Title

Authors , affiliations

# Abstract

# Introduction

Mouse Pupil Project

Introduction:

The study of pupil dynamics has long been a vital area of research within the fields of neuroscience and psychology, providing critical insights into the autonomic and central nervous systems. The pupil, controlled by the iris, dilates and constricts in response to a variety of stimuli, including changes in light, cognitive load, and pharmacological agents. This dynamic response serves as a non-invasive indicator of underlying brain activity and emotional and conscious states.

Recent advancements have expanded our understanding of pupil dynamics in the context of pharmacological interventions. Drugs such as atropine, which blocks parasympathetic input, lead to pronounced pupil dilation, while agents like pilocarpine, which stimulate parasympathetic activity, cause constriction. Psychoactive substances, including psychedelics like psilocybin, have been shown to induce significant changes in pupil size, reflecting their profound effects on brain function and consciousness.

Furthermore, the advent of high-resolution eye-tracking technology has enabled more precise measurements of pupil responses, facilitating detailed studies on how different stimuli affect pupil size. This technology has uncovered nuanced patterns of pupil dynamics in response to cognitive and emotional tasks, highlighting the role of the locus coeruleus, a brainstem nucleus involved in physiological responses to stress and panic.

Studies on non-human animals, particularly mice, have provided valuable models for understanding the neural mechanisms underlying pupil dynamics. Research involving mice and rats has shown that their pupil responses are not only affected by light and pharmacological agents but also by behavioral states such as running, resting, and different stages of the sleep-wake cycle. These findings underscore the complexity of the factors that influence pupil size and the potential for using pupil dynamics as a biomarker for various neurological and psychiatric conditions.

Despite these advances, existing methods for analyzing pupil dynamics in animal models often rely on proprietary software or rigid pipelines that limit scalability, reproducibility, and adaptability across experimental setups. Many of these tools require manual intervention, are sensitive to variations in lighting and occlusion, or lack the flexibility needed for high-throughput analysis. Furthermore, few open-source solutions offer robust, frame-level tracking of the pupil across diverse behavioral conditions with integration into modern deep learning frameworks. To address these limitations, we present a customizable and scalable pupil tracking pipeline built on top of DeepLabCut. Our pipeline enables high-resolution, markerless tracking of the pupil in head-fixed mice, incorporating post-processing tools to enhance tracking accuracy and minimize noise. This work bridges a critical gap by providing experimentalists with a transparent, adaptable framework for studying pupil dynamics across a wide range of experimental paradigms.

# Methods

Experimental procedures followed closely to those described in Claar, Rembado, et al 2023). The methods are summarized below and additional details are provided for novel procedures.

**Mice**

C57BL/6J wild-type (n=?) were purchased from Jackson Laboratories (JAX sock #000664) on postnatal day 28-35. Mice were housed in the Allen Institute animal facility and used in accordance with protocols approved by the Allen Institute’s Institutional Animal Care and Use Committee under protocol 2212 (and 2003?). All mice were housed in a shared facility with room temperatures between 20 and 22°C and humidity between 30 and 70%. Mice were maintained on a reverse 12-h light cycle and were single-housed following surgery and had ad libitum access to food and water.

