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Technical Report

Chronic *in vivo* imaging of tau-induced neurodegeneration and microglial response using two-photon microscopy

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ABSTRACT

Investigations into neurodegenerative progression have traditionally relied on pathological sectioning and intergroup analysis, with outcomes heavily dependent on sample size. Emerging *in vivo* imaging techniques now enable dynamic, long-term tracking of the same subjects, enhancing comparability and reducing variability. In this study, we use two-photon microscopy to visualize neurons and microglia through fluorescent markers introduced by adeno-associated virus (AAV) labeling and transgenic mice. Additionally, AAV-driven tau pathology induces neurodegeneration in the brain. A head ring is attached to the mouse's skull for reversible mounting on a head-fixed treadmill. This setup allows us to image neurodegeneration progression without anesthesia, quantifying changes in the number, morphology, and spatial positioning of tau-overexpressing neurons and microglia. This approach provides spatiotemporal insights into neurodegeneration previously unattainable.

KEYWORDS

neurodegeneration, microglia, two-photon *in vivo* imaging, tau

1 Introduction

Tau pathology plays a pivotal role in the pathogenesis of a wide range of neurodegenerative diseases [1–3], including, but not limited to, Alzheimer's disease, Pick's disease, progressive supranuclear palsy, corticobasal degeneration, agyrophilic grain disease, Huntington's disease and frontotemporal dementia with parkinsonism-17. The hallmark of tau pathology in these diseases is the presence of intracellular and extracellular neurofibrillary tangles (NFTs) and paired helical filaments (PHFs), formed from misfolded tau protein subunits that have dissociated from microtubules. Microtubules are essential for neuronal morphogenesis and function, as neurons are highly polarized cells whose structure and operations critically depend on intact microtubule networks. The misfolding and dysfunction of tau proteins can lead to microtubule destabilization, contributing to neurodegeneration.

In both cellular and animal models, tau-mediated neurodegeneration has been linked to loss of function, toxic gain of function, and mis-localization of tau proteins. Furthermore, clinical and preclinical evidence suggests that tau-related neurodegeneration evokes an immune response both centrally and peripherally, with microglia emerging as key players in the central neuroimmune regulation. Tau pathology and the consequent neuronal degeneration activate microglia, resulting in heterogeneous responses tailored to localized brain abnormalities. While microglial heterogeneity is typically characterized morphologically through immunostaining, the dynamic behaviors of microglia have remained largely unexplored due to technical challenges.

Recent advancements in *in vivo* imaging techniques have

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revolutionized the study of dynamic neurodegenerative processes [4, 5]. Multi-photon microscopy, particularly two-photon imaging, enables long-term, dynamic tracking of neurodegeneration and microglial responses within the same subjects. By visualizing fluorescently labeled neurons expressing mutant tau alongside microglia, we can gain unprecedented insights into the spatiotemporal progression of neurodegeneration (Fig. 1). Notably, the use of an awake imaging rig eliminates the need for anesthesia during recording, thereby avoiding its potential influence on the progression of neurodegeneration. These advanced imaging techniques allow for the chronic tracking of tau pathology-induced neurodegeneration and the corresponding microglial response *in vivo*, offering a novel and comprehensive perspective on these complex processes (Fig. 2).

2 Experimental design

2.1 Design of AAV vectors for labeling neurons and inducing tau-related neurodegeneration for chronic *in vivo* two-photon imaging

To develop a robust model for chronic *in vivo* two-photon imaging that allows simultaneous visualization of neurons and induction of tau pathology, we engineered adeno-associated virus (AAV) to express both a fluorescent marker and the A152T-variant tau. The selection of Tau^{A152T} is driven by its established role in promoting neurodegeneration, making it a

