Chapter 2

Surgical Procedures to Study Microglial Motility in the Brain and in the Spinal Cord by In Vivo Two-Photon Laser-Scanning Microscopy

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Abstract

Microglia are the innate immune cells of the central nervous system (CNS). As such, they contribute to neural inflammation in a large number of neurodegenerative diseases. The processes of microglia are continuously moving to survey the brain parenchyma. Depending on local signals, microglia can almost instantaneously change their morphology. Two-photon laser-scanning microscopy (2P-LSM) has become the method of choice to study microglia in vivo. Here, we will describe in detail surgical procedures to prepare transgenic mice with fluorescent protein expression for 2P imaging of microglia in the cortex and the spinal cord. In addition, we will give some advice how to optimize image quality.

Key words Microglia, In vivo two-photon imaging, Spinal cord, Brain, Surgery, Transgenic mice

1 Introduction

Microglia were first described by Pio del Rio Hortega in 1932, and most of his functional postulations are still valid today [1]. Microglia are evenly dispersed within the CNS. Classically, they have been described to have two different activity states. In the so-called resting state or quiescent state, the microglia have a small cell body and numerous extended processes. More recent research, however, showed that the term "resting" is an insufficient description. All the processes are continuously moving and surveying the adjacent parenchyma, and the term "surveillant" has been introduced as a more appropriate term [2–4]. Interestingly, the cell somata largely remain at fixed positions. In the "activated" state microglia change into an amoeboid morphology, i.e., a large cell body and less processes. Developmental situations as well as disease conditions let microglia switch between states. During early development activated

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microglia use vessels and white matter tracts as guiding structures to enter and migrate through CNS, where they transform into the surveying state [5].

1.1 Surveillant Microglia

In the surveying state, microglia have a housekeeping function. The processes are continuously moving without any apparent direction. Despite their high process motility, the cell body remains largely immobile. Microglia are positioned 50–60 μm apart from each other [3]. The different processes of neighboring cells appear not to touch each other while they are scanning the environment. This setup allows microglia to explore any volume of the CNS every few hours [3]. However, during tissue scanning the processes establish brief contacts with neuronal synapses [6, 7], thereby probably monitoring the functional status of synapses. It is conceivable to speculate that these synapse-process interactions contribute to remodeling of the neuronal cytoskeleton during episodes of plasticity [8]. Besides their surveillant function, microglia also clear accumulating metabolic products and cell debris of deteriorated cells by phagocytosis.

1.2 Activated Microglia

When surveillant microglia encounter injured cells or debris, they quickly become activated. It starts with extension of microglial processes towards the target, followed by migration of their cell bodies and eventually phagocytosis [9]. There is no general difference between the microglia reaction in the gray and white matter as observed in the cortex or spinal cord. The speed, however, in which the processes are sent to the target site in the white matter, appears to be slightly accelerated [10]. After experimental lesions, for example, if local tissue is damaged by a high-power laser pulse, microglia will react to within 3–5 min (Fig. 1A, B). The responsive microglia do not target a specific area, but form a "spherical shell" around the lesion site (Fig. 1C).

1.3 In Vivo Two-Photon Laser-Scanning Microscopy (2P-LSM) of Microglia

Any form of CNS damage will initialize a cascade of cellular and molecular events over several days. Therefore, microglia in acutely isolated brain slices or even in tissue cultures have to be regarded as being in the activated state. With the recent advent of 2P-LSM of the CNS in living animals, we are now equipped with an excellent tool to investigate microglia both in the surveillant and in the activated state. By increasing our knowledge about microglia, we obtain better chances to develop treatment strategies for neural inflammation in neurodegenerative diseases. In the following we will describe how we investigate transgenic mice by (long-term) 2P-LSM in the cerebral cortex and spinal cord. We will compare two different methods of surgery and provide advice to troubleshoot problems (*see* **Note 2**).