**Headframe Implantation Surgery**

Prior to undergoing experimental procedures, all mice were implanted with a titanium headframe to facilitate head fixation *in vivo* (surgical procedures adapted from [Groblewski et al 2020)](https://pdf.sciencedirectassets.com/271055/1-s2.0-S0165027020X00161/1-s2.0-S0165027020303459/main.pdf?X-Amz-Security-Token=IQoJb3JpZ2luX2VjEKX%2F%2F%2F%2F%2F%2F%2F%2F%2F%2FwEaCXVzLWVhc3QtMSJIMEYCIQDFAyss9DNImizkMKJ%2Fk6jHwlVwQSUGKmyzVPCRCzGP8gIhAOVRM9FjrtwRU9w5Sylhtiz4KXni3LKqZcmmOyoH6nypKrMFCC0QBRoMMDU5MDAzNTQ2ODY1Igxgs3zJabaw3ReOFK4qkAXJGmBEPsicahQ52J3%2FfUBOhuou%2BHzcz6zcEV94JDi0y05WpY30cpyAltH7YL%2B3xTKVFhi2rrru0GOCA%2BT14tqdwJxjjlpI56YfPAmXA6N7P%2BaCoXT580xOvcij0nN1jl7BLt25b0wSFTFAOHdFh1nUbpiGKi9uGJ%2BvwpX3FwT9ey%2B4Wz3bF2tXoeLab95IrDkFGSw066jSL2dldh%2FrlNVey8TyGB36fvQwafVnBbG9XW54UaaEIATcAW6ZUegHEapTejUDnnE3o%2FMV4RKtPgb2TKLy1brjNc3UgMLQay0C7vlo3uEto7E8kdUZRCHo68%2BTOa6VW8LxOuFHhJJPGC5K8o5PWJswfDOyMHzqKLsc5DatvWI6f90ojjxuLMT0Wa2ke6seSQAEiOTcWQ%2FadpYZvkWxdfaPQlczCzXUj4TIIIqoxby8X%2Fi7wBkdnzREDU1YeREMLN1LNlydXhv6LgaresMuc8HBYa23IbEPFYFyeqDIX4AutotrykDIKu8dFB%2BJuRVtmoQ6q9V9X1H6U2B9bvJGuolmwSqagWuTccYpnTRDU3rYnkNxF4Cid2gWy4p9TnrYLSXweqME8FU393R5FY0yH84mBhUXAy7xiJVDVNYw%2BCqzZ8c4PzVZRvsaiEgBsGLgLySGhsCfh0ETDW4qJScZQxIyufGsqFhzkKjpTfgr3%2FpSr%2BJvj2qeIqzNR%2BxY5Yk3wDWpRNtwK4Q%2Bfg7Dvkd3hKpwutCvroLrIkagmerTxAT0MhvehfuMqi98WSOTFSi7SpUl5p0s%2FBZst%2B0ipTVF3YbvWie1uFyaBGf5LqYXb1gEo9PKC5AgPkgxzIGCWUgo0b0YWYwl5W2RtK%2FcNvhSm4tUVc1%2BmPWk1eQzJzCR%2FZWsBjqwAW5aDP%2FgrTYtJ1kAXy4u7byhCXwLhM%2FQ2eHu75tdeZFwbYHXTFBdxAWAX0xeNivK2zuXzgibXIL083IxR5LbPLZa73cfSq98Ijps3aX82DvN%2Bon7Piev8%2BED8Bm%2BrPL5P7UO379SQ0ZJUrAY4RC5GAwZpt6YygsbtJRrq3TmpV93v%2B%2FN8RzIBdQfHD29zmBKIhdlIpsLS7KTKgDM1zitNXVav%2BqU4hP11Sr3XP3HXltU&X-Amz-Algorithm=AWS4-HMAC-SHA256&X-Amz-Date=20231222T141307Z&X-Amz-SignedHeaders=host&X-Amz-Expires=300&X-Amz-Credential=ASIAQ3PHCVTY2NVLNWHR%2F20231222%2Fus-east-1%2Fs3%2Faws4_request&X-Amz-Signature=6ffc20ade40c02b623f4d5ee50e11ff6c41a47a3ca1f81fe1fa9330ebb948c22&hash=a19d56c1d705c4e4cb31cd1e1cc30c9f653786a793ca2d4af5f5af08380d3b9f&host=68042c943591013ac2b2430a89b270f6af2c76d8dfd086a07176afe7c76c2c61&pii=S0165027020303459&tid=spdf-d2a7f077-05fb-4cbb-8fb9-894ae50da720&sid=e801beab6b98514bf58ac765703cea3b3316gxrqa&type=client&tsoh=d3d3LnNjaWVuY2VkaXJlY3QuY29t&ua=0f135a515f5f02570252&rr=8398f6b3a9ba9c6c&cc=us). Surgery took place when mice were 4-11 weeks old. Pre-operative injections of dexamethasone (3-4 mg/kg, IM) and ceftriaxone (100-125 mg/kg, SC) were administered one to three hours prior to surgery. Mice were deeply anesthetized with isoflurane (5%) in an induction chamber and then placed on a stereotaxic rig (Model# 1900, KOPF; Tujunga, CA) and maintained at a surgical level of anesthesia using isoflurane (1.5-2.5%) via nose cone. Breathing was monitored and body temperature was maintained at 37.5 °C with a heating pad under the animal (TC-1000 temperature controller, CWE, Inc). Ocular lubricant (Systane, Alcon Inc., Geneva, Switzerland) was applied to the eyes during anesthesia to maintain hydration. During surgery, skin was removed to expose the skull, and the skull was leveled with reference to bregma. Using white C&B Metabond (Parkell, Inc., Edgewood, New York), a custom titanium headframe was secured to the skull and the rest of the exposed skull was covered. After surgery, the mouse was given an injection of lactated Ringer’s solution (up to 1mL, SC.) and was placed on a heating pad to recover. All animals received analgesics and antibiotics for two days post-surgery.

