

# Noninvasive 40-Hz light flicker to recruit microglia and reduce amyloid beta load

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**Microglia**, the primary immune cells of the brain, play a key role in pathological and normal brain function. Growing efforts aim to reveal how these cells may be harnessed to treat both neurodegenerative diseases such as Alzheimer's and developmental disorders such as schizophrenia and autism. We recently showed that using noninvasive exposure to 40-Hz white-light (4,000 K) flicker to drive 40-Hz neural activity transforms microglia into an engulfing state and reduces amyloid beta, a peptide thought to initiate neurotoxic events in Alzheimer's disease (AD). This article describes how to construct an LED-based light-flicker apparatus, expose animals to 40-Hz flicker and control conditions, and perform downstream assays to study the effects of these stimuli. Light flicker is simple, faster to implement, and noninvasive, as compared with driving 40-Hz activity using optogenetics; however, it does not target specific cell types, as is achievable with optogenetics. This noninvasive approach to driving 40-Hz neural activity should enable further research into the interactions between neural activity, molecular pathology, and the brain's immune system. Construction of the light-flicker system requires ~1 d and some electronics experience or available guidance. The flicker manipulation and assessment can be completed in a few days, depending on the experimental design.

## Introduction

Since the discovery of amyloid beta (A $\beta$ ) aggregates in the brains of AD patients, hundreds of studies have examined the production and clearance mechanisms of these peptides, based on the theory that their accumulation initiates a series of neurotoxic events in AD<sup>1–5</sup>. Primary therapeutic strategies for AD have focused on reducing amyloid load by reducing A $\beta$  production or by using antibodies to remove it from the brain<sup>6</sup>. However, these pharmacological approaches have failed to produce effective therapies for the disease<sup>6,7</sup>. To date, therapeutic strategies have not made use of the brain's endogenous clearance mechanisms such as the engulfment of A $\beta$  by microglia. Clearance via microglia, the brain's primary immune cells, or other endogenous mechanisms may provide a more efficacious therapeutic approach to AD. The role of microglia in AD is currently an active area of investigation, but this research is limited by a lack of noninvasive methods to manipulate these cells<sup>8,9</sup>. Thus, noninvasive methods for stimulating the brain's immune cells and other endogenous clearance mechanisms are needed, both as a potential therapeutic strategy and as a foundation for further basic and translational research.

## Development of the approach

Recently, we discovered a noninvasive method to drive neural activity at 40 Hz, or gamma oscillations, that recruits microglia to a state associated with clearance and reduces A $\beta$  in mouse models of AD<sup>10</sup>. We found that driving 40-Hz neural activity for 1 h leads to the transformation of microglial morphology to an engulfing state, an increase in amyloid/microglia colocalization, and 40–50% reduction in A $\beta$  levels in young pre-plaque mice<sup>10–13</sup>. We achieved these effects by simply exposing animals for 1 h to white light (4,000 K) flickering at 40 Hz, similar to a high-speed strobe light. This method of brain stimulation via sensory exposure is based on prior studies showing that visual cortices entrain to flickering light at a range of frequencies<sup>14</sup>. We implemented 40-Hz light flicker

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after initially discovering that driving 40-Hz oscillations reduced amounts of A $\beta$  and altered microglia while using optogenetic stimulation of parvalbumin (PV) interneurons in the hippocampus<sup>10</sup>. The effects of light flicker on A $\beta$  were frequency specific: 20-Hz and 80-Hz light flicker did not reduce amyloid levels, whereas 40-Hz light flicker reduced amyloid levels in multiple well-validated mouse models of AD. We have successfully performed light-flicker exposure, as well as microglial and A $\beta$  assays, with 5XFAD mice at 3 months of age, when these animals have elevated amyloid levels, and at 6 months of age, after plaque development<sup>15</sup>. 5XFAD mice are a well-established model of AD that carries five familial AD mutations, including human transgenes for APP with the Swedish (K670N and M671L), Florida (I716V), and London (V717I) FAD mutations, as well as human PS1 harboring two FAD mutations, M146L and L286V. We have also found reduced A $\beta$  levels in APP/PS1 mice and in aged wild-type (WT) mice. APP/PS1 mice are another well-validated AD model in which human transgenes for both APP bearing the Swedish mutation and PSEN1 containing an L166P mutation are expressed under the Thy1 promoter<sup>16</sup>. In all cases, the animals had sufficient A $\beta$  levels such that changes were detectable, an important consideration in study design. We examined these effects in the visual cortex, where we have shown that light flicker entrains 40-Hz oscillations. These results show that our approach can be generalized to multiple transgenic mouse lines and to amyloid expressed under endogenous conditions as in WT mice<sup>10,15,16</sup>. Many researchers interested in mechanisms to recruit microglia, reduce amyloid levels, and drive brain oscillations could readily use this simple light-flicker approach.

### Overview of the procedure

Here we describe the construction of a light-flicker-exposure apparatus, methods of light exposure, appropriate control stimuli, and assays of the effects of light flicker. The frequency of light flicker is critical to alteration of microglia and A $\beta$ ; therefore, the on and off timing of lights must be controlled with millisecond precision. We provide a complete description of how to build such a precise light-flicker apparatus (Steps 1–23). As in many experiments, control conditions are also critically important and will depend on the exact experimental goals (Steps 24–28 and Experimental design). Thus, we include a discussion of appropriate control groups and the conditions for their implementation (Experimental design). The effects of 40-Hz light flicker can be studied with many different assays. In this protocol, we present a few key assays that we have performed with reliable results, focusing on histological analysis of microglia using standard immunohistochemistry (IHC; Step 29A) and Clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue-hydrogel (CLARITY) tissue clearing with system-wide control of interaction time and kinetics of chemicals (SWITCH) immunolabeling (Step 29B)<sup>17,18</sup>. Many other methods of analyzing microglia and A $\beta$  can also be used to examine the effects of 40-Hz light flicker.

### Advantages and limitations of the approach

To our knowledge, this is the first method to promote microglial engulfment without inducing neural damage. A noninvasive approach is important when studying microglia because these immune cells respond to tissue damage and small changes in their environment<sup>12,13</sup>. Therefore, this light-flicker approach could be used to study neuroimmune interactions broadly. Microglia are implicated in multiple diseases, both neurodegenerative and developmental, and driving gamma oscillations via flicker could elucidate the effects of microglial engulfment in a wide range of diseases. This approach could also be used to study how reducing A $\beta$  alters neural function. Whether the observed effects extend to brain regions beyond the visual cortex has yet to be determined. Indeed, one of the primary limitations of this approach is that it may not extend to every brain region desired. Furthermore, it has yet to be determined whether this approach will have the same effects in other species besides mice. Another primary disadvantage of this approach is that the mechanisms by which entraining 40-Hz oscillations recruits microglia and reduces A $\beta$  are unknown. However, these mechanisms that remain to be identified provide extensive opportunities for new research.

Although 40-Hz activity can also be driven using optogenetic stimulation of PV interneurons, optogenetics is typically invasive, requiring injection of a virus and insertion of an optical fiber<sup>19,20</sup>. Other noninvasive forms of brain stimulation, such as transcranial alternating current stimulation, may achieve similar results; however, it has yet to be shown whether they can produce robust 40-Hz activity, microglial transformation, and A $\beta$  reduction. Beyond being noninvasive, light

flicker is relatively easy and fast to implement. Once the flicker apparatus is set up and all materials are acquired, a flicker manipulation can take only a few hours and results can be obtained in days, depending on the assay used. In short, this light-flicker approach is simple, fast, and noninvasive.

## Experimental design

### Selection and grouping of animals

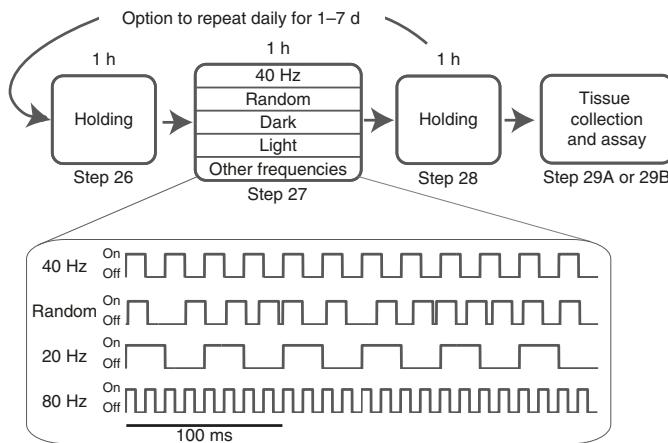
The selection and grouping of animals must be carefully considered before starting an experiment. Animals must have adequate A $\beta$  levels such that changes are detectable (e.g., aged WT mice or transgenic mice such as 5XFAD and APP-PS1, as we have used previously). When planning to make a comparison between groups, animals from one litter should be evenly distributed between the groups that will be compared. Males and females should be tested separately, as we do not yet know how the effects of flicker treatment differ by sex. In many cases, multiple litters or cohorts of animals will need to be combined to have enough animals per condition. In such cases, mice should be age-matched across the cohorts as closely as possible, and animals of different ages should be evenly dispersed between condition groups. We recommend normalizing to control conditions within each cohort to account for variability between cohorts. For normalization, the average value of the control condition is calculated as baseline (or 100%). Controls and experimental groups within the cohort are then expressed relative to that baseline.

### Experimental and control groups

In designing a study using light flicker, appropriate experimental and control groups must be determined. Importantly, experimenters must be blind to treatment conditions, especially during tissue processing, imaging, and analysis. The primary experimental group contains the animals that are exposed to 40-Hz light flicker, which entrains the visual cortex to 40 Hz. Other stimulation groups may include animals exposed to other frequencies of light flicker, such as 20 Hz or 80 Hz, to see whether the effects are frequency specific. Note that very fast flicker frequencies, such as 80 Hz, are generally too fast to be perceived as flickering. Controls may include continuous light on (light), continuous light off (dark), or randomized stimulation. In the light or dark conditions, animals are exposed to constant ambient light or lack thereof, and the visual cortex does not show elevated 40-Hz power<sup>10</sup>. In the randomized stimulation condition, the intervals between light flashes when the lights are off are randomly selected, with intervals  $> 0$  and that average 25 ms (40 Hz). In this condition, animals are exposed to the same average number of light pulses over time, but the visual cortex neurons do not entrain to 40 Hz. Therefore, the randomized stimulation condition controls for the overall amount of stimulation. In our initial study, we found that firing rates did not significantly differ ( $P > 0.09$ ) between random and 40-Hz flicker, indicating that this condition can control for overall changes in activity levels due to sensory stimulation.

