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Analysis of optical properties of the mouse cranium—Implications for *in vivo* multi photon laser scanning microscopy

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ABSTRACT

Trans-cranial imaging is the least invasive method for optical *in vivo* studies of structures in the mouse brain and has found wide application over the last few years. An important issue is how and to what extent the cranium and the tissue between the cranium and the focal point detract from the quality of the recorded images. Here we address this issue by recording transmission images in wild type mice at five wavelengths in the visible and near-infrared spectrum. The recorded laser scanning microscopic images were analyzed pixel by pixel in order to quantify the light attenuation and shading as function of the location of the focal point relative to the cranium. Additional images demonstrate the effects of the mouse crania on the images of fluorescent microspheres in the low micrometer range.

The results of this study demonstrate that light attenuation by the cranium, though with typical losses of less than 20% of the incident light, induces shading effects during the imaging process. Geometrical shapes and sizes in the images of the recorded objects may differ substantially depending on whether they have been recorded trans-cranially or not. This is true even for comparatively large structures such as cell somata.

Our results call for a more realistic appraisal of the potential of the trans-cranial imaging approach, particularly when it comes to absolute measurements of sizes and shapes of small objects. As trans-cranial imaging has found wide use in contemporary research it is important that the results be interpreted with due caution.

1. Introduction

When performing *in vivo* observations (Trachtenberg et al., 2002; Grutzendler et al., 2002; Klunk et al., 2002; Ottersen and Helm, 2002; Nase et al., 2005, 2008a,b) and image recordings on structures in the brains of small rodents by means of multi photon laser scanning microscopy (MPLSM) (Denk et al., 1990; Denk and Svoboda, 1997), one faces a dilemma if the observed structures are located so close to the cranium that trans-cranial observation and image recording (Christie et al., 2001; Klunk et al., 2002; Tsai et al., 2004; Nase et al., 2005, 2008a) are possible.

Either, at the position of the planned observation, a small disk of bone and the corresponding underlying area of the dura can be removed from the cranium and replaced with an implant including a microscope cover slip, or the cranium can be milled to a thickness of approximately 30 μm to prepare it for trans-cranial microscopy. While the former method provides a better optical access to the region of interest (ROI) within the brain, the latter is less invasive and leaves the brain basically undisturbed, avoiding long term pathological reactions in the central nervous system during chronic studies (Xu et al., 2007), albeit at the price of less favorable optical conditions.

Here, a study of some of the optical properties of the thinned cranium of the mouse is presented, outlining the light attenuation and shading properties of the cranium and describing qualitatively how it induces changes to the point spread function (PSF) of a given microscope objective. The most important consequence of the presented study is that, while *relative* microscopic geometrical or photometric trans-cranial measurements are feasible, the measurement of *absolute* values is hampered by systematic errors that have to be duly considered in any optical imaging study of the intact brain.

2. Materials and methods

Slices of the cranium, milled down *in situ* as done for trans-cranial observations to thicknesses between approximately 20 μm and 70 μm, were micro-surgically explanted from the skull of the mice at the usual locations for trans-cranial observations during the type of experiments described earlier (Nase et al., 2005, 2008b).