**Habituation**

Five days after the headframe implantation surgery, all mice began a 3-week training schedule. Researchers first habituated mice to handling for 2 days, and on all subsequent days, mice were placed on a running wheel where the headframe was fixed in place with two sets of screws ([Groblewski et al 2020)](https://pdf.sciencedirectassets.com/271055/1-s2.0-S0165027020X00161/1-s2.0-S0165027020303459/main.pdf?X-Amz-Security-Token=IQoJb3JpZ2luX2VjEKX%2F%2F%2F%2F%2F%2F%2F%2F%2F%2FwEaCXVzLWVhc3QtMSJIMEYCIQDFAyss9DNImizkMKJ%2Fk6jHwlVwQSUGKmyzVPCRCzGP8gIhAOVRM9FjrtwRU9w5Sylhtiz4KXni3LKqZcmmOyoH6nypKrMFCC0QBRoMMDU5MDAzNTQ2ODY1Igxgs3zJabaw3ReOFK4qkAXJGmBEPsicahQ52J3%2FfUBOhuou%2BHzcz6zcEV94JDi0y05WpY30cpyAltH7YL%2B3xTKVFhi2rrru0GOCA%2BT14tqdwJxjjlpI56YfPAmXA6N7P%2BaCoXT580xOvcij0nN1jl7BLt25b0wSFTFAOHdFh1nUbpiGKi9uGJ%2BvwpX3FwT9ey%2B4Wz3bF2tXoeLab95IrDkFGSw066jSL2dldh%2FrlNVey8TyGB36fvQwafVnBbG9XW54UaaEIATcAW6ZUegHEapTejUDnnE3o%2FMV4RKtPgb2TKLy1brjNc3UgMLQay0C7vlo3uEto7E8kdUZRCHo68%2BTOa6VW8LxOuFHhJJPGC5K8o5PWJswfDOyMHzqKLsc5DatvWI6f90ojjxuLMT0Wa2ke6seSQAEiOTcWQ%2FadpYZvkWxdfaPQlczCzXUj4TIIIqoxby8X%2Fi7wBkdnzREDU1YeREMLN1LNlydXhv6LgaresMuc8HBYa23IbEPFYFyeqDIX4AutotrykDIKu8dFB%2BJuRVtmoQ6q9V9X1H6U2B9bvJGuolmwSqagWuTccYpnTRDU3rYnkNxF4Cid2gWy4p9TnrYLSXweqME8FU393R5FY0yH84mBhUXAy7xiJVDVNYw%2BCqzZ8c4PzVZRvsaiEgBsGLgLySGhsCfh0ETDW4qJScZQxIyufGsqFhzkKjpTfgr3%2FpSr%2BJvj2qeIqzNR%2BxY5Yk3wDWpRNtwK4Q%2Bfg7Dvkd3hKpwutCvroLrIkagmerTxAT0MhvehfuMqi98WSOTFSi7SpUl5p0s%2FBZst%2B0ipTVF3YbvWie1uFyaBGf5LqYXb1gEo9PKC5AgPkgxzIGCWUgo0b0YWYwl5W2RtK%2FcNvhSm4tUVc1%2BmPWk1eQzJzCR%2FZWsBjqwAW5aDP%2FgrTYtJ1kAXy4u7byhCXwLhM%2FQ2eHu75tdeZFwbYHXTFBdxAWAX0xeNivK2zuXzgibXIL083IxR5LbPLZa73cfSq98Ijps3aX82DvN%2Bon7Piev8%2BED8Bm%2BrPL5P7UO379SQ0ZJUrAY4RC5GAwZpt6YygsbtJRrq3TmpV93v%2B%2FN8RzIBdQfHD29zmBKIhdlIpsLS7KTKgDM1zitNXVav%2BqU4hP11Sr3XP3HXltU&X-Amz-Algorithm=AWS4-HMAC-SHA256&X-Amz-Date=20231222T141307Z&X-Amz-SignedHeaders=host&X-Amz-Expires=300&X-Amz-Credential=ASIAQ3PHCVTY2NVLNWHR%2F20231222%2Fus-east-1%2Fs3%2Faws4_request&X-Amz-Signature=6ffc20ade40c02b623f4d5ee50e11ff6c41a47a3ca1f81fe1fa9330ebb948c22&hash=a19d56c1d705c4e4cb31cd1e1cc30c9f653786a793ca2d4af5f5af08380d3b9f&host=68042c943591013ac2b2430a89b270f6af2c76d8dfd086a07176afe7c76c2c61&pii=S0165027020303459&tid=spdf-d2a7f077-05fb-4cbb-8fb9-894ae50da720&sid=e801beab6b98514bf58ac765703cea3b3316gxrqa&type=client&tsoh=d3d3LnNjaWVuY2VkaXJlY3QuY29t&ua=0f135a515f5f02570252&rr=8398f6b3a9ba9c6c&cc=us). During the course of the training, mice were kept head-fixed on the running wheel for an increasing amount of time, starting with 5-minute sessions and ending with 90-minute sessions. At the end of the second week of training, mice began receiving 2 intraperitoneal (IP) injections during each training session (method described below). All training and experimental sessions were performed during the dark cycle.

