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Two-Photon Imaging

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Abstract

During the past two decades, two-photon microscopy has become a powerful tool in neuroscience. Unlike other imaging methods like MRI, its spatial resolution is micrometric and enables the observation of structures at the subcellular scale. In this chapter, the physical principles and the way to study phenomenon occurring in the living animal are summarized. Then, we describe the methods to observe the different components of the nervous system like neurons, glia and brain microvasculature. Finally, we discuss the advantages of two-photon microscopy in neuro-oncology and in the study of diseases.

Introduction

During the past two decades, two-photon microscopy has become a powerful tool to image biological structures with a higher depth than confocal microscopy. Its high spatial and temporal resolution, down to a micrometer and less than a second, enable imaging of subcellular structures on cell cultures, on tissue slices and also directly in the living animal.
Its potential applications in the neuroscience field were rapidly demonstrated and intravital studies in the brain were conducted allowing morphological and physiological observations with a spatial resolution never obtained with other methods of neuroimaging such as MRI or CT-Scan.
Here, we will discuss the physical principles behind two-photon microscopy and the methodology for studying structures or phenomena occurring in the brain of living animals. Then, we will see how the different elements of the nervous system like neurons, glial cells and cerebral microvasculature can be imaged. Finally, we will describe some applications of two-photon microscopy in neuro-oncology and in research on neurological diseases.

Methods

Instrumentation

Two photon microscopy belongs to a larger family where non-linear optics (NLO) and scanning microscopy are conjugated.

NLO phenomena such as multiphoton absorption, mainly two-photon (2P) absorption, multiharmonic generation, mainly Second Harmonic Generation (SHG) and Coherent Antistokes Raman Scattering (CARS) are now commonly used. NLO phenomena occur at high light intensity, even during a short time, but biological tissues cannot support excessive heating. These contradictory requirements explain the choice of laser excitation in the pulsed regime, with high instantaneous intensity but low average power and in the near infrared, a spectral window where tissue absorption is minimal. This choice is judicious for other reasons: the light scattering by tissue inhomogeneity is minimized, the resulting light emission is in the visible spectral range where sensitive detectors are available and finally, very stable and robust laser sources, in particular the Titanium Sapphire (Ti:Sa) femtosecond lasers, are now commercially available at non-prohibitive prices.

The laser scanning technology is derived largely from the confocal microscope: the laser beam is usually scanned by a pair of oscillating mirrors and focused on the sample by an objective with a large optical aperture (Fig 1). The oscillations of the mirrors introduce a X,Y scanning of the focused beam in the focal plane. The light resulting from the excitation is usually collected by the same objective and de-scanned before reaching confocal pinholes in front of detectors. In fact, multiphoton or multiharmonic scanning is simpler, since the emitted light comes from only a small region surrounding the focal point and has not to be de-scanned and conjugated with a pinhole in front of the detector. For confocal microscopy, the light scattering and the presence of the pinhole are at the origin of photon waste and limit the depth of observation to e.g. ~50 µm in the mouse brain cortex. Near-infrared excitation and non de-scanned light collection as close as possible to the microscope objective minimize the photon waste by scattering and the depth of observation could be increased to 600 µm. A last advantage of a non de-scanned mode is the possibility to have simultaneously trans and epi-collection as shown in Figure 1.

Like confocal microscopy, multiphoton or multiharmonic microscopy is a three dimensional (3D) imaging technique. The Z dimension is given by the displacement of the focused laser beam in the Z-direction by means of a motor driving the microscope objective. Three dimensional images are made up of stacks of 2D (XY) images.
The speed of image acquisition is mainly limited by the collected light intensity, itself limited by the laser excitation power which must not cause excessive tissue damage. In general, one image (512X512 pixels) per second or a stack of ~60 images per minute, are currently obtained if the dye is adapted to two-photon microscopy.

NLO phenomena are inefficient for scattered laser photons and only the ballistic laser photons contribute to the light emission. So the spatial resolution is the same for scattering and non scattering tissues. The resolution depends first on the spatial dimensions of the luminescent (or SHG) spot where the laser is focused. The smallness of the spot is governed by the laser beam quality and by the aperture of the microscope objective. For a Ti:Sa laser beam, an objective aperture of 1 and a NLO second order process (two-photon or SHG) the resolution is about ~3µm in the Z direction and 0.5µm in the XY plane.
Other causes such as animal movements (bulk movements or local movements) are at the origin of a lack of spatial resolution. Animal holders are specially designed to limit bulk displacement and good anesthesia methods should be used to limit excessive local movements.

Two-photon microscopy requires endogenous or exogenous emitting molecules. Unfortunately, while endoluminescence can be used for many tissues, it is rarely sufficient for imaging brain tissues, thus staining is necessary. For intravital brain microscopy three strategies can be used: local staining by dye injection, staining via the blood circulation after an intravenous injection and transfection of genes coding for fluorescent proteins. For instance, calcium sensitive dyes are infused locally to follow neuron activity, while non permeant plasmatic dyes are used to image the vascular network. Some dyes can cross the blood-brain-barrier (BBB) and stain the adjacent tissues. Other physico-chemical properties have also to be considered in the choice of a dye, such as the luminescent and the non linear absorption cross section efficiencies, the photochemical stability and the non toxicity. The large number of requirements explains why the development of new dyes remains an important area for fundamental research.

