Video Article

Simultaneous Two-photon *In Vivo* Imaging of Synaptic Inputs and Postsynaptic Targets in the Mouse Retrosplenial Cortex

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Abstract

This video shows the craniotomy procedure that allows chronic imaging of neurons in the mouse retrosplenial cortex (RSC) using *in vivo* two-photon microscopy in Thy1-GFP transgenic mouse line. This approach creates a possibility to investigate the correlation of behavioural manipulations with changes in neuronal morphology *in vivo*.

The cranial window implantation procedure was considered to be limited only to the easily accessible cortex regions such as the barrel field. Our approach allows visualization of neurons in the highly vascularized RSC. RSC is an important element of the brain circuit responsible for spatial memory, previously deemed to be problematic for *in vivo* two-photon imaging.

The cranial window implantation over the RSC is combined with an injection of mCherry-expressing recombinant adeno-associated virus ($rAAV^{mCherry}$) into the dorsal hippocampus. The expressed mCherry spreads out to axonal projections from the hippocampus to RSC, enabling the visualization of changes in both presynaptic axonal boutons and postsynaptic dendritic spines in the cortex.

This technique allows long-term monitoring of experience-dependent structural plasticity in RSC.

Video Link

The video component of this article can be found at https://www.jove.com/video/53528/

Introduction

Two-photon microscopy revolutionized the observation of brain activity in living and behaving animals. Since its introduction in 1990 it quickly gained popularity and is now implemented as one of the most interesting and innovative approaches towards examination of numerous aspects of brain activity *in vivo* [1,2]. These applications include blood flow measurements, neuronal activation (*e.g.*, using calcium level indicators or immediate early genes expression) and the morphology of neuronal cells. An increasing number of laboratories use two-photon microscopes, implementing the technique throughout the scientific world as a new standard for *in vivo* brain imaging.

The standard approach involves implantation of the cranial window (a round hole in the cranium covered with a cover glass) over the barrel or visual cortex of the mouse brain [3]. Next, depending on the experimental protocol, the mouse undergoes a series of visualization and behavioral training sessions, allowing to monitor the changes in the brain activity and neuronal morphology over time [4,5]. In both cases the craniotomy only affects the parietal bone, without crossing the sutures. It is largely believed that the main drawback of the technique is its limited application to easily accessible cortexes such as the barrel or visual cortex. Implantation of the cranial window over other regions poses a lot of difficulties, due to excessive bleeding and/or spatial hindrance.

In this paper we propose the implantation of the cranial window above the retrosplenial cortex (RSC) as another possible region of interest for two-photon *in vivo* microscopy [6]. RSC is an important element of the brain circuit responsible for spatial memory formation. Anatomically, RSC is a part of a neuronal network connecting cortical, hippocampal, and thalamic regions [7]. It is heavily involved in a range of behaviors, such as spatial learning and extinction as well as spatial navigation [6].

In order to visualize the morphological changes of the neurons we use a transgenic mouse line expressing green fluorescent protein (GFP) under the *thy1* promoter. In these mice, GFP is expressed in approximately 10% of the neurons in the brain allowing for clear visualization of the cortical axons and dendrites using two-photon microscopy [8]. Another innovation that we propose is the injection of a recombinant adeno-associated virus serotype 2/1 (rAAV2/1) coding a red fluorescent protein (mCherry) under a neuron-specific *camkii* promoter [9] into the deeper

structures of the brain projecting to RSC, such as the hippocampus. The expression of rAAV2/1^{mCherry} in the hippocampus of Thy1-GFP mouse allows for simultaneous visualization of pre- and postsynaptic elements of the hippocampo-cortical synapses [10]. The rAAV-driven expression of mCherry requires two to three weeks for the protein to reach sufficient level in the axonal terminals. This period is consistent with the usual time required for recovery from craniotomy.

Protocol

All experimental procedures described below were approved by Local Ethical Committee at the Nencki Institute of Experimental Biology, Polish Academy of Sciences.

Note: Some of the scenes in the associated video are accelerated. Speed factor is indicated in these scenes.

