Consolidated Chamber Design and Protocol for Olfactory Conditioning Assay with *Drosophila melanogaster*

Sasha Bronovitskiy[2], Andres Castillo[1], Michael Yan[3], and Fang Ju Lin[1]*

[1]Department of Biology, Coastal Carolina University, Conway, SC; [2] School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA; [3]Arnold School of Public Health, University of South Carolina, Columbia, SC

The olfactory conditioning assay is widely used in Alzheimer's disease research to quantify learning and memory in *Drosophila melanogaster*. The assay tests ability to recall an aversive conditioned stimulus of scent paired with electrical shock when presented a choice between shock-associated and unrelated scents. The T-maze, a commonly used apparatus for olfactory conditioning assays, employs an elevator mechanism to transfer live flies from the shock-delivering training chamber to the scent selection point. This elevator mechanism is known to cause fly casualty. T-mazes are not commercially available and often difficult to reproduce. Other existing variations of olfactory conditioning apparatuses use airflow or automated machinery to transfer flies in place of the elevator. These alternative methods are known to inflict stress on flies during transfer, potentially altering conditioning effectiveness. A new, single-chamber apparatus was designed to address these concerns. The design consolidates the training chamber and scent selection point into one space, eliminating the need for transfer. The chamber features a flexible copper printed circuit board, which is powered off to convert the space to the non-shocking selection point. A multi-opening slider component provides controlled access to the chamber, streamlining fly insertion, training, testing, and removal. All structural elements are 3D printed, allowing for simple reproduction and alteration if desired. In preliminary trials, the single-chamber design displayed both minimal fly casualty and promise in functioning as a suitable alternative for traditional olfactory conditioning apparatuses.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by neuron loss-of-function, yielding impairments in cognition and memory. Amyloid β-42 (Aβ-42) is a toxic fragment cleaved from amyloid precursor protein and is associated with the pathogenesis of AD (1). *Drosophila melanogaster*, the fruit fly, is a commonly used transgenic model for the research of human AD. When *Drosophila* expresses the human Aβ-42 peptide, it is known to display the phenotypic pathologies associated with human AD, including memory loss and cognitive impairment. As such, *Drosophila* has been established as a valuable model to understand the neurodegenerative effects of Aβ-42 plaque aggregation in the brain (2).

The olfactory conditioning assay quantifies learning and memory in *Drosophila*, testing the ability to recall an aversive conditioned stimulus of scent paired with electrical shock when presented a choice between shock-associated and unrelated scents (3). The assay has been successfully used to observe Aβ-42 induced memory impairment in *Drosophila*. In a test of short-term memory, flies expressing Aβ-42 peptides exhibited less correct recall of the conditioned scent stimulus, when compared to that of control flies (4). The T-maze is a commonly used apparatus for olfactory conditioning, employing an elevator mechanism to transfer live flies from the shock-delivering training chamber to the scent selection point (5). This elevator mechanism is known to cause excessive fly casualty, as flies are often trapped and crushed between the elevator walls during transfer (6). It is shown that extended exposure to dead conspecifics is aversive to *Drosophila* (7), indicating that T-maze induced fly casualty may alter the effectiveness of olfactory conditioning by presenting an immeasurably variable stimulus. Other existing variations of olfactory conditioning devices use airflow or automated machinery to make the transfer between training and testing points (8, 9). These alternative methods potentially confer stress associated with mechanical agitation, as *Drosophila* is shown to release a stress-indicative odorant upon sustained periods of mechanical agitation (10), which may further disrupt the conditioning protocol.

T-mazes are not readily available for purchase, causing individual laboratory configurations to vary widely and experimental replication to be limited. In addition, some components used to construct a T-maze are prohibitively costly, inaccessible, or difficult to reproduce. Taking inspiration from Risner's 3D printed air flow-mediated conditioning device (8), we aimed to construct an easily reproducible, cost-friendly olfactory conditioning chamber that reduces fly casualty and minimizes all external forms of stress.