Non linear optical neuro-imaging is not only used for morphological information, quantitative information on brain physiology can also be extracted, *e.g.*, the local blood volume or the blood-brain-barrier permeability to specific dyes. For these purposes the emitted light intensity is treated as a parameter representative of the local volume or the local dye concentration. Image stacks then constitute a 3D representation of these parameters.

Animal preparation

Rodents are the main animal models used in neurological two-photon imaging studies. Mice are the most encountered because of the availability of a wide range of transgenic animals expressing fluorescent proteins.
In general, studies on rats are conducted on young animals only (up to one month) whereas on mice, they can be accomplished during adulthood.
To minimize the movements during image acquisition, the choice of the anesthesia is fundamental. A mixture of xylazine and ketamine is a good candidate but volatile anesthetic are generally better, for example, isoflurane (2%) in a mixture of O_2 and N_2O (30% : 70%).

Immobilization of the skull is necessary. During the whole experiment the temperature of the animal must be monitored and maintained at 37°C using a heating blanket. The next step is to obtain good optical access to the cortex. This surgical procedure can be done in three different ways:

- the bone of the skull is removed and the objective of the microscope is immersed in physiological saline at the surface of the brain (Fig 2A).
- the bone of the skull is replaced by a quartz window allowing longitudinal studies on the same animal (Fig 2B).
- the bone of the skull is just thinned and observations are made right through. This method limits the observation depth to the first 200µm of the cortex due to the high scattering of the photons by the skull (Fig 2C and Fig 3).

In adult rats, the dura is either removed, since his opacity is higher than in mice.

Imaging the different components of the nervous system

Neurons

The prime function of neurons is to transmit electrical signals in a network that undergoes constant modification as synapses are formed, strengthened or eliminated. Compared with the traditional techniques of *in vivo* recording of electrical potentials with micropipettes, and analysis of ultrastructure on fixed sections of tissue, two-photon microscopy allows very great advances on the information that can be obtained. What can be achieved depends on the fluorophores that are introduced in the neurons. The most notable imaging to date has been in the upper layers of the mouse cerebral cortex *in vivo*, and on brain slices.

Calcium imaging

Electrical activity in neurons is almost always associated with changes in free [Ca^{2+}] in the cytoplasm. Changes in free [Ca^{2+}] are readily detected optically because free [Ca^{2+}] can change by up to a thousandfold, and an extensive range of fluorescent Ca^{2+} indicators is available.

Ca^{2+} indicators, such as Calcium Green 1 or Fluo 4, can be injected into a single neuron through a fine-tipped micropipette or through a patch pipette.

Since only one neuron is labeled, useful information can be obtained with only moderate spatial resolution. To increase the depth of penetration in the tissue, it may be useful to reduce the convergence of the excitation beam by illuminating only the center of the exit pupil of the objective. Calcium signals are generally slow compared with electrical ones (hundreds of ms) so low time resolution allows more photons to be collected from each point in the neuron. Since the injection technique requires an intracellular microelectrode or a patch pipette, the electrical activity in the cell soma can be recorded simultaneously with the Ca^{2+} activity in dendrites, or the cell resting potential can be displaced by injecting current.

Uptake of dye by neurons

Many Ca^{2+} indicators are available as esters which cross cell membranes and are de-esterified by endogenous enzymes. When applied to nervous tissue (for example to the surface of the cortex, or in the bath of a tissue slice) Ca^{2+} indicators are usually taken up by astrocytes as well as neurons, although some appear to be taken up mainly by neurons (e.g., Oregon Green Bapta-AM). If the astrocytes are simultaneously labeled with sulforhodamine 101 it is possible to distinguish the two types of cell and select recordings from neurons. This simple

technique of applying Ca^{2+} indicator to tissue enables large populations of neurons to be labeled so that waves of activity can be observed.

Voltage-sensitive dyes

Dye molecules are available with lipophilic moieties that insert in the cell membrane and whose fluorescence varies with membrane potential. So far, the poor signal-to-noise ratio has been a constraint on the use of these dyes in the thick tissues for which two-photon excitation is useful; However, the same intense infra red illumination that produces two-photon excitation of voltage-sensitive dyes also generates a second harmonic (e.g., with excitation at 900 nm a fraction of the emitted light is at 450 nm). This second harmonic generation is more sensitive to changes in membrane potential than is the fluorescence and has been used to detect electrical signals in segments of a single dendrite in a brain slice.

Labeling by expression of fluorescent proteins

Mutant mice in which a sub-population of neurons expresses Green Fluorescent Protein (GFP) have made it possible to image individual features of a neuron through a cranial window or thinned skull over periods of months. Expression of fluorescent label has also been achieved in monkey by injection in the cortex of an adenovirus bearing the gene for Enhanced Green Fluorescent Protein. These techniques are extremely productive in studying the development of brain circuits and their plasticity associated with modifications of axon geometry and formation and elimination of dendritic spines (Fig 3).

Glial cells

Glial cells are with neurons the two major cell types encountered in the nervous system. The 3 main classes are astrocytes, oligodendrocytes and microglial cells.

Astrocytes were first described as metabolic supporting cells for neurons as well as an intimate association with the BBB. However, their potential implication in communication by means of Ca^{2+} signaling was discovered and documented only during the past two decades.

First studies were conducted on cell cultures and on living slices. Despite, the high quality of these preparations, *in vivo* investigations are the only ones that can minimize the preparation artifacts encountered on slices. Further, intravital studies allow the observation of the interactions between neurons and astrocytes with the best physiological state. That is why two-photon microscopy is applied in numerous studies on astroglia.