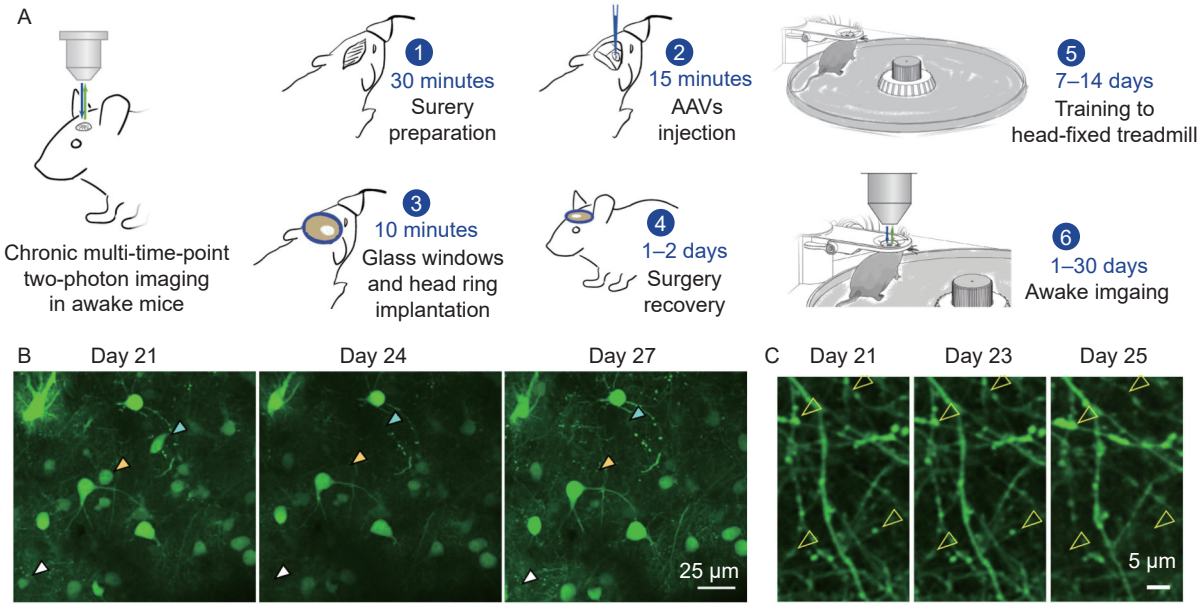

Figure 1 Two-photon *in vivo* imaging setup for awake mice, surgical preparation, and longitudinal imaging of neurons and dendrites across multiple time points. (A) Schematic of the chronic multi-time-point two-photon imaging setup in awake mice, highlighting the six major steps. Following AAV injection and cranial window implantation, a head ring is securely bonded to the skull. After the mouse recovers, the head ring allows the mouse to be fixed to the treadmill during imaging sessions. (B) and (C) Representative images of neurons and dendrites labeled with EGFP and expressing the Tau^{A152T} protein via AAV vectors in the cortex, captured at multiple time points. Arrows of different colors indicate the positions of different neurons.

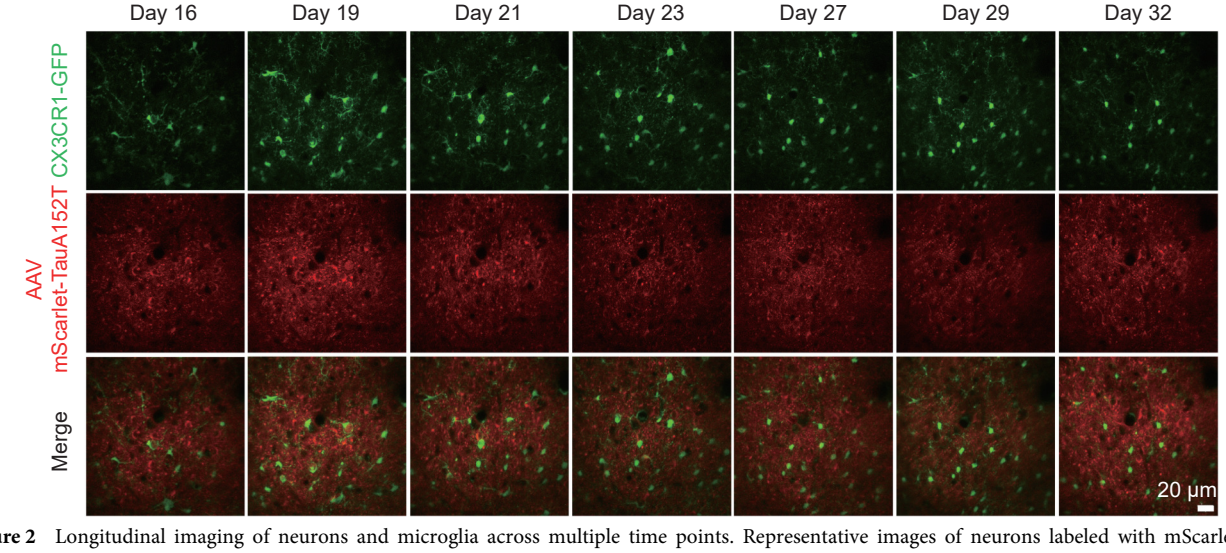

Figure 2 Longitudinal imaging of neurons and microglia across multiple time points. Representative images of neurons labeled with mScarlet and expressing the Tau^{A152T} protein via AAV vectors in the cortex, captured at multiple time points. Microglia were labeled using CX3CR1-GFP mice, allowing for simultaneous visualization of both neurons and microglia over time.

