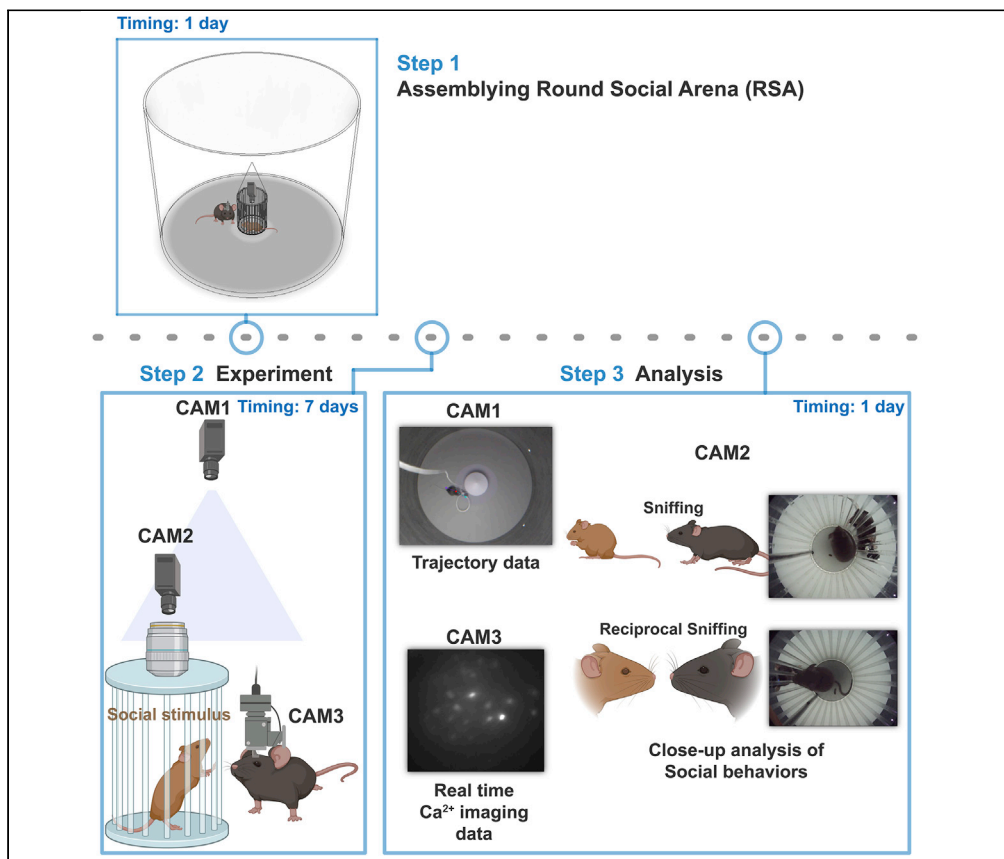


Protocol

Simultaneous analysis of social behaviors and neural responses in mice using round social arena system



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Highlights

A round-shaped rodent social behavior testing arena with multiple cameras

Enables detailed capture and analysis of social interactions with minimal blind spots

Induces longer duration of social interaction than conventional sociability test

Compatible with simultaneous acquisition of neural data or optogenetic manipulations

Stable and accurate capturing of detailed social behaviors is essential for studying rodent sociability. Here, we introduce a round social arena (RSA) system that enables close-up monitoring of detailed social behaviors in mice. We describe the steps to build RSA apparatus and set up the wiring for video synchronization. We then detail how to conduct RSA experiment with simultaneous Ca^{2+} imaging or optogenetics. This protocol also includes a custom MATLAB script for aligning the behavioral dataset to Ca^{2+} trace data.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Simultaneous analysis of social behaviors and neural responses in mice using round social arena system

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SUMMARY

Stable and accurate capturing of detailed social behaviors is essential for studying rodent sociability. Here, we introduce a round social arena (RSA) system that enables close-up monitoring of detailed social behaviors in mice. We describe the steps to build RSA apparatus and set up the wiring for video synchronization. We then detail how to conduct RSA experiment with simultaneous Ca²⁺ imaging or optogenetics. This protocol also includes a custom MATLAB script for aligning the behavioral dataset to Ca²⁺ trace data.

For complete details on the use and execution of this protocol, please refer to Kim et al. (2022).

BEFORE YOU BEGIN

Comparison to conventional sociability tests

Analyzing neural responses during social behavior is a challenging approach due to the limitations of the conventional sociability tests that have been designed with few options for contemporary neural investigations including *in vivo* Ca²⁺ imaging and optogenetics. For instance, the 3-chamber sociability test (3CST; Figure 1A) (Moy et al., 2004) is difficult to be used for Ca²⁺ imaging or optogenetics due to the cables tethered to the head of a mouse. The mouse with a cable cannot travel through the gated barriers (Figure 1B). On the other hand, a social dyadic test that monitors free interaction between two mice without barriers (Winslow, 2003) has a potential risk of the opponent mouse being distracted by or biting the cable (Figure 1C). As a compromise, the sociability test (ST) without chamber walls can be used for simultaneous analysis of social behavior and neural activity (Figure 1D) (Liang et al., 2018; Kim et al., 2020). However, ST also has a potential risk of the subject mouse being distracted by non-social stimuli or corner areas of the arena by innate spatial preference which significantly reduces the overall amount of social interaction (Figure 1D-bottom). Moreover, ST focuses on measuring proximity to social stimulus as an index for social behavior, yet it should be noted that proximity itself is not a direct indicator of social behavior. Exploratory sniffing, which has higher face validity for social behavior, requires observation of head orientation and posture to evaluate (Figure 1E). These factors then can be used to further analyze the reciprocity of social behavior. For instance, two mice sniffing each other facing nose to nose indicates reciprocity of the encounter (Figure 1E-right) while only one mouse sniffing the other indicates a non-reciprocated social encounter (Figure 1E-left). Despite the enriched behavioral patterns during social interaction, the conventional ST has limitations in capturing these details reliably due to blind spots of the cameras (Figure 1F).