### Light flicker exposure

To implement light flicker, we used white light-emitting diodes (LEDs), which include a combination of visible wavelengths (390–700 nm). Specifically, we used LEDs that have a correlated color temperature (CCT) of 4,000 K at intensities between 100 and 480 lux. We have not tested other wavelengths, CCTs, or intensities. We have typically programmed lights to turn on for 50% of each duty cycle (percentage of each cycle that the lights are on) because that produces a clear and bright flickering stimulus. Furthermore, lights are on for the same total amount of time over the course of flicker exposure (50% of the time), even though the frequency of stimulation varies. Example stimuli with **50% duty cycles** include a 40-Hz stimulus (25-ms period) with lights on for 12.5 ms and off for 12.5 ms, a 20-Hz stimulus (50-ms period) with lights on for 25 ms and off for 25 ms, and an 80-Hz stimulus (12.5-ms period) with lights on for 6.25 ms and off for 6.25 ms (see Fig. 1 and the Supplementary Data for source code). We have not explicitly tested how the duty cycle impacts the results; however, we have observed that if the lights are on for only 1 ms per cycle, the flicker appears dimmer. One study that examined the effects of light-flicker duty cycle on EEG responses found that for flicker at 11–22 Hz, 50% duty cycles tended to more reliably induce entrainment in the human **occipital lobe** than 25% or 10% duty cycles<sup>21</sup>. However, whether the duty cycle has similar effects for flicker at  $\geq 40$  Hz was not addressed. To control for the total amount of time the light is on between the 40-Hz and random conditions, during random stimulation, lights are on for 12.5 ms in each duty cycle, and intervals of dark between light pulses are randomized and are  $> 0$  so



**Fig. 1 | Diagram of exposure timing and sample stimulus conditions.** Top, animals are held in a quiet area for at least 1 h and then exposed to 1 h of stimuli, followed by 1 h in the holding area. Animals are then killed and tissue is collected for experiments that require a single exposure session. Alternatively, to assess the effects of longer stimulation, the stimulus procedure may be repeated every day at the same time for multiple days before tissue is collected. Bottom, traces of transistor-transistor logic (TTL) pulses that turn the lights on (high) or off (low). Example stimuli are shown, including 40-Hz, random, 20-Hz, and 80-Hz flicker with 50% duty cycles. For random stimulation, lights are on for 12.5 ms and off for a randomized interval > 0 that averages 12.5 ms. For example, we have used random stimulation with off intervals that ranged from 0.2 to 34.1 ms.

that light pulses do not overlap. The duration of exposure to these stimuli will depend on the experimental goals of the project. We have found that 1 h of stimulation followed by 1 h of darkness reduces A $\beta$  levels and alters microglial morphology in animals with elevated amyloid levels<sup>10</sup>. By 24 h after light flicker, A $\beta$  levels return to baseline. In animals with plaques, we have found that 1 h of stimulation per day for 7 d reduces levels of insoluble A $\beta$ , plaque size, and plaque number. For repeated exposure, the time of day should be kept consistent<sup>10</sup>. During all types of exposure, animals should be singly housed. Broadly speaking, we expect the effects of more stimulation to accumulate and thus have a more marked effect; however, this must be tested and will depend on the experiment performed.

### Sources of variability

Experimenters should be aware of several key sources of variability. The use of improper controls or imprecise flicker frequencies can attenuate differences between groups. To prevent these issues, the flicker frequency precision must be tested. Furthermore, animals should be grouped appropriately, with litters split between conditions as evenly as possible. Animals of different ages should be assessed separately, and males and females should be assessed separately because levels of amyloid or responses to stimulation conditions may differ by age and sex. Because amyloid levels can vary within litters, we recommend using within-group normalization. Finally, small sample size could contribute to variability if not enough tissue or animals are used.

## Materials

### Reagents

- Mice: The exact strain and age depend on the experimental design (see ‘Experimental design’ section in Introduction). In the procedures described in this protocol, we used 5XFAD and APP/PS1 mice from established breeding colonies at MIT. **! CAUTION** All procedures involving animals must be performed in strict compliance with institutional and federal regulations concerning laboratory animals. The procedures that we describe here were approved by the Division of Comparative Medicine (DCM) at the Massachusetts Institute of Technology. **▲ CRITICAL** The disease model must have adequate A $\beta$  levels such that changes are detectable. This will depend on the mouse line of interest; for example, 5XFAD animals >3 months old have adequate levels of A $\beta$ .
- Paraformaldehyde (32% (wt/vol); PFA; Electron Microscopy Sciences, cat. no. 15714-S) **! CAUTION** This solution can be potentially harmful to the skin, eyes, and respiratory and alimentary tracts. Wear eye/face protection, gloves, a lab coat, and a respirator or equivalent and use a fume hood when handling.

- Triton X-100 (Fisher Biotech, cat. no. BP151-100) !**CAUTION** This liquid can be potentially harmful to the skin, eyes, and respiratory and alimentary tracts. Handle with caution in a well-ventilated room and wear eye/face protection and gloves.
- Normal donkey serum (EMD Millipore, cat. no. S30-100ML)
- Phosphate-buffered saline (PBS; Sigma Life Sciences, cat. no. P5368-10PAK)
- Hoechst 33258 (Sigma-Aldrich, cat. no. 94403) !**CAUTION** This liquid can be potentially harmful to the skin, eyes, and respiratory and alimentary tracts. Handle with caution and wear eye/face protection and gloves.
- Fluoromount G mounting medium (Electron Microscopy Sciences, cat. no. 17984-25)
- Sodium azide (Millipore Sigma, cat. no. S2002) !**CAUTION** This liquid can be potentially harmful to the skin, eyes, and respiratory and alimentary tracts. Handle with caution in a well-ventilated room and wear goggles and gloves.
- Acrylamide (40% (wt/vol); Bio-Rad, cat. no. 161-0140) !**CAUTION** This liquid can be potentially harmful to the skin, eyes, and alimentary tracts. It may cause cancer, genetic defects, and organ damage through prolonged or repeated exposure. Handle with caution in a well-ventilated room and wear eye/face protection and gloves.
- Glutaraldehyde (10% (wt/vol); Electron Microscopy Sciences, cat. no. 16110) !**CAUTION** This liquid is combustible and can be potentially harmful to the skin, eyes, and respiratory and alimentary tracts. Handle with caution in a well-ventilated room and wear eye/face protection and gloves.
- Sodium dodecylsulfate (Sigma-Aldrich, cat. no. L3771) !**CAUTION** This powder is flammable and can be potentially harmful to the skin, eyes, and respiratory and alimentary tracts. Handle with caution in a well-ventilated room and wear eye/face protection and gloves.
- Lithium hydroxide monohydrate (Sigma-Aldrich, cat. no. 254274-50G)
- Boric acid (Sigma-Aldrich, cat. no. B7901) !**CAUTION** This powder can potentially lead to reproductive toxicity and can cause respiratory irritation. Handle with caution in a well-ventilated room and wear gloves, eye/face protection, and a respirator or equivalent during use.
- Double-distilled H<sub>2</sub>O
- This liquid can be potentially harmful to the skin, eyes, and respiratory and alimentary tracts. Handle with caution in a well-ventilated room and wear gloves, goggles, and a respirator or equivalent during use.
- Glycine (Sigma-Aldrich, cat. no. G7126)
- Sodium hydroxide (Sigma-Aldrich, cat. no. 795429) !**CAUTION** Sodium hydroxide is highly corrosive to skin, can cause serious eye damage, and can irritate the lungs if inhaled. Handle with caution in a well-ventilated room and wear gloves, goggles, and a respirator or equivalent during use.
- Sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>; Sigma-Aldrich, cat. no. S7907)
- Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>; Mallinckrodt Chemicals, cat. no. 7100-12)
- BSA (Gemini Bio-Products, cat. no. 700-100P)
- Histodenz (Sigma-Aldrich, cat. no. D2158-100G)
- Neural Tissue Dissociation Kit (P) (Miltenyi Biotec, cat. no. 130-092-628)

### Antibodies

- ▲**CRITICAL** Different primary or secondary antibodies specific to the research purposes may be used. For example, we have successfully used the secondary antibodies Cy2 (488) donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, cat. no. 711-225-150) and Cy5 (647) donkey anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, cat. no. 715-605-150).
- Anti-Iba1 (rabbit) (Wako Chemicals, cat. no. 019-19741). See Box 1 on characterizing microglia.
  - Purified anti-β-amyloid, 1–42 (12F4) antibody (mouse) (BioLegend, cat. no. 805501). See Box 2 on selection of the appropriate anti-Aβ antibody.
  - β-amyloid (D54D2) XP rabbit mAb (Cell Signaling Technologies, cat. no. 8243)
  - Allophycocyanin (APC)-conjugated CD11b mouse clone M1/70.15.11.5 (Miltenyi Biotec, 130-098-088)
  - Phycoerythrin (PE)-conjugated CD45 antibody (BD Pharmingen, cat. no. 553081)
  - Alexa Fluor 488 fragmented donkey anti-rabbit (Abcam, cat. no. ab181346)
  - Alexa Fluor 568 fragmented donkey anti-mouse (Abcam, cat. no. ab175694)
  - Alexa Fluor 488 AffiniPure donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch, cat. no. 711-545-152)
  - Alexa Fluor 647 AffiniPure Donkey anti-mouse IgG (H + L) (Jackson ImmunoResearch, cat. no. 715-605-151)

**Box 1 | Characterization of microglial activity**

Microglia are the resident immune cells of the brain and fulfill a wide variety of tasks related to active immune defense and homeostatic maintenance<sup>12,33</sup>. Previous work has primarily categorized microglia as either ‘resting’ or ‘activated’, the former considered a static phase in healthy brain tissue and the latter a reactive phase responding to an immune challenge (i.e., bacterial infection)<sup>34</sup>. However, current evidence suggests that microglial responses are not all-or-none; instead, microglia shift through a spectrum of activated states, ranging from tissue surveillance, phagocytosis, and synaptic pruning to cytotoxic and inflammatory states<sup>12,33,35</sup>. Because of their functional and phenotypic diversity, microglial activity should be analyzed through a characterization of morphology, cell markers, and transcriptional changes. Microglial markers include Iba1, used in this protocol, as well as CD11b, F4/80, CD68, CD40, and others; however, many of these markers are nonspecific and may stain for other macrophage cells or subsets of microglia in the brain.