Their staining was reported to be done with specific dyes, such as the Sulforhodamine 101 injected in the neocortex. Transgenic animals using fluorescent proteins such as the GFP under the astrocyte specific GFAP promoter are also excellent models for visualizing these cells directly *in vivo* or on living brain slices.

Studies on the Ca^{2+} signaling encountered in astrocytes have been conducted using fluo-4 AM, an indicator whose fluorescence emission is dependent of the intracellular concentration of Ca^{2+}. Such experiments allow the observation of the propagation of Ca^{2+} waves on brain slices and more interestingly *in vivo* where the neuronal/glial interactions can be highlighted. For example, an increased neuronal discharge was associated with increased Ca^{2+} concentration in neighboring astrocytes.

Last, the double staining of astrocytes and vasculature using a specific dye and an intravascular fluorescent marker is a novel approach to study the glio-vascular interface *in vivo*.

Other glial cells such as oligodendrocytes can be stained using transgenic animals with a fluorescent protein under the control of the proteolipid protein promoter for example.

However, intravital imaging of the white matter remains difficult in rodents due to its depth. Such observations must be considered on living brain slices.
Finally, microglial cell motility has been extensively studied using time-lapse two-photon microscopy. Morphological observations, such as rapid movements of microglial processes and cell migration after a focal injury were observed with a high spatial and temporal resolution. In such experiments, the same laser used for the excitation of fluorescent proteins can be used to generate the injury. Thanks to the two-photon properties, the high power excitation is restricted to the focal point, as a consequence the injury is limited on a small volume and induces less damage than a mechanical or chemical one.

Cerebral microvasculature

The central nervous system, composed of the brain and the spinal cord, controls the majority of our organs. Cerebral activity is guaranteed by a neuronal network which requires a large and well regulated supply of energetic substrates. Indeed, the human brain represents about 2% of the total body mass but it consumes 20% of the respired oxygen. These nutrients, mainly oxygen and glucose, are transported to brain by the blood, through a dense and complex vascular system, the cerebral microvasculature.
The low diffusivity of the substances transported by the blood makes essential the presence of a large network of capillaries (4 to 8 µm in diameter) within the whole tissue volume. It is at this scale that exchanges between blood and brain take place.

The cerebral blood perfusion (CBP) is defined as the volume (ml) of blood which irrigates 100 g of brain tissue during one minute. This parameter is of crucial importance. It has been proved that a strong correlation exists between CBP and cerebral (neuronal) activity. Changes of up to 50% in CBP have been observed in the grey matter in regions activated during a motor task or perception (taking an object, speaking, visual exercise…). In case of brain pathologies, differences in CBP have been described. For instance, some hypoperfused regions were detected in epileptic focal regions (intercritical phase) or in posterior regions of the brain in Alzheimer's disease. Hyper and hypo perfused zones can be found in high grade glial tumors. Changes in CBP are often related to alterations in cerebral blood volume (CBV). Both parameters can be estimated on clinical imaging systems, such as positron emission tomography (PET) or magnetic resonance imaging (MRI). However, none of these methods has sufficient resolution to describe the events at a capillary level. Recently, intravital two-photon microscopy has been proposed to investigate local variations of CBV in pre-clinical protocols with very high temporal and spatial resolutions. Indeed, a direct analysis of a 0.16 mm^3 sample can be study in 2 min with a sub-micromic resolution.

Two-photon microscopy allows observation of the brain microvasculature after the intravenous injection of fluorescent dyes such as Fluorescein or Rhodamine dextran conjugates (Fig 4). Depending on the age and the animal species, it is possible to image the microvasculature down to 600 µm below the dura with a micrometric resolution. Furthermore, three dimensional images of the cerebral cortex vasculature can be reconstructed using Z-stacks.

Using this method, studies on the permeability of the blood brain barrier (BBB) can be conducted *in vivo*. It can be assessed after the intravenous injection of a cocktail of two fluorescent dyes, one with a high molecular weight (Rhodamine dextran 70 kDa for example) and the second one with a low molecular weight (FITC dextran 4 kDa for example). Simultaneous imaging of the distribution of these two dyes in the vascular compartment and

in the brain parenchyma is then obtained with two-photon microscopy (Fig 5). The wide availability of fluorescent dyes conjugated with different molecular weight dextran molecules enables a precise evaluation of the BBB permeability. This method is of particular interest in preclinical studies of new radiotherapeutic or chemotherapeutic approaches.
Finally, it is possible to combine in one two-photon microscopy experiment a triple staining to study neuronal-glial-vascular interactions.

Applications to pathologies

In vivo **two photon and second harmonic generation microscopy for imaging the brain tumor micro-environment and therapy effects**

Primary brain tumors, in particular glioblastomas, are among the most therapy-resistant tumors. Until now, no therapy has yet been found to arrest their fast growth. Therefore the development of new therapies on tumor animal models is primordial. In this preclinical research, detailed knowledge on e.g. the different transport barriers for drugs in brain tumors will help, since a drug that is injected in the blood circulation encounters many obstacles before arriving in the tumor cell. These obstacles in the heterogeneous tumor micro-environment are among others: the tumor vasculature, the more or less leaky vessel walls, the extra-cellular matrix (ECM) and the cell membrane. In the next paragraph, we will show that *in vivo* two photon microscopy and second harmonic generation imaging (SHG) allows deep imaging of transport barriers in tumors growing in living animals. In the last paragraph, we will explain how these optical imaging techniques may further be useful in monitoring therapy effects.