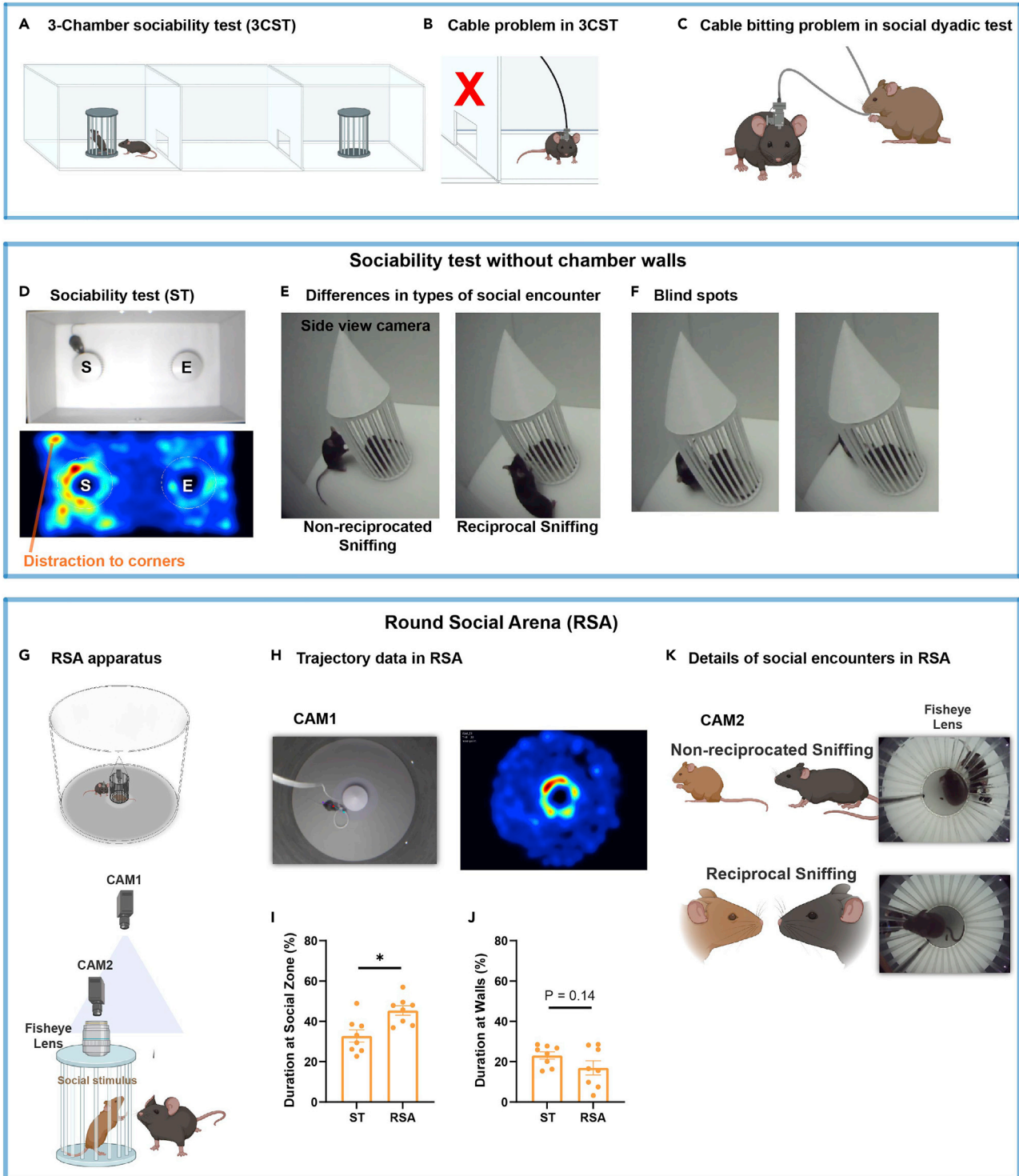


Figure 1. Comparison of conventional sociability tests and round social arena (RSA)

(A) A schematic illustration of the 3-chamber sociability test (3CST).

(B) Cable problem that limits the use of neural investigation tools in 3CST.

(C) Social dyadic test and potential problem of social stimulus mouse being distracted to cable.

(D–F) Sociability test (ST) without barriers between chambers. (D) (Upper) Overhead view of the ST. The letter ‘S’ indicates a cage containing social stimulus, and ‘E’ an empty cage. (Bottom) Occupancy heat-map created from the trajectory of a subject mouse showing the preference of S over E cage.

Figure 1. Continued

Distraction by the corner area can be observed. (E) Different types of social encounters captured by a side-view camera in ST. Subject mouse either shows non-reciprocated sniffing (left) to the social stimulus or reciprocal sniffing (right). (F) Blind spots of the side-view camera where details of the social interaction are masked by the inner cage or the social stimulus mouse. (G–K) Round social arena (RSA). (G) A schematic of RSA apparatus (upper) and positions of cameras in RSA environment (bottom). RSA has two cameras: one on the ceiling (CAM1) and another inside the inner cage (CAM2). (H) Overhead view of the RSA (left). Occupancy heat-map from animal’s trajectory tracked by CAM1 in RSA (right). No corner areas exist in RSA, which maximizes the subject animal’s stay in the social zone. (I) Duration at Social zone (3 cm range around social cage) compared between ST and RSA. An asterisk (*) indicates $p < 0.05$. (J) Duration at walls (5 cm range around walls) compared between ST and RSA. (K) Details of social encounters in RSA. A fisheye lens camera is used as CAM2 to enable observation of a uniform 360-degree view around the inner cage. Examples of observable social behaviors are non-reciprocated sniffing and reciprocal sniffing. CAM2 securely records these interactions with minimized risk of being masked by social stimulus or inner cage. Data are presented as mean values \pm SEM (standard error of the mean).

To overcome the limitations of the conventional methods, we have developed the round social arena (RSA) (Figure 1G). RSA consists of a round arena and an inner cage at the center holding the social stimulus mouse (Figure 1G-upper). One camera (CAM1) installed on the ceiling monitors the overall trajectory of the subject mouse, and another camera (CAM2) with a fisheye lens monitors the interaction of two mice in a close-up view (Fig. G-bottom). The round-shaped arena prevents cable disturbance by minimizing obstacles; and its non-distractive uniform environment significantly increases time spent in the Social zone compared to the ST (paired t-test; $t(7) = 3.605$, $p = 0.008$; Figures 1H and 1I). RSA also reduces the time spent near walls compared to ST, although not statistically significant ($p = 0.14$; Figure 1J). Utilization of a fisheye lens camera (CAM2) inside the inner cage enables the detailed and reliable monitoring of social interaction with minimum blind spot (Figure 1K). Thus, complex social behaviors such as non-reciprocated sniffing and reciprocal sniffing can be better observed and scored in RSA.

In the present report, we describe all the details of the RSA system including (1) the assembling process of the RSA system, (2) sociability analysis using the RSA system, (3) Ca^{2+} imaging analysis during social behaviors in the RSA, and (4) optogenetic manipulations using the RSA system.

Institutional permissions

All procedures were conducted with a protocol approved by the University of Tennessee Institutional Animal Care and Use Committee in accordance with US National Institutes of Health guidelines.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Cre-dependent GCaMP carrying AAV virus: pGP-AAV2/9-Syn-Flex-jGCaMP7f-WPRE	Addgene	Addgene viral prep #104492-AAV9
Cre-carrying AAV virus: AAV2/9-hSyn-Cre-WRRE-hGH	Addgene	Addgene viral prep #105553-AAV9
Cre-dependent ChR2-carrying AAV virus: pAAV-hSyn-hChr2(H134R)-EYFP	Addgene	Addgene viral prep #26973-AAV9
Deposited data		
Files & codes for building RSA	This paper	https://doi.org/10.5281/zenodo.6687251
Supplemental figures for this paper	This paper	Mendeley Data: https://doi.org/10.17632/r5f3jkf2s5.1
Experimental models: Organisms/strains		
C57BL/6J mouse, 8–10 weeks old, male.	The Jackson Laboratory	Jackson Laboratory Stock #000664
Software and algorithms		
Ethovision XT Software Suite	Noldus	https://www.noldus.com/ethovision-xt
Arduino Sketch	Arduino	https://www.arduino.cc/
Inscopix nVista Data Acquisition Software	Inscopix	https://www.inscopix.com/nvista
MATLAB R2020 or above	MathWorks	https://www.mathworks.com/products/matlab.html

