Protocol

Two-Photon Imaging of Neural Networks in a Mouse Model of Alzheimer's Disease

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In humans, Alzheimer's disease (AD) develops over many years. It comprises a chain of subtle yet irreversible alterations in brain function, finally leading to impairment of memory and cognition. Pre-symptomatic and thus invisible in humans, these alterations can be studied in the animal models of AD. Mouse models of the disease expressing AD-related proteins with familial mutations reproduce several pathological hallmarks of AD. Although the models do not recapitulate the abundant neuronal loss seen in humans, they offer a unique opportunity to learn more about synaptic and cellular mechanisms underlying the disease (both in their essence and in their temporal sequence) through in vivo analyses of brain function. This, however, requires in vivo monitoring of brain function in aged living animals at both a single-cell and network level. Tools developed over the last several decades can be used to selectively mark and to visualize in vivo many important elements of the diseased brain parenchyma, such as amyloid plaques, individual neurons, and glial and microglial cells. Here we describe a method in which cell-type-specific labeling of neurons and glia is combined with in vivo two-photon calcium imaging and fluorescent labeling of amyloid plaques to study functional properties of cortical circuits in a mouse model of AD.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Agarose, low melting point
Cyanoacrylate glue
Dimethylsulfoxide (DMSO)
Eye ointment (e.g., Bepanthen)
Isoflurane, applied through a vaporizer, vaporized in pure oxygen
Lidocaine or equivalent local anesthetic agent
Membrane-permeable calcium indicator dye (e.g., Oregon Green 488 BAPTA-1 AM [OGB-1]; Molecular Probes)
Pluronic F-127 (Sigma-Aldrich), 20% (w/v) in DMSO

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Standard external saline for mouse <R>
Standard pipette solution <R>
Sulforhodamine 101 (SR101; Sigma-Aldrich)
Thioflavin-S (e.g., Thioflavine S, Sigma-Aldrich)

Equipment

Anesthesia unit including a chamber for preanesthetic medication, a flow meter, and a vaporizer
(latter items are for volatile anesthetic agents only)
Consult the literature (e.g., Flecknell 2000) for the best choice of anesthesia for your species.
Animal monitoring system for monitoring respiration and pulse rate, body temperature, and blood
pressure (e.g., ADInstruments)
Centrifugal filters (Ultrafree-MC; Millipore)
Dental hand drill (e.g., NSK-Nakanishi), drill bits with 0.5-mm diameter
Glass capillaries (e.g., Hilgenberg GmbH)
Imaging setup

Any commercially available two-photon imaging system can be used. Such systems are available from several providers (e.g., Carl Zeiss, Olympus, Leica Microsystems, and Prairie Technologies). We currently use a custom-built setup based on a mode-locked Ti:sapphire laser with automated dispersion compensation (Mai Tai HP DeepSee, Spectra-Physics) and a laser-scanning system (Olympus Fluoview) coupled to an upright microscope (BX51WI, Olympus) and equipped with a 40 $\times$, 0.80-numerical aperture (NA) water-immersion objective (CFI Apo 40 $\times$, Nikon). A custom-built system such as this can be assembled following instructions found in Majewska et al. (2000) and Nikolenko and Yuste (2005). OGB-1 can be excited at any wavelength between 800 and 930 nm. The emitted fluorescence of the dye is then collected between 400 and 720 nm. For simultaneous visualization of OGB-1 and the amyloid plaque marker Thioflavin-S, the dyes are excited at 800 nm and the emitted light is separated at 515 nm using a beam splitter. The collected images are background-corrected and analyzed off-line with the ImageJ program (<http://rsb.info.nih.gov/ij/>) and a LabView-based software package (National Instruments).

Micromanipulator (e.g., Luigs & Neumann GmbH)
Needles, 30-gauge (attached to either a syringe or a hand-driven micromanipulator)
Pasteur pipettes, plastic
Patch-clamp amplifier (e.g., HEKA)
Pipette puller (e.g., PP830; Narishige)
Pressure application system (e.g., npi electronic GmbH or Toohey Company)
Recording chamber with central access opening: custom-made from a standard tissue-culture dish
(35-mm diameter; for details, see Garaschuk et al. [2006])
Stereo dissecting microscope equipped with variable magnification lens ($\sim 1\times$ – $4\times$)
Stereotaxic apparatus (e.g., TSE-Systems)
Surgical equipment
Warming blanket (e.g., TSE-Systems)

METHOD

Surgical Procedure

1. Anesthetize the animal. Test the toe pinch withdrawal reflex to ascertain that the surgical level of anesthesia has been reached.

