Introduction

Various novel microscopical techniques have given new impulses to biological sciences in the last decades. One of these new imaging techniques is ultramicroscopy (UM), which recently has been shown to allow three-dimensional reconstructions of even cm-sized specimen with micrometer resolution.1-3 Thus, UM bridges a gap between confocal microscopy and macroscopic imaging techniques like computer tomography (CT), making it a versatile tool for anatomical studies of numerous biological and medical specimens.

In UM, the specimen is illuminated perpendicular to the observation pathway by a very thin sheet of laser light, formed by optical elements (Fig. 1). Because illumination of out of focus layers is avoided in UM, no light has to be excluded by a pinhole later on, like in confocal microscopy. By stepping the specimen chamber vertically through the laser light sheet optical sectioning is obtained. Since in UM the light has to travel horizontally along the whole width of a specimen, it is necessary that specimens are sufficiently transparent. This is achieved by a chemical clearing procedure, which is based on replacing the water in the specimen by a liquid having approximately the same refractive index as protein. As a consequence, light scattering is strongly reduced, and the specimen becomes translucent.4

Lectins are proteins that highly specifically bind to sugar complexes, attached to proteins and lipids.5 Presently, lectins are widely used in research, particular in serology, drug targeting and histopathology. In the recent decade, lectins became especially important as markers for microvascular labeling.6-9

Using UM, we three-dimensionally reconstructed several millimeter sized microvascular networks in whole mouse organs. The topology of filigree branches of the microvasculature was visualized. Since tumors require an extensive growth of blood vessels to survive, this novel approach may open up new vistas in neurobiology and histology, particularly in cancer research.

Results and Discussion

We imaged lectin labeled mouse brains by UM and computed three-dimensional reconstructions from the obtained image stacks according to Becker, Jährling et al.2 Figure 2A shows a top view of the brain hemispheres and the cerebellum. As can be seen from the figure, even the finest branches of the microvascular network are visualized. The major vessels generally show less staining. Figure 2B depicts a single cortical blood vessel, surrounded by complexly branched capillaries.

We also obtained three-dimensional images from the spinal cord (Fig. 3A), and of the auricles of the heart (Fig. 3B). In the 3D-reconstructions of the spinal cord some of the thin capillaries supporting the spinal nerves are apparent. The spinal canal (Canalis spinalis) is visible. In its center any vascularization is clearly completely lacking. The reconstructions of the heart (Fig. 3B) illustrate the microvascular architecture in the cardiac auricle (Auricula atrii), formed by close loops of capillaries.

UM of lectin-FITC labeled tissue enables three-dimensional imaging of vast vascular networks in whole mouse organs like...
Figure 1. Principle of UM. A transparent specimen is illuminated perpendicular to the observation pathway by a laser forming a thin sheet of light. Concave lens (1), convex lens (2), slit aperture (3), cylindrical lens (4). Thus, fluorescent light is only emitted by a thin plane and no out of focus light has to be excluded by a pinhole like in confocal microscopy. The emitted fluorescence light is projected to a camera target by an objective, while the excitation light is blocked by a matched optical filter. Objective (5), tube lens (6). By moving the specimen chamber through the light sheet a stack of images is obtained. Afterwards, a 3D-reconstruction is calculated by software.

Figure 2. 3D-reconstructions of vascularization in mouse brains. (A) Top-view of the brain in maximum intensity projection (MIP) (Olympus objective XL-Fluar 2x, N.A. 0.14, reconstructed from 567 images, 2,048 x 2,048 pixel), scale bar 1 mm. (B) Single cortical blood vessel surrounded by complexity branched capillaries (Olympus objective UPlanFL N 10x, N.A. 0.30, reconstructed from 191 images, 2,048 x 2,048 pixel), scale bar 30 μm. Monochrome images were transposed into false colors using the color map shown at the left side of (A).
works in contrast to SPIM with a vertical orientation of the optical detection pathway, it can be easily adapted to standard upright or inverted microscopes. Three dimensional reconstructions of microvascular structures as presented in Figure 2 are impossible to obtain with conventional histological techniques as mechanical sectioning by a microtome. This is due to unavoidable mechanical distortions by the microtome knife, which make the appropriate spatial alignment of hundreds of single sections a nearly unsolvable problem. Since in UM mechanical slicing is substituted by optical slicing, this drawback is completely eliminated. Standard confocal microscopy is limited to specimen sizes of a few hundred micrometers. Therefore it is also not applicable for 3D-reconstruction of large specimen like whole mouse organs and blood vessel networks. Since tumors require an extensive growth of blood vessels to survive, and thus detailed morphological studies of the 3D structure microvasculature are crucial in cancer research, our approach may open up new vistas in this field of research.

Materials and Methods

Six week old mice (Bl6) were killed by CO₂ and perfused transcardially with 5 ml PB and heparin for rinsing the blood out of the vessels until the fluid became clear. Afterwards, we perfused with 10 ml 1% Paraformaldehyde (PFA). All animal preparation procedures were performed according to the regulations of the Austrian animal-care committee. In a next step, 10 ml FITC-Lectin (10 μg FITC-Lectin per ml PB) from Lycopersicon esculentum (Sigma-Aldrich, L0401), specific for N-acetyl-D-glucosamine and N-acetyl-polylactosamine oligomers, was perfused. After 2 min incubation further 30 ml 4% PFA were perfused. We removed the brains from the skull and incubated them in 4% PFA.
at 4°C for 4 hours. Afterwards, they were rinsed 3 times in PBS. For adult mice the volume of the fluids should be doubled. We dehydrated the organs using an ascending ethanol series (50%, 80%, 96%, 100%). Then, the organs were cleared in a solution containing 2 part benzyl benzoate and 1 part benzyl alcohol (BABB).

For UM, we used the setup described in Becker, Jährling et al. 23D-reconstruction was carried out with the 3D-reconstruction software Amira 5.2 (Visage Imaging, San Diego, USA).

For optimal resolution, the data underlying Figure 3A were deconvolved by Huygens deconvolution software (Huygens, Hilversum, Netherlands) using an empirically determined point spread function (PSF). The PSF was determined by imaging sub resolution sized fluorescent latex beads of 1 μm diameter (Invitrogen, Oregon, USA).

References