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Preform	Formlabs	https://formlabs.com/software/
Dremel Digilab 3D slicer	Dremel	https://www.dremel.com/gn/en/digilab/software
Other		
GigE color camera (Basler Ace color camera with Gigabit Ethernet interface)	Basler	https://www.baslerweb.com/en/products/cameras/area-scan-cameras/ace/
SVPRO Fisheye Lens 180 Degree USB Camera Module	SVPRO	https://www.amazon.com/SVPRO-Illumination-Camera-Module-Fisheye/dp/B07C1N624T
Inscopix nVista system (DAQ + microendoscopic camera)	Inscopix	https://www.inscopix.com/nvista
Proview GRIN lens (1 mm diameter, 4 mm length, 0.5 NA)	Inscopix	1050-004605
Prizmatix optogenetics LED-Dual	Prizmatix	https://www.prizmatix.com/Optogenetics/Optogenetics-LED-Dual.aspx#Header
Prizmatix USB pulser	Prizmatix	https://www.prizmatix.com/Optogenetics/PulserPlus.htm
Fiber optic cannulae with 1.25 mm diameter ceramic ferrule, 400 μm core, 0.5 NA	RWD Life Science Inc.	R-FOC-L400C-50NA
Mini USB I/O Box	Noldus	https://www.noldus.com/ethovision-xt/io-box
Arduino Uno	Arduino	https://www.arduino.cc/
Form2	Formlabs	https://formlabs.com/
3D20	Dremel	https://www.dremel.com/gn/en/p/dremel-3d20-idea-builder-3d20-01-v680
Formlabs Clear Resion v4	Formlabs	RS-F2-GPCL-04
PLA-White Filament, 1.75 mm Diameter	Dremel	PLA-WHI-01
PC817 opto-coupler	Gikfun	N/A
Resistors (220 ohm, 10k ohm)	BOJACK	N/A

STEP-BY-STEP METHOD DETAILS

Part 1. Building RSA apparatus

⌚ Timing: 2 h

The apparatus for the RSA can be made using a commercial LLDPE trash can (Figure 2A). Cut a cylinder-shaped LLDPE trash can (outside HXD: 33 × 27") in given dimensions (inner diameter: 490 mm, height: 450 mm from the can floor). Note that the apparatus must have enough contrast with the color of animals that will be tracked inside the arena. Gray is optimal for both dark and light-colored animals.

Then modify the floor of the apparatus as follows (Figure 2B):

1. Drill a hole (inner diameter: 10 cm) at the bottom of the apparatus. This enables the connection of the camera cable to the operating system.
2. Make a circle-shaped floor plate (inner diameter: 490 mm, thickness: 5 mm) using plexiglass of the same color as the apparatus. Sand the surface to make the surface non-reflective.
3. Draw a smaller circle (inner diameter: 8 cm) on the center of the plexiglass floor.
4. Drill a rectangular hole (W: 2 cm, H: 7 mm) at the edge of the circle with a shorter side facing toward the center of the circle. This will fit the cable hole of the inner cage that will be installed in the later step.

Part 2. Assembling RSA inner cage

⌚ Timing: 1 day

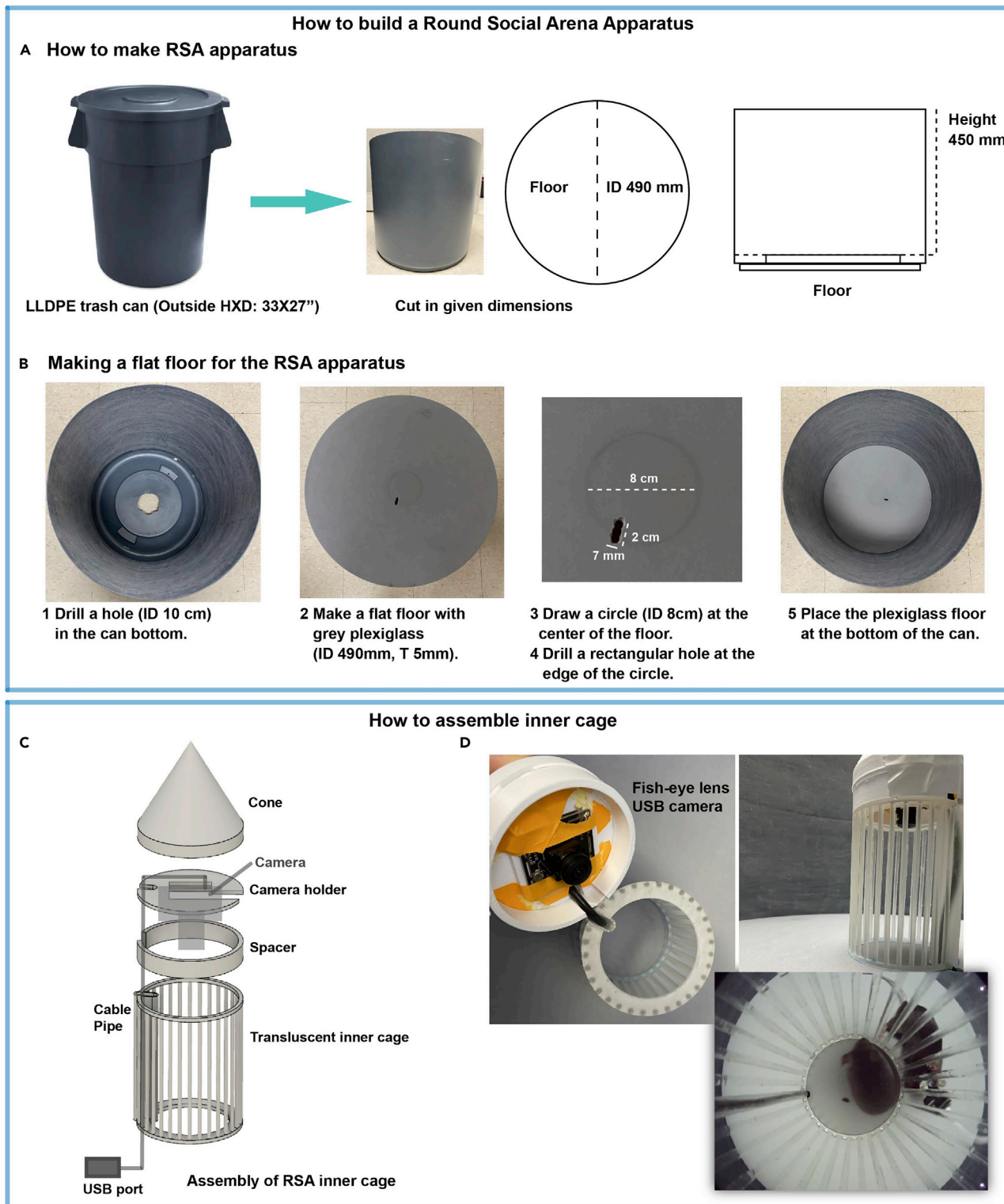


Figure 2. How to build a round social arena apparatus

(A) RSA apparatus can be made by cutting a commercial LLDPE trash can in given dimensions.