Note that the required amount of anesthetic can vary with age and/or the individual state of the animal. For easily adjusting the amount of anesthetic, use the volatile anesthetic isoflurane. To induce anesthesia, we use 2.5% isoflurane (in the anesthesia induction chamber) and, for surgery, 1.5% isoflurane.

2. Place the animal on a warming blanket preheated to 38°C and monitor the animal's temperature and breathing rate. To prevent the animal's eyes from drying out, apply eye ointment.
3. Prepare the area that will be recorded from:
 - i. Inject a local anesthetic under the skin above the animal's skull. Wait for the anesthetic to take effect.
 - ii. Carefully remove the skin. Start with a horizontal cut at the base, followed by two oblique cuts toward the eyelids of the mouse converging at the midline.
 - iii. Gently scrape any resting tissue on top of the animal's skull with a surgical blade. Dry the bone completely using compressed air.
 - iv. Use a stereotaxic device to identify the location of the brain area to be targeted for recordings.
4. Attach the recording chamber:
 - i. Apply cyanoacrylate glue around the defined recording area.
 - ii. Glue a recording chamber to the dried skull. Immediately remove any glue covering the recording area.
 - iii. Allow the glue to dry (wait for 10–20 min).
 - iv. Fix the recording chamber in an appropriate holder under a dissecting microscope.
5. Thin the skull:
 - i. Use a dental drill with appropriate drill bits to evenly thin the skull under the opening of the recording chamber. Use a high drilling speed (50,000 rev/min) and avoid pressing onto the skull while drilling.

The skull of older mice often contains many small blood vessels. It is not necessary to avoid them, just continue drilling evenly.
 - ii. Regularly use compressed air to remove the bone dust and a few drops of standard external saline to absorb the heat produced by drilling.
 - iii. Stop drilling when the brain vessels located under the dura become visible.

The skull is sufficiently thinned if you can clearly see the pattern of blood vessels located under the dura (apply standard external saline on top of the skull to improve skull transparency), and you see the skull bending when it is touched with thin tweezers.
6. Transfer the animal to the experimental setup. Connect it to a monitoring system for continuous monitoring of respiration and pulse rate, body temperature, and blood pressure.
7. Perfuse the recording chamber with 37°C standard external saline.
8. Perform a craniotomy, making a rectangular hole that is 0.5–1 mm on each side. For good optical control of your manipulations, perform the craniotomy under a 10× objective.
 - i. Keep the chamber filled with standard external saline.

Covering the skull with the fluid increases tissue transparency and keeps the dura/cortex from drying out.
 - ii. Choose an area devoid of large blood vessels. Open the skull with a 30-gauge needle attached to either a syringe (and guided by your steady hand) or a hand-driven micromanipulator. Form a rectangle by making four straight, slightly overlapping cuts through the bone

only; do not cut the dura. Ensure that the edges of the rectangle do not adhere to the rest of the skull.

- iii. Lift and remove the cut rectangle of bone. It should come out easily. Do not remove the dura.

*Leaving the dura in place protects and stabilizes the underlying tissue.
See Troubleshooting.*

9. Depending on your experimental strategy, either proceed to Step 10 (for staining plaques) or skip to Step 16 for staining neurons and glial cells.

Plaque-Staining Procedure

Stain plaques in the area of interest using Thioflavin-S, a selective marker.

10. Dissolve Thioflavin-S in DMSO to 1%. Dilute this stock solution with standard pipette solution to a final Thioflavin-S concentration of 0.001%. Filter the staining solution using an Ultrafree-MC centrifugal filter.
11. Pull glass micropipettes so that they have a resistance of 5–6 M Ω when filled with the staining solution.
12. Fill a micropipette with the Thioflavin-S staining solution. Use a 4 $\times$ objective and an LN-Mini manipulator to place the dye-containing pipette above the skull opening. Switch to a 40 $\times$ long-working-distance objective and maneuver the pipette directly onto the cortical surface.
It is preferable to insert the pipette at the steepest angle allowed by the geometry of the system.
13. Insert the pipette into the cortical tissue by moving it along the pipette axis until it is 200 μ m under the cortical surface. Inject the Thioflavin-S for 30 sec at 55 kPa.
14. Repeat the application every 50 μ m while withdrawing the pipette until a depth of 50 μ m under the dura is reached (Fig. 1B). Insert the staining pipette in up to four different locations to stain all plaques under the craniotomy.
Thioflavin-S does not diffuse as well as the calcium indicator dyes, so only a small amount should be administered per injection site.
15. Wait for up to 25 min for the unbound Thioflavin-S to wash out before assessing the quality of plaque staining by means of two-photon imaging (Fig. 1).