**Box 2 | Selection of the appropriate anti-β-amyloid antibody**

Multiple antibodies react with A $\beta$ , and different antibodies are optimal for different experimental goals. For example, the purified anti-β-amyloid, 1-42 (12F4) antibody works well for IHC and is A $\beta$  specific<sup>25–28</sup>. Unlike some other A $\beta$  antibodies, the 12F4 antibody does not cross-react with amyloid precursor protein (APP) because it binds specifically to the C terminus of A $\beta$ , in particular to the A $\beta$ -42 isoform<sup>25–28</sup>. In our experience, the 12F4 antibody seems to primarily stain for nonaggregated A $\beta$ , although plaques are occasionally stained. The 12F4 antibody (mouse) also allows for co-staining with the recommended anti-Iba1 antibody (rabbit). Alternatively, anti-β-amyloid 1-16 antibody (6E10, BioLegend, cat. no. SIG-39300) and anti-β-amyloid, 17–24 antibody (4G8, BioLegend, cat. no. SIG-39200) can also be used to label A $\beta$ ; however, these antibodies may cross-react with APP. β-amyloid (D54D2) XP monoclonal antibody stains for plaques, reacting with several isoforms of human and mouse A $\beta$ , including A $\beta$ -37, A $\beta$ -38, A $\beta$ -39, A $\beta$ -40, and A $\beta$ -42<sup>31,32</sup>. However, the D54D2 antibody is cultivated in the same species (rabbit) as the anti-Iba1 antibody, and therefore they cannot be used concurrently. In addition to the antibodies described here that we have used successfully, several other antibodies for A $\beta$  and microglia are available and may serve the experimental goals of the project.

**Equipment**

- Tantalum capacitors, 47 μF, 20%, 16 V, radial (DigiKey, part no. 478-8851-ND)
- Resistors, 25 Ω, 5 W, 5% axial (DigiKey, part no. 25J25RE-ND)
- MOSFET N-CH, 60 V, 2.5, 4-DIP (DigiKey, part no. IRLD024PBF-ND)
- Z&T solderless flexible jumper wires (Amazon, cat. no. B005TZJ0AM)
- Flexible LED strip lights (white) (SuperBrightLEDs.com, part no. NFLS-NW30X3-WHT-LC2)
- Solderless breadboard (DigiKey, part no. 438-1045-ND)
- Arduino Uno SMD R3 microcontroller (model no. A000066; Amazon, cat. no. B008GRTSV6)
- Connecting wires for the Arduino: USB 2.0 Type A Male to Type B Male (Arduino, code no. M000006; Amazon, cat. no. B00NH11KIK)
- 12-V power supply: battery holder with eight AA cells, wire leads (DigiKey, part no. BH48AAW-ND) or other power supply of choice (may require AC/DC converter)
- 8 AA batteries (any; rechargeable may be preferred)
- Large-area mounted silicon photodiode (Thorlabs, part no. SM05PD1A)
- Electrical tape (any)
- Scissors (any)
- Wire stripper (corresponding to wire size AWG 16)
- Oscilloscope (any)
- Transparent box to hold mice (~11–15 inches long × 7–8 inches wide × 5–6 inches high); can be an empty mouse housing cage without bedding
- Opaque black fabric or plastic (any)
- Tape (any)
- Vibratome (Leica, model no. VT100S)
- Glass slides and coverslips (VWR VistaVision; VWR International)
- 12-Well plates (any)
- 24-Well plates (any)
- Confocal microscope (Zeiss, model no. LSM 710) for immunohistochemical imaging
- Confocal microscope (Zeiss, model no. LSM 710) with the accompanying Zen Black 2011 SP4, 64-bit software (Carl Zeiss Microscopy), for immunohistochemical imaging

- Confocal microscope (Zeiss, model no. LSM 880) with the accompanying Zen Black 2.1 SP1, 64-bit software (Carl Zeiss Microscopy), for immunohistochemical and CLARITY imaging
- Plan-Apochromat 63 $\times$ /1.4 oil, NA 0.17, differential interference contrast objective (Carl Zeiss Microscopy)
- Plan-NEOFLUAR 40 $\times$ /1.3 oil, NA 0.17, differential interface contrast objective (Carl Zeiss Microscopy)
- 40- $\mu$ m cell strainer (Falcon cell strainer, sterile; Corning, prod. no. 352340)

### Software

- Arduino software (<https://www.arduino.cc/en/Main/Software>)
- ImageJ (<http://imagej.nih.gov/ij/download.html>)
- Coloc2 plugin ([https://imagej.net/Coloc\\_2](https://imagej.net/Coloc_2))
- Bitplane Imarisx64 8.3.1 (<http://www.bitplane.com/releasenotes.aspx>)
- Source code (Supplementary Data)

### Reagent setup

#### 4% (wt/vol) PFA

Dilute 32% (wt/vol) PFA in 1 $\times$  PBS (vol/vol). Prepare PFA within 24 h of perfusion and store it at 4 °C.

#### IHC blocking buffer

IHC blocking buffer is 0.3% (vol/vol) Triton X-100 and 10% (vol/vol) normal donkey serum in 1 $\times$  PBS (vol/vol). Prepare the solution fresh on the day of the experiment and store it at 4 °C when not in use.

#### IHC primary antibodies

IHC primary antibodies consist of anti-Iba1 (rabbit) diluted 1:500 (vol/vol) in blocking buffer to label microglia (see Box 1 on characterizing microglia); purify anti- $\beta$ -amyloid, 1–42 antibody (12F4) (mouse) diluted 1:500 (vol/vol) in blocking buffer to label A $\beta$  (see Box 2 on selection of the appropriate anti-A $\beta$  antibody); and  $\beta$ -amyloid (D54D2) (rabbit) antibody diluted 1:500 (vol/vol) in blocking buffer to label plaques. Prepare the primary antibody dilutions fresh on the day of the experiment. Store the solutions at 4 °C when not in use. If co-labeling microglia and A $\beta$ , combine the primary anti-Iba1 and anti-A $\beta$  12F4 antibodies. The anti- $\beta$ -amyloid (D54D2) cannot be combined with the anti-Iba1 antibody because they are both produced in rabbits. If labeling separately, make separate primary antibody dilutions.

#### IHC secondary antibodies

IHC secondary antibodies consist of Alexa Fluor 488 AffiniPure donkey anti-rabbit IgG (H + L) diluted 1:1,000 (vol/vol) in blocking buffer, and Alexa Fluor 647 AffiniPure donkey anti-mouse IgG (H + L) diluted 1:1,000 (vol/vol) in blocking buffer. Prepare the secondary antibody dilutions fresh on the day of the experiment. If co-labeling microglia and A $\beta$ , combine the secondary Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 647 donkey anti-mouse antibodies. If labeling separately, make separate secondary antibody dilutions. Store the solutions at 4 °C when not in use and protect fluorescent antibody solutions from light.

#### CLARITY inactivation solution

CLARITY inactivation solution is 4% (vol/vol) acrylamide, 1 M glycine, and 0.1% (vol/vol) Triton X-100 in 1 $\times$  PBS. Store the solution at room temperature and replace if the solution becomes cloudy or a precipitate forms.

#### CLARITY clearing buffer (pH 8.5–9.0)

CLARITY clearing buffer is 200 mM sodium dodecylsulfate, 20 mM lithium hydroxide monohydrate, and 4 mM boric acid in double-distilled H<sub>2</sub>O. Store the solution at room temperature and replace if the solution becomes cloudy, the pH changes, or a precipitate forms.

#### CLARITY PBST (1 $\times$ )

CLARITY PBST is 0.1% (vol/vol) Triton X-100 in 1 $\times$  PBS. Store the solution at room temperature when not in use. Replace if the solution becomes cloudy.

**SWITCH blocking solution**

SWITCH blocking solution is 2% (wt/vol) BSA in PBST. Prepare the solution fresh on the day of the experiment and store at 4 °C when not in use.

**SWITCH weak binding buffer (pH 8.5–9.0)**

SWITCH weak binding buffer is 37.75 mM Na<sub>2</sub>HPO<sub>4</sub>, 3.53 mM KH<sub>2</sub>PO<sub>4</sub>, and 0.02% (wt/vol) sodium azide in PBST. Store the solution at room temperature and replace if the solution becomes cloudy, the pH changes, or a precipitate forms.

**SWITCH primary antibodies**

SWITCH primary antibodies consist of purify anti-β-amyloid, 1–42 (12F4) (mouse) and anti-Iba1 (rabbit) primary antibodies diluted to 1:100 (vol/vol) in PBST to label Aβ and microglia concurrently (see Box 2 on selection of the appropriate anti-β-amyloid antibody and Box 1 on characterizing microglia). Prepare the primary antibody dilutions fresh on the day of the experiment. Store the solutions at 4 °C when not in use.

**▲ CRITICAL** If co-labeling microglia and Aβ, combine the primary anti-Iba1 and anti-Aβ 12F4 antibodies. If individually labeling microglia or Aβ, do not combine the primary anti-Iba1 and anti-Aβ 12F4 antibodies.