(B) How to make a flat floor for the RSA apparatus (Inner diameter 490 mm, 5 mm thickness). Holes are made to install camera cables.

Figure 2. Continued

(C) Blueprint for assembling 3D printed model parts of the RSA inner cage. Camera (fish-eye lens webcam) is attached below the camera holder with tapes. Its cable is inserted above the camera holder and travels through cable holes and pipes of the parts, RSA apparatus hole, and extends to the USB port.

(D) Images of assembled RSA inner cage, and the close-up view from the inner cage camera.

3D printable parts of the RSA inner cage are available in the Zenodo depository: 6687251 (see also [key resources table](#)). [Table 1](#) shows which type of 3D printer and material are optimal for printing each part.

5. Print 3D STL files in the Zenodo depository. Refer to [Table 1](#) for optimal printing.
6. Insert the JST end of the USB camera cable through the bottom of the RSA arena, plexiglass floor, and the cable pipe of the translucent cage, then insert it into the camera board.
7. Assemble parts and the camera (SVPRO Fisheye Lens 180 Degree USB Camera Module HD 1080P; see also [key resources table](#)) as illustrated in [Figure 2C](#).

Note: The roof part (cone + camera + camera holder + spacer) can be fixed together with hot glue and tapes, but should not be fixed to the cage ([Figure 2D](#)).

Part 3.1. Wiring for video synchronization

⌚ **Timing: 2 h**

⚠ **CRITICAL:** The temporal synchronization of two cameras used in the RSA experiment is a crucial step for later analysis. This step is not required if two GigE cameras are used for CAM1 and CAM2, which can be simultaneously controlled by EthoVision software.

8. Drill four holes (size of the infrared LED head) around the RSA wall not higher than 6 cm above the floor ([Figure 3B](#)). Arbitrary positions can be used as long as at least two infrared LEDs can be simultaneously viewed through CAM2 (inner cage camera).
9. Insert infrared LEDs in the holes from the outside apparatus, facing toward the inner cage. Check if IR LEDs can be visible through CAM2.
10. Connect IR LEDs to an Arduino Uno board’s digital output pins (choose from 3–20) and GND ([Figure S1](#)). The remaining Arduino output pins can be used for additional visual indicators ([Figure 3C](#)).
11. Connect an output channel and GND of Noldus mini IO Box to an optocoupler (PC817)’s anode (+) and cathode (-), respectively. Then connect the optocoupler to Arduino pin 2 by making a pull-down resistor circuit. See [Figures 3C](#) and [S1](#) for details.
12. Upload Arduino sketch file (see Zenodo depository: 6687251) onto Arduino board.
13. Program EthoVision trial and control settings to auto-start experiment when an animal is detected for longer than 1 s in the arena, then trigger mini IO Box to send TTL output to Arduino, triggering to turn on infrared LEDs. An example trial control setting is shown in [Figure 3E](#).

Table 1. Optimal 3D printing for each part of RSA inner cage

Part	3D printer	Material
Cone.stl	Dremel 3D20 (Dremel) or any type of FDM 3D printer	White PLA or ABS filament (Black if animal is white)
CameraHolder.stl	Dremel 3D20 (Dremel) or any type of FDM 3D printer	White PLA or ABS filament (Black if animal is white)
Spacer.stl	Dremel 3D20 (Dremel) or any type of FDM 3D printer	White PLA or ABS filament (Black if animal is white)
TranslucentCage.stl	Form2 (formlabs) or any type of SLA or polyjet 3D printer	Transparent clear resin

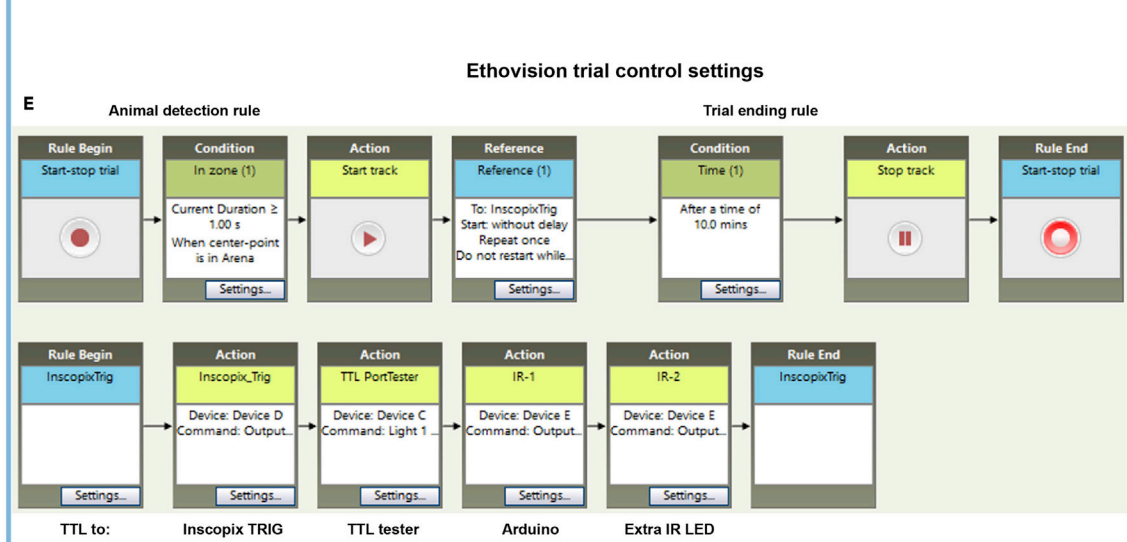
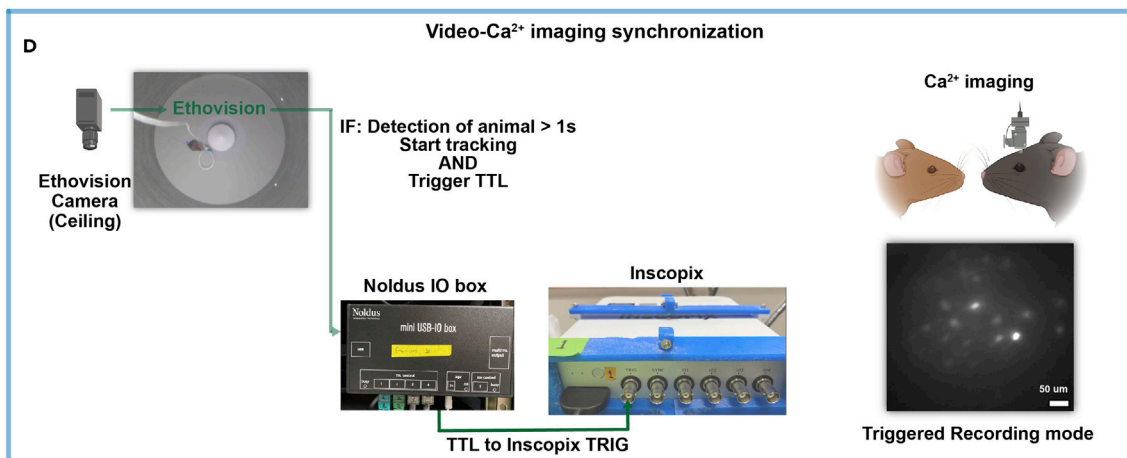
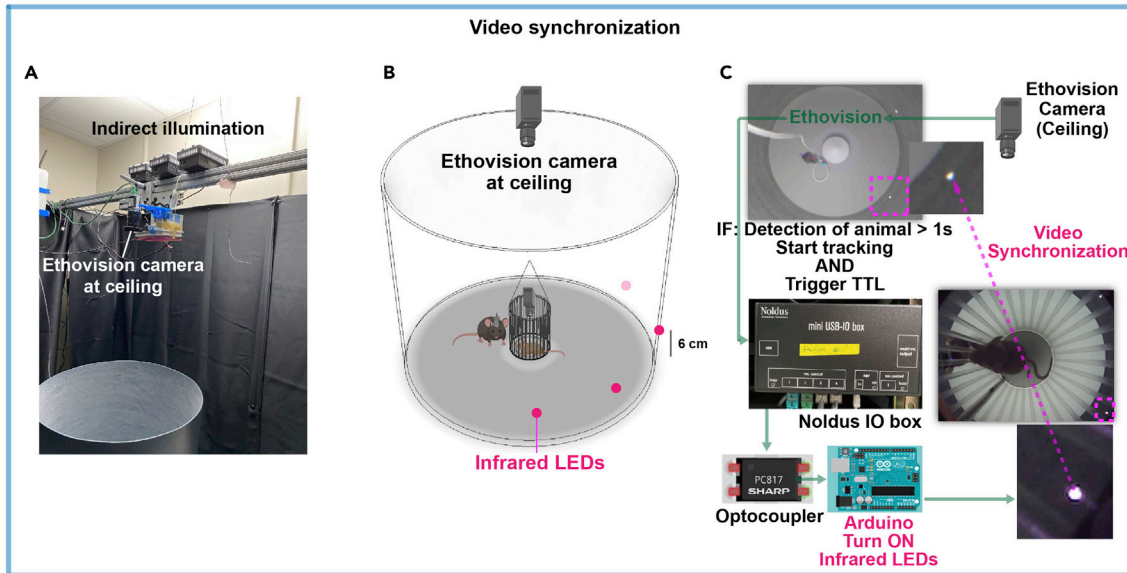