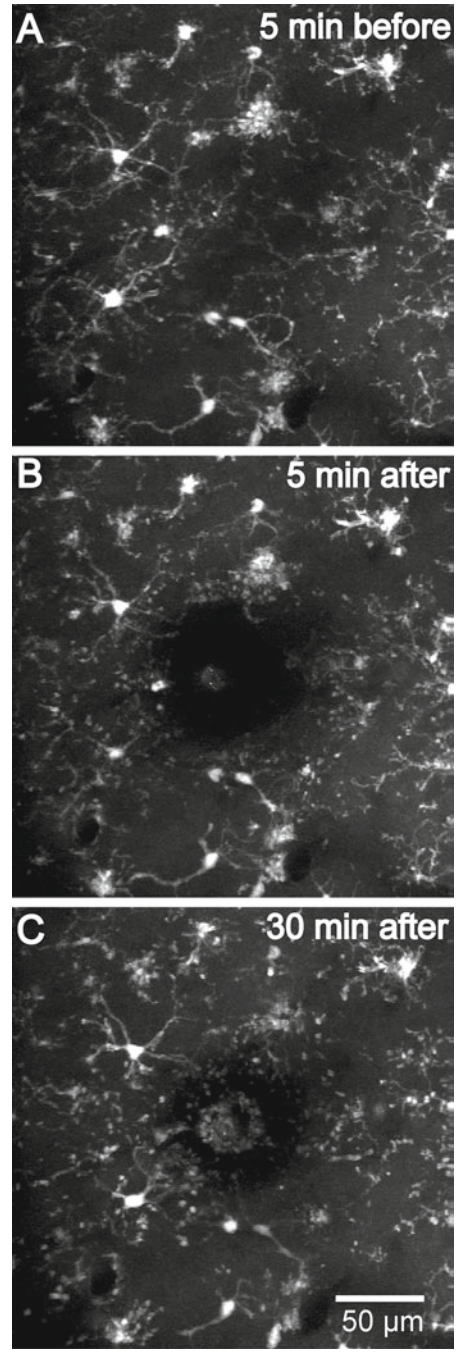


Fig. 1 Two-photon images of green fluorescent microglia in an adult $CX_3CR1^{GFP/+}$ mouse. (**A**) Image recordings taken before and after the lesion at indicated times. A lesion is made by increasing the power of the laser for 1.5 s and changing the wavelength from 900 to 800 nm. (**B**) The microglia react within 5 min to the lesion in the middle of the image. (**C**) After 30 min the microglia have formed a "spherical shell" around the lesion

2 Materials

2P-LSM in vivo commonly uses cell-specific labeling with fluorescent dyes. The labeling can occur using low-molecular-weight fluorescent dyes and taking advantage of cell-specific uptake mechanisms [11, 12]. However, more frequently transgenic mice with cell-type-specific fluorescent protein expression are used [3, 13]. For microglia there are two of such mouse lines available: in TgH(CX_3CR1-EGFP) mice, EGFP (enhanced green fluorescent protein) is expressed from the gene locus encoding the fractalkine receptor CX_3CR1 [14]. The other mouse line has been generated by nonhomologous recombination. The transgene is composed of the Iba1 promoter and the EGFP open-reading frame [15]. Iba1 is a calcium-binding protein, specifically expressed in microglia and upregulated in the activated state. According to the experience of most colleagues, the TgH(CX_3CR1-EGFP) mouse shows a more complete, uniform, and brighter labeling of microglia than the TgN(Iba1-EGFP) mouse. However, one has to take into account that the TgH(CX_3CR1-EGFP) misses one allele for CX_3CR1 expression.

2.1 2P Imaging Setup

Any commercial or custom-made two-photon laser-scanning microscope (2P-LSM) can be used. The setup requires a separate channel for bright-field imaging which is equipped with a CCD camera. The bright-field channel visualizes blood vessels at the surface above the region of interest (ROI). The respective vessel pattern facilitates the localization of the ROI in subsequent imaging sessions. It is quite difficult to do the same with a fluorescent channel only. It is trivial to say, but if this channel does not detect fluorescence, it is completely dark and does not give any information regarding the position of the microscope. For 2P-LSM, a long-distance 20× objective with high numerical aperture (NA) is required (e.g., Zeiss W Plan-Apochromat 20× 1.0 NA DIC). We have good experiences with Pockels cells (Conoptics) to control the laser intensity. As software we are using the open source ScanImage written in Matlab [16].