**Experimental sessions**

After mice completed 3 weeks of habituation to the experimental setup, they underwent 1 recording a day for 3 consecutive days. Half of the animals received isoflurane on the first day and psilocybin on the second day, and the other half experienced the reverse order. The urethane recording always took place on the third day because it is a terminal procedure (cite?). During each recording session, mice were head-fixed on a wheel and data was collected for 30-90 minutes. On the isoflurane and psilocybin recording days, there was a period of x minutes of baseline activity recorded during the awake state before administering the drug, followed by x minutes after drug administration. On the urethane day, there was no baseline period recorded due to constraints inserting the intravenous catheter while the mouse was head-fixed on the running wheel.

**Video Recordings**

Videos of the eye and body were acquired at 30hz and 60hz, respectively, during the experimental sessions. The angular position of the running wheel was acquired by a dedicated computer with a National Instruments card acquiring digital inputs at 100 kHz, which was considered the master clock. A 32-bit digital “barcode” was sent with an Arduino Uno (DEV-11021, SparkFun Electronics, Niwot, Colorado) every 30 seconds to synchronize all devices (cite Siegle, Jia et al., 2021?).

**Psilocybin and Ketanserin Administration**

To administer psilocybin, a protocol was developed to give an intraperitoneal (IP) injection while the animal was head-fixed on the wheel. The researcher would scruff the loose skin over the mouse’s back and immobilize the 2 hind legs to prevent the mouse from moving. While the animal was restrained, the researcher administered the injection into the peritoneal cavity on the lower right quadrant of the mouse’s abdomen. During psilocybin recordings, mice received one injection of sterile saline (1 mg/kg, IP; check brand) followed 10-12 minutes later by an injection of psilocybin (1mg/kg, IP; Usona Institute). During psilocybin+ketanserin recordings, the saline injection was substituted with a ketanserin (1 mg/kg, IP; S006, Sigma-Aldrich) injection.

**Anesthesia Scale**

To ensure a reliable and reproducible state of consciousness during both isoflurane and urethane states, a behavioral scale was developed based on Devor and Zalkind, 2001 and implemented to test the depth of anesthesia (Devor and Zalkind, 2001). The scale quantifies the mouse’s posture and response to noxious stimuli on a scale of 0-4, where a higher number corresponds to the response seen during a deeper level of anesthesia (reference table here). The four tests included an assessment of muscle tone and voluntary movement (posture), withdrawal reflexes of tail and foot when a firm pinch was applied (300-500 grams of force), and response to the presence of a noxious alcohol swab near the whiskers. The sum of the scores from each individual test ranged from 0-16 and represented 4 states on the continuum of consciousness. A summed score < 6 corresponded to a “conscious” state, while a score of 6-10 represented a “mildly anesthetized” state. To be considered an anesthetized state, a mouse needed to achieve a score ≥ 11 on the anesthesia scale, which corresponds to either a “completely unconscious” state (11-13) or a “deep anesthesia” state (14-16).

**Isoflurane Administration**

To induce isoflurane anesthesia while head-fixed on the wheel, the mouse received inhalant isoflurane delivered through a small tube placed in front of the mouse’s nose. After 2-5 minutes at 5% isoflurane, the flow was reduced to 1-2% for the remainder of the recording. 10 minutes after the start of isoflurane, the mouse was given a score based on the anesthesia scale. If the score was lower than 11, the percentage of isoflurane was increased (up to 3% - check data?) and the test was administered again 10 minutes later until the mouse scored 11 or higher on the scale. Throughout the recording, the precise concentration of isoflurane being administered was recorded. During anesthesia, a heating pad was placed under the animal and set at 37.5 °C to maintain thermoregulation. At the end of the recording, the isoflurane was switched off and the mouse recovered before being placed back in its homecage.