Figure 3. How to synchronize multiple systems involved in the RSA experiment

- (A) An example picture shows the installation of the EthoVision camera on the ceiling (CAM1).
 (B) A schematic for RSA apparatus, positions of cameras (CAM1 and CAM2), and infrared LEDs.
 (C) Synchronization of two different types of video images using TTL signals and infrared LEDs. When the EthoVision software detects the animal in the arena for more than 1 s, it triggers the Noldus mini USB IO Box to send TTL output to an Arduino Uno board via an optocoupler circuit which then turns on multiple infrared LEDs. These lights can be later used as a temporal reference point to synchronize two video images.
 (D) Synchronization of video recordings and Ca²⁺ data. EthoVision's mini USB IO Box can be programmed to trigger Inscopix to start recording upon initiation of the experiment.
 (E) An example EthoVision trial and control setting, which is programmed to start tracking upon detection of an animal for more than 1 s, then trigger multiple devices, and end the session after 10 min.

Note: There are two reasons for wiring infrared LEDs to Arduino but not directly to Noldus mini USB IO Box: (1) Saving digital output channels of Noldus mini USB IO Box. (2) As EthoVision has two simultaneous tasks running (mouse tracking and IO box controlling), its processing time is slower than Arduino. Turning on multiple IR LEDs via the IO box may occasionally result in a temporal delay between LEDs, whereas such delay is only at the micro-second level in Arduino.

Part 3.2. Wiring for synchronization with Ca²⁺ imaging

⌚ Timing: 2 h

This step is required when conducting Ca²⁺ imaging simultaneously with behavioral experiments.

14. Connect an output channel of the Noldus mini USB IO box to the TRIG port of Inscopix DAQ (or other TTL input channel when using a different DAQ system).
15. Program EthoVision Trial and Control settings to send TTL output to TRIG upon initiation of tracking (Figure 3E).
16. Change Inscopix DAQ configuration to "Triggered recording mode".

Part 4. Conducting RSA experiment with simultaneous Ca²⁺ imaging

⌚ Timing: 7 days

Given that GCaMP viral injection and GRIN lens/baseplate implantation have been complete, below is the protocol for conducting an RSA experiment with simultaneous Ca²⁺ imaging. Detailed protocols for viral injection and GRIN lens/baseplate implantation can be found in these references: (Kim et al., 2015, 2020, 2022; Liang et al., 2018; Kennedy et al., 2020). For a step-by-step protocol for these processes, please refer to (Carrier-Ruiz et al., 2021).

The overall schedule for preparing and conducting RSA (Figure 4A):

17. Start single housing (1 week prior to the RSA experiment).
18. Handling (10 min/3 days) for both subject mice and social stimulus mice (female C3H/HeJ mice; p30-50). Two strains should be handled separately.
19. Habituation (10 min per mouse × 3 days).
 - a. Insert a dummy microendoscope on the baseplate and allow each subject mouse to freely explore the arena with an empty inner cage.
 - b. After a batch of daily habituation sessions for subject mice is finished, habituate each social stimulus mouse inside the inner cage (10 min per mouse × 3 days).
 - c. Illuminate the arena with indirect light with ~35 lux luminance throughout the experiment.
20. RSA experiment with consecutive Empty (10 min) and Social (10 min) stages (5 min interval). During the social stage, a social stimulus mouse is placed inside the inner cage. Use 3 to 5 social stimulus mice in rotation for multiple subject animals. See below for the procedural details.