**SWITCH reversal buffer (pH 7.4)**

SWITCH reversal buffer is 37.75 mM Na<sub>2</sub>HPO<sub>4</sub> and 3.53 mM KH<sub>2</sub>PO<sub>4</sub> in 0.02% (wt/vol) sodium azide in PBST. Store the solution at room temperature and replace if the solution becomes cloudy, the pH changes, or a precipitate forms.

**SWITCH secondary antibody**

SWITCH secondary antibody consists of Alexa Fluor 488 fragmented donkey anti-rabbit and Alexa Fluor 568 fragmented donkey anti-mouse secondary antibodies diluted to 1:100 (vol/vol) in PBST. Prepare the secondary antibody dilutions fresh on the day of the experiment. Store the solutions at 4 °C when not in use and protect fluorescent antibody solutions from light. **▲ CRITICAL** To individually label microglia or Aβ, do not combine the secondary Alexa Fluor 488 fragmented donkey anti-rabbit and Alexa Fluor 568 fragmented donkey anti-mouse antibodies. **▲ CRITICAL** For 100-μm sections (Step 29B), standard full-chain secondary antibodies may be substituted (i.e., Alexa Fluor 488 AffiniPure donkey anti-rabbit IgG (H + L)). Fragmented secondary antibodies are recommended for adequate diffusion into larger-volume tissues, such as whole brains or sections >1 mm thick.

**SWITCH refractive index matching solution**

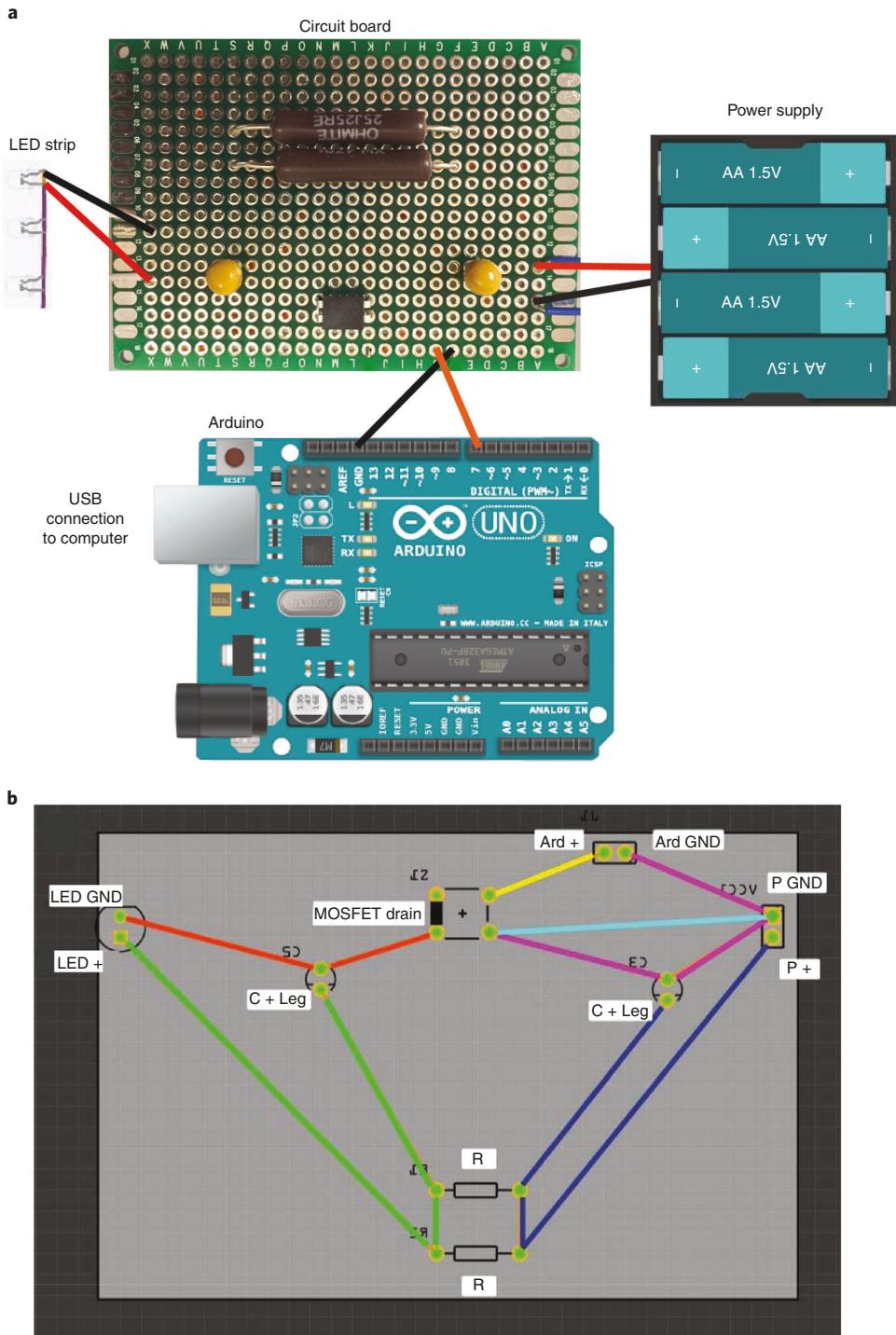
SWITCH refractive index matching solution (RIMS) is 75 g of Histodenz, 20 mL of 0.1 M phosphate buffer, and 0.02% (wt/vol) sodium azide in 60 mL of double-distilled H<sub>2</sub>O. Store the solution at room temperature and replace if the solution becomes cloudy or a precipitate forms.

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**Procedure****Circuit configuration ● Timing ~1 d**

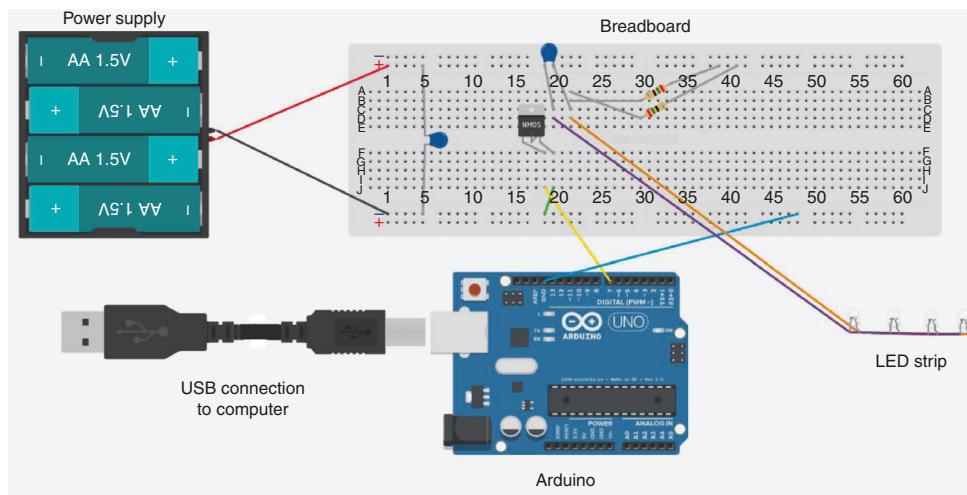
**▲ CRITICAL** This section describes how to assemble the breadboard circuit required to generate the 40-Hz flicker. The breadboard circuit can be easily disassembled, which is optimal for testing and debugging, but not ideal for long-term use. To produce a more durable flicker circuit, construct a soldered circuit as shown in Fig. 2. This soldered circuit requires the same materials as the breadboard circuit and additional items described in the figure legend. Note that a soldered circuit will require more time to construct than a breadboard circuit.

1. Connect the power supply by connecting the red wire to the red bus (12-V power) on the breadboard (second row from the top in the schematic shown in Fig. 3). Note that the top and bottom two rows of the breadboard are the red bus (+) and blue bus (-), and are typically used to supply power to the circuit. All holes within one bus are connected to each other; therefore, one can connect a wire to any row within the bus.
2. Connect the black wire to the blue bus (12-V ground) on the breadboard (second row from the bottom in the schematic in Fig. 3).
3. Connect the capacitors. Start by identifying the positive leg of each capacitor (usually, the longer leg).
4. In row 5 (Fig. 3), connect the positive leg of one capacitor to the red bus and connect the negative leg to the blue bus. Use a jumper wire to extend the reach of the capacitor, if necessary.
5. Using another capacitor, connect the positive leg to 22C and connect the negative leg to 20C (Fig. 3).
6. Connect one of the legs of one resistor to 22B and connect the other leg to the red bus (Fig. 3).



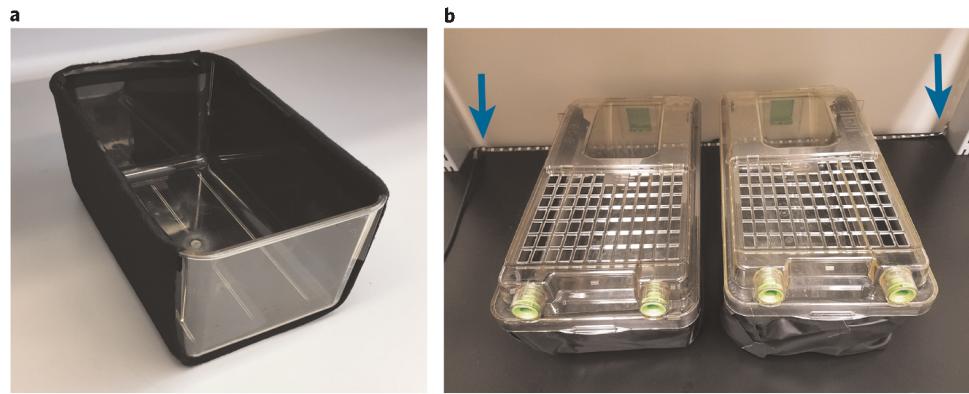
**Fig. 2 | Alternative circuit option: soldered circuit.** **a**, Schematic view of circuit elements on the top side of a perforated circuit board (top left; Digi-Key, prod. no. V2025-ND) connected to a power supply (top right), lights (top, far left), and Arduino (bottom). **b**, Schematic view of the underside of the same circuit board. Colored lines represent the connections to be soldered together using wire (insulated, PVC-coated, 30-AWG (American wire gauge) wire-wrapping wire; prod. no. DM-30-1000; Amazon, cat. no. BOOSUVMYLO), soldering lead, and a soldering iron (any).