1. Surgery Preparation

1. Sterilize all tools, glass containers for liquids and cotton swabs in the autoclave. Use dispensable gloves. Clean the surgical table, the stereotaxic frame and all the surrounding area with 70% ethanol. Use a sterile surgical pad to create a sterile space for all the sterilized equipment. Cut gelfoam into small pieces and soak them in sterile saline.
   Note: According to the Guide for the Care and Use of Laboratory Animals, ethanol is neither a sterilant nor a high-level disinfectant. It should only be used as a cleaning/defatting agent on previously sterilized surfaces.
2. Put the animal in the induction chamber and set the isoflurane level to 5% and oxygen flow to 2 L/min. This procedure should take about 3 min.
3. Take the animal out of the induction chamber. Use tail or toe pinches in order to ensure that the animal is fully sedated.
4. Using a precise trimmer shave the hair from the back of the head (between the ears) up to the eyes.
5. Place the animal in the stereotaxic frame and stabilize the head with ear bars.
6. Set anesthesia levels to 1.5-2% isoflurane and 0.3 L/min oxygen.
7. Apply the eye ointment.
8. Inject the animal subcutaneously with Tolfedine (4 mg/kg), Butomidor (2 mg/kg) and Baytril (5 mg/kg) to prevent inflammation, pain and infection, respectively.
9. Inject the animal intramuscularly with Dexamethasone (0.2 mg/kg) to prevent brain swelling.
   Note: It is possible to inject Dexamethasone subcutaneously or intraperitoneally to prevent muscle damage.
10. Clean the skin using sterile cotton swabs with Betadine followed by 70% ethanol.
11. Change the gloves and spray them with 70% ethanol.
    Note: While using disposable gloves do not touch the sterile field. Touch the animal only with the tips of sterile surgical instruments and sterile swabs.

2. Cranial Window Surgery

1. Lift the skin with forceps and using micro scissors incise the skin horizontally along the base of the head and then obliquely to the front point between the eyes. Remove the skin flap.
2. Apply lidocaine ointment with a sterile swab on the periosteum to prevent excessive bleeding and pain.
3. Use sterile cotton swabs or a scalpel to remove the periosteum. Dry the skull with sterile swabs.
4. Using a sterile needle apply dense cyanoacrylate glue on the skin edges to immobilize them and to prevent from contact with dental cement. Wait for the glue to dry.
5. Lay a sterile 3 mm coverglass over the skull anteriorly to the lambdoid suture. Center the coverslip at RSC coordinates: AP, bregma -2.8; ML, bregma 0. Mark the coverslip edges by scratching the skull surface with a sterile needle. Put the coverglass back into the sterile container with 70% ethanol.
6. Use a high-speed dental drill with small diameter burr to outline a 3 mm diameter circle. Clean the drilling site from the bone dust with sterile saline dipped swabs. Use the gelfoam and swabs to stop the occasional bleeding and clean the bone.
7. In between drilling check the bone thickness with fine forceps by gently touching the bone circle and checking its mobility. Keep in mind that the bone is thicker on the suture's area. Stop the drilling when the bone circle is mobile and only an even, thin layer of bone is left on the circumference. Clean the operational field of all the remaining bone dust with saline dipped swabs.
8. Drop the sterile saline on the drilling area, covering the drilled circle. Carefully pry the bone circle with fine forceps and then gently but firmly remove the bone by lifting it upwards. Be careful not to skew the bone circle while lifting it to prevent possible damage to the dura.
9. Gently apply the gelfoam soaked in sterile saline on the dura to help stop the bleeding. Wait until all bleeding is fully stopped. Carefully remove the gelfoam not to disturb the clotting process.
   Note: The suture area is highly vascularized, so the bleeding at this point might prove to be profound. It is essential to wait for the sufficient time for the bleeding to stop completely. It is helpful to keep the saline-soaked gelfoam cooled by placing it on ice.