Staining Neurons and Glia with Calcium Indicator Dyes

To stain neurons and glia in mouse models of AD with calcium indicator dyes, we modified a multicell bolus loading technique (MCBL) (Stosiek et al. 2003).

16. Dissolve OGB-1 AM in a solution containing 20% (w/v) Pluronic F-127 in DMSO to yield 10 mM OGB-1 AM. Dilute this solution with standard pipette solution to yield a final dye concentration of 0.5 mM. Filter the staining solution through an Ultrafree-MC centrifugal filter directly before application.
17. Pull glass micropipettes so that they have a resistance of 5–6 M Ω when filled with the staining solution.
18. Fill a micropipette with the OGB-1 AM solution. Use a 4 $\times$ objective and an LN-Mini manipulator to place the dye-containing pipette above the skull opening. Switch to a 40 $\times$ long-working-distance objective and maneuver the pipette directly onto the cortical surface.
19. To obtain good staining of cortical layer 2/3 neurons in AD mice, use up to four staining locations per 0.5–1 mm². Place the pipette directly on top of the dura at the steepest possible angle and advance it slowly to a depth of ~250–300 μ m. Inject the dye for 1–2 min at 55–60 kPa.

FIGURE 1. Labeling of amyloid plaques. (A) Amyloid plaques in the cortex of an APP23 $\times$ PS45 mouse (Busche et al. 2008) stained by pressure injection of both Pittsburgh compound B (left) and Thioflavin-S (middle). The image to the right is an overlay of the two images. The arrowhead points to fiber-like structures, which have selectively bound Thioflavin-S. (B) (Left) Amyloid plaques stained by topical application of 0.005% Thioflavin-S for 20 min. (Right) Plaques are stained by pressure injection of Thioflavin-S as described in Steps 13 and 14. Both three-dimensional reconstructions show a $60 \times 60 \times 260$ - μ m volume of the mouse cortex. (C) Emission spectra of Thioflavin-S (two-photon excitation) and OGB-1 (one-photon excitation, taken from Molecular Probes Catalog). (A,B, Reproduced, with permission from Springer Science + Business Media, from Eichhoff et al. 2008.)

20. Wait at least 60 min for diffusion, de-esterification, and wash out of the extracellular dye before imaging. Check the quality of the obtained staining.

See Troubleshooting.

21. If plaques were previously stained, then split Thioflavin-S and OGB-1 fluorescence using a 500-nm beam splitter (Fig. 1C and Fig. 2A).

For technical reasons, we use a 515-nm beam splitter in our setup.

22. To assure that your preparation is viable, monitor spontaneous neuronal activity as described in Busche et al. (2008).

See Troubleshooting.

23. Good-quality recordings of spontaneous neuronal activity (Fig. 2B) can be routinely obtained for up to 5 h. To resolve the time course of neuronal calcium transients, use a recording speed of at least 10 Hz.

24. If plaques are not yet stained with Thioflavin-S, deduce their location based on OGB-1 staining (Fig. 2A,B).

In the brain tissue labeled with OGB-1, only plaques appear as large, bright, sphere-shaped areas (arrow in Fig. 2A, upper image) often surrounded by dark areas devoid of labeled cells (asterisks in Fig. 2B). Thus, it is possible to record neuronal or glial activity first and then stain the preparation with Thioflavin-S. There are two possible advantages of this scenario. First, recordings can begin earlier and are thus made during the time when the animal is in the best shape; second, any possible Thioflavin-S-mediated modification of cellular activity is excluded. If one chooses this approach, we recommend a careful three-dimensional reconstruction of the imaged cells/area at the end of the recording session. The reconstructed volume should contain characteristic landscape marks, like blood vessel pattern and bright cells with processes. This enables easy identification of the imaged area after plaque staining (Fig. 2B, bottom).