7. Connect the other resistor to 22A and the red bus (Fig. 3).
  8. Identify the side of the MOSFET where the two pins are not connected to one another. These two pins are the source and the gate. On the other side of the MOSFET is the drain, which is the side where the two pins are connected to each other.
  9. Connect the source and the gate to 19F and 20F, respectively, and the drain to 19E and 20E (Fig. 3).



**Fig. 3 | Schematic of the breadboard circuit.** Circuit elements needed to flicker lights are connected on the breadboard as shown (top right). Power is supplied by a 12-V battery pack (top left). The timing of lights turning on or off is controlled by an Arduino (bottom right) that is connected to a computer via a USB A/B cable (bottom left). Note: The breadboard design can differ from that shown as long as the same connections are maintained.

10. Connect the jumper wires and the Arduino.  
**▲ CRITICAL STEP** One can also use a data-acquisition board (DAQ) and corresponding code to control the lights, instead of using an Arduino. We have used a DAQ from National Instruments and MATLAB code successfully.
  11. Using the shorter jumper wire (green in the schematic), connect one end to the blue bus and connect the other end to 20J (Fig. 3).
  12. Using one of the longer jumper wires (yellow in the schematic), connect one end to 19J and connect the other end to 7J on the Arduino (Fig. 3).
  13. Using another longer jumper wire (blue in the schematic), connect one end to the blue bus and connect the other end to the ground (GND) on the Arduino (Fig. 3).
  14. To connect the LED strip, first separate the two wires of the LED cable and then strip the ends of each wire.
  15. Identify the ground wire (the ground wire has white dashed lines along its length and is indicated by an orange line in the schematic) and connect it to 22D (Fig. 3).
  16. Connect the other wire (purple in the schematic) to 20D (Fig. 3).
  17. Connect the four-pin connection at the other end of the LED cable to one side of the LED strip.
  18. Connect multiple LED strips together using four-pin connectors.
  19. Connect the Arduino to the computer via the USB cable.
  20. To test whether the LED lights flicker, run the Arduino code that turns them on and off (see the Supplementary Data for code for 40-Hz stimuli) and turn off any overhead lights. The human eye can perceive 40-Hz flicker; therefore, when programmed to produce 40-Hz flicker, lights should visibly turn on and off. Additional Arduino code can be found in the Supplementary Data.
- ? TROUBLESHOOTING**
21. Test all stimuli included in the experimental design (see ‘Experimental Design’ section in the Introduction and the Supplementary Data for source code files).
- ? TROUBLESHOOTING**
22. To test the pulse frequency that is sent to the lights, use an oscilloscope or other equipment to record 5-V square waves (TTL pulses) and measure the intervals between square waves.
- ? TROUBLESHOOTING**
23. To test the light-flicker frequency, use a photodiode to measure the light power over time.  
**▲ CRITICAL STEP** The photodiode must be placed  $\leq 1$  cm from the LED strip in order for the flicker frequency to be accurately assessed.
- ? TROUBLESHOOTING**



**Fig. 4 | Flicker exposure setup.** **a**, Exposure boxes are covered on three sides, with the transparent side facing the LED strip lights. Box is shown here without a lid in order to show both the inside and the outside of the box. **b**, The flicker room setup consists of LED strip lights lined along a white wall on a table (blue arrows indicate the start and end of the strip lights, which run behind the cages). Exposure boxes face the LED strip lights and are covered with lids during use. A computer connected to a flicker circuit (not shown) controls light stimuli.

### Box 3 | Example stimuli that can be used with this protocol

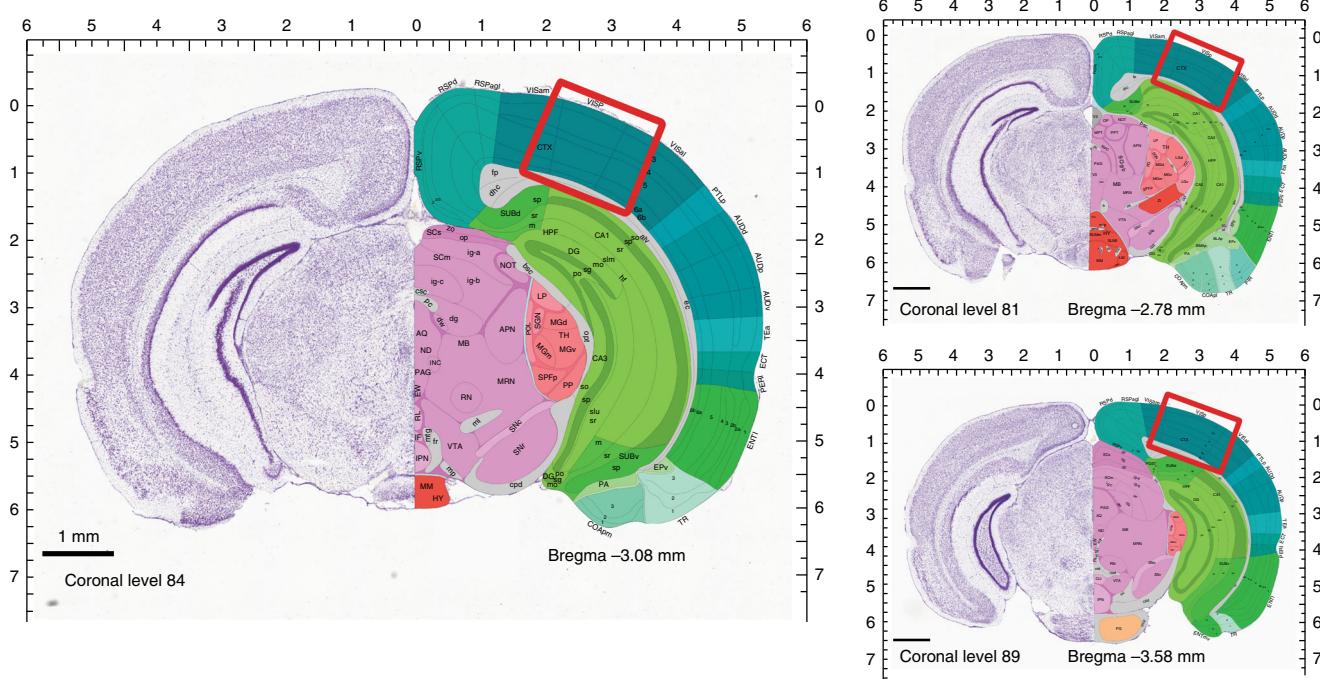
- **40-Hz flicker.** A 40-Hz flicker means that light pulses on for 12.5 ms and off for 12.5 ms. This can be used to reduce A $\beta$  levels, alter microglial morphology, and increase colocalization of A $\beta$ /microglia (Fig. 1).
- **Random stimulation.** Light pulses (12.5 ms) are delivered with a randomized interval that averages 40 Hz. This serves as a control that delivers the same amount of light pulses without generating 40-Hz oscillations in the brain (Fig. 1).
- **Lights on.** Lights are continuously on as a control.
- **Lights off.** Lights are continuously off as a control.
- **Other frequencies.** 20 Hz or 80 Hz (for example) are used to determine whether the effects observed are frequency specific (Fig. 1).

### Flicker exposure ● Timing ~4 h–7 d

24. Wrap the exposure boxes on three sides with black plastic or cloth for all exposure conditions (Fig. 4a). Coverage of three sides ensures that the main source of light is from the LED light source and not from reflection. Covering the sides facing other exposure boxes also visually separates the animals from one another and the experimenter.
25. Set up the boxes along a white wall with transparent sides facing the LED strip (Fig. 4b). Light levels should measure ~480 lux in the exposure box at the side closest to the lights (~6 in 15 cm from the strip lights) and >100 lux at the other end of the exposure box.
26. Bring the mice to the lab and allow them to rest for ~1 h in a quiet holding area (see Fig. 1 for a schematic overview of the experimental design of Steps 26–29).
27. Put the mice in the individual empty exposure boxes (without bedding), place breathable lids on the boxes, turn off ambient lights, and commence light flicker or control stimuli for 1 h (Supplementary Video). During stimulation, animals may ambulate, explore (sniff, rear), and rest. The choice of stimuli depends on the experimental aims and appropriate controls. Box 3 details stimuli that can be used with this protocol.
28. After the exposure, return the mice to their home cage in the holding area for 1 h.