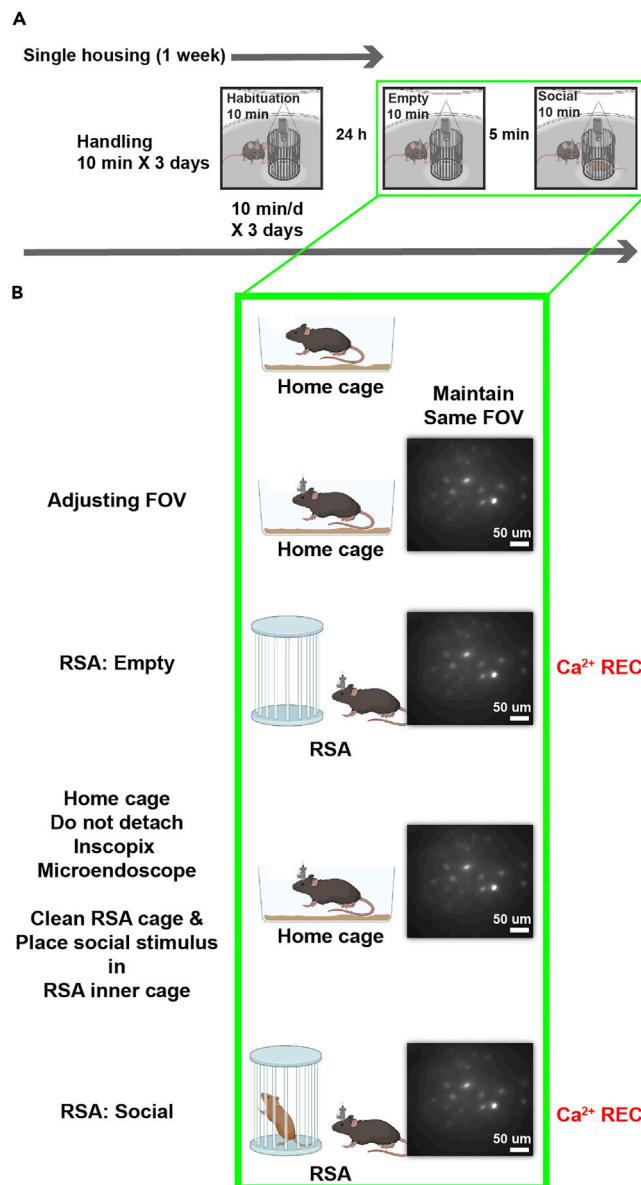


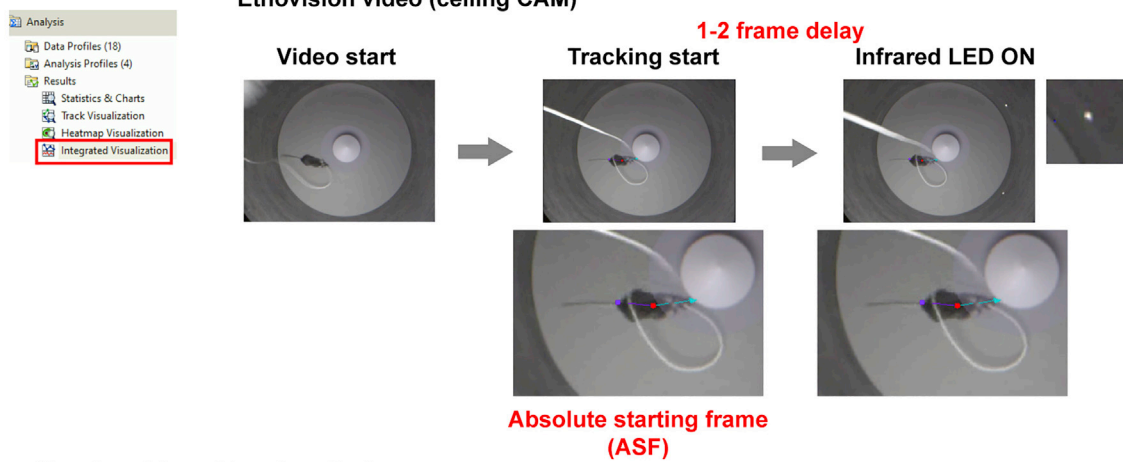
Figure 4. Conducting RSA experiment with simultaneous Ca²⁺ imaging

(A) Schedule for preparing and conducting RSA experiment: 1 week of single housing prior to the experiment, 3 days of handling, 3 days of habituation, and 1 day of RSA experiment. RSA experiment consists of Empty and Social stages. (B) Procedures to conduct RSA experiment with simultaneous Ca²⁺ imaging. Microendoscope insertion and adjusting for optimal field of view (FOV) precedes the RSA experiment in the home cage. Once optimal FOV is found, the microendoscope should not be detached throughout the experiment in order to maintain the same FOV.

Procedural details during RSA experiment (Figure 4B):

21. Transport the single-housed home cage to the experimental setting.
22. Insert the microendoscope (Inscopix) to the baseplate and adjust the field of view (FOV) from Inscopix software to obtain the most optimal plane with the highest image quality of neurons and their activity.
23. Empty stage (10 min):
 - a. Click "Record" in Inscopix data acquisition (triggered recording mode).

A



B Merging videos (Premiere Pro)

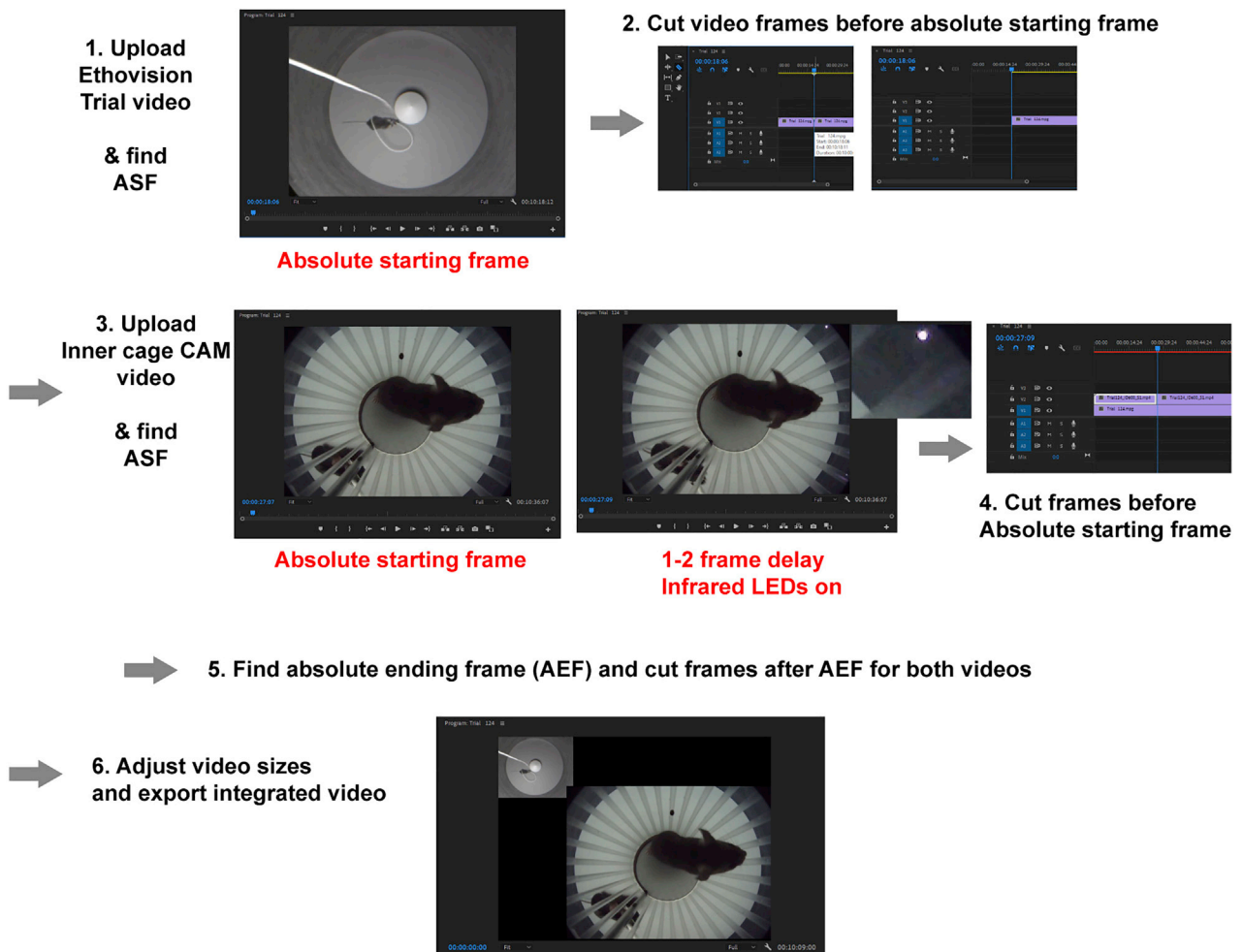


Figure 5. Video preprocessing for temporal synchronization of two video images

(A) Finding absolute starting frame (ASF) in the EthoVision trial video. ASF is the frame where animal's coordinates were first tracked. Infrared LEDs are usually turned on 1 or 2 frames after ASF. Thus ASF is a more precise temporal reference point for the initiation of the trial.