Imaging the tumor micro-environment
In two-photon microscopy the tumor micro-vasculature can be observed directly *in vivo* after intravenous injection of fluorescent dyes that stay in the intravascular space (Figure 6). In this figure, the neo-vasculature of the tumor is sprouting from the normal host vasculature (see arrow) at the surface of the tumor and shows abnormalities, such as: dilatations, and numerous bifurcations. These changes have an impact on the blood pressure and perfusion, which often decrease in tumor tissue. If a drug is injected in the blood circulation, then the supply to the tumor may be reduced, intermittent or even absent in chronic hypoxic areas. These areas often contain viable but radio- or chemotherapy resistant tumor cells and are of interest for possible tumor re-growth after treatment. The local blood perfusion rate can be estimated by measuring the linear velocity (mm/s) of single red blood cells in the micro-vessels using two-photon microscopy. Further, the local vessel permeability determines the transport of drugs across the vessel wall and can be measured in two-photon microscopy using diffusible fluorescent dyes or drugs. But this is not the end of possible transport barriers (see introduction). Collagen fibers in the ECM, which can be detected by SHG imaging, might influence the intra-tumoral diffusion. Indeed, the enzymatic breakdown of these fibers has been shown to increase drug diffusion rates in the ECM.

Monitoring therapy
In a successful treatment, a marked reduction of the tumor volume or even total ablation of the tumor is observed. But this appears often at weeks or months after the onset of a treatment. Information on pathophysiological and cellular processes such as: the induction of tumor cell death or necrosis and a possible shut down of the vasculature that precede volume reduction or tumor ablation can be observed by two-photon microscopy. These two-photon

microscopy analyses will help in the fine tuning and/or modification of a therapy in an early phase when it fails to induce tumor cell death. For these longitudinal *in vivo* two-photon microscopy experiments, scientists need a permanent window in an animal for direct microscopic observations. For grafted brain tumors, two window models exist, namely a dorsal skin fold chamber and a cranial window (see fig. 2B). In the first model, a glioma grows inside a skin fold on the back of animal, which is hold upright by two metal plates that include a window. The cranial window approach includes the positioning of a window at the top of the brain cortex after craniotomy of small area just above the glioma. The latter model is preferred over the skin fold chambers, because the tumor development and physiology strongly depend on the host tissue bed and vasculature.
In summary: two-photon microscopy and SHG imaging are very promising for the *in vivo* microscopic evaluation of the tumor micro-environment and might therefore predict and monitor therapy outcome.

Applications to nervous system diseases

The understanding of neurodegenerative diseases such as Alzheimer's disease is an important goal for fundamental and pre-clinical research. The study of their pathophysiology can be done *in vitro* but to analyze their mechanisms as a whole, in vivo observations are necessary. Two-photon microscopy has the potential to allow such observations. For example, a fluorescent molecule which has the ability to cross the BBB and bind specifically to amyloid plaques after an intravenous injection was recently tested. The intravital staining of these structures as well as the validation of the specificity of these molecules is of particular interest in the development of targeted drugs.
In Alzheimer's disease, there seems to be a correlation between cognitive dysfunctions and a loss of synapses. It was further shown using time-lapse two-photon microscopy that dendrites exhibit spine loss and atrophy in the vicinity of amyloid deposits. Astrocytes and activated microglia that are often found around amyloid plaques have been simultaneously labeled using transgenic constructions to give a better overview of the neuropathology *in vivo*.

Two-photon microscopy is also of particular interest in the study of cerebrovascular diseases such as cerebral ischemia. Short and long-term effects of stroke can be assessed with time lapse imaging using cranial window models. Morphological changes of cells and vasculature can be observed in the penumbra (the area surrounding the core lesion where the blood flow is reduced and where delayed cell death can occur) leading to a better understanding of the injury and the mechanisms of partial recovery. One of the most outstanding studies of vascular occlusion using two-photon imaging was done using photothrombosis of single vessels in the living mouse cortex. Hemodynamics parameters, as well as, flow modifications in the neighbouring vessels were studied with a high accuracy.

New insights into the pathophysiology of epilepsy have been obtained in the cortex of the living animal. It has been shown that antiepileptic drugs such as valproate and gabapentin reduce the ability of astrocytes to transmit Ca^{2+} signals. The potential key role of astrocytes in seizure activity was hypothesized in this study, as well as their possible use in therapeutic targeting.
Two-photon imaging is used in the study of many diseases of the nervous system. The last three examples just explain the major breakthroughs in the understanding of neuropathologies.
In summary, the study of a neuropathology using two-photon imaging requires: 1) to find the best animal model for the disease, 2) to choose the best way to label the pertinent biological

elements, (stain, transgenic animal expressing a fluorescent protein, ...), 3) to determine how the data will be processed after image acquisition for quantifying the phenomena.

Conclusions and perspectives

Two-photon microscopy appears a key method for neuroscience. We have seen some applications on cell cultures, ex vivo on tissue slices and more interestingly, directly in the brains of living animals. Currently observations can be made to a depth of at least 600 µm below the dura depending on the species, age and preparation of the animal model. In mice, nearly all the neocortex is accessible including layer V. However, deeper structures such as basal ganglia, thalamus or hippocampus can not be observed with intravital two-photon microscopy.
Research is underway to improve the imaging depth by two different approaches:

- the application of optic fibers that can be directly inserted in cerebral structures using stereotaxic coordinates. First trials have been done in rats allowing the imaging of the microvasculature in the hippocampus.
- the development of new fluorescent molecules with a higher quantum yield.