3. Virus Injection

1. Attach the infusion pump to the stereotactic tower and connect the controller.
2. Insert the 35G needle into the syringe. Flush the syringe 10 times with ethanol to sterilize it and 10 times with sterile saline to remove traces of ethanol. Remove air bubbles from the syringe. Insert the syringe into the pump.
   Note: Consider using other disinfectants.
3. Thaw a single dose of rAAV2/1^{mCherry} preparation (10^{12} pfu is recommended) and keep it on ice. Fill the syringe with the virus solution.
4. Center the needle on the bregma and then gently insert into the hippocampus using the following coordinates: AP -2, ML +/- 1.0, DV -1. These coordinates will be located near the edge of the craniotomy. Wait for 5 min for the tissue to stabilize.

5. Inject 0.7 µl of the rAAV2/1^{mCherry} solution at the rate of 50 nl/min. Wait 10 min for the virus to fully adsorb. Gently remove the needle. Blot with gelfoam if bleeding occurs. Repeat with the contralateral site.

4. Cranial Window Implantation

1. Lay the sterile, dried coverglass on the top of the dura in the drilled circle frame. Hold the coverglass with the forceps to gently flatten the dura and bring coverglass' edges closer to the skull surface.
   Note: It is possible that the coverglass disrupts the clot and the bleeding resumes. If that is the case, lift the coverglass, place it in alcohol, dry and return to step 2.9.
2. Using a sterile needle apply the dense cyanoacrylate glue on the coverglass edges to attach them to the skull. Wait for the glue to dry.
3. Place a fixation bar (M2 nut or a custom made design) in the front part of the skull. Apply the cyanoacrylate glue over the edges of the bar. Wait for the glue to dry.
   1. Place the fixation bar in a position that will enable horizontal positioning of the cranial window during imaging session. Place it as distant as possible from the window. If it is placed too close to the window, the bar and the screw connecting it with the custom-made holder might pose as an obstacle for the objective during the imaging process.
4. Prepare the dental acrylic and apply it on the skull surface around the glass. It is helpful to form a crater-like shape around the window. It will create a cavity for water applied later for imaging with the water objective.
5. Create a cap with the dental acrylic, covering the rest of the operational area, skin edges, fixation bar, reinforcing the crater around the cranial window. Wait for the dental cement to harden.
6. Remove the animal from the stereotaxic frame and put it into the recovery chamber.
7. Wait for the animal to recover from the surgery while observing the physiological functions.
8. Apply post-operative analgesia (carprofen, 10 mg/kg) and antibiotics treatment (baytril, 5 mg/kg) for 48 hr.

5. Imaging

1. Start the Ti:Sapphire laser, power up the microscope. The system used in this experiment is equipped with a two-photon laser, OPO system and dual GaAsP PMT.
2. Put the animal in the induction chamber and induce anesthesia.
3. Remove the animal from the induction chamber and place in the gas anesthesia mask under the microscope. Decrease the oxygen flow to 0.3 L/min and the isoflurane concentration to 1.5-2%.
4. Fix the animal to the custom microscope frame with a M2 screw (or another custom system). Level the cranial window.
   Note: It is possible to use the microscope manufacturer's head fixation system, although the specific custom frame gives better results (improved head stability, constant positioning in multiple sessions during a chronic experiment).
5. Using the widefield microscope settings and a low magnification objective center the view on one of the sides of retrosplenial cortex and focus it on the coverslip surface.
6. Apply a droplet of water into the crater-like acrylic well. Switch to a long distance water immersion objective. Move the objective towards the cranial window until the water meniscus connects the specimen and objective.
7. Switch to two-photon settings and begin scanning the specimen top to bottom using lowest zoom. The crossing of dura mater will be visible as a glare of high non-specific signal.
8. Adjust the microscope acquisition settings in both channels (GFP and mCherry) according to the signal strength from fluorescent cells in order to cover the entire dynamic range.
9. After finding a suitable neuron (with the dendritic tree separated from other cells) perform an initial scan using only the GFP filterset with lowest zoom and z-distance of 5 microns.
10. Obtain a maximum projection of the scanned stack and print it for annotations (using inverted colors).
11. Set zoom to a value that will allow to image the desired morphological details. Image the entire dendritic tree in the GFP and mCherry channels using maximum projection as guide.