FIGURE 2. In vivo imaging of different cell types in a mouse model of AD. (A) Images of cortical layer 1 stained with pressure application of OGB-1 (top) and Thioflavin-S (middle). The bottom panel shows an overlay of the two images. The dyes are excited at 800 nm and the emission light is split at 515 nm. Note that large plaques are visible even in the OGB-1 channel (arrow); small fiber-like structures, however, can be recognized only when stained with Thioflavin-S (arrowhead). (B) Intracellular calcium transients (middle) recorded from cells marked with corresponding numbers in the top panel. In this preparation, stained with OGB-1 only, amyloid plaques appear as bright spherical areas with surrounding dark regions (asterisks). Subsequent staining of the same area with Thioflavin-S (bottom) confirms the deduced location of the plaques. (C) Microphotograph of the cortical layer 2/3 stained with OGB-1 (green), sulforhodamine 101 (red), and Thioflavin-S (blue). Neurons, astrocytes, and plaques are visualized by consecutive splitting of the emission light at 515 and 570 nm. (D) An image of cortical layer 1 in a triple mutant mouse made by crossing APP23 x PS45 mice with CX₃CR1 mice expressing eGFP-labeled microglia (Jung et al. 2000). OGB-1 staining is shown in green, Thioflavin-S in blue, and eGFP in red. OGB-1 and Thioflavin-S were excited at 800 nm and their fluorescence was separated by a 515-nm beam splitter. eGFP was excited at 930 nm and the emitted light was sampled between 475 and 515 nm. (B, Reproduced, with permission from AAAS, from Busche et al. 2008.)

Multicolor Imaging

25. Using cell-type-specific markers and appropriate optics, visualize and monitor different cells types within the cortical network (Fig. 2C,D). For example, label astrocytes with SR101, a specific astroglial marker (Nimmerjahn et al. 2004). Inject SR101 simultaneously with the calcium indicator dye using a protocol described in Garaschuk et al. (2006).

Because of its red-shifted fluorescence spectrum, SR101 can be easily separated from OGB-1 and Thioflavin-S by using, for example, a 570-nm beam splitter (Fig. 2C).

26. Visualize microglial cells in vivo using transgenic mice in which this cell type is labeled with enhanced green fluorescent protein (eGFP) (Jung et al. 2000; Hirasawa et al. 2005).

Using multicolor two-photon imaging, microglial cells can be visualized in transgenic mice containing both eGFP-labeled microglia and amyloid plaques (Fig. 2D). To separate the eGFP fluorescence, we use excitation splitting, making use of the fact that eGFP is very efficiently excited at 930 nm but not at 800 nm (Sohya et al. 2007).

TROUBLESHOOTING

Problem (Step 8): Bleeding is seen during the craniotomy.

Solution: Consider the following:

1. Take care to cut the skull only and not to touch or pull or damage the underlying blood vessels. Because blood vessels are usually located under the dura, take care not to cut the dura. If you pull blood vessels when cutting, stop immediately and reposition the cutting blade. The skull should be sufficiently thinned beforehand to minimize the risk of blood vessel damage during the craniotomy.
2. If bleeding occurs, try to stop it by repeatedly rinsing the bleeding spot with standard external saline applied with a plastic Pasteur pipette. This will also prevent red blood cells from adhering to the dura and thus reducing the optical transparency of the preparation.
3. Should blood circulation in a large blood vessel be disrupted as a result of damage during the craniotomy, stop and attempt another craniotomy in a sufficiently remote location.

Problem (Step 20): Poor or blurry staining is seen.

Solution: Consider the following:

1. Make sure that no large blood vessels were damaged during the craniotomy, that the surface of the brain is not covered by erythrocytes, and that you are not imaging below a large blood vessel. Trying to dye-label a damaged brain area results in so-called “salt-and-pepper” staining (see Fig. 3 in Garaschuk et al. 2006).
2. If the preparation looks healthy but unstained, the following possibilities exist:
 - i. The dye-injection pipette may be clogged. Monitor pipette resistance during the staining procedure.
 - ii. Application of the dye may be too superficial. Monitor pipette resistance to estimate when the pipette is touching the dura. Especially when labeling neurons in adult and aged tissue, it is sometimes necessary to wait longer (e.g., for an additional 30 min) for the staining to develop. In general, the more plaques the animal has, the more challenging it is to obtain high-quality labeling of layer 2/3 neurons. Therefore, first familiarize yourself with the technique in control littermates and then in animals that have not yet reached their maximal plaque load.

