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4D in in vivo 2-photon laser scanning fluorescence microscopy with sample motion in 6 degrees of freedom

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ABSTRACT

2-Photon laser scanning microscopy (TPLSM) is often used for chronic in vivo studies. Small deviations in the sample orientation, however, make comparison of three-dimensional image stacks taken at different time-points challenging. When analysing changes of three-dimensional structures over time (4D imaging) this fundamental problem is one of the main limitations when complex structures are studied repetitively.

We used an upright two-photon microscope complemented with a software-controlled stage-rotation instead of a conventional stage for chronic in vivo imaging in the brain of transgenic mouse models of Alzheimer's disease. Before every session an optimal imaging condition was successfully created by aligning the surface of the cranial window perfectly perpendicular to the laser beam. Deviations in the sample orientation between consecutive imaging sessions could be eliminated which improves conditions for chronic in vivo studies.

1. Introduction

As the most non-invasive technique for the exploration of the brain two-photon laser scanning microscopy (TPLSM) is the method of choice of neuroscientists for deep-tissue imaging in slices (Yuste and Denk, 1995; Rose et al., 1999) and in living animals (Svoboda et al., 1997; Helmchen et al., 1999; Stosiek et al., 2003). Analysis of mechanisms that cause long-term changes in the brain of transgenic mouse models of neurodegenerative diseases requires repetitive high-resolution optical imaging over weeks or months (Misgeld and Kerschensteiner, 2006; Svoboda and Yasuda, 2006; Fuhrmann et al., 2010; Burgold et al., 2011; Hefendehl et al., 2011). Transgenic animals expressing fluorescent proteins in the cells of interest allow their observation in vivo (Tsien, 1998; Feng et al., 2000).

Performing in the near-infrared spectrum to minimize the effects of scattering and hence increasing tissue penetration two-photon microscopy is particularly eligible (Denk et al., 1990; Zipfel et al., 2003; Helmchen and Denk, 2005). Long-term imaging is achieved by implanting a chronic window into the mouse's skull and thus allowing in vivo studies on modifications of small structures like neurons and their dendrites or even dendritic spines over time (Holtmaat et al., 2009). However, deviations in the sample alignment from one imaging session to the next can lead to a change in the angle of view large enough to slightly disarrange structures that had been seen earlier. This demands a rotation of the skull in order to turn the structure of interest inside the brain repeatedly into the same orientation to permit comparison of the images taken in different imaging sessions. This is especially important if long-term changes have to be quantified. This calls for a new method providing highly accurate angular orientation of the sample of interest.

So far, not much information on mouse mountings for in vivo mouse brain imaging is available. Publications report on ear-bar methods (Skoch et al., 2005), bars with threaded holes attached to the skull by dental cement (Holtmaat et al., 2009) or just "custom-built mountings". All of them, however, are attached to conventional microscope stages and allow more or less precise and reproducible orientation of the sample, or the mouse brain respectively.

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Unlike common stages a hexapod (Physik Instrumente, Karlsruhe, Germany) provides both translational and rotational motion of the sample. The hexapod was controlled by custom-written software adjusting the sample angle based on spatial reference points on the glass surface of the cranial window. This setup permitted repetitive imaging at precisely controlled angles. The hexapod was implemented into the highly flexible "Intravital" microscope from TILL Photonics.

2. Materials and methods

2.1. Mouse models

APPPS1 mice are double transgenic for $APP_{KM670/671NL}$ and $PS1_{L166P}$ (Radde et al., 2006). Heterozygous mice of this line were crossed with mice heterozygous for YFP-H (Feng et al., 2000) (B6.Cg-Tg(Thy1-YFPH)2Jrs/J from The Jackson Laboratory, Bar Harbor, USA). For these experiments, however, the pathological background was irrelevant. Only the expression of yellow fluorescent protein (YFP) in a subset of cortical neurons is necessary for visualisation. The mouse was available for an experiment in our lab, so no additional animals were required for the present demonstration. All experimental procedures were performed in accordance with an animal protocol approved by the University of Munich and the Government of Upper Bavaria (55.2-1.54-2531-110-06).

2.2. Surgery

As previously described by Holtmaat et al. (2009) a cranial window was created for chronic imaging in the mouse brain (Fig. 1). To avoid inflammation dexamethasone was injected intraperitoneally (4 mg/kg body weight) just before surgery. After anaesthetization with ketamin/xylazin (130 mg ketamin and 10 mg xylazin per kg body weight intraperitoneally) the skin on the skull was removed and a circular hole was drilled into the cranial bone using a dental drill (Schick-Technikmaster C1; Pluradent; Offenbach, Germany). A circular coverslip with a diameter of 5 mm was then placed onto the craniotomy and glued to the bone using dental acrylic (Cyano-Veneer fast; Heinrich Schein Dental Depot, Munich, Germany). Only little pressure should be applied to avoid disturbance of the brain blood circulation. For fixation on the microscope stage a metal bar is glued next to the window. For pain relief the mouse is administered carprophen (Rimadyl; Pfizer, New York, USA) subcutaneously for 3 days (5 mg/kg body weight).