Methods

Chamber Fabrication

A 3D model of the chamber and slider was designed using TinkerCAD open-source modeling software (11). The design was saved as a .STL file and processed on Ultimaker's Cura (12), an open-source slicing software, prior to 3D printing. The final model was printed using a Dremel 3D DigiLab 3D45 printer and PETG translucent filament, though any neutral-colored opaque filament will suffice. A thin layer of electrical tape was placed on the outer wall of the slider to provide an airtight seal between the slider and chamber walls. A layer of smooth cardstock was pasted on the inner wall of the chamber to reduce friction between the slider and the chamber walls. The openings of slider settings 2 and 3 on the slider were covered with thin copper mesh.

Shocking Mechanism

A flexible copper printed circuit board was inserted into the inner chamber (Fig. 1) and connected to a 60V DC power source. The circuit board was designed using ExpressPCB open-source circuit software (13). This design was printed onto toner paper, transferred to a flexible copper sheet with a clothing iron, and etched with ferric chloride copper etchant solution (MG Chemicals, Ontario, CA). The power source delivered twelve 60V shocks at 1.25 seconds each, with 3.75 second pauses in between shocks. The shock sequence was determined by an Arduino UNO R3 microcontroller programmed by Risner's group (8).

Scent Delivery and Removal

Scent tubes contained 50 µL each of 63 mM 3-Octanol and 80 mM 4 -Methylcyclohexanol. In each scent tube, cotton held the scent dilution and a thin copper wire was wrapped around the cotton for easy removal. A 200 µL pipette tip was glued in a small hole cut from the bottom of each tube for an airtight connection to an assigned air pump tube. The air pump delivered a gentle airflow controlled by a precise valve-flexible tube system designed by Risner's group (8), and each scent was assigned its own flexible tube to prevent scent mixing. This airflow was essential to ensure each scent reached from its tube to flies in the conditioning chamber. A gentle vacuum was used to remove each scent from the inner chamber at designated times throughout conditioning. The vacuum was fitted with a parafilm nozzle for an airtight seal.