suitable candidate for modeling tauopathy in neuronal circuits [6]. The promoter driving expression within the AAV construct was carefully chosen to ensure specificity to excitatory neurons, which are predominantly involved in synaptic activity within the neocortex and hippocampus. To achieve this, the CaMKIIa promoter was utilized, given its strong and selective expression in excitatory neurons, thereby aligning with the experimental focus on excitatory synapse-related tau pathology.

To visualize the transduced neurons, we included a fluorescent reporter gene, either mScarlet or EGFP, positioned adjacent to—but not fused with—Tau^{A152T} within the AAV construct. This dual expression system not only facilitates real-time imaging of neuronal morphology but also ensures that the onset and progression of tau-induced neurodegeneration can be monitored in a temporally controlled manner.

The design aims to balance fluorescence intensity—sufficient for clear imaging—while minimizing any potential cytotoxic effects, thereby preserving neuronal health throughout the experimental timeline. Additionally, when determining the amount of AAV to deliver, it is important to consider that severe inflammation from neurodegeneration can reduce parenchymal clarity. Therefore, striking a balance between maintaining tissue clarity and achieving the desired intensity of neurodegeneration is crucial for your research. This approach enables precise control over both expression levels and the timing of induced neurodegeneration, ensuring the model's suitability for longitudinal studies.

2.2 Generation of mice expressing fluorescent microglia for chronic *in vivo* two-photon imaging

To study the interactions between microglia and transduced neurons *in vivo*, we employed transgenic mouse models that enable fluorescent labeling of microglia for chronic two-photon imaging. For experiments requiring mScarlet-labeled neurons, CX3CR1-GFP mice are an appropriate choice due to the complementary fluorescence of GFP. Conversely, for experiments using EGFP-labeled neurons, CX3CR1-CreER:: Rosa26-tdTomato mice provide distinct red fluorescence in microglia, ensuring clear differentiation between cell types.

To generate the necessary experimental animals, homozygous CX3CR1-GFP mice should first be bred with C57BL/6 wild-type mice. The heterozygous offspring from this cross can then be used for imaging studies. Concurrently, homozygous CX3CR1-CreER and Rosa26-tdTomato mice should be obtained and crossed to produce double heterozygous offspring, which will express the desired fluorescent markers for microglia.

2.3 Surgery for AAV injection and cranial window implantation

The surgical procedure for AAV injection and cranial window implantation is a critical step in enabling chronic *in vivo* two-photon imaging of neuronal degeneration and microglial behavior in the mouse brain. This method involves the precise delivery of AAV into specific cortical regions, allowing for the targeted expression of fluorescent proteins and pathogenic genes within neurons. By establishing a stable cranial window, long-term imaging of these genetically modified cells becomes feasible, providing invaluable insights into the dynamics of microglial behavior and the progression of neurodegenerative diseases over time.

The success of this procedure hinges on meticulous surgical techniques, including the careful preparation and sterilization of instruments, precise drilling of the cranial window, and the controlled injection of viral vectors. The cranial window must remain clear and stable throughout the experiment, ensuring consistent imaging quality. Moreover, the injection of AAV must be accurately targeted and delivered at a rate that prevents viral leakage, minimizes tissue damage, and preserve the integrity of the dura.

2.4 Chronic *in vivo* two-photon imaging of neurons and microglia

By providing high-resolution, real-time imaging of cellular processes deep within the cortical tissue, this method enables the study of synaptic changes, neuronal degeneration and microglial responses in a way that static imaging techniques cannot match. The significance of this approach lies in its ability to track the same neuronal circuits and microglial cells across multiple imaging sessions, providing insights into the temporal progression of neurodegenerative diseases, synaptic plasticity, and neuroinflammatory responses. The technique's ability to record in four spatiotemporal dimensions—x, y, z, and t—makes it particularly valuable for studying complex processes such as neuronal connectivity, microglial surveillance, and their interactions during pathological conditions.