Representative Results

The expression of GFP in a subset of neurons in the Thy1-GFP reporter mouse allows *in vivo* imaging of the cortical dendrites and local axonal projections in RSC. **Figure 1A** shows maximum projection of a stack of images with multiple GFP-positive dendrites visible. The cell body is obscured by an artery. **Figure 1B** shows a single plane zoomed image (digital zoom 3x) of the dendritic branch indicated in 1A. Details of dendritic morphology (spines, filopodia) are clearly visible. The GFP channel is acquired by using the band pass emission filter 500-550 nm.

An injection of the rAAV2/1^{mCherry} into the dorsal hippocampus allows visualization of the hippocampal axons and synaptic boutons terminating in RSC. These terminals can be detected in the mCherry channel (the band pass emission filter 570-610 nm).

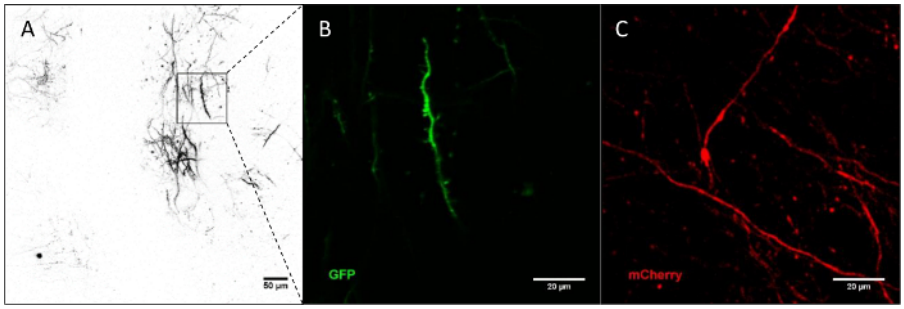

Figure1. Two channel *in vivo* two-photon imaging of RSC neurons and hippocampal projections to RSC. (**A**) An overview of the GFP-expressing cells in a fragment of RSC (image shown in inverted colors). Maximum projection shown of a 100 µm thick stack taken at low magnification (0.7x digital zoom). (**B**) Single optical plane of the fragment indicated in (**A**) acquired at high magnification (3x digital zoom) in the GFP channel. (**C**) Single optical plane fragment indicated in (**A**) using the mCherry acquisition settings. See Protocol for details of the detection filters. Please click here to view a larger version of this figure.

Discussion

In the current paper we present a protocol for simultaneous two-photon *in vivo* imaging of the synaptic inputs and postsynaptic targets in RSC through a cranial window. The implantation procedure consist of several key steps. First, the animal is deeply anesthetized and fixed in the stereotactic frame, then the skull over RSC is thinned with a drill along the marked circular lines and the circular bone is removed. After the bleeding is stopped, the rAAV2/1^{mCherry} is injected into the hippocampus, and the cover glass is fixed to the skull over the drilled area. Finally the fixation bar is secured on the head and the animal is placed in the recovery chamber for 48 hr. After approximately 2-3 weeks needed for the virus expression, the RSC can be visualized. The imaging protocol comprises of the following steps. First, the animal is anesthetized and fixed under the microscope. The focus is then set using the widefield microscope, the system is switched into the two-photon mode and the channels of interest (GFP and mCherry) are visualized.

The presented technique offers a major improvement over the previously described protocols. In the standard approach, only one type of a label could be detected. It could be used to image local axonal projections and dendritic trees, but no long range connectivity studies were possible. By combining two fluorescent proteins we enabled simultaneous tracing of pre- and postsynaptic elements. This allows long term *in vivo* monitoring of putative synaptic connections.