2.2 Reagents

Agarose (low melting point) and artificial cerebrospinal fluid (ACSF; 125 mM NaCl, 25 mM $NAHCO_3$, 2.5 mM KCl, 1.25 KH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM glucose). Bubble with 5 % CO_2 and 95 % O_2 to generate and stabilize a pH of 7.3, Bepanthen®(Bayer), buprenorphine (Temgesic), cyanoacrylate (Superglue), dental cement, ethanol, ketamine, methohexital sodium (e.g., Brevimytal® Hikma, 6.67 mg/ml in 0.9 % NaCl containing 0.7 mg/ml glucose), norepinephrine, 0.2 % pancuronium bromide (dilute 1 volume in 9 volumes of 0.9 % NaCl), pentobarbital sodium (5 mg/ml in 0.9 % NaCl), Quick-Sil (WPI), respiration gas mixture 1 (N_2, O_2, CO_2), respiration gas mixture 2 (N_2O, O_2, Isoflurane), vaseline, and xylazine.

2.3 Equipment

For some parts of the different surgeries, some custom-made items are needed, and we describe them in detail in the figures of paragraph 3.

Anesthetic bracket for two gases (Harvard Apparatus); anesthesia equipment (injection pump for application of small volumes (e.g., Programmable Syringe Pump AL-1000, WPI)); 5-ml syringe with Luer lock connection fitting and rubber piston, intravenous cannula (polythene, 0.28 mm inner and 0.61 mm outer diameter), and hypodermic needle (0.3 mm outer diameter) to fit the intravenous cannula; binocular dissection microscope; coverslips; drill; differential preamplifier; filter tips; gelatin sponges (Spongostan); adjustable heat pad; hypodermic needles; oscilloscope; Pasteur pipettes; platinum electrode; pulse oximeter; rectal temperature sensor; respiration equipment (high-pressure gas bottle with reduction valve for respiration of gas mixture, fittings, adjustable flow meter to measure and adjust respiration gas flow (Platon GTF 1 BHD, for air, 20–250 ml/min), solenoid valve (ASCO scientific 4224) for artificial respiration, control logic for solenoid valve, tracheal tube (T-shaped, custom-made), pressure tubing to connect to reduction valve and flow meter, silicon tubing (1 mm inner and 3 mm outer diameter) to connect flow meter with tracheal tube, and silicon tubing (2 mm inner and 4 mm outer diameter) to connect tracheal tube with solenoid valve); spinal cord clamps (Narishige STS-A); surgical instruments (curved forceps (FST 11051-10), dissector scissors (FST 14081-09), Dumont forceps (#5 Biology, #3c, and #7), Michel suture clips (FST 12040-01) and forceps (FST 12018-12), micro curette (FST 10081-10), micro scissors, needle holder (FST 12002-12) and needles (FST12050-03), scalpel handle (FST 10007-12) and blades (FST 10010-00), Wecker-type scissors (FST 15010-11), silk suture (FST 18020-30), and straight scissors (FST 14088-10)); surgical staples; tape; temperature probe; thin cotton sewing thread; and vacuum pump.

3 Methods

Here, we will give detailed information of different surgery procedures for both the brain and the spinal cord of anesthetized mice to be imaged by 2P-LSM. First, we will describe some procedures which apply to surgery in general and thus to all other sections of this paragraph.

3.1 General Procedures

All surgical procedures should be performed under a binocular dissection microscope with adjustable magnification and sufficient working distance. During the surgery anesthesia has to be carefully controlled (*see* **Note 3**). Criteria for sufficient anesthesia are decrease in body temperature and the absence of central reflexes like pinna reflex or the corneal reflex. In this state, the natural

respiration is slowed down to less than two breaths per second; the pulse and respiration will normally not accelerate during surgical procedures. During surgery these criteria should be monitored continuously and anesthesia should be adjusted accordingly. For adequate surveillance of vital functions, we record the electrocardiogram (ECG) and the O_2-saturation level. For ECG recording, we insert a platinum electrode in each forelimb, ground the animal, connect the electrodes with a sensitive preamplifier, and visualize the signal with an oscilloscope. For recording the O_2-saturation level, we use pulse oximetry (e.g., MouseOx®, Starr Life Sciences Corp.).