Post-surgery, mice were singly-housed.

2.3. Imaging and post-processing

For chronic imaging (Grutzendler and Gan, 2006; Bittner et al., 2010) the mouse is anesthetized using isofluran (approximately 1% in pure oxygen). It was placed on a heating mat during the whole imaging process. Its eyes were covered with Bepanthen salve (Bayer, Leverkusen, Germany) to prevent drying.

Re-imaging was performed using the blood vasculature as a map as previously described (Grutzendler et al., 2002).

Images were acquired with a size of $500\,\mu m \times 500\,\mu m$ (1000×1000 pixels with $500\,nm \times 500\,nm$ each). The distance in z was $2\,\mu m$ for stacks.

YFP was excited by the Ti:Sapphire laser (Mai Tai HP DeepSee, wavelengths 690–1020 nm, Spectra-Physics, Darmstadt, Germany) at $\lambda = 880\,nm$ and all fluorescence $\lambda > 500\,nm$ was collected. To avoid phototoxicity the power applied to the tissue was kept at $P < 50\,mW$. Laser intensity was adjusted with increasing imaging depth.

ImageJ (freely distributed by the US National Institues of Health) was used to adjust contrast and brightness after acquisition. No z-projections are shown. Single images always only represent one plane. For three-dimensional depiction of image stacks Imaris 6.2.1 (Bitplane, Zurich, Switzerland) was used.

2.4. Imaging system

TILL Photonics' (Gräfelfing, Germany) microscope platform "more" was used to create a highly compact upright two-photon system (Fig. 2). This configuration is then called "Intravital". It features 2 sensitive non-descanned GaAsP-detectors (H7422P-40 modified for larger entrance angles at the photocathode, Hamamatsu Photonics, Japan) collecting emission light directly behind the $20\times$ Zeiss (Jena, Germany) water-immersion objective with a NA = 1.0 numerical aperture. The system is run in Colibri, a LabVIEW (National Instruments, Austin, Texas, USA) open source software which allows the easy implementation of additional hard- and software by the user. A Ti:Sapphire laser (Mai Tai HP DeepSee, wavelengths 690–1020 nm, Spectra-Physics, Darmstadt, Germany) was used for two-photon excitation. Its pulse-width in the object plane is 130 fs. It is prechirped by the DeepSee unit of the MaiTai. The point spread function (FWHM) in the x/y plane at $\lambda = 880\,nm$ was $404\,\mu m$; in z direction $1201\,\mu m$ were achieved. A piezo-driven focussing device (PIFOC, Physik Instrumente, Karlsruhe, Germany) with $400\,\mu m$ vertical travel is combined with a hexapod (M-810, Physik Instrumente, Karlsruhe, Germany), providing both translational and rotational motion of the sample.

2.5. Angle adjustment

For our analysis we used a cranial window prepared according to the protocol of Holtmaat et al. (2009). To give the glass surface a better contrast it was marked at the window's rim. Waterproof pen is a quick and easy option. The mouse was then fixed to the hexapod directly under the objective (Fig. 2).

The in-focus z-positions of three different points P_1, P_2 and P_3 in the marked rim on the glass surface were then acquired. This was done in widefield mode by adjusting the focus manually, see flow chart in Fig. 3. Up to this point the stage angles have not been manipulated. To keep errors small x, y-positions of all three points were chosen as far apart from each other as possible. In our case this was in the range of $\Delta r \sim 3\,mm$. Because of a small uncertainty in the exact focus position due to the focal volume's z-dimension large distances between the points are crucial. As small pieces of dust or dirt on the glass falsify the result clean areas have to be chosen for the points P_1, P_2 and P_3. This ensures they are located directly on the surface.

The hexapod's x, y and z coordinates of all three points' in-focus positions were stored upon mouse click by our custom-made LabVIEW software (Supplementary). Those coordinates (Fig. 4a) are then processed to find the angles for each axis. This is accomplished by first seeking the vector normal $\vec{n}$ to the surface which is the cross product of the two vectors connecting P1 with P2 and P3, respectively (Fig. 4b). Then its angular difference to the z-unit vector $\vec{e}_z$ is trigonometrically derived (Fig. 4c and Supplementary). After this procedure is performed for both x- and y-axis these angles are passed on to the LabVIEW driver of the hexapod. The driver is provided by its manufacturer (Physik Instrumente, Karlsruhe, Germany). The hexapod subsequently rotates to align the glass surface exactly perpendicular to the laser beam (Fig. 4d).

The marked rim can also be used to check the adjustment: it should be focussed in all positions of the rim when the z-position of the hexapod and the piezo-focussing device is kept constant.

With this method angles of up to $\theta < 0.05^\circ$ can be reached. In the present publication a surface showing a difference of less than $10\,\mu m$ in focus position of the marked rim at opposing sides of the window is considered horizontal. Considering the window size of