### Analysis of light-flicker effects on A $\beta$ and microglia

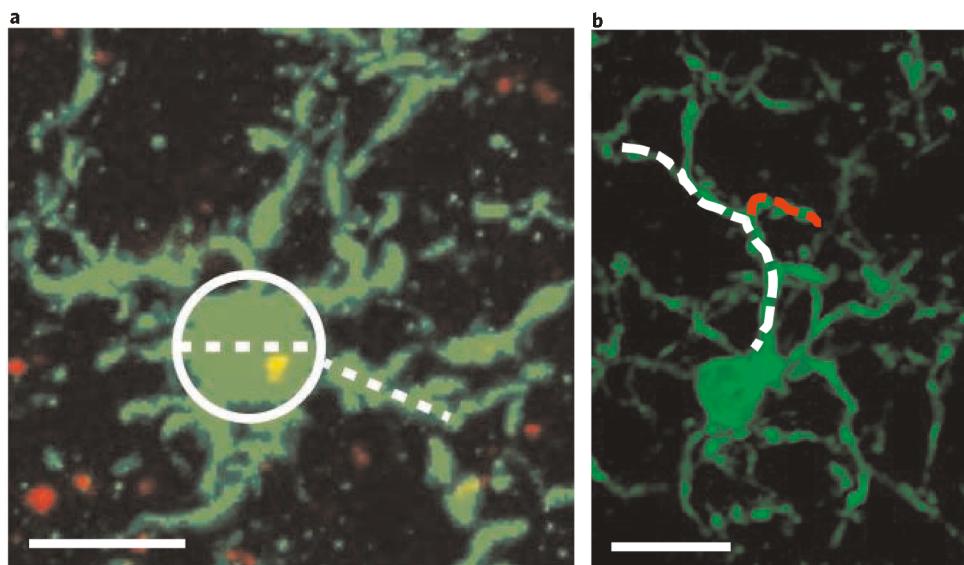
29. For analyzing A $\beta$  and microglia using IHC, follow option A. To use CLARITY and SWITCH, follow option B.
  - (A) **Immunohistochemistry** ● **Timing** ~7 d
    - (i) Euthanize the mice by exposing the animals to carbon dioxide with a flow rate of <20% of chamber volume per min to induce asphyxiation. Other forms of euthanasia may also work.
    - (ii) Perfuse mice with ice-cold 1× PBS, followed by ~40 mL of fresh ice-cold 4% (vol/vol) PFA (at ~5 mL/min). Carefully dissect out each brain (as described by Treuting and Snyder<sup>22</sup>) and postfix them overnight in 4% (vol/vol) PFA.
    - (iii) Wash each brain in 1× PBS three to four times for 5 min each before slicing.



**Fig. 5 | Coronal sections showing mouse visual cortex.** Coronal sections from 2.78 (top right), 3.08 (left), and 3.58 (bottom right) posterior to the bregma from the Allen Brain Atlas are shown. The primary visual cortex is circled in red. Scale bars, 1 mm. Images reproduced with permission from the Allen Brain Atlas, © copyright 2008 Allen Institute for Brain Science. Available from <http://mouse.brain-map.org>.

- (iv) Slice the brain using a vibratome, collecting 40- $\mu\text{m}$  coronal sections from ~3.0 mm posterior to the bregma to target the visual cortex (see example sections from the Allen Brain Atlas in Fig. 5 and at <http://mouse.brain-map.org/>).
- (v) Place two to five slices per well (filled to 500  $\mu\text{L}$  with PBS) in a 12-well plate, serially. We typically use every third 40- $\mu\text{m}$  section for a particular stain.
- (vi) Remove the PBS and add 500  $\mu\text{L}$  of blocking buffer. Incubate the slices for 1–2 h, with shaking at room temperature.
- (vii) Remove the blocking buffer from the slices and replace with ~500  $\mu\text{L}$  of the primary antibody solution (anti-Iba1 antibody and anti- $\beta$ -amyloid, 1–42 (12F4) diluted to 1:500 in blocking buffer). Shake overnight at 4 °C.
- ▲ **Critical Step** Use anti- $\beta$ -amyloid (D54D2) antibodies alone if assessing plaques.
- (viii) Wash the slices with 500  $\mu\text{L}$  of PBS three to four times for 5 min each, shaking gently at room temperature.
- (ix) Remove the PBS from the slices and replace it with secondary antibody solution (Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 647 donkey anti-mouse secondary antibodies diluted to 1:1,000 (vol/vol) in blocking buffer). Shake for 2 h at room temperature while protecting sections from light.
- ▲ **Critical Step** Use Alexa Fluor 488 donkey anti-rabbit secondary antibody alone if assessing plaques.
- (x) Wash the slices one to two times with 500  $\mu\text{L}$  of PBS for 5 min on a shaker. Wash with Hoechst 33258 diluted 1:1,000 (vol/vol) in PBS for 20–30 min on a shaker. Wash the slices again in PBS for 5 min on a shaker.
- (xi) Mount the slices on a glass slide, remove excess PBS from the slide, add Fluoromount (or a similar product), cover the slices with a glass coverslip, and store the slides overnight at 4 °C in the dark.
- **Pause Point** Ideally, image the slides the next day because fluorescence degrades over time. However, the slides can be stored and protected from light at 4 °C until one is ready to image.
- (xii) Image the slides using a confocal microscope with a 40 $\times$  objective by obtaining z-stacks of the entire thickness of the slice (~30–35  $\mu\text{m}$  of tissue can be imaged).
- ▲ **Critical Step** Perform imaging and analysis while blinded to treatment groups.
- ? **TROUBLESHOOTING**

- (xiii) Compress the z-stack using the ‘maximum intensity projection’ tool in the Zen Black 2011 SP4 software or using the ‘stack Z-projection; MAX intensity’ tool on ImageJ.
- (xiv) To measure Iba1<sup>+</sup> cell body diameters and the length of primary processes (Fig. 6 and Box 1), use ImageJ while blinded to treatment groups. Primary processes are the major projections directly coming off from the soma body. Nonprimary processes are projections that split from primary processes and are usually thinner and faint (Fig. 6).
- (xv) Go to ‘Image’ > ‘Color’ > ‘Split Channels’ (this will split your original image into separate images based on the number of different channels you imaged with, i.e., 488, 568, 647, and Hoechst).
- (xvi) To measure the cell area, use the ‘Freehand selections’ tool to trace around the cell body, or use the circle shape to surround the cell body and select ‘Analyze’ > ‘Measure’ and the area measurement will be reported.
- (xvii) To measure the cell diameter, draw a straight line across the cell body and select ‘Analyze’ > ‘Measure’ and the diameter measurement will be reported.
- (xviii) To measure the primary process, use the ‘Segmented Line’ tool to trace the length of the process and obtain the measurement length.
- (xix) To measure soluble Aβ intensity, select ‘Analyze’ > ‘Measure’. The mean value is the intensity value.
- (xx) To assess colocalization of Iba1 and Aβ, use the Coloc2 plugin.
- (xxi) (Optional) Observe and count each microglial cell by eye to determine whether there is amyloid colocalization within the cell.
- (xxii) To reconstruct three-dimensional (3D) images of microglia and Aβ, use the surfaces module in Bitplane Imarisx64 8.3.1 or similar software. Open the acquired raw z-stack image file (not a maximum-intensity projection) with Imaris and establish parameter settings that best model the cells of interest.
- (xxiii) Count the number of plaques by identifying Aβ deposits of  $\geq 10 \mu\text{m}$ .
- (xxiv) Using the ImageJ software, select an image and choose ‘MAX intensity images from z-stack image composites’.
- (xxv) Select ‘Image’ > ‘Color’ > ‘Split Channels’ (this will split your original image into separate images based on the number of different channels you imaged with, i.e., 488, 568, 647, and Hoechst).
- (xxvi) Close all images except for the plaque image (the channel will depend on which secondary antibody is used to detect Aβ, i.e., if you use Alexa Fluor 488/Cy2, then this will correspond to the green channel).



**Fig. 6 | Measurement of microglia cell body diameter and process length using ImageJ.** **a**, Illustration showing how microglia cell body (white circle), diameter (white dashed line in circle), and primary processes (white dashed line emanating from circle) are identified for quantification on a 40× image of a microglial cell stained with Iba1 (green). Scale bar, 10 μm. **b**, Identification of primary processes (white dashed line) and secondary processes (red dashed line). Scale bar, 10 μm. The procedures used to obtain these images were approved by DCM at the Massachusetts Institute of Technology.

- (xxvii) To measure the intensity of your plaque staining, select ‘Analyze’ > ‘Measure’. The mean value is the intensity value.
- (xxviii) To measure the number, total area, and average size of plaques, select ‘Image’ > ‘Adjust’ > ‘Threshold’ to adjust or remove any irrelevant, background noise by controlling the intensity of the plaques or the whole image. Adjust the settings until you find the best resolution. Once you have the optimal settings, note them and keep them consistent throughout your quantification process.
- (xxix) Select ‘Analyze’ > ‘Analyze Particles’, and enter “ $10 \mu\text{m}^2$ ” (this tells the program to automatically count the number of plaques  $>10 \mu\text{m}^2$ ).
- (xxx) Check ‘Clear Results’, ‘Summarize’, and ‘Exclude on edges’.
- (xxxi) Select ‘Enter’ to obtain the results for the respective image. This will let you determine the area and size of the plaques.
- (B) CLARITY and SWITCH** ● **Timing** ~10 d
- CLARITY (Step 29B(i–viii))**. Euthanize the mice by exposing the animals to carbon dioxide with a flow rate of  $<20\%$  of chamber volume per min to induce asphyxiation. Other forms of euthanasia may also work.
  - Perfuse the mice with ice-cold 1× PBS, followed by ~40 mL of ice-cold 4% (vol/vol) PFA and 1% (vol/vol) glutaraldehyde in 1× PBS (at ~5 mL/min). Carefully dissect out each brain (as described in ref. <sup>22</sup>).
  - Post-fix the brains in 10 mL of 4% (vol/vol) PFA and 1% (vol/vol) glutaraldehyde solution, and rock for 72 h at 4 °C.
  - Terminate fixation by incubating the brains in 10 mL of inactivation solution for 48 h, rocking at room temperature.
  - Wash the brains for 12 h in 10 mL of PBST, rocking at room temperature.
  - Using a vibratome, slice the fixed brains into 100-μm coronal sections and transfer to 1 mL of 0.02% (wt/vol) sodium azide in 1× PBS in a 24-well plate.
- ▲ CRITICAL STEP** The SWITCH immunolabeling protocol outlined below works well with 100-μm sections. We could clearly reconstruct microglia with sections of this thickness. Because tissue clears faster with sections of brain instead of whole brains, this protocol may require modification for use with thicker sections or whole brains (see, e.g., Chung et al. <sup>17</sup>, Chung and Deisseroth <sup>23</sup>, and Tomer et al. <sup>24</sup>).
- PAUSE POINT** Sections can be stored at 4 °C until one is ready for clearing and immunostaining.
- Select sections identified to contain the visual cortex (refer to Fig. 5 and the Allen Brain Atlas, <http://mouse.brain-map.org/>) or other brain regions of interest, and incubate in clearing buffer for 2–4 h, with shaking at 55 °C until white matter tracts are translucent.
  - ? TROUBLESHOOTING**
  - Wash the cleared sections three times for 15 min in 1 mL of PBST, with shaking at room temperature.
  - SWITCH immunolabeling (Step 29B(ix–xxi))**. Transfer the cleared sections to 500 μL of blocking solution and leave overnight, with shaking at room temperature.
  - Wash the sections three times for 1 h in 1 mL of PBST, with shaking at room temperature.
  - Incubate the sections in 1 mL of weak binding buffer for 1 h at room temperature.
  - Transfer the sections to 400 μL of primary antibody (anti-β-amyloid and anti-Iba1 primary antibodies diluted to 1:100 (vol/vol) in weak binding buffer) with shaking at 37 °C for at least 12 h.
  - Add 71 μL of reversal buffer to each section each hour for 6 h, with shaking at 37 °C, to allow for gradual homogeneous primary antibody binding throughout the tissue. After final addition, let the samples incubate for another hour, with shaking at room temperature.
  - Wash the sections three times for 1 h in 1 mL of PBST to remove any unbound primary antibody, with shaking at room temperature.
  - Transfer the sections to 400 μL of secondary antibody (Alexa Fluor 488 fragmented donkey anti-rabbit and Alexa Fluor 568 fragmented donkey anti-mouse secondary antibodies diluted to 1:100 (vol/vol) and 1 mg/mL Hoechst 33258 (diluted 1:500 (vol/vol)) in PBST, with shaking at room temperature for 12 h.
  - Wash the sections for 12 h in PBS, with shaking at room temperature.
  - Incubate the slices in 500 μL of RIMS for 4 h until the sections are transparent, with shaking at room temperature.