**Urethane Administration**

Prior to intravenous (IV) injection of urethane, the mouse was warmed under an infrared heat lamp (add supplier) for 4 minutes to increase body temperature and promote vasodilation. The animal was then anesthetized using isoflurane inhalant (3-5%) and placed on a gel heating pad to maintain thermoregulation. The tail was dipped in hot water to increase vasodilation and then an alcohol swab was administered to sterilize the tail and increase visibility of the lateral tail vein. A beveled 31G needle attached to infusion catheter tubing (SAI [product](https://www.sai-infusion.com/collections/mouse-catheters/products/mouse-tail-vein-catheters)) was held parallel to the mouse’s tail and inserted into the lateral vein. Correct placement of the needle was confirmed by the presence of blood in the infusion tube and smooth injection of fluid without any resistance on the plunger of the syringe. Once the needle was in the vein, Vetbond Tissue Adhesive (Patterson Veterinary, Loveland, CO) was applied to the exposed area of the needle to hold it in place. Isoflurane was discontinued and the mouse was moved to a restrainer where the urethane (1.5-1.8g/kg; sigma? brand) was administered via the tail vein catheter at a rate of 0.02mL/min. The depth of anesthesia was tested 60-90 minutes after the initial urethane injection and if the animal scored 10 or below, supplemental doses (20% of initial dose) were administered (up to 60% of initial dose) until the animal achieved a score of 11 or higher. When the IV injection was unsuccessful (6 out of 19 animals), urethane (1.5-1.8g/kg) was administered in two IP injections, each containing half of the full dose, 15 minutes apart. During anesthesia, a heating pad was placed under the animal and set at 37.5 °C to maintain thermoregulation.

**Behavior states / locomotion**

Across each experiment, the behavior state of the animal was categorized into 3 groups: awake resting, awake running, and anesthetized. Awake resting was defined as a period when the mouse's speed (measured by the wheel’s angular velocity) was between 0-0.1 cm/s. If the mouse’s speed was greater than 0.1 cm/s it was classified as a period of awake running. The anesthetized period began once the animal reached a score of 11 or higher on the anesthesia scale. Transitional states between awake resting/running and anesthesia were excluded from analyses.

We employed DeepLabCut (DLC), a markerless pose estimation toolbox based on deep convolutional neural networks (Nath et al., 2019), to track pupil dynamics in mouse eye videos. Each frame was annotated with 12 equidistant points around the pupil’s contour, arranged in a clockwise orientation, and 4 additional reference points along the horizontal and vertical axes. These annotations served as training data for two model types: a General Model and an Individual Model.

## **DLC General Model**

The General Model was trained on a large, diverse dataset comprising 13,959 annotated frames collected from over 90 videos spanning five distinct states of consciousness: awake, urethane anesthesia, isoflurane anesthesia, psilocybin, and psilocybin with ketanserin pretreatment (Not yet). This training dataset included both active (e.g., running) and passive states, thereby equipping the model with the ability to generalize across variable lighting conditions, eye orientations, and pupil shapes. Training was performed using DLC’s ResNet-50 architecture for 1,000,000 iterations. Once trained, this General Model was used to analyze new videos without requiring per-session retraining, offering high-throughput and reproducible pupil tracking across experimental paradigms.

## **DLC Individual Model**

For experiments requiring session-specific precision, a tailored Individual Model was used. For each new video, 150 frames were randomly sampled and manually annotated using the same 12-point pupil scheme. A separate ResNet-50 model was then fine-tuned for 500,000 iterations. This approach ensures high accuracy even in videos with atypical lighting, eye positioning, or artifact presence. The resulting model was then used to analyze the corresponding video.

## **Post-processing and Ellipse Fitting**

After analysis, only points with confidence (likelihood) ≥ 0.9 were retained. For each frame, if six or more high-confidence pupil edge points were available, an ellipse was fit using a least-squares method to approximate the pupil contour. The fitted ellipses were used to extract metrics such as pupil area, diameter, and eccentricity, providing robust quantitative measures for downstream statistical analysis.

## **Eye-Loop**

# Results

# Discussion