In chronic experiments (i.e., repeated imaging sessions over weeks or months), we anesthetize the animals with the volatile anesthetic isoflurane. Animals are placed into a confined space (preferably a box in which you can observe the animal) with a mixture of 5 % isoflurane and 47.5 % O_2 and 47.5 % N_2O (nitrous oxide, also known as laughing gas, it has analgesic properties) using Harvard Apparatus equipment. When the animal has passed out, we move the animal to the surgery table and reduce the isoflurane to 2 % and the flow of the O_2 and the N_2O to 0.4 l/min (0.8 l/min in total). Make sure that the isoflurane is directed at the nose of the mouse and that the excess anesthesia is sucked away (we do it with a vacuum pump), so it does not contaminate the lab. With i.v. anesthesia in acute experiments, a tracheotomy may be performed to allow artificial respiration. With this procedure we can relax the muscles with curare-like substances to suppress active respiratory movements of the ribcage, which are responsible for most of the unwanted movements of the spinal cord, and subsequently improve the quality of the images. It is self-evident that volatile anesthesia can be combined with a tracheal tube and that a tracheal tube may also be inserted through the mouth; thus it is possible to perform chronic experiments also in combination with muscle relaxation. A temperature probe soaked in vaseline has to be inserted rectally, and the body temperature has to be kept close to 37 °C by an adjustable heat plate. The eyes should be covered with an eye protective substance (e.g., Bepanthen) to prevent the cornea from drying. In chronic experiments, all procedures have to be done under conditions as sterile as possible, and the wounds have to be kept as small as possible. We use 70 % ethanol to disinfect the skin and the instruments. Stop bleedings with gelatin sponges (Spongostan) (*see* **Note 4**). Postoperative analgesia should be given. We normally give 3.3 µl buprenorphine (0.9 mg/ml in 0.9 % NaCl) per g body weight of the mouse. Be careful not to overdose buprenorphine; it also acts as a respiration depressive.

3.2 Spinal Cord Surgery: Variant 1

The animals are initially anesthetized by i.p. injection of 77 mg sodium pentobarbital (5 mg/ml in 0.9 % NaCl) per kg body weight of the mouse. The dose should be suitable to allow all procedures including the placement of the central vein catheter for the successive anesthesia. In a supine stretched position, expose the jugular

vein by a longitudinal cut of the skin of the neck providing access to the trachea. Then insert the central vein catheter into the jugular vein. We use polyethylene tube, 0.28 mm (inner diameter) and 0.61 mm (outer diameter), which is fixed to the vein by two ligatures and sewed to the skin of the chin to keep the proper direction. The tube is connected with a 5-ml syringe via a Luer lock connection to a hypodermic needle (0.3 mm outer diameter). The syringe is operated by a programmable syringe pump (e.g., AL-1000, World Precision instruments). This syringe is filled with sodium methohexital (synonyms Brevital, Brietal, Brevimytal), 6.67 mg/ml in 0.9 % NaCl. The dose is 55 mg/(kg × h). Starting the pump infusion of methohexital, the dose has to be enhanced by about 50 % for about half an hour, because the effect of pentobarbital is fading and the recommended dose of methohexital is just the required maintenance dosage.

3.3 Intubation

The trachea is exposed by dividing the salivary glands and the ventral neck muscles in the midline. Caudally of the thyroid gland, we position two suture loops round the trachea for fixation of the tracheal tube. Be careful not to injure the thyroid gland, the vagus nerve, or the carotid artery. Apply a transverse half incision and a short (1 mm) longitudinal incision in caudal direction between the two suture loops. Then insert the custom-made connector (Fig. 2A) and fix it with the lower suture loop and with the excess ends of the suture loops. Close the skin of the neck with surgical staples. For image recordings of the spinal cord without motion artifacts, a certain degree of paralysis is obligatory. Therefore, we relax the animal with 8 μg pancuronium in 100 μl NaCl. Relaxation has to be repeated every hour. Then the connector (Fig. 2A) has to be connected with the respiration tubes. Experience shows that a mixture of 47.5 % O_2, 2.5 % CO_2, and 50 % N_2 is appropriate. The relatively high percentage of CO_2 prevents respiratory alkalosis. The high level of oxygen allows for flat respiration and reduction of respiratory movements. Here, we use pressure inspiration and passive expiration. We adjust the inspiration time to 100 ms while about 140 μl of gas mixture is pressed into the lungs, and the passive expiration time is set to 400 ms. The gas flow is adjusted to about 84 ml/min. Inner diameters of the tubes of the connector (Fig. 2A) are designed according to this kind of artificial respiration, small connection to the respiration gas tube, larger inner diameter in the trachea, and very large inner diameter to release the respiration gas. Finally, respiration occurs with about 2 breaths per second. Adjustments of respiration should be done by solenoid valve control and gas flow according to values of blood oxygenation.