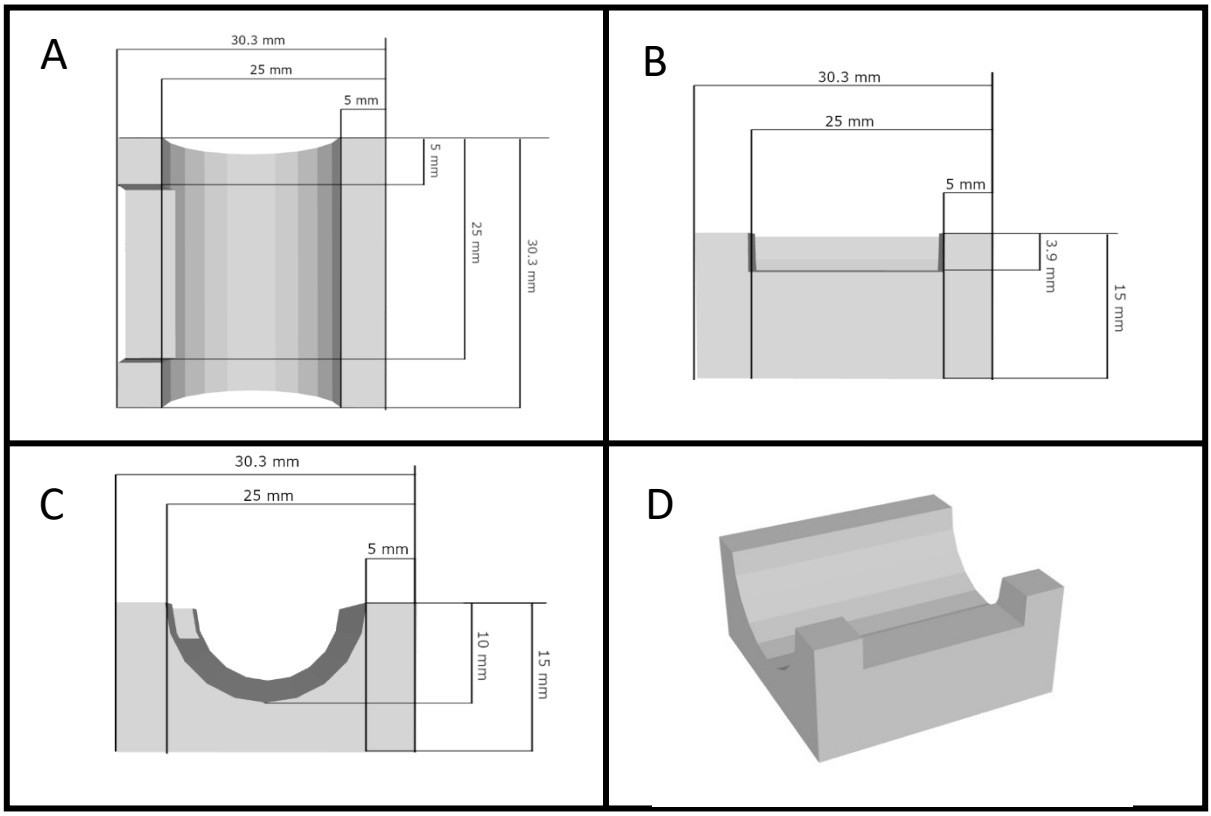

Figure 1: 3D model of flexible printed circuit board carrying inner chamber. A: upper half chamber, top view; B: upper chamber, side view; C: lower half chamber, side view; D: lower chamber, overview.

Results

Assembling and Setting Design

The testing and training points are consolidated into one space, eliminating need for transfer. The inner chamber (Fig. 1) housed the wired circuit board, which is inserted to the adaptor chamber (Fig. 2) for the slider (Fig. 3). Assembled setup is shown in Fig. 4. The inner chamber consists of upper and lower halves for easy cleaning and assembling with circuit board. The slider has 4 settings. On setting 1, live flies are transferred through funnel into the inner chamber. The solid walls of each half-setting on the slider are for rest periods during conditioning. Setting 2, the training point, contains a single copper mesh opening, so flies can be exposed to scent during conditioning without exiting the chamber. Setting 3, the scent removal point, contains two copper mesh openings, one on each side, for optimal airflow. Setting 4, the scent selection point, contains two unobstructed openings so flies can freely choose between scents.

Operational Protocol

First, 20 live flies are knocked into the chamber using a funnel on slider setting 1. Flies are given 90 seconds to acclimate on slider setting 1.5. Flies are then introduced to the first scent and shocked simultaneously for 60 seconds on slider setting 2. The scent is promptly removed with a vacuum for 30 seconds on slider setting 3. Flies are exposed to the second scent without a shock for 60 seconds on slider setting 2, and the second scent is removed with a vacuum for 30 seconds on slider setting 3. After another 90 second rest period on slider setting 3.5, flies were given a 120 second selection period on slider setting 4 (14). The protocol is conducted in dim red light to minimize any influential visual stimuli that may affect conditioning. The procedure is also documented on YouTube (14).

Discussion

The chamber design consolidates the shocking-delivering training point and the scent selection testing point into one space, eliminating the need for transfer between the two. The design allows consolidation by using a multi-opening slider mechanism that transforms the functionality of the internal chamber. Slider setting 1 contains a singular unobstructed opening, so live flies can be directed into the chamber. Setting 2, the training point, contains a single fine copper mesh opening, so flies can be exposed to scent delivered via air pump during conditioning without exiting the chamber. Setting 3, the scent removal point, contains two aligned fine copper mesh openings so that scent can be removed via vacuum from the internal chamber without disrupting the flies. Setting 4, the scent selection point, contains two aligned unobstructed openings, allowing flies to freely choose between scents after conditioning. The solid walls of each half setting on the slider are for rest periods during conditioning.

In conditioning trials with live flies, it is imperative to preserve the initial sample group and limit unintentional stimuli as much as possible. Fly casualty is prevalent with the T-maze due to its elevator mechanism which transfers flies from the shock-delivering training point to the scent selection point. In lab-made T-mazes, there is often a gap between the elevator walls that tends to trap and crush flies during transfer (6). By eliminating the need for transfer and designing the point of contact between the slider and main chamber to be airtight, fly casualty is minimized. *Drosophila* is observed to avoid dead conspecifics when naive flies are exposed to both live healthy flies and dead flies in a binary choice assay (7). If flies are killed during transfer, it is difficult to tell if a live fly makes a particular scent choice because of effective olfactory conditioning or the presence of dead conspecifics. Since the consolidated chamber eliminates the need for transfer, fly casualty is reduced, minimizing disruptions in conditioning due to the presence of dead flies and maintaining the initial sample size.