Before imaging, it is essential to acclimate the mice to the fixation apparatus to minimize stress and movement during the procedure, ensuring consistent and reliable data collection. The two-photon imaging system, equipped with a water immersion objective lens, allows for the precise focus on target regions within the brain. By carefully aligning the imaging setup and consistently referencing the same field of view, researchers can repeatedly capture high-quality images from identical brain regions over time, enabling longitudinal studies.

2.5 Analysis of images from *in vivo* recordings

Analyzing images from *in vivo* recordings is a critical step in uncovering the intricate cellular behaviors associated with neurodegeneration. This process involves applying comprehensive, objective, and in-depth analyses to the raw imaging data, allowing researchers to extract meaningful insights into the dynamic processes occurring within the brain. Through meticulous image analysis, it is possible to identify subtle changes in neuronal and microglial activity, track the progression of pathological features, and quantify the effects of experimental interventions.

This analysis lies in its ability to transform vast amounts of imaging data into actionable knowledge, revealing patterns and correlations that are not immediately apparent. By leveraging advanced analytical techniques, researchers can create detailed models of cellular interactions, assess the impact of neurodegenerative processes at a granular level, and provide empirical evidence to support or refine existing hypotheses. Ultimately, this analysis is essential for advancing our understanding of the underlying mechanisms in

Neuroimmunology driving neurodegeneration and for developing targeted therapeutic strategies.

3 Materials

3.1 AAV

All AAVs were packaged by a local company (Packgene, Guangzhou, China), with plasmid designs based on sequences obtained from Addgene. The DNA sequences and plasmid maps are provided in the supplementary file. The AAVs used in this study, all of serotype 9, utilized promoters including CamKIIα and CAG. They were designed to express Tau^{A152T} fused with mScarlet or EGFP, with EGFP or mScarlet serving as controls.

3.2 Animals

All experiments were conducted using 2-month-old male mice, housed in groups of 3–5 per cage with constant access to water and maintained on a 12-hour light/12-hour dark cycle. All animal procedures were approved by the Animal Care and Use Committee of the South China University of Technology. The mice used in this study included wild-type C57BL/6 mice obtained from the Guangdong Medical Laboratory Animal Center. Additionally, we utilized CX3CR1-GFP mice (JAX# 005582), which express GFP under the control of the CX3CR1 promoter, enabling visualization of CX3CR1-expressing cells, and CX3CR1-CreER mice (JAX# 020940), which express Cre recombinase under the CX3CR1 promoter to drive expression specifically in CX3CR1-expressing cells; CreER activation is achieved through tamoxifen administration, providing temporal control of Cre activity. Rosa26-tdTomato mice (JAX# 007914, Ai14) were also used; these mice carry a loxP-flanked stop cassette that prevents tdTomato fluorescent reporter expression until Cre recombinase activation, at which point the stop cassette is excised, resulting in tdTomato expression in Cre-expressing cells.

3.3 Essential reagents, instruments, equipment and setup

The following reagents, instruments, and equipment are essential for the procedure. Reagents include single bond universal adhesive (3M, cat# 41282) and flowable restorative dental adhesive (3M, cat# 7032A2). Key surgical instruments include glass micro-electrodes with an outer diameter of 1.5 mm and an inner diameter of 0.86 mm (GEOLOGY, cat# 1586), 4 mm diameter cover glasses (SITRANTECH), and head rings (SITRANTECH, cat# 9001). Surgical equipment comprises a micropipette puller (Sutter, cat# P-97) and the Drummond Nanoject III programmable nanoliter injector (Drummond, cat# 13-681-460). Other required equipment includes a head-fixed mouse treadmill (SITRANTECH, cat# DTM-100R) with the head ring for fixation (SITRANTECH, cat# 9001) and a two-photon microscope system (Leica, STELLARIS 8 DIVE). For instrument setup, head rings should be preserved in 75% ethanol and can be recycled. Stripped rings must have any solidified adhesive removed at the start of the process, with an ultrasonic cleaner used for further cleaning if needed. Cover glasses should also be stored in 75% ethanol for preservation.