3.4 Spinal Cord Preparation

To get access to the spinal cord, the mouse has to be turned to a prone position. Open the back skin in the midline at the location of the lumbar spines and fix the spinal column rostral to the L1 spine and caudally to L2 spine with spinal cord clamps (Narishige STS-A).

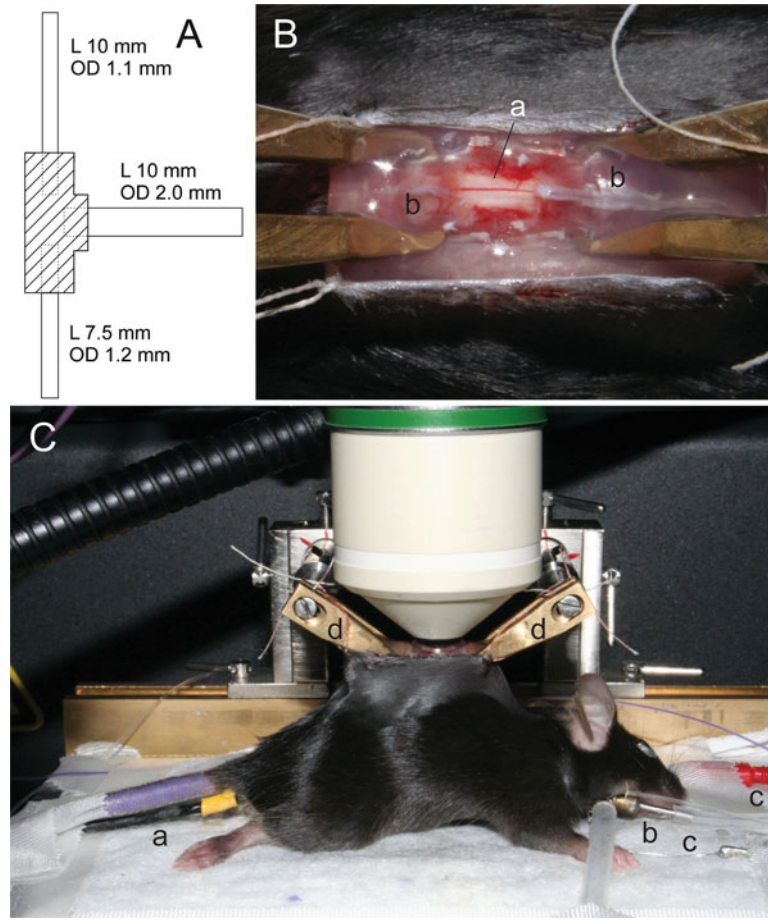


Fig. 2 Spinal cord surgery—variant 1. (**A**) Schematic view of the custom-made connector. The part with outer diameter (OD) 2.0 mm is respiration out, 1.2 mm is connected to the trachea, and 1.1 mm is respiration in. For more details on the respiration gas mixture, see text. (**B**) The region of interest (*a*) has to be kept wet with ACSF. Therefore we make a pool of agar (*b*), so the ACSF will not flow away. (**C**) Overview. The mouse is positioned on an adjustable heat plate (*a*) and has a rectally inserted thermo-probe (*a*). The respiration connector (T piece) is indicated with *b*; the schematic view is in (**A**). The wires we use to record the EEG are indicated with (*c*). The spinal clamps are indicated with (*d*)

Remove the muscular and tendon material between the clamps with a small curette (1–1.5 mm, Fine Science Tools, FST). Remove the vertebral arches of L1 and L2 with a blunt stainless steel forceps #7 (Dumont). These are strong enough to break off the bone between the articular processes and the spinal process. The remaining spinal process can be removed. All steps can be done without damaging the dura mater. Remove the debris on top of the dura with a fine Wecker-type scissors (e.g., FST #15010-11). Be careful to keep the spinal cord wet with artificial cerebrospinal fluid (ACSF).