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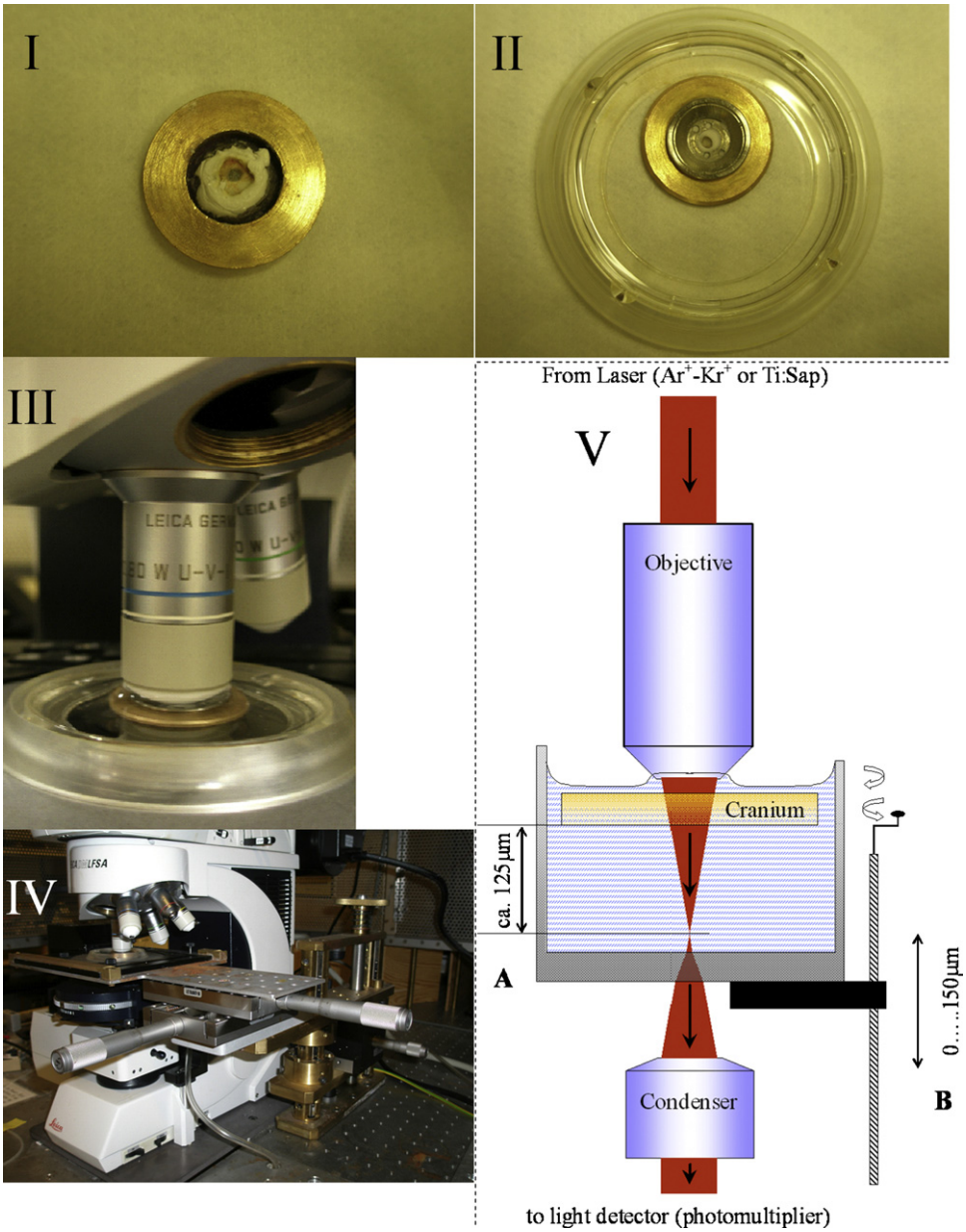

Fig. 1. (I) A titanium implant as used for *in vivo* observations of the mouse brain is reversibly attached to a ring made from brass. A slice of mouse cranium, surgically thinned to approximately 30 μm, is fixed to the implant by means of dental cement. (II) The preparation is placed upside down in a glass Petri dish filled with saline. The thickness of the bottom glass of the Petri dish deviates by less than 0.1 mm from the nominal thickness throughout the entire area of the bottom glass. It is important that the preparation is positioned on the periphery, and not in the centre of the Petri dish. (III) This Petri dish is then fixed on the galvanometric z-stage of a model "DM LFSA" fixed stage microscope equipped with a model "TCS SP" confocal scanning laser microscope unit (Leica Microsystems Heidelberg GmbH, Mannheim, FRG). As light sources, both an Ar^+–Kr^+ laser (model "643", Omnichrome – cvi Melles Griot, Carlsbad, CA, USA) and a Titanium Sapphire laser system (Coherent Inc., Santa Clara, CA, USA) are available. The scanning unit is equipped with a transmitted light photomultiplier tube (PMT, model R6357, Hamamatsu Photonics K.K., Hamamatsu City, Japan). (IV) The galvanometric z-stage of the microscope is in itself fixed to a home made microscope stage (Helm et al., 2001), which allows to position the preparation in *x*, *y* and *z* controllably at a precision of better than 1 μm. (V) The preparation can be moved relative to the objective and condenser at which objective and condenser do *not* move relative to each other. This, of course, implies that the condenser can be moved for focussing purposes and adjustment of proper Köhler illumination while the stage and preparation remain totally un-touched. "A" and "B" refer to two of the measurement series; see Section 2.

These slices, one at a time, were then fixed to an implant of titanium as commonly used for trans-cranial observations and mounted on a microscope stage as described in Fig. 1 I and II. The observations were done rapidly after the explantation in order to ensure that the changes of the material properties of the cranium slice were minimal. During the time interval of 2 min after explantation and a couple of hours after the explantation, the measured optical properties of the cranium slice in the saline did not change. The regions of interest for our observations were, on the microscopic scale, far away from the cutting zone of the explanting procedure. The region of interest was centred in the 20 mm field of view of a 40× objective, while the border line of the cranium slice was not visible in the entire field of view, so that the distance between the border line of the cranium slice and the ROI centre was at least 500 μm. Altogether, 10 cranium slices were analyzed (five animals, two cranium slices out of each animal).

The cranium of the adult mouse will, in the regions of interest analyzed in this manuscript, be at least 100 μm in thickness. Milling down the cranium to an average value of approximately 30 μm by experience renders the best *imaging* properties for chronic measurements. Milling the cranium even thinner will result in the cranium becoming opaque during weeks or months after the oper-