4 Procedure

4.1 Surgery for AAV injection and cranial window implantation

4.1.1 Preparation before surgery

Begin by preparing the operating table, cleaning it thoroughly with 75% alcohol and wiping it down, followed by a 30-min ultraviolet light disinfection. Cover the table with a disposable sterile surgical drape. Prepare the necessary surgical instruments, including ophthalmic scissors and curved forceps (thicker forceps for grasping the scalp and fine forceps ≤ 0.1 mm for lifting the skull), ensuring all tools are sterilized by autoclaving. Adjust the stereomicroscope for the procedure, which requires microscopic observation. Prepare reagents, including 3% hydrogen peroxide, iodine disinfectant, sterile saline (0.9% NaCl), and AAV constructs (CAG-EGFP-T2A-Tau^{A152T} and camkIIa-mscarlet-T2A-Tau^{A152T}), keeping the AAV on ice. Finally, prepare the glass electrode using an electrode puller to split it and trim the tip with ophthalmic scissors. Fill the electrode with corn oil using a syringe, avoiding air bubbles, and mount it onto the injection pump. Expel a small amount of oil to create space for subsequent AAV uptake.

4.1.2 General anesthesia and preoperative preparation for mice

To initiate anesthesia, place the mouse in a pre-anesthesia box containing two cotton swabs soaked in isoflurane. Once fully anesthetized, secure the mouse's head in a stereotaxic frame and activate the gas anesthesia system with a 0.5 L/min oxygen flow rate. Use 2% isoflurane for induction and reduce to 1.5% for maintenance. To ensure the mouse's body temperature remains stable during surgery, place it on a 37°C heating pad. Shave the hair on the mouse's head to expose the scalp, ensure adequate lighting, and apply erythromycin eye ointment to protect the eyes.Shave the hair on the mouse's head with a blade to expose the scalp. Ensure adequate lighting throughout the surgery. Apply erythromycin eye ointment to protect eyes.

4.1.3 Exposure of cerebral cortex

Disinfect the scalp with iodine solution, then lift and remove the scalp using curved forceps and ophthalmic scissors to expose the skull. Minimize the size of the incision to prevent excessive bleeding, using hemostatic sponges if necessary. After removing the thin membrane covering the skull, apply 3% hydrogen peroxide to cauterize the periosteum, then clean the surface with saline. Ensure the skull is dry and clean using a cotton swab. Create a circular bone flap (~4 mm in diameter) on the right hemisphere by thinning the bone with a drill. Carefully lift the thinned skull with forceps to expose the brain. Rinse the area with sterile saline to control bleeding, applying hemostatic sponges as needed until the window is clear and free of visible bleeding.

4.1.4 AAV injection

Using the injection pump, draw AAV (1×10^{13} GC/mL) into the prepared glass electrode, ensuring space is available after expelling excess corn oil. Secure the electrode in the stereotaxic apparatus and target the injection site at the center

of the window, avoiding prominent blood vessels. Set the Z coordinate to zero upon touching the dura mater. Gently pierce the dura and inject 300 nL of AAV at 1 nL/s to a depth of 0.4 mm. After completing the injection, wait 10 minutes before removing the electrode to allow proper diffusion. During this time, keep the area around the window dry with a cotton swab.

4.1.5 Implantation of glass window

Ensure the window site is free of bleeding and the skull surface is completely dry before proceeding with implantation. In dim lighting, apply a thin layer of base adhesive to the skull surface using a fine brush, avoiding contact with brain tissue. Cure the adhesive with a dental curing light (420–480 nm) for 40 seconds. Apply a thin circle of dental adhesive along the edge of the window, ensuring minimal excess adhesive. Place a 4 mm diameter, 0.13 mm thick glass cover over the window, gently pressing it into place. Secure the glass with additional adhesive and cure with the dental light until fully sealed.

4.1.6 Implantation of head ring adapting to head-fixed treadmill

Apply dental adhesive to the entire skull surface, then position the head ring so that the glass window is centered and the ring is parallel to the window. Gently press the head ring while curing the adhesive for 60 seconds using a dental curing light. After the procedure, return the mouse to its cage and monitor its recovery closely. Adhere strictly to aseptic principles throughout the surgery to minimize infection risks. Allow the window to heal for 14 days before proceeding with two-photon imaging.

4.2 Chronic *in vivo* two-photon imaging of neurons and microglia

4.2.1 Training mice in the head-fixed treadmill

Before imaging, mice should be acclimated to the head-fixed treadmill to minimize stress and ensure reliable data collection. This involves securing each mouse in the fixation apparatus for 30 minutes per session, repeated three times. This training familiarizes the mice with the setup, promoting natural behavior during subsequent imaging sessions.