FIGURE 3. Quality of neuronal staining in juvenile and aged mice. (A,B) Images of cortical layer 2/3 cells stained with OGB-1 in a juvenile (A) and an aged (B) mouse. (C,D) Histograms (C) and a corresponding bar graph (D) show the distributions and the mean values of the neuropil/cell brightness ratio in the juvenile (black) and aged (red) mouse cortex. The brightness of the neuropil was measured by using a region of interest identical to the one used to analyze the corresponding cell placed in the immediate neighborhood of the cell of interest (see asterisks in A). (B–D, Reproduced, with permission from Springer Science+Business Media, from Eichhoff et al. 2008).

Problem (Step 22): There is a lack of spontaneous neuronal activity despite good labeling with the indicator dye.

Solution: Check the condition of the experimental animal. Breathing rate should not decrease below 85 breaths/min and the body temperature should stay above 37°C. These criteria should be applied throughout the experiment (e.g., surgery, staining, and recording). Surgical and staining procedures should not exceed 2 h before the commencement of recording.

Problem (Step 22): Unstable recording conditions and/or movement artifacts are seen.

Solution: Movement artifacts observed during high-resolution in vivo imaging can be subdivided into two classes: a slow drift of the image plane and fast movement artifacts caused either by breathing or by the heartbeat of the animal. Consider the following:

1. To prevent slow drifts, keep both body temperature and the temperature of the superfusing standard external saline constant.
2. To reduce breathing artifacts, stabilize the animal's breathing rate above 85 breaths/min and assure that the recording chamber is tightly attached to the skull and tightly fixed in the experimental setup.
3. Heartbeat pulsations can cause artifacts at a frequency of ~400 beats/min. These are very prominent in the vicinity of large blood vessels and are rather difficult to avoid. Therefore, we prefer to record from brain regions devoid of large blood vessels.
4. In general, movement artifacts are less of a problem when imaging cortical areas in adult/aged animals compared with young/juvenile ones. To control movement artifacts, it is often sufficient simply to leave the dura intact. As in young animals, movement artifacts can be further minimized by covering the skull with 2% low-melting-point agarose in standard external saline and a glass coverslip (Svoboda et al. 1999).

DISCUSSION

With more than 15 million affected individuals worldwide, AD is the most prevalent and costly neurodegenerative disorder. It causes progressive deterioration of mental capabilities and cognitive and functional impairments accompanied by severe memory loss. The disease is clearly age dependent, with advancing age being the number one risk factor for developing AD. The probability of being diagnosed with AD nearly doubles every 5 yr after the age of 65. Around 95% of all AD cases are sporadic and only ~5% are due to autosomal-dominant (familial) mutations, mostly in three AD-related genes: amyloid precursor protein (APP) and presenilins 1 and 2 (Waring and Rosenberg 2008). Mouse models of the disease expressing AD-related proteins with familial mutations reproduce several pathological hallmarks of AD, including (i) accumulation of amyloid β -containing plaques, (ii) intraneuronal aggregation of hyperphosphorylated protein tau, (iii) inflammatory response present in AD, and (iv) learning and memory deficits (Morrisette et al. 2009). The models, however, do not recapitulate the abundant neuronal loss seen in humans. Nonetheless, these mouse models offer a unique opportunity to learn more about synaptic and cellular mechanisms underlying the disease (both in their essence and in their temporal sequence) through in vivo analyses of brain function.

In Vivo Labeling of Amyloid Plaques

Several fluorescent compounds can be used to label amyloid plaques in vivo. These include Thioflavin-S (or Thioflavin-T; Bacskai et al. 2001), Pittsburgh compound B (PiB; Bacskai et al. 2003), and methoxy-X04 (Klunk et al. 2002), as well as its parent substances Congo Red and Chrysamine-G (Nesterov et al. 2005). These compounds bind to fibrillar β -sheet amyloid deposits (Klunk et al. 2002; Rak et al. 2007) and thus recognize dense core plaques but not diffuse ones. However, when applied in the same concentration to the same plaques, PiB labeled only the very core of the plaque (note that similar images were obtained by Klunk et al. [2002] when using methoxy-X04), whereas Thioflavin-S also labeled surrounding fibril-like structures (Fig. 1; Eichhoff et al. 2008). There are several possible explanations for this finding: Thioflavin-S may bind in vivo with higher affinity, have better fluorescence quantum yield or be capable of recognizing structures with lower β -sheet content. Surprisingly, the core of the plaque is also reasonably well stained with OGB-1 (Fig. 2) and with other fluorophores, such as calcium-insensitive dye Alexa Fluor 594 (not shown). Although the precise mechanism of this binding remains unclear, such binding allows for convenient and early identification of plaques in the preparations stained (for example) with OGB-1 only. It has to be stressed, however, that small fibril-like, Thioflavin-positive structures (arrowheads in Figs. 1 and 2) cannot be identified in the OGB-1-labeled tissue.