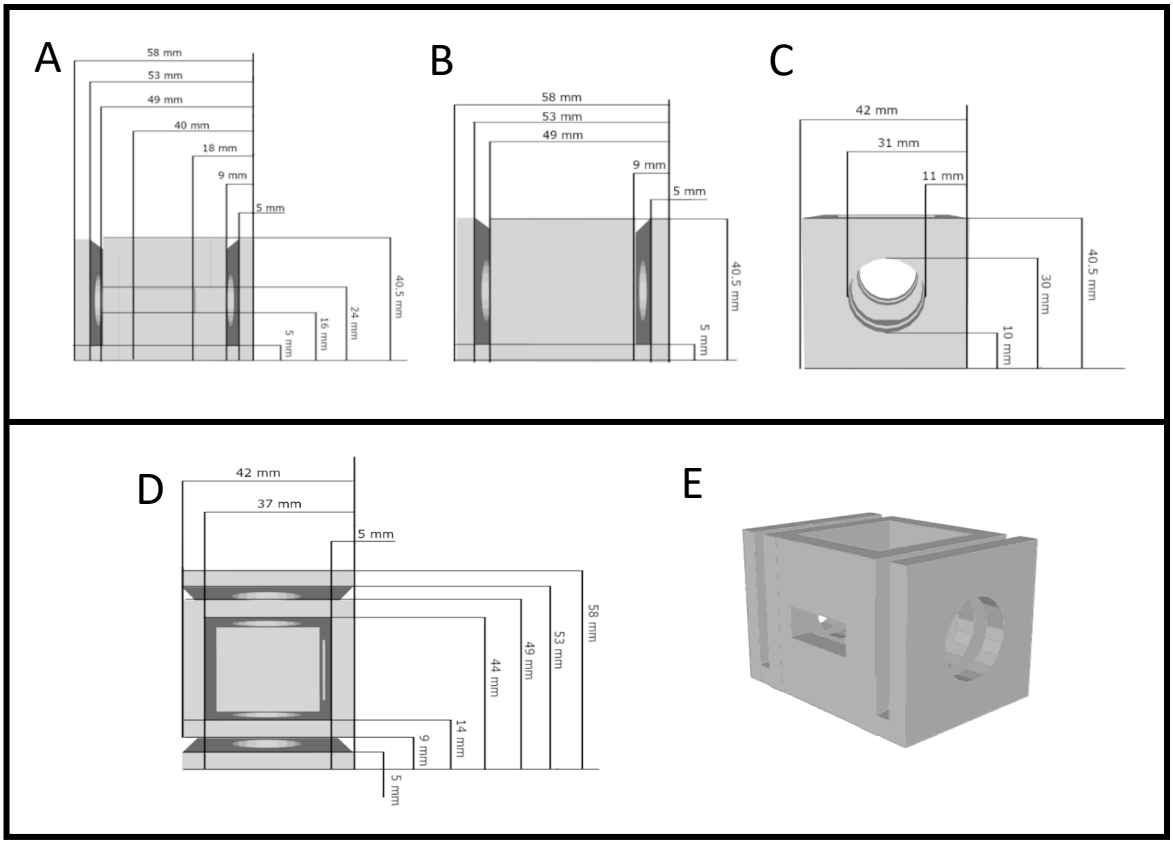

Figure 2: 3D model of adaptor chamber. A, B: side views; C: 90-degree turn from A to show openings that align with slider; D: top view with center accommodates inner chamber; E: overview, with rectangular opening to allow circuit wire to connect with power supply (not shown).