4.2.2 Two-photon brain imaging in awake mice freely running on head-fixed treadmill

Begin by powering on the two-photon imaging system, including the microscope and computer software, and initializing configurations such as image resolution, scanning speed, laser settings, and optical receiver. Secure the mouse in the fixation apparatus and apply Vaseline around the head ring to hold clean water for the water immersion objective lens. Lower the objective lens into the water carefully, turn on fluorescence, and focus on the target region. Capture an initial reference image through the ocular lens to ensure consistent imaging of the same location in future sessions. Once the field of view is identified, use the camera connected to the computer to capture images, adjusting imaging parameters as needed. Record spatiotemporal dimensions (x, y, z, and t) based on the experimental requirements to obtain optimal results.

4.3 Analyzing images

4.3.1 Screening of raw data

Begin by browsing the raw data in LasX software to identify suitable images for further analysis. Compare data across different time points and select series with complete datasets for processing. The selected images will then be imported into ImageJ (version 1.54f) for subsequent analysis.

4.3.2 Screening of raw data

Load the Z-Stack images in ImageJ by opening the raw Lif format files identified in Step 1. Correct any motion artifacts caused by mouse movement during two-photon imaging using the "StackReg" plugin, ensuring proper alignment of the Z-Stack slices. Create a maximum intensity projection for a 2D representation of the 3D data by navigating to Image > Stacks > Z Project and selecting "Maximum Intensity." For improved visibility, adjust the contrast by going to Image > Adjust > Brightness/Contrast and fine-tuning the sliders as needed. Save the processed images in TIFF format and import them into Illustrator for integration and presentation. For detailed and easily followable procedures, please refer to the Electronic Supplementary Material.

5 Discussion

The progression of neuronal death and the interaction between microglia and degenerating neurons are fundamental to understanding neurodegenerative diseases. *In vivo* two-photon chronic imaging offers a unique opportunity to capture these processes in real time, providing dynamic insights that are unattainable with traditional static methods. In this study, we developed and implemented a protocol that utilizes this advanced imaging technique to explore neuronal death in Tau model mice and track the dynamic behavior of microglia.

Our protocol leverages key tools, including transgenic reporter mice and AAV-Tau^{A152T} with a mScarlet reporter. Microglia-specific GFP labeling under the Cx3cr1 promoter allows for precise visualization of microglial dynamics during neuronal degeneration. Concurrently, the AAV carrying the mutant Tau^{A152T} gene and the mScarlet reporter induces progressive neuronal toxicity, enabling long-term imaging of neurodegeneration. This dual-labeling system distinguishes microglia and Tau^{A152T}-overexpressing neurons during two-photon imaging.

The protocol is divided into three comprehensive procedures tailored for specific experimental applications. The first procedure involves surgery for AAV injection and cranial window implantation, which establishes the platform for imaging. The second focuses on chronic *in vivo* two-photon imaging of neurons and microglia, enabling longitudinal observation of dynamic processes. The third procedure is image analysis, which facilitates the quantification of cellular behaviors and interactions.

Unlike traditional histopathological techniques, which capture only static snapshots of disease progression, our protocol enables long-term tracking of morphological changes during neuronal degeneration and death. This approach allows for the monitoring of microglial activation from a resting state to their phagocytic role in clearing degenerating

neurons. By capturing these dynamic interactions, our method provides a clearer understanding of the distinct roles and functions of various cell types during neurodegeneration.

Beyond neurodegenerative studies, this platform has broader applications in neuroscience. It can be used to investigate synaptic plasticity, neuronal circuit dynamics, glial–neuronal interactions, and brain-wide activity during complex behaviors, such as sensory processing and decision-making.

Despite its strengths, our protocol has certain limitations. While it effectively captures long-term changes, it may miss very rapid or transient cellular events critical to some processes. The spatial resolution, while sufficient for many applications, may not allow for detailed observations of subcellular interactions, potentially limiting insights into closely located cellular processes. Furthermore, the protocol focuses primarily on neurons and microglia, potentially overlooking the contributions of other cell types, such as astrocytes, that are also involved in neurodegeneration. Lastly, the method requires specialized equipment, technical expertise, and significant resources, which may limit its accessibility and reproducibility in other laboratories.

In summary, our protocol provides a dynamic, long-term approach to studying neurodegeneration and microglial behavior, advancing our understanding of these complex processes while offering versatility for broader applications in neuroscience.

Author Contributions

X.W., H.Z., Z.X. and Y.U.L. developed the research concept and designed the experiments; X.W., H.Z. and Z.X. performed the experiments and analyzed the data; X.W., H.Z., J.S., Z.X. and Y.U.L. wrote the manuscript with critical inputs and edits by all the co-authors.

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Conflict of Interest

The authors declare no conflicts of interest.

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