Form a reservoir by tying the back skin flaps up. To reduce motion artifacts, fill the reservoir with low-melting agarose (2 % in ACSF; be careful to let the agarose cool down sufficiently) and excise some agarose gel after hardening to form a sufficiently large hole for the water immersion lens (Fig. 2B, C). Before the reservoir is filled with agarose, protect the spinal surface with a small piece of ACSF-soaked cotton ball at the ROI. This will also facilitate the excision of hardened agarose. Fill the hole with ACSF. The whole procedure may be expanded to more than only two spinal arches. To get access to the dorsal roots, all lumbar spinal arches (L1–L5) have to be removed. The spinal clamps have to be positioned accordingly.

3.5 Preparation for Repeated Imaging

In principle, this procedure is similar to the one in acute experiments. However, the incision of the back skin is kept as small as possible. Spinal cord clamps are positioned with only two spines in between the clamps. Remove the longitudinal muscle fibers and the tendon material only between these two spines to open a small but sufficiently large window to the spinal cord. Beneath, the intact dura will be visible. It contains a lot of collagen causing significant backscatter during 2P-image recordings. Therefore, the fiber bundles on top of the dura have to be thinned by fine forceps (#5 Dumont).

3.6 Spinal Cord Surgery: Variant 2

This type of surgery has been developed by the group of Franck Debarbieux [17]. Here, we will provide a short summary of their approach. This variant requires more animal handling, and therefore we do not use volatile anesthesia, but inject i.p. ketamine (100 mg/ml) and xylazine (20 mg/ml) in 0.9 % NaCl (100 µl per 20 g body weight). To get access to the spinal cord, make a dorsal midline incision over the T12-L2 vertebras of about 1.5–2 cm. Remove subcutaneous fat, so that a clear view of the dorsal processes of T12-L2 is established. Make two cuts parallel to the dorsal processes, in between the spinal and transverse processes. Remove the muscles covering the lateral part of the T12-L2 vertebrae with a micro curette. After this dissection four individual vertebral bodies should be visible. Drill a small puncture lateral to the bodies of the T12 vertebrae and L2 vertebrae. Put two modified staples (Fig. 3Bb) in such a way that they fit along the T12 and L2 pedicles. Stabilize the spinal window with cyanoacrylate. Pressing a little on the extensions will guarantee a bigger imaging window. Cyanoacrylate has to be applied to all tissue, even under the cut skin, but the vertebral bodies should remain free of cyanoacrylate. Implant in the cyanoacrylate a modified paperclip (Fig. 3Ba). After the cyanoacrylate is polymerized, apply dental cement over the cyanoacrylate layer and the modified paperclip. This is needed to form a rigid foundation base around the vertebrae. While waiting for the cement to harden, keep the vertebrae moist with ACSF.