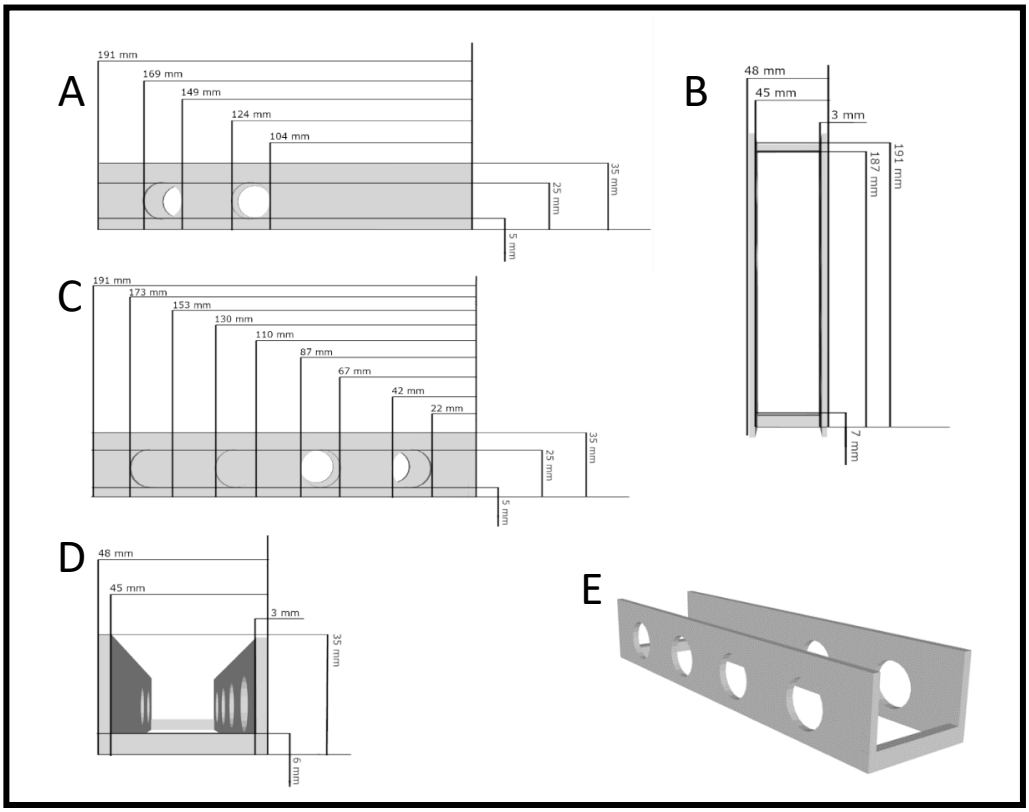

Figure 3: 3D model of slider mechanism. Four openings on one side arm and two openings on the other, for position settings 1, 2, 3 and 4.

Other iterations of olfactory conditioning devices employ airflow or mechanization to make the transfer (8, 9). Both methods may alter conditioning effectiveness by introducing the additional adverse stimulus of mechanical agitation, as *Drosophila* is known to have an adverse reaction to sustained mechanical disturbances (9). The only considerable mechanical agitation flies encountered during conditioning trials with the new chamber design was being knocked into the chamber from their storage vial during insertion on slider setting 1. While the transfer process is generally quick regardless of method, it is best to minimize all unintentional aversive stimuli that may affect the conditioning process.

Cost is an important factor to consider in the implementation of lab equipment. Inequality in Research Project Grants distributed by the National Institute of Health has increased in recent years, with the top 1% of Principal Investigators receiving about 10% of available funding and the bottom 50% receiving only 20% of funding (15). Variability in funding across labs yields the initiative to create low-cost, accessible alternatives to popular lab equipment, such as the T-maze. The T-maze is not commercially available, and construction of the device often requires custom made parts (6) that may not be financially feasible to produce for all labs. Variations in T-maze configurations resulting from funding inequality could potentially cause inconsistencies in olfactory conditioning data across labs. The consolidated chamber is designed, processed, and modifiable entirely with open-source software and can be easily distributed via .STL file. The chamber can be fabricated with any opaque PETG filament, and 3D printers are becoming increasingly available at academic institutions. All other equipment involved in the olfactory conditioning protocol was derived from Risner's airflow-mediated transfer chamber and found to be relatively low-cost (8).

Through preliminary trials, however, many *Drosophila* were not compelled to make a choice between scents after conditioning. Most flies remained in the chamber when flies were given full access to either scent tube during scent selection. Initially it was believed that the training phase of conditioning was not effectively long enough to induce learning in flies, though a published training protocol for olfactory conditioning was used (6). Lengthening the exposure time to each scent during training and later repeating exposure was not observed to encourage scent selection. Initial testing of a fully automated olfactory conditioning chamber noted most flies remaining in the central chamber due to large internal chamber volume, which may have limited odor detection sufficient for scent selection (9). Future iterations of the consolidated chamber design will see a decreased width of the inner chamber to ensure odor is reaching flies and may crowd flies enough to compel a choice between scents after conditioning.