- (xviii) Mount the tissue sections on microscopy slides with coverslips using RIMS.
- (xix) Image the slides on a Zeiss LSM 880 upright confocal microscope with accompanying Zen Black 2.1 SP1 software. For imaging cellular morphology in the regions of interest, use a Zeiss Plan-NEOFLUAR 40 $\times$ /1.3 oil DIC, NA 0.17 objective. Acquire z-stack images with a step size of 0.4–0.5  $\mu$ m, pixel dwell of 4.1 ms, averaging of 2, and resolution of 1,024  $\times$  1,024, suitable for 3D reconstruction. For larger areas, take a bidirectional tile scan, with 15% overlap and 0.7 rotation. For higher-resolution images to analyze cellular structures such as endosomes, use a Plan-APOCHROMAT 63 $\times$ /1.4 oil DIC, NA 0.17 objective for 3D reconstruction.
- ▲ CRITICAL STEP** Perform imaging and analysis while blinded to treatment groups.
- (xx) Use Bitplane Imarisx64 8.3.1 for 3D rendering and analysis of a z-stack image file. The surface module is used to create a 3D reconstruction of the cell morphology on the basis of thresholds set for various parameters, such as the size, shape, intensity, and location of the signal of interest. Once an accurate set of signal detection and rendering parameters is established, these settings should be kept constant across all the images within an experiment.

## Troubleshooting

Troubleshooting advice can be found in Table 1.

**Table 1 | Troubleshooting table**

Step	Problem	Possible reasons	Solution
20 and 21	Lights do not flicker	Arduino code is not running properly Breadboard connections are incorrect  Connections have become loose Batteries are not charged or the power source is not generating sufficient voltage If lights are too slow, they may not be capable of following the TTL pulse Circuit relay is not functioning properly	See error in Arduino code interface for details and contact Arduino support for help Check that the circuit follows the schematic. Check that positive and ground wires have not been reversed for the power source (Step 1), the Arduino (Step 5), or the lights (Step 6). Check that the MOSFET was placed in the appropriate direction (Step 4) Check each connection and ensure that it is intact Use a multimeter to ensure that the power draw is 12 V  Buy the lights listed in the ‘Materials’ section
22 and 23	Lights flicker at the wrong frequency		Record the TTL output of the circuit (e.g., with a DAQ) and measure the interpulse interval to determine whether the TTL pulses are correct. If the pulses are incorrect, fix the code. If the pulses are correct, failure is in the circuit: build a new circuit with the parts mentioned in the ‘Materials’ section
29A(xii)	A $\beta$ levels are not detectable	Age of mice is wrong (e.g., mice are too young for changes in A $\beta$ levels to be measurable), strain of mice is incompatible with the protocol, or the amount of tissue is too small	Use mice with higher baseline A $\beta$ levels and/or use more mice or more tissue
	Background noise in IHC	Poor perfusion  Poor blocking Poor washing	Use fresh, ice-cold PFA and practice excellent perfusion technique (e.g., the resulting brain tissue is off-white with no trace of pink) Use fresh blocking buffer; block for longer Use blocking buffer (1 $\times$ PBS, 0.2% (vol/vol) Triton X-100, and 5% (vol/vol) normal donkey serum) for washing
29B(vii)	Tissue does not clear properly	Poor perfusion  Clearing buffer is not fresh and is no longer at the correct pH Fixation was not properly inactivated by the inactivation solution	Use fresh PFA and practice excellent perfusion technique (e.g., the resulting brain tissue is off-white with no trace of pink) Incubate in clearing solution for a longer period of time and/or use fresh clearing buffer Keep the slices in inactivation solution for a longer period of time and refresh the inactivation solution

## Timing

The experimenter should plan for the initial setup (including circuit configuration and exposure setup) to take 1 d, but this setup could take longer, depending on the troubleshooting required. If all components have been acquired, initial assembly of the breadboard will take ~30 min (Steps 1–18). Installing the Arduino software, connecting the Arduino to the computer, and opening the provided code takes ~10 min (Steps 19 and 20). The time to test the code for each condition will depend on the number of stimuli (~2 min per stimulus; Steps 21 and 22). The time to assess the accuracy of each frequency will also depend on the number of conditions to be used in the experiment (Step 23). Time spent debugging the flicker will vary, depending on the problem. Wrapping the flicker boxes takes 5–10 min each (Step 24). Setting up the exposure area will take ~10 min (Step 25). Exposure of the animals to the desired stimuli will take 1–7 d, depending on the experimental design (Steps 26–28).

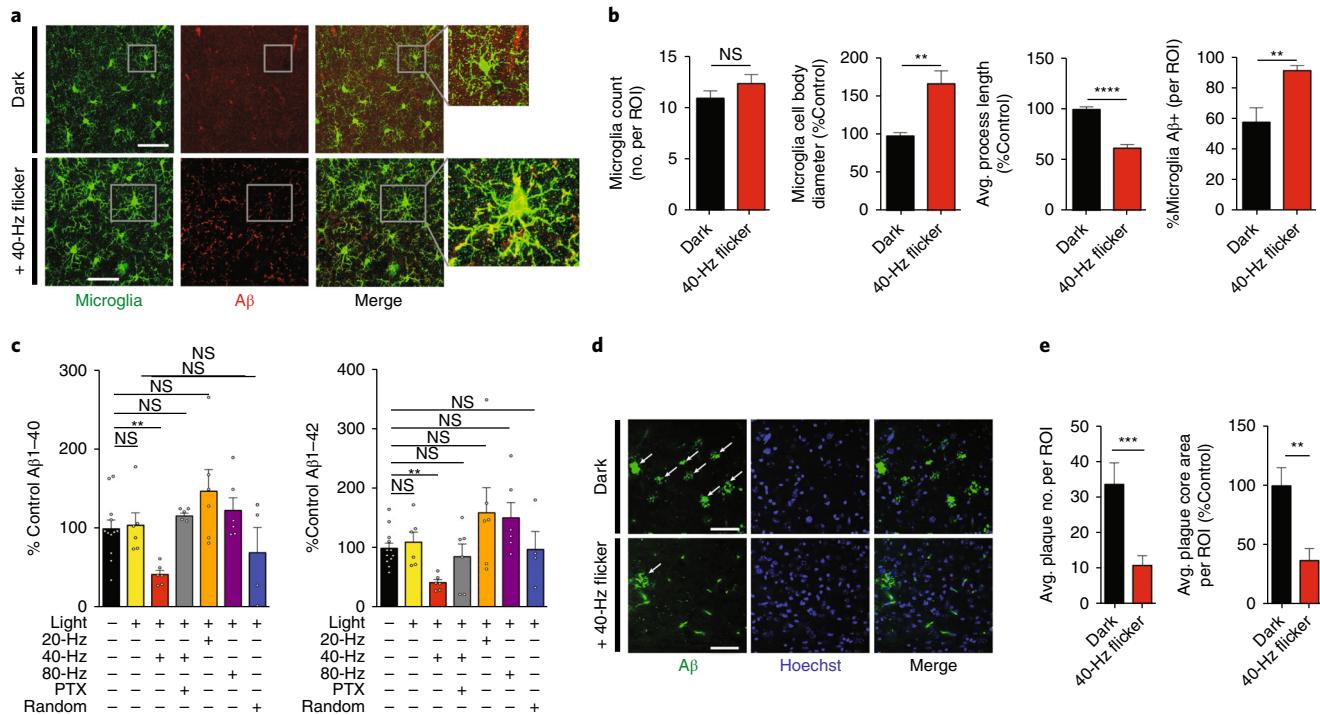
Step 29A: The experimenter should budget ~7 d for IHC. Preparing solutions, euthanizing and perfusing animals, and postfixing brains can generally be done over 24 h, depending on the number of animals perfused (expect 15–30 min per single animal perfusion, followed by overnight fixation and ~15 min of washes) (Step 29A(i–iii)). Similarly, the time it takes to slice sections with the vibratome depends on the number of brains being sliced and the number of slices collected (35–40 min per brain; Step 29A(iv,v)). The staining process itself will take ~2 d (Step 29A(vi–x)). The time spent mounting and imaging tissue and analyzing the images will be based on the specific experimental goals and the amount of tissue imaged. For three channels at 40 $\times$  for 40- $\mu$ m sections using z-stacks and tiled imaging, it should take ~8 min per section at a fast imaging speed and up to 45 min per section for a slow imaging speed (Step 29A(xi–xxxii)).