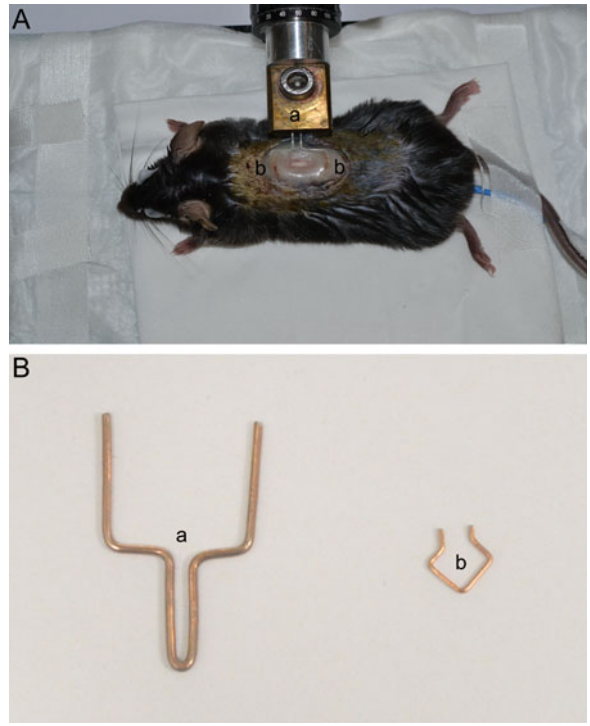


Fig. 3 Spinal cord surgery—variant 2. (**A**) Surgery table. The holder is the *golden colored plate* with the screw. The mouse is attached to the holder with a modified paperclip. *b* indicates the modified staples. Both the holder and the table can be moved so that the ROI can always be perpendicular to the vertical axis of the objective. (**B**) Modified paperclip (*a*) and modified staple (*b*). The paperclip and the staple are modified with a plier. The staple is modified in such a way that they can fit along the T12 and L2 pedicles. The bottom part of the modified paperclip can be fitted in the holder, which is depicted in (**A**)

After the dental cement has dried, place the mouse into a holder (Fig. 3A) by putting the top of the modified paperclip in the holder. Perform a laminectomy on the two vertebrae in between the T12 and L2 using a drill (FST). If necessary access cement should be removed. Clean the dura carefully after the laminectomy. Cover the exposed area with Quick-Sil (WPI). Put immediately a coverslip on the Quick-Sil. Remove superfluous Quick-Sil. Apply cyanoacrylate to the edges of the coverslip. After the cyanoacrylate has dried, put dental cement over the cyanoacrylate to reinforce the structure. Wait at least 12 h before the first imaging session (*see* **Note 5**).

3.7 Brain Surgery: Variant 1

Expose the part of the brain you want to image. Remove all tissue (skin, hairs, and muscle). Clean the skull with ACSF and make sure the skull is dry before you start drilling. Make a craniotomy; be careful not to damage the dura. Clean the dura with ACSF and put

a small drop of Quick-Sil on the dura. Make sure the dura is as dry as possible. Put a coverslip on top of the Quick-Sil and press the Quick-Sil to the edges of the craniotomy. Apply cyanoacrylate to the edges of the coverslip, and allow a little bit of the cyanoacrylate to go underneath the coverslip. When the cyanoacrylate is dried, put dental cement over the cyanoacrylate and the bone. Before the dental cement has hardened, put a modified paperclip (Fig. 3Ba) in the dental cement in such a way that the mouse can be placed in the holder (Fig. 3A). Wait at least 2 h before the first imaging session (*see* **Note 6**).

3.8 Brain Surgery: Variant 2

Anesthetize the animal with isoflurane (see General Procedures). Place the animal on the surgery table (Fig. 4), fix both ear bars, and put the nose in the anesthesia tube. The ear bars, the anesthesia



Fig. 4 Brain surgery—variant 2. (**A**) Overview. The mouse is placed on an adjustable heat plate. The body temperature is monitored by a rectally inserted thermoprobe. The position of the head can be adjusted by moving both ear bars (indicated with *a*) or by moving the anesthesia tube (indicated with *c*) in which the nose of the mouse is positioned. With *b* the head plate is indicated and with *d* the holder of the head plate. (**B**) Head plate with holder. The holder can be moved up and down by loosening and tightening the big *black screw*; it can be moved backwards and forwards by adjusting the *red screw*. The head holder itself can be moved by the small screw. The pedestal is positioned in such a way that the surface under the pedestal is completely flat. The craniotomy is made in the middle of the *open circle* of the head holder. The craniotomy is then filled with Quick-Sil and a coverslip, which is put on the edges of the *circle* to push the brain back and make the preparation as stable as possible

tube, and the head plate are all connected to holders that can be moved up and down. The anesthesia tube, the ear bars, and the head plate can be moved from left to right. By adjusting the position of all the holders, the ear bars and the anesthesia tube, it is possible to get the best access to the ROI. It is obvious that the mouse has to be in a completely different position for imaging the cerebellum than the cortex. It is also important that the ROI is as leveled as possible (i.e., rectangular to the vertical axis), as this significantly increases the quality of the images. Since the skull is curved, it is not trivial to get your ROI in such a position. Remove all tissue (hairs, skin, and muscle tissue), clean the skull with ACSF and subsequently dry it. Put dental cement on the head plate and lower the head plate on the skull. Wait until the dental cement is polymerized. Then make a craniotomy, do not disrupt the dura, carefully clean and dry skull and dura, fill the craniotomy with Quick-Sil, and close it with a coverslip. Image recordings can start straight away. This procedure is also suitable for long-term and repeated image recordings (*see* **Note 6**).