In conclusion, two-photon microscopy has opened a new era in the morphological and (patho)physiological studies of the nervous system. Its multidisciplinary approach required strong interactions between biologists, physicists and chemists to provide a unique imaging tool for a better integrative approach of neuroscience.

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Figure Legends

Figure 1: Basics of multiphoton and multiharmonic microscopy.
Upper part :
- On the left : energy levels and optical transitions involved in a two photon process, in red the simultaneous absorption of two photons bringing the optical species from ground to excited level; in green the photon emission from the relaxed excited state.
- On the right : ray tracing for two photon excitation (red) and emission (green) in presence of scattering. Scattering reduces the number of focussed photons but the non-scattered (ballistic) are still well focussed, the spatial resolution is preserved. A large part of the scattered (and back-scattered) emitted photons can be collected.

Lower part :
schematic of a multiphoton or multiharmonic microscope with epi and trans light collection with an example of an image of the cortex vasculature.

Figure 2: Schematization of the three surgerical approches for the imaging of the brain cortex in vivo with two-photon microscopy.
A. A craniotomy with a 3-4 mm diameter is carried out above the cortex. The hole is filed with physiological saline and the objective is positionned in immersion.
B. A craniotomy with a 3-4 mm diameter is carried out above the cortex, the hole is filed with artificial cerebrospinal fluid and a quartz window is fixed above the craniotomy. This method allows longituninal studies.
C. The skull is thinned above the cortex and the objective is positionned in immersion on the area of interest. This method is less invasive but the imaging depth is more limited.

Figure 3: Two-photon microscopy allows repeated imaging of structural details in vivo over periods of weeks. The images are from the barrel cortex of transgenic mice expressing yellow fluorescent protein in pyramidal neurons of Layer V. **a**, fixed section of barrel cortex, made after intravital two-photon imaging, showing the outlines of the barrels, each of which receives input mainly from one whisker, and a red patch indicating the area imaged in vivo. **b**. low magnification intravital image of apical dendrites of pyramidal neurons. **c – f**, repeated imaging, over a period of 2 weeks, of the dendrite boxed in **b**. Some dendritic spines were

eliminated (arrowheads) and some were formed (large arrows).Filipodia also appeared and disappeared (small arrows). The images were obtained through an area of skull thinned to 30 –50 µm, at a depth of about 150 microns below the pia. Scale bars: 500 µm (**a**); 10 µm (**b**); 2 µm (**c-f**). Reproduced with permission from Zuo, Y, Yang G, Kwon E, Gan W-B (2005) *Nature* 436: 261-265.

Figure 4: Mouse cerebral microvasculature. The image a represents the whole vascular network of the mouse cortex ranged between 0 and 600 µm under the dura. The 3 smaller vignettes show individual images at different depths in the brain. At the surface (b, 65 µm) large arteries can be detected. They form cortical columns which plunge in the brain and divide into capillaries (c and d, 225 and 475 µm under the dura respectively). Images were acquired *in vivo* using two photon microscopy after intravenous injection of fluorescent dye (FITC-dextran 70 kDa). Scale bars represent 100 µm.

Figure 5: Vizualisation of the permeability of the Blood-Brain-Barrier. Simultaneous acquisition of Rhodamine dextran 70kDa (A), FITC dextran 4kDa (B) and merge of the two pictures (C) at 170µm below the dura in a healthy mouse.
Merge (D) of simultaneous acquisition of Rhodamine dextran 70kDa (red) and FITC dextran 4kDa (green) at 170µm below the dura in a mechanically BBB induced disruption model. Note the "black holes" (arrows) which are cells not labelled by the extravased dye. In that case, the FITC dextran 4kDa crossed the BBB whereas Rhodamine dextran 70kDa didn't.

Figure 6: 3D image of the tumor vasculature at the surface of a glioma growing subcutaneously in the hind limb of a living nude mouse. The vasculature is stained after intravenous injection of FITC-dextran (70 kDa). 20X objective, 600 x 600 µm image in 0.9 seconds. The 3D image is a z-projection of z-stack with a depth of 100 µm and a step-size 2 µm. It is clearly shown how the new tumor vessels originate from the muscle vasculature (white arrow) and form a heterogeneous structure with important bifurcations and dilations.