(B) Finding absolute starting frame (ASF) in the EthoVision trial video. ASF is the frame where animal's coordinates were first tracked. Infrared LEDs are usually turned on 1 or 2 frames after ASF. Thus ASF is a more precise temporal reference point for the initiation of the trial.

- b. Start recording USB camera (CAM2), and set EthoVision XT as "Ready" for automatic detection.
- c. Place the animal in RSA with an empty inner cage.

Note: Detection of the subject >1 s will trigger the EthoVision to start tracking (CAM1), initiate Inscopix recording, and turn on the infrared LEDs.

24. After the Empty stage session, remove the animal from the RSA and place it back in the home cage. Do not detach the microendoscope from the baseplate in order to maintain the same FOV. Clean up the RSA and inner cage with 70% ethanol and place a social stimulus mouse (C3H/HeJ; p30-50) inside the inner cage.
25. Repeat the process described in (3) to conduct the Social stage, but with a social stimulus mouse (10 min).

Part 5. Video preprocessing and synchronization

⌚ Timing: 30 min

To temporally align the data from two different types of videos, a preprocessing step is needed. This step can be neglected if the same GigE cameras are used for both ceiling and inner cage cameras (CAM1 & 2, respectively).

Finding absolute starting frame (ASF; [Figure 5A](#)):

26. Open EthoVision's integrated visualization tab, and select the trial to be analyzed.
27. The ASF is the frame where tracking first started and in which the markers for the animal's position first appear. This frame usually precedes the first IR-LED ON frames by 1 or 2 frames.
28. Find an absolute ending frame (AEF) using a similar approach.

Merging two videos ([Figure 5B](#)):

29. Open 'new project' in Adobe Premiere Pro and upload the EthoVision trial video. This will set the EthoVision trial video as a reference for the frame rate. Find ASF using the information from EthoVision integrated visualization.
30. Remove the frames of the EthoVision trial video prior to ASF.
31. Upload the inner cage video to the same project, and also find ASF.
32. Remove the frames of the inner cage video prior to ASF.
33. Remove the frames after AEF from both videos.
34. Adjust the sizes of the videos to view two videos on one screen. It is ideal to enlarge the size of inner cage video and reduce the EthoVision trial video. Check the temporal synchronization of two videos and export the integrated videos into one file.

Part 6. Manual scoring of social behaviors

⌚ Timing: 1 h

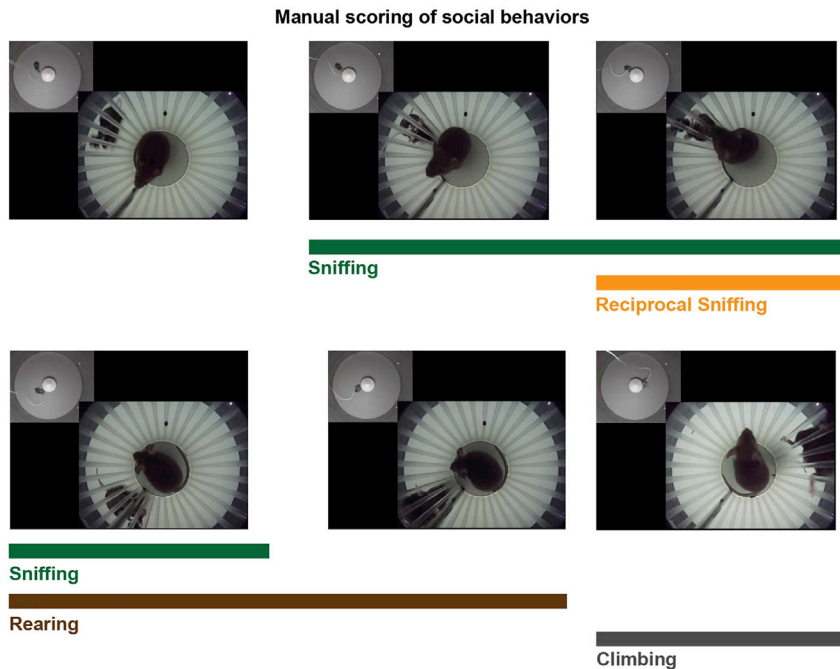


Figure 6. Manual scoring of detailed behaviors

Manual scoring of social (sniffing, reciprocal sniffing) and non-social behaviors (rearing, climbing) using EthoVision manual scoring function. Occasionally, more than two behaviors can overlap as mice often engage in multiple behaviors simultaneously.

Using the integrated video from part 5, detailed social behaviors and non-social behaviors can be manually scored in EthoVision manual scoring section (Figure 6). Examples of social behaviors are shown in [Methods video S1](#).

35. Create a new EthoVision project, and upload the integrated videos exported in [Part 5. Video preprocessing and synchronization](#).
36. Open the manual scoring tab in EthoVision's acquisition panel.
37. Allocate keys for the following behaviors: sniffing, reciprocal sniffing, grooming, rearing, climbing, and other behaviors of interest.
38. Slow down the videos to 0.5× speed and manually score the allocated behaviors. More than two behaviors can temporally overlap. All reciprocal sniffing is involved in sniffing. Non-reciprocated sniffing can be analyzed later by subtracting reciprocal sniffing from all sniffing events.

Part 7. Temporal alignment of social behavior data to Ca²⁺ trace data

© Timing: 10 min

While EthoVision video is normally sampled at 25 Hz, the Ca²⁺ imaging system (Inscopix) has a different sampling rate (20 Hz) and is recommended to downsample into 10 Hz during preprocessing of the Ca²⁺ trace data. Thus, we made a custom MATLAB script that downsamples and temporally aligns the behavioral dataset to the preprocessed Ca²⁺ trace data. Unzip and open the 'Behavior_to_Calcium_MATLAB' folder from the Zenodo depository (6687251) and run 'Behavior_Ca_match.m' file in MATLAB. Example behavior file is named as 'Ethovision_behavioral_data.csv' and example Ca²⁺ trace data is named as 'Example_Calcium_data.csv'.