Step 29B: The experimenter should budget ~11 d for CLARITY experiments. Solution preparation, euthanasia, and perfusion should take ~1 d (Step 29B(i,ii); see above). Fixation and inactivation require 5.5 d (Step 29B(iii–v)). The time it takes to slice tissue in the vibratome will depend on the number of brains to be analyzed and the thickness of the sections (15–20 min per brain for 100- $\mu$ m sections) (Step 29B(vi)). The tissue-clearing process will take up to 4 h, after which the cleared sections should be washed and incubated in blocking solution overnight (Step 29B(vii–ix)). The SWITCH immunolabeling process itself will take ~4 d (Step 29B(x–xvi)). Sections will then be incubated in RIMS for 4 h and mounted (Step 29B(xvii,xviii)). The time for imaging and analyzing images will be based on specific experimental goals and will vary, depending on imaging parameters such as the thickness of the tissue, imaging speed, number of channels, and area imaged. For example, when imaging four channels in a 100- $\mu$ m section, using a one-tile z-stack at a step size of 0.5  $\mu$ m, at 40 $\times$  magnification, with high resolution, one can expect a 30- to 40-min acquisition time at a fast imaging speed and up to 3 h at a slow imaging speed (Step 29B(xix,xx)).

## Anticipated results

This 40-Hz light-flicker exposure protocol can be used to noninvasively decrease A $\beta$  levels, alter microglial morphology, and increase the proportion of microglial co-labeling with A $\beta$  in the visual cortex. Under appropriate conditions, one can expect the overall number of microglia to remain unchanged, cell body diameter to increase, and process length to decrease in the visual cortex after 1 h of 40-Hz light-flicker exposure (Figs. 6 and 7)<sup>10</sup>. These morphological changes to the microglia indicate enhanced engulfment activity<sup>11–13</sup>. Concurrent staining for A $\beta$  and microglia demonstrates that colocalization of A $\beta$  within microglia increases in the 40-Hz flicker group as compared with dark controls (Fig. 7)<sup>10</sup>. Microglial morphology and A $\beta$  colocalization are assessed using anti-Iba1 antibody to label microglia and anti- $\beta$ -amyloid antibody (12F4) to label A $\beta$  in microglia<sup>25–28</sup>. The 12F4 anti- $\beta$ -amyloid antibody does not cross-react with APP and is therefore specific to A $\beta$  (see Box 2 for discussion of A $\beta$  antibodies)<sup>25–28</sup>. 3D renderings of microglia from 100- $\mu$ m sections of the visual cortex using CLARITY allow for increased resolution of morphological changes to microglia (see Box 1 for discussion of microglial characterization)<sup>10,17</sup>.

Many other assessments can be used to assess cellular and molecular changes after a 40-Hz light flicker. One can expect A $\beta$  levels to be significantly reduced after 40-Hz flicker, as compared with control conditions (Fig. 7,  $P < 0.01$ )<sup>10</sup>. A $\beta$  levels can be assessed by ELISA or IHC. Isolation of microglia using FACS and then assessment of overall A $\beta$  levels within microglia using ELISA demonstrates microglial engulfment of A $\beta$  (ref. <sup>29</sup>). Using this method, we have shown that levels of A $\beta$  associated with microglia are significantly greater in 5XFAD animals than in WT controls ( $P < 0.01$ )<sup>10</sup>. In addition, we have found that 1-h 40-Hz flicker repeated daily over 7 d reduced



**Fig. 7 | Expected results.** **a**, Sections of visual cortex stained with anti-Iba1 (green) and anti-A $\beta$  (12F4; red) antibodies in 3-month-old 5XFAD mice exposed to dark (top) or a 40-Hz flicker (bottom). Scale bars, 50  $\mu$ m. Magnified images on the right reflect 120 $\times$  zoom relative to primary images. **b**, The number of Iba1 $^{+}$  microglia per region of interest (ROI; far left), Iba1 $^{+}$  microglia cell-body diameter (center left), Iba1 $^{+}$  microglia primary process length (center right), and percentage of Iba1 $^{+}$  microglia co-labeled with A $\beta$  (far right) after 1 h of 40-Hz light flicker (red) or dark (black) exposure followed by 1 h in a holding room in 3-month-old 5XFAD mice (Student's *t* test, unpaired,  $n = 4$  mice per group). **c**, Relative A $\beta$  1-40 (left) and A $\beta$  1-42 (right) levels normalized to those in dark-exposed controls, in visual cortex of 3-month-old 5XFAD mice exposed to dark (black); light (yellow); 40-Hz (red), 20-Hz (orange), or 80-Hz (purple) flicker; 40-Hz flicker with picrotoxin (PTX) (gray); or random (blue) conditions ( $n = 12$  for dark;  $n = 6$  for light, 40-Hz, 20-Hz, and 80-Hz flicker, and 40-Hz flicker with PTX;  $n = 4$  for random condition; one-way ANOVA). **d**, Plaques (white arrows) in the visual cortex of 6-month-old 5XFAD mice after 7 d of 1 h per day in dark or exposure to 40-Hz flicker (stained with anti-A $\beta$  (D5452; green) antibody (scale bars, 50  $\mu$ m)). **e**, Number (left) and core area (right) of A $\beta$  $^{+}$  plaques (Student's *t* test, unpaired,  $n = 8$  mice per group). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ; circles in **c** indicate  $n$ ; mean  $\pm$  s.e.m. is shown in bar graphs. NS, not significant. Image adapted with permission from ref. <sup>10</sup>, Springer Nature.

amyloid plaque number and size in 6-month-old 5XFAD mice and reduced another primary hallmark of AD pathology, tau phosphorylation, in 4-month-old TauP301S mice, a tauopathy mouse model<sup>10,30</sup>. During flicker stimulation, one should expect animals to ambulate about the cage, explore, or rest. Flicker may also induce changes in behavior; however, this has yet to be determined. Light flicker at 40 Hz has transformed microglia and reduced AD pathology of two molecules (A $\beta$  and tau phosphorylation) in multiple mouse models, indicating that this approach generalizes across animal strains and molecular pathology. Thus, one can expect 40-Hz flicker to reduce A $\beta$  levels and tau phosphorylation in mouse models of amyloidosis and tauopathy, respectively. Further research may reveal other neuroprotective effects. Importantly, this light-flicker approach is relatively easy to implement, and therefore we expect that it will spur further research into the interactions between neural activity, molecular pathology, and the brain's immune system.

Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

### Data and code availability

All data and code used in this study are included in this published article and its Supplementary Information files.

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### Author contributions

A.C.S. developed the light-flicker methods; A.C.S., J.M.D., A.J.M., F.A., H.M., and C.A. performed the experiments; A.C.S., J.M.D., M.K.A., and J.T. constructed and tested the flicker circuitry; and A.C.S., J.M.D., A.J.M., F.A., and L.-H.T. wrote the manuscript.

### Competing interests

L.-H.T. is the scientific founder and serves on the scientific advisory board of Cognito Therapeutics, and A.C.S. owns shares of Cognito Therapeutics. The remaining authors declare no competing interests.

### Additional information

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2. Mathys, H. et al. *Cell Rep.* **21**, 366–380 (2017) <https://doi.org/10.1016/j.celrep.2017.09.0393>
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### Antibodies

#### Antibodies used

- This protocol paper describes a method which could make use of several antibodies:
- Anti-Iba1 (Rabbit) (Wako Chemicals, cat. no. 019-19741)
  - Purified anti- $\beta$ -Amyloid, 1-42 (12F4) Antibody (Mouse) (Biolegend, cat. no. 805501)
  - $\beta$ -amyloid (D54D2) XP® Rabbit mAb (Cell Signaling Technologies, cat. no. 8243)
  - Allophycocyanin (APC)-conjugated CD11b mouse clone M1/70.15.11.5 (Miltenyi Biotec, 130-098-088)
  - Phycoerythrin (PE)-conjugated CD45 antibody (BD Pharmingen, 553081)
  - Alexa Fluor® 488 Fragmented Donkey Anti-Rabbit (Abcam, cat. no. ab181346)
  - Alexa Fluor® 568 Fragmented Donkey Anti-Mouse (Abcam, cat. no. ab175694)
  - Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch, cat. no. 711-545-152)
  - Alexa Fluor® 647 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, cat. no. 715-605-151)

#### Validation

See Box 1: SELECTING THE APPROPRIATE ANTI- $\beta$ -AMYLOID ANTIBODY and REAGENTS sections

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#### Laboratory animals

- This protocol paper describes a method which could make use of animals
- Mice: exact strain and age depends on experimental design (see ‘Experimental Design’ section in Introduction). In the procedures described in this Protocol, we used 5XFAD and APP/PS1 mice from established breeding colonies at MIT. 5XFAD mice

are a well-established model of AD that carries five familial AD mutations including human transgenes for APP with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) FAD mutations, as well as human PS1 harboring two FAD mutations, M146L and L286V. APP/PS1 mice are another well-validated AD model in which human transgenes for both APP bearing the Swedish mutation and PSEN1 containing an L166P mutation are expressed under the Thy1 promoter. Animals must have adequate A $\beta$  levels such that changes are detectable (for example, aged wild-type mice or transgenic mice like 5XFAD and APP-PS1, as we have used previously). When planning to make a comparison between groups, animals from one litter should be evenly distributed between the groups that will be compared. Males and females should be tested separately as we do not yet know how the effects of flicker treatment differ by sex. In many cases, multiple litters or cohorts of animals will need to be combined to have enough animals per condition. In such cases, mice should be age matched as closely as possible across the cohorts and animals of different ages should be evenly dispersed between condition groups. We recommend normalizing within each cohort to control conditions to account for variability between cohorts. For normalization, the average value of the control condition is calculated as baseline (or 100%). Controls and experimental groups within the cohort are then expressed relative to that baseline.

Wild animals

None

Field-collected samples

None