville, NC - https://www.getscw.com/) systems which have proved incredibly robust and reliable:

- Admiral Pro 16 Channel 4K NVR – ADMP16P16
- Deputy 2.0 2MP Fixed Wide Lens Turret Dome Camera – 26DF2
- 10TB Surveillance Grade Hard Drive – 10TB-HD

Two-Bottle Choice (2BC)

- 50mL Conical Tube – #22-170-199, Fisher Scientific
- Rubber (black) stopper, Size 6 – #6R, Ancare, Bellmore, NY

Solutions/Chemicals

- Quinine Hydrochloride Dihydrate – CAS# 6119-47-7, Sigma-Aldrich, Q1125
- 190 Proof Undenatured Ethanol – Decon Labs, #2801, Ethyl Alcohol CAS# 64-17-5
- Acetic Acid, Glacial – CAS# 64-19-7, Fisher Scientific, A38-500

Operant Behavior SOP

Overview

Our protocol consists of 4 training/acquisition phases each with distinct criteria that must be met to advance. This happens at each animal's own pace depending how their performance each day. After learning the task, the animals run a Pre-test consisting of 3 days of baseline ethanol consumption followed by 4 days of escalating punishment via quinine adulteration of the ethanol solution. After the Pre-test, animals will have another period in which they are exposed to different conditions (depends on experiment and what's being tested) and then run the same 7-day experiment from Pre-test as a Post-test. How each animal's behavior changes from the Pre- to Post-test can give us insights into how the middle exposure period can affect animal's compulsive drinking behavior.

> **Experimenter Note:** Operant ethanol reinforcement is a defining feature of STAR. Once established, this provides a great deal of experimenter control; however, behavior during initial acquisition of the operant reflects a mixture of learning rate and stochastic/incidental interactions with the operandum in addition to the reinforcing properties of ethanol. We have developed a controlled acquisition protocol which allows assessment of ethanol reinforcement during animals' first opportunity to drink to intoxication independent of variance in operant acquisition. For assessment of longitudinal phenotype dynamics this protocol is highly recommended but can be altered if assessments after phenotypes have stabilized are the only outcome of interest.

- Each animal is always tested in the same operant box each day – time of day should also be kept as consistent as possible for each subject. Boxes should be cleaned between each animal to ensure that the conditions are as similar as possible from day to day
- **CRITICAL:** As mentioned in the experimental overview, animals should be run at the same time every day, during the active period (dark phase for rodents). Operant self-administration, like most behaviors, exhibits robust circadian regulation
- Feeding should also be at a consistent time in relation to the testing sessions. We feed at the end of experiments each day, so that the time between feeding and start of operant behavior the following day will be consistent for each animal

Step-by-Step Guide

1. Bring animals to experimental area at least 30 minutes prior to when experiment should start to allow animals to acclimate to environment
2. Set up boxes by screwing in flooring and changing any parts out as needed
3. Clean all boxes and floors thoroughly with 0.03% acetic acid before any animals run. This will also be done between animals, but by wiping down first with acetic acid, all animals get the same conditions
    a. **Important:** Avoid spraying acetic acid near/into the boxes as the aerosol can mess up the electronics in the chambers, especially lickometers. Instead, spray onto paper towel and then wipe down necessary areas
4. Put a paper towel down in the bottom of each metal tray underneath the grid flooring to make for easier cleanup (and easier visualization of the animal if trying to do tracking)
5. Fill up and test sipper tubes
    a. See lickometer filling guide for specifics on how to do this and additional notes
6. Carefully put tubes onto retractable sipper tube and tighten the screw to ensure contact between tube and the lickometer wire
7. Load the program that will be run that day and start it. Test each nosepoke as well as the lickometer (once extended) using the lickometer testing guide. Make sure nose-pokes are illuminated and correctly counted and that contacts are counted accurately

8. **CRITICAL:** Before and after each session, record the volume of solution in the graduated sipper. This should be done after sippers are placed on the motorized extender and all other cleaning/testing has been completed to ensure accurate assessment of subjects' consumption. Because the sipper is held at an angle on the extender, the meniscus will not align with the graduations. This is not an issue because the absolute volume in the sipper is irrelevant; however, volume must be recorded in a consistent fashion (e.g. closest graduation to the top of side of the fluid surface) to ensure that difference between pre-post values accurately reflect the change in volume
9. End program, reload it using proper information for each animal, and place animal in box to run. Once program is started, it will run for 1 hour (3600 seconds) OR until the criteria is met for the day
10. If using video recording, make sure to start the video(s) prior to animals going into the operant chamber. That will ensure that all behavior is captured on video and in case there is any mistake with the box operation, the animal's behavior can still be observed and measured
11. **Important:** When animal has completed experiment for the day, weigh immediately upon removing from operant box before returning to home cage. The animal's weight will be used to normalize any alcohol intake and thus is critical to write down as close to when the animal ran as possible
12. Stop video(s) once animals have been removed from the box
13. **Important:** After the animal is done, but before retesting any of the tubes, write down the final volume of solution to the nearest 0.05mL. If volume isn't recorded, total consumption for the day cannot be calculated
    a. **Experimenter Note:** As a general rule of thumb, once the animal has learned the task well, 100 licks is ~0.1mL consumption. If the amount consumed is very disproportionate to that (e.g. 100 licks and negligible volume difference OR 0.2mL volume change and only 50 licks) check that spout is working correctly
14. Remove the paper towel from the tray and clean out box again using 0.03% acetic acid
15. **CRITICAL:** Retest lickometer between animals to ensure that counting and flow are still working as expected

Overview of Phases, Criteria, and Programs

	Magazine Training	Operant Training 1	Operant Training 2	Operant Discrimination	STAR Test
Trial Structure	Sipper constantly extended, nose pokes have no consequence	FR1-30s schedule; 1 active nose poke → 30s extension of tube containing 15% EtOH	FR1-10s schedule; 1 active nose poke → 10s extension of tube containing 15% EtOH	FR5-10s schedule; 5 active nose pokes → 30s extension of tube containing 15% EtOH	FR10-10s schedule; 10 active nose pokes → 10s extension of tube containing 15% EtOH or in water or 250-1000uM Quinine
Session End Criteria	100 licks or 1 hour	100 licks or 1 hour	100 licks or 1 hour	100 licks and ≥ 70% nose pokes on active side	1 hour
Phase End Criteria	Reaches lick criteria for 1 day	Reaches lick criteria for 2 consecutive days	Reaches lick criteria for 2 consecutive days	Reaches lick and discrimination criteria for 2 consecutive days	Moves on after each day, regardless of performance
Program Run	00_STAR_ MAGTRAINING_ CAPPED	01_STAR_ACQ_ FR1_30s_CAPPED_ LEFT/RIGHT	02_STAR_ACQ_ FR1_10s_CAPPED_ LEFT/RIGHT	03_STAR_ACQ_ FR5_10s_CAPPED_ LEFT/RIGHT	04_STAR_TASK_ FR10_10s_NOCAP_ LEFT/RIGHT

Important: If an animal fails to meet criteria for 3 consecutive days, it moves back to the previous phase and must meet those criteria again to continue on. If an animal moves back 3 times ever during the experiment, they are considered a "non-learner" and are dropped from the rest of the experiment