Conclusion

In preliminary conditioning trials with live flies, minimal fly casualty was observed. The operational protocol minimized mechanical agitation, where the only noticeable agitation, other than shocking, was fly insertion. The chamber can be replicated with readily available, low-cost materials and its dimensions can be easily adjusted with open-source online software. However, the size of the inner chamber was found to be too large, and flies were not adequately crowded to compel a distinct choice during scent selection. With slight dimensional modifications, this consolidated chamber shows a promising alternative to traditional olfactory conditioning assay apparatuses.

Acknowledgements

This project was supported by grant P20GM103499-20 (SC INBRE) from the National Institute of General Medical Sciences, National Institutes of Health. Thanks to Alyssa Risner, Shawn Scialabba, Phillip Strickland, and Alvina Emran for their fundamental contributions to this project and SC-INBRE for their support. * corresponding author email: flin@coastal.edu

Notes and References

**Corresponding author email: flin@coastal.edu*

[1] Sun, X., Chen, W-D., & Wang, Y-D. (2015). β-Amyloid: the key peptide in the pathogenesis of Alzheimer's disease. Front Pharmacol. 6:221.

[2] Iijima, K., Liu, H-P., Chiang, A-S., Hearn, S. A., Konsolaki, M., & Zhong, Y. (2004). Dissecting the pathological effects of human Aβ40 and Aβ42 in *Drosophila*: a potential model for Alzheimer's disease. Proc Natl Acad Sci USA, 101(17): 6623.

[3] Guven-Ozkan, T., & Davis, R. L. (2014). Functional neuroanatomy of *Drosophila* olfactory memory formation. Learn Mem, 21(10), 519.

[4] Kaldun, J. C., Lone, S. R., Humbert Camps, A. M., Fritsch, C., Widmer, Y. F., Stein, J. V., Tomchik, S. M., & Sprecher, S. G. (2021). Dopamine, sleep, and neuronal excitability modulate amyloid-β–mediated forgetting in *Drosophila*. PLOS Biol 19(10), e3001412.

[5] Malik, B. R., & Hodge, J. J. (2014). *Drosophila* Adult Olfactory Shock Learning. J Vis Exp, 90:e50107

[6] DNA Learning Center. (2010, September 10). Training Flies in a T-Maze [Video]. Josh Dubnau: https://dnalc.cshl.edu/view/1720-Training-Flies.html

[7] Chakraborty, T. S., Gendron, C. M., Lyu, Y., Munneke, A. S., DeMarco, M. N., Hoisington, Z. W., & Pletcher, S. D. (2019). Sensory perception of dead conspecifics induces aversive cues and modulates lifespan through serotonin in *Drosophila*. Nat Commun, 10: 2365.

[8] Risner, A., Scialabba, S., Strickland, P., Emran, A., Limpasuvan, V., & Lin, F.J. (2017). A New Generation of Maze for a *Drosophila* Olfactory Memory Assay. Proceedings of National Conference on Undergraduate Research (NCUR). Also on YouTube: https://www.youtube.com/watch?v=K4UKbN1QDc4

[9] Jiang, H., Hanna, E., Gatto, C. L., Page, T. L., Bhuva, B., & Broadie, K. (2016). A fully automated Drosophila olfactory classical conditioning and testing system for behavioral learning and memory assessment. J Neurosci Methods, 261: 62.

[10] Yost, R. T., Liang, E., Stewart, M. P., Chui, S., Greco, A. F., Long, S. Q., McDonald, I. S., McDowell, T., McNeil, J. N., & Simon, A. F. (2021). Drosophila melanogaster stress odorant (dSO) displays the characteristics of an interspecific alarm cue. J Chem Ecol., 47(8–9): 719.

[11] *Tinkercad* (version 2021). 2021. Autodesk.

[12] *Ultimaker CURA* (version 4.0). https://ultimaker.com/software/ultimaker-cura

[13] *ExpressPCB/SCH* (version 7.5.0). 2021. Windows. ExpressPCB.

[14] CCU INBRE. (2021, August 4). Olfactory Conditioning Assay Singular Chamber Design [Video]. YouTube: https://www.youtube.com/watch?v=DiHt9aVSbhc.

[15] Michael S Lauer, & Roychowdhury D. (2021) Inequalities in the distribution of National Institutes of Health research project grant funding. eLife 10:e71712.