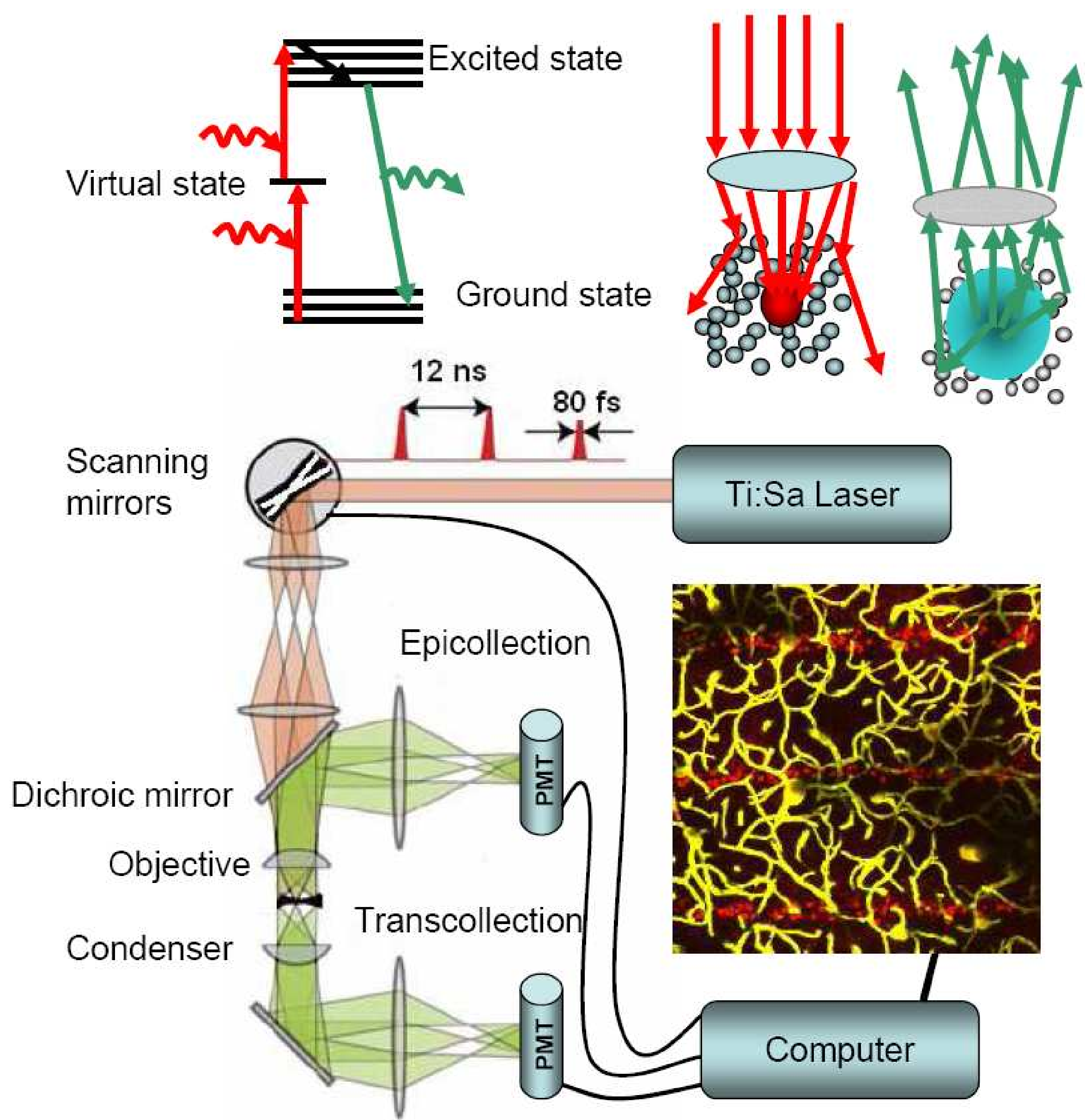

Fig 1

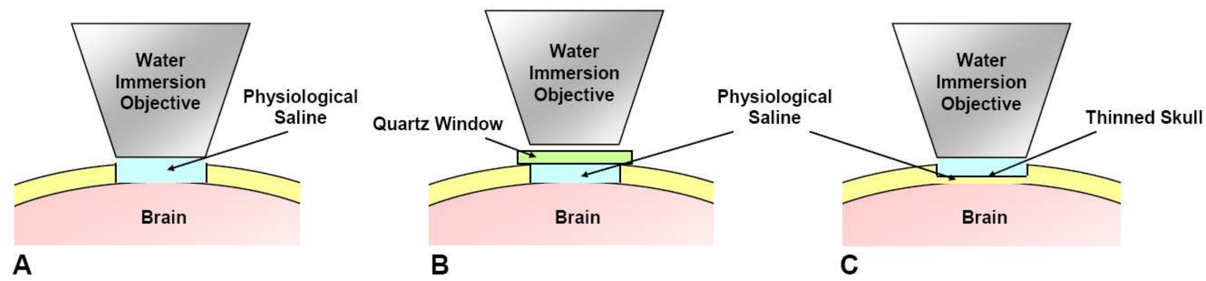

Fig 2