4 Notes

1. All animal studies of the authors were carried out at the University of Saarland in strict accordance with the recommendations to European and German guidelines for the welfare of experimental animals, approved by the Saarland state's "Landesamt für Gesundheit und Verbraucherschutz" in Saarbrücken/Germany (animal license number: 72/2010).
2. Image quality can be optimized by considering four aspects. (1) The laser should be aligned as good as possible. (2) The surgery has to be performed with minimal damage to the tissue, simultaneously reaching the closest possible access to the ROI. (3) The surface above the ROI should be placed rectangular to the objective to ensure optimal spatial resolution. In living tissue this is not so straightforward, since most structures are curved. (4) All types of movements have to be minimized.
3. It is often recommended to deepen aesthesia until the peripheral reflexes (like the withdrawal reflex of a limb) do not function any more. However, this anesthesia state is already very close to the exitus with periods of agonal respiration that can end up in a breathing arrest. Our own measurements have indicated arterial oxygen saturation of the blood to be often less than 70 % when the central reflexes are absent; therefore, we recommend depth of anesthesia not until peripheral reflexes fail.

   The body weight is only a first very rough indication for the dose of i.p or i.v. anesthetic. Every mouse needs an individual dose of anesthetic; use the abovementioned criteria for

fine tuning. To get relevant data, we have to keep vital functions as close as possible to natural conditions and values. Therefore, a maximum surveillance of the vital data is indispensable and enables us to react to irregularities. Every mouse is different, and the doses of drugs are individual. Start with the recommended dose and inject more if necessary.

Infused volumes (methohexital) may lead to extreme motion during imaging. An additional dose of pentobarbital given subcutaneously reduces the infusion of methohexital while keeping the anesthesia on a sufficient level.

Sometimes when you have anesthetized the animal with isoflurane, the mouse can start gasping. It will start breathing through its mouth and not only through its nose. This has to be addressed immediately. One can increase the flow of oxygen or move the position of the mouse until the gasping disappears. Note that it is not wise to move the mouse when the dental cement is drying; the holder can loosen. Another possibility to reduce gasping is to hydrate the anesthetic mixture. This can easily be done by leading the tube before it reaches the animal through a bottle of 0.9 % NaCl.

4. Surgery sometimes causes strong bleedings. A mouse weighing 25 g has only a blood volume of about 2.5 ml. In emergency case, press a swap on the bleeding. Always have a stockpile of small pieces of Spongostan to use them as swabs. After pressing it on the bleeding, leave it there. You may remove it after some minutes if necessary. Remove the blood immediately with ACSF from the ROI; otherwise it is difficult to get rid of the coagulated blood.

   If the oxygenation of the arterial blood decreases, the artificial respiration in an acute experiment can be deepened by extending inspiration and expiration times. The result may be controlled by pulse oximetry.

5. The easiest access to the spinal cord with extended white matter is spinal cord segment L4, the region where the dorsal roots of the leg innervation enter the spinal cord; be aware that this region is located below the spinal arches lower L1 to upper L2.

   Unwanted movements during imaging caused by respiration may be reduced by flattening the artificial respiration by reduction of the respiration gas flow and enhancing the respiratory frequency. This has also to be controlled by pulse oximetry. Moreover, the spinal cord clamps may be lifted until thorax and abdomen have less or no more contact to the heated support; consequently, heating has to be enhanced.

   To obtain best-quality images, the region of interest should have an angle of 90° with the objective. The easiest way to achieve this is to put the mouse on table that can be altered in x, y, and z direction.

6. Microglia can respond to a craniotomy, so one could also choose to thin the skull as much as possible instead of removing it. In this way the microglia will not react to the craniotomy, but the quality of the images will be reduced. We normally remove the skull. If the processes of the microglia are not moving after the craniotomy, it is impossible to further investigate the motility and dynamics of microglia. The microglia will not react at all.

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