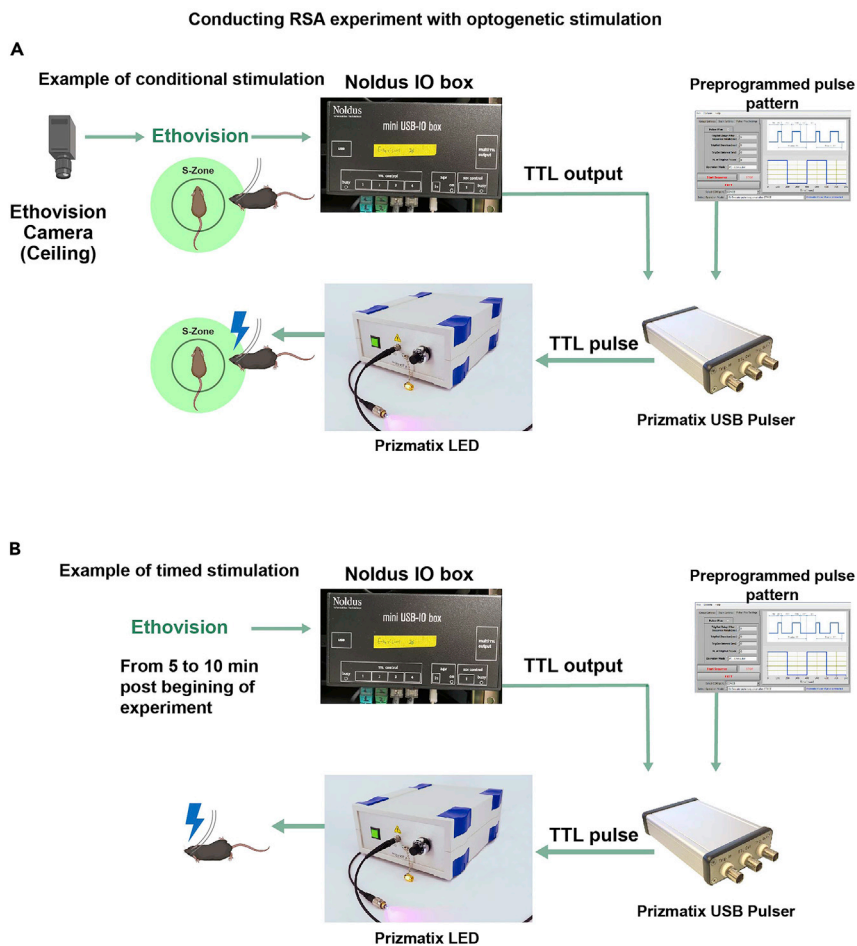


Figure 7. Conducting RSA experiment with optogenetic stimulations

(A) Optogenetics experiment with conditional stimulation. LED light is delivered when the head of the mouse enters the S-Zone around the inner cage.

(B) Optogenetics experiment with timed stimulation. LED light is given via a pre-determined schedule regardless of the actions of the mouse.

Part 8. Conducting optogenetics experiment in RSA

⌚ Timing: 9 days

RSA can be conducted with an optogenetics experiment. An example of connecting the optogenetics system to RSA is shown in Figure 7. Estimated time required for optogenetic experiment with RSA is about 9 days, including 3 days of handling, 3 days of habituation, 1 day of baseline RSA test, 1 day of RSA with optic stimulation in RSA, and 1 day of optic stimulation without social stimulus. For detailed experimental procedures, see: (Kim et al., 2020, 2022). In the current section, how to connect RSA with optogenetic stimulation system is explained.

39. Connect an output channel of the Noldus mini USB IO box to the trigger input of Prizmatix USB pulser. This channel controls the initiation and termination of optogenetic stimulation. Define the conditions for stimulation in 'Trial and Control settings' in the Ethovision.
40. Connect Prizmatix USB pulser and computer with USB cable.
41. Connect the Prizmatix USB pulser to the external input channel of the Prizmatix LED system.

42. Prizmatix USB pulser can be programmed for the configuration of pulse-width and intervals between pulses.

Note: Conditions for stimulation can be adjusted depending on the experimental purpose. For instance, whether the subject mouse enters into S-Zone (area near the inner cage) can be the condition for stimulation (Figure 7A). Another example is timed stimulation where stimulations are given according to the pre-programmed schedule regardless of the actions of the subject mouse (Figure 7B).

EXPECTED OUTCOMES

RSA provides a secure platform for simultaneous analysis of social behaviors and neural investigation. RSA induces longer social interaction than conventional sociability paradigms and allows observation and reliable scoring of enriched social behavior patterns and underlying neural responses. In summary, these advantages make RSA a useful tool for studying neural mechanisms underlying social behavior.

LIMITATIONS

RSA does not directly show social preference over a non-social object nor preference for social novelty unlike in the conventional sociability tests. Although running a multi-stage experiment with both 'Empty' and 'Social' stages can provide this information, the order effect may not be completely ruled out. However, RSA has only been recently designed and tested, and has more space for development. Alternative experimental design such as randomizing session orders may be beneficial for solving the order effect.

TROUBLESHOOTING

Problem 1

Related to [Part 1. Building RSA apparatus](#), building RSA apparatus.

If the floor plate of the RSA apparatus has a reflective surface, it can interfere with animal tracking.

Potential solution

- Sand and grind the floor plate with sanding paper until it is no longer reflective.

Problem 2

Related to [Part 2. Assembling RSA inner cage](#), assembling RSA inner cage.

After printing the translucent cage with an SLA 3D printer, supporting parts should be removed by nipper. During this process, cage bars can be damaged.

Potential solution

- Use a sharp nipper. If the cage bars are damaged, repair the parts with super glue.

Problem 3

Related to [Part 3.1. Wiring for video synchronization](#), wiring for video synchronization.

Either incorrect wiring or errors in the Ethovision protocol can result in irresponsive IR LEDs at the initiation of an experiment.

Potential solution

- Prior to the experiment, always check whether EthoVision digital outputs and IR LEDs are working.

Problem 4

Related to [Part 3.2. Wiring for synchronization with Ca²⁺ imaging](#), wiring for synchronization with Ca²⁺ imaging.

Incorrect wiring or setting Inscopix in a 'manual recording mode' will not enable the TTL-triggered recording.

Potential solution

- Prior to the experiment, always check whether all the wirings are correct, and the Inscopix is in 'triggered recording mode'.

Problem 5

Related to [Part 6. Manual scoring of social behaviors](#), manual scoring of social behaviors.

In rare cases, the social stimulus mouse rears or climbs onto the cage bars which can create a blind spot, blocking the subject mouse from the camera ([Figure S2](#)). This interferes with the manual scoring process.

Potential solution

- Use video frames before and after the blocking event to infer the behavior of the subject mouse.
- Use simultaneously recorded EthoVision trial video taken from the CAM1 (ceiling camera) to obtain additional information about the actions of the subject mouse within the given frames.

RESOURCE AVAILABILITY

Lead contact

Questions and requests for resources and materials should be directed to and will be fulfilled by the lead contact, Il Hwan Kim (ikim9@uthsc.edu).

Materials availability

This study did not generate new unique reagents. All materials developed in this study will be available from the [lead contact](#) upon request.

Data and code availability

Original data have been deposited to Zenodo depository :6687251 (see also [key resources table](#)). Supplemental figures are available in the Mendeley Data: <https://doi.org/10.17632/r5f3jkgf2s5.1>.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101722>.

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AUTHOR CONTRIBUTIONS

S.K. and I.H.K. designed and developed the RSA system. S.K. conducted experiments including behavior, Ca²⁺ imaging, and optogenetics. Y.K. produced adeno-associated viruses for Ca²⁺ imaging and optogenetics. This paper was written by S.K. and I.H.K.

DECLARATION OF INTERESTS

The authors declare no competing and financial interests.

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