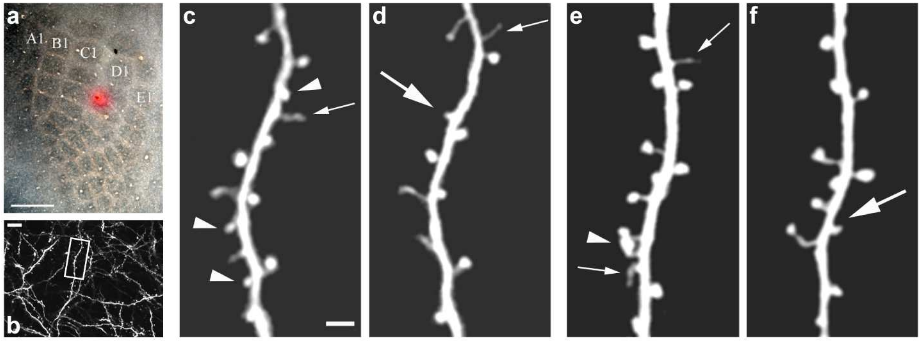

Fig 3

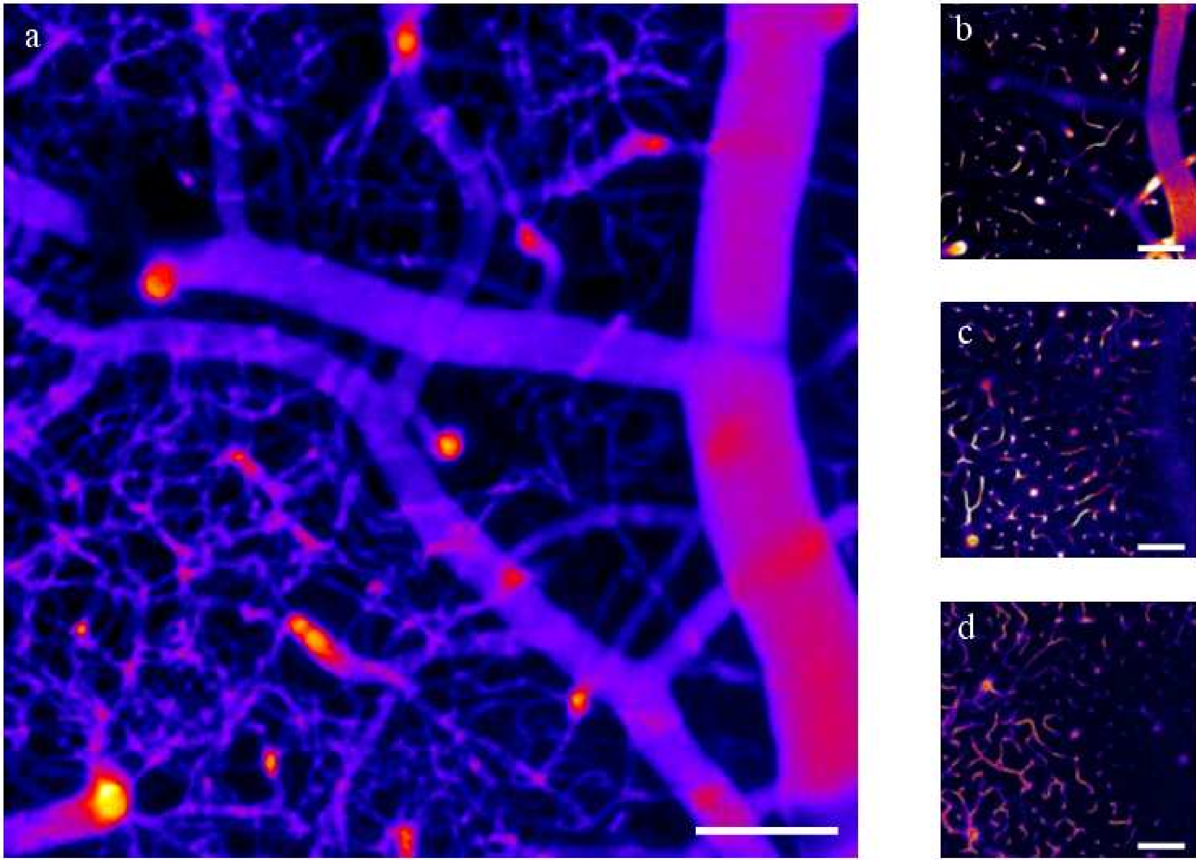

Fig 4