When deciding which dye to use, it is necessary to also consider the method for administering the dye. PiB and methoxy-X04 can cross the blood–brain barrier and therefore can be applied intravenously or, in the case of methoxy-X04, even intraperitoneally. In contrast, Thioflavin-S must be applied directly to the area of interest either topically (on the top of the cortex) or by pressure-injection. Thus, the choice of dye critically depends on the experimental design. Experiments aiming at monitoring plaques over prolonged periods of time will preferably use methoxy-X04 or PiB for plaque staining. However, bear in mind that intravenous/intraperitoneal application of any drug or dye results in its delayed delivery to the brain area of interest. The kinetics of dye delivery as well as the concentration of the dye remain unknown and may vary from animal to animal and from brain area to brain area depending, for example, on inhomogeneities of blood circulation. Especially in aged mouse models of AD with substantial deposition of cerebrovascular and parenchymal amyloid, differences in blood flow may substantially influence the labeling pattern and quality (see Zou et al. 2008 for additional concerns regarding intraperitoneal dye administration). Therefore, we prefer to use Thioflavin-S for combined imaging of neurons and amyloid plaques. Because a craniotomy is a necessity in such experiments, labeling plaques with Thioflavin-S requires little additional effort. Because Thioflavin-S shows limited diffusion (Fig. 1B), we prefer to target dye delivery directly to the area of interest, as described in Steps 10–15 above.

Using MCBL to Label Adult and Aged Brain Tissue

Originally, MCBL was developed to stain juvenile (Stosiek et al. 2003) and newborn (Adelsberger et al. 2005) tissue. As shown in Figures 2 and 3, MCBL can also be used to label adult and aged cortical tissue. To our knowledge, this is the only technique for intravital labeling of aged brain tissue with small molecule calcium indicators. However, image contrast is decreased in the aged compared to juvenile tissue (Fig. 3). This reduction in labeling quality is most probably caused by (i) less effective diffusion of the dye within the tissue, (ii) reduced activity of intracellular esterases, and (iii) impeded wash out of the dye from the extracellular space. These factors make the use of MCBL in adult/aged mice more challenging. The following strategy was used to enable MCBL-based labeling of aged tissue: the amount of the dye within the staining pipette and the duration of the dye injection were reduced (to reduce the amount of the dye delivered at once), and the number of injections was increased with injection spots homogeneously distributed all over the area of interest. In addition, longer waiting periods were used to allow better dye wash out and de-esterification.

Another factor to consider when imaging adult/aged tissue is the “aging pigment” lipofuscin. Lipofuscin is the product of the breakdown and oxidation of unsaturated fatty acids (Terman and Brunk 2004). It accumulates in lysosomes throughout the life. However, the speed of its intraneuronal accumulation is increased by neurodegenerative diseases, such as AD and Parkinson’s disease (Brunk and Terman 2002; Meredith et al. 2002). Lipofuscin is strongly fluorescent and has a broad emission spectrum ranging from 450 to 700 nm (Bindewald-Wittich et al. 2006; Eichhoff et al. 2008). Thus, lipofuscin interferes with many commonly used indicator dyes. When using OGB-1, however, it is possible to make use of the long-wavelength part of the lipofuscin emission spectrum to color-code and thus to visualize lipofuscin granules (see Fig. 4 in Eichhoff et al. 2008). It has to be noted, however, that the largest portion of photons emitted by lipofuscin has the same spectral properties as OGB-emitted photons (Fig. 4 in Eichhoff et al. 2008). Therefore, to avoid interference between lipofuscin and OGB-1 fluorescence, we do not study neurons containing large lipofuscin granules.

With all of these precautions in mind, the combination of MCBL, two-photon microscopy, and multicolor imaging provides a versatile technique for monitoring in vivo activity of many different elements of the cortical network in the aging and diseased brain.

RECIPES

Standard external saline for mouse

125 mM NaCl
4.5 mM KCl
26 mM NaHCO₃
1.25 mM NaH₂PO₄
2 mM CaCl₂
1 mM MgCl₂
20 mM glucose

The pH should be 7.4 when the solution is bubbled with 95% O₂ and 5% CO₂.

Standard pipette solution

10 mM HEPES
2.5 mM KCl
150 mM NaCl

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