In order to obtain the optimal results in the described procedure, it is important to pay attention to several critical steps. During lifting the bone circle in step 2.8 any damage to the dura may cause inflammation and impair transparency of the cranial window. Insufficient stoppage of bleeding in step 2.9 or at any other step of the procedure results in blood accumulation under the window and significantly reduces the field of view. After injecting the virus it is vital to wait for at least 10 min before removing the needle, in order for the virus to infuse into the tissue at the injection site only. It limits the possibility of unwanted infection of the cortex with the virus during needle removal. The area of the viral transfection should be examined with a *post hoc* histological analysis of the brain tissue. Any mCherry expression in cells along the needle trace should be avoided. The standard recovery time is 3 weeks. This period is sufficient for the virus to reach stable expression in the synaptic projections and for the cranial window to fully heal and stabilize. The animals should be single housed in order to prevent removal of the cranial window implant by the cage mates. The use of an enlarged cage might be considered in order to avoid accidental damage of the cranial window by hitting the metal bars. Stable fixation of the mouse under the microscope is essential. Any head movement, including breathing movements, may cause significant reduction in quality of the obtained images. It is also helpful to position the cranial window horizontally to the objective, to limit possible problems with acquiring equal focus for the entire plane of the window. Clear criteria for resolving spines and boutons should be applied. Generally, boutons are defined as axonal swellings having a diameter at least 3 times bigger than the preceding fiber [7]. Spines are defined as clearly distinguishable protrusions from the dendrite shaft that contain a bulbous head. Further division into populations of thin, stubby, mushroom and branched spines is possible [3]. Due to the relatively poor axial resolution of the two-photon microscopy, analysis of spines that project along the optical axis should be avoided [3]. In order to clearly distinguish functional boutons and spines, a *post hoc* immunolabeling can be performed in order to identify pre- and postsynaptic markers [7].

Although the presented protocol proved to be the most favorable in our experimental designs, it is possible to modify it in order to fit different experimental goals. Sometimes it is more suitable to use injectable anesthesia (such as ketamine-xylasine) instead of isoflurane, but it is important to adequately adjust the dosage, keeping in mind differences in drug susceptibility between mice of different strains, age and sex. It is possible to use a trepan bur instead of a spherical one for the drilling, but it could increase the risk of damage to the dura. Sterile saline may be successfully replaced with artificial cerebrospinal fluid (ACSF), but it is important to keep it sterile at all times during preparing and operation. ACSF is stable for 3-4 weeks after preparation, and if any contamination occurs before this time it should be immediately discarded. Different head fixation devices may be applied, depending on the experimental design, including the possibility of observing the awake mouse under the two-photon microscope.

As any other technique, this one also has its limitations. The two-photon microscope allows for visualization of the brain tissue up to 500 µm deep from the dura surface. For examination of the deeper brain structures additional modifications must be applied. Our protocol allows access to most of RSC, but the part of the structure hidden under the superior sagittal sinus is still not accessible. The resolution of the two-photon microscope is not sufficient for the identification of a specific synapse as well as detailed morphological analysis of dendritic spines. Additional techniques, such as correlative electron microscopy must be applied in order to confirm the existence of a suspected structure. It is also important to mention that this is a relatively difficult surgical technique and it is not recommended for an unexperienced operator.

The presented technique may be applied in a wide range of experiments. It may be modified for investigation of different brain regions accessible with the two-photon microscope. It enables simultaneous monitoring of axonal and dendritic alterations during variety of physiological and

pathological states including cognitive processes and aging or progression of neurologic and psychiatric conditions. It allows the use of cell-specific promoters to visualize projections originating at precisely defined neuronal subsets. It may also be adjusted to fit the protocols of experiments on awake and behaving animals. Furthermore, calcium- or pH- sensitive proteins can be expressed in the brain in order to visualize not only the neuronal morphology but also changes in cell activity and function. Another possible modification of the approach is the use of a different rAAV serotype for mCherry expression. The chimeric 2/1 serotype provides robust expression at the injection site with sufficiently rapid onset (2-3 weeks). The mCherry levels remained stable for at least 12 weeks after initial onset and no retrograde labeling was detected in our experiments. In order to obtain retrograde labeling, a different serotype might be used, such as rAAV9. The imaging sessions can be performed at any frequency, however at least 24-hr interval is recommended in order to allow proper recovery of the animal after anesthesia. If applied properly, this technique allows performing multiple imaging sessions of the same region over the course of several months. For long-term experiments (longer than 6 months), a Cre/LoxP system can be used with the recombinase delivered with the AAV vector into a floxed GFP mouse line.

Disclosures

The authors (K.L., M.R., K.R.) have rights for patent pending (PL410001) for Holder frame used in this Article.

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