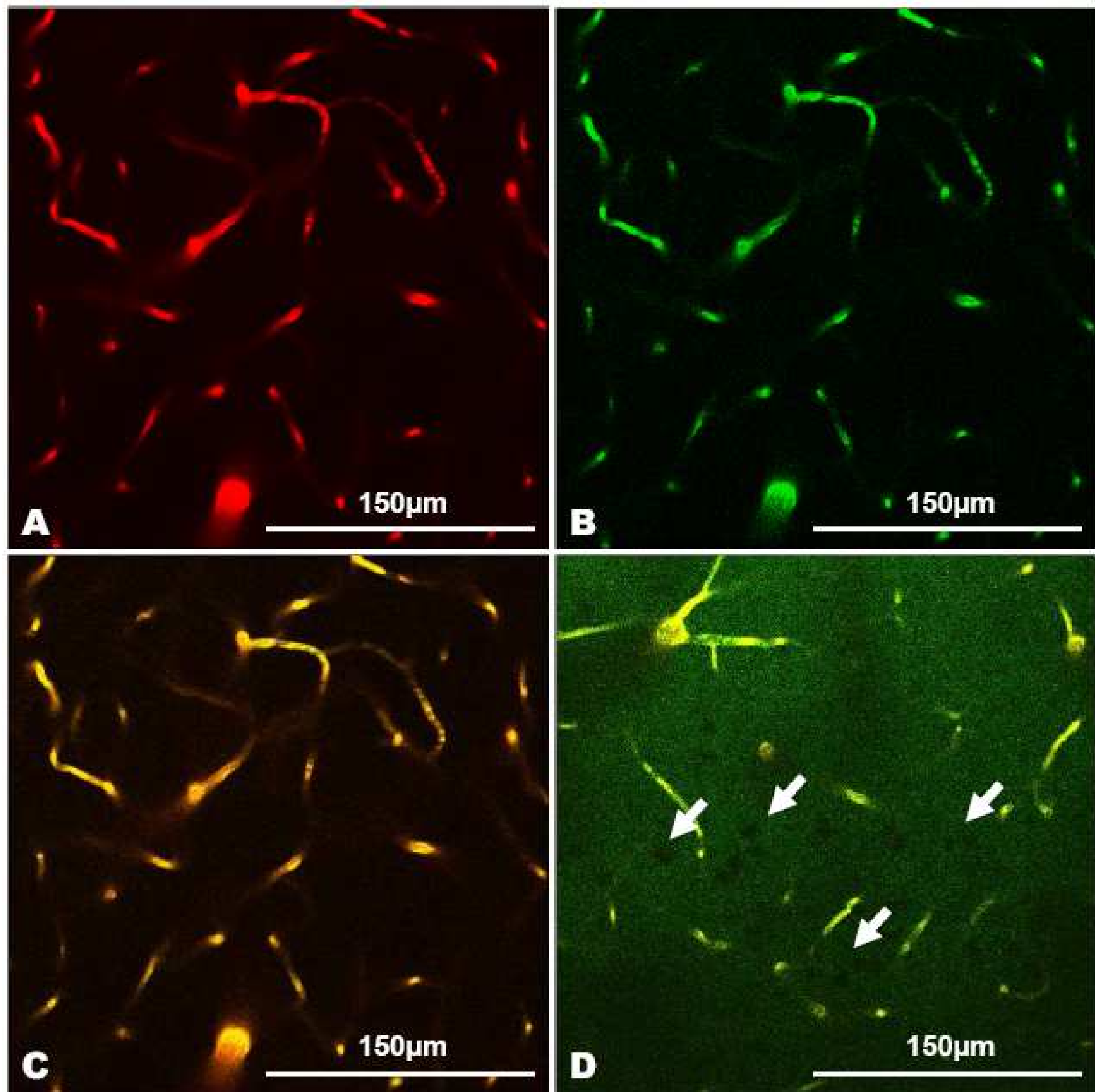

Fig 5

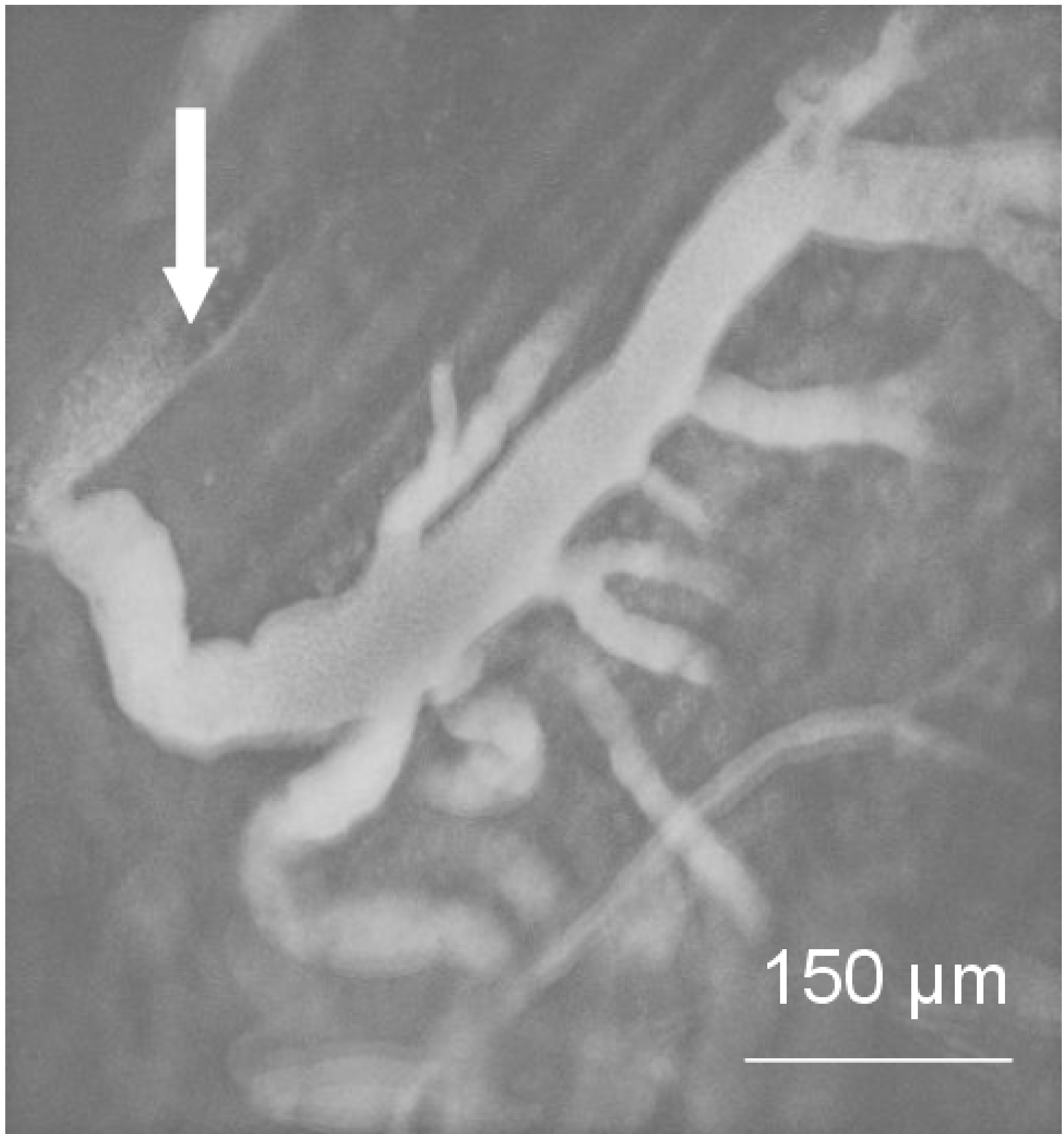

Fig 6