

# Hierarchical micro imaging for multiscale analysis of large vascular networks

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## Introduction / Customer need

Over the last decade, the accelerated development and advance in  $\mu$ -computed tomography ( $\mu$ CT) enabled the replication of large volumes of microvasculature *ex vivo*, allowing for quantitative assessment of vessel architecture in three dimensions. Besides the descriptive and functional studies of vasculature in healthy organs and tissue, vascular alterations associated with pathology has gained increased attention, additionally fuelled by the availability of genetically manipulated animals as disease models (Beckmann et al., 2003). Recently, neurodegenerative diseases, other than vascular dementia or stroke, have been associated with vascular alterations. Anatomical abnormality of the hippocampus has been suggested as a preclinical predictor for dementia onset in the elderly (Redwine et al., 2003; Vanhoesen and Hyman, 1990). In Alzheimer's disease, ischemic lesions and alterations in the micro vascular network seem to play a major role in the progression of the pathology (de la Torre, 2002). It has been stated that vascular imaging systems could have a high potential to significantly accelerate the process of drug discovery and development (Beckmann et al., 2004; McDonald and Choyke, 2003). Although *in vivo* experiments are the ultimate goal for clinical applications, *ex vivo* studies based on animal models offer some significant advantages, in particular when going to high-resolution imaging. Because of the limited detector size and the cubically increasing amount of image data produced as going to smaller voxel sizes, there is a trade-off between resolution and volume that can be acquired in a single scan.

A desktop  $\mu$ CT system operated at 16- $\mu$ m voxel size, for example, captures an entire mouse brain with a volume of  $10 \times 16 \times 8 \text{ mm}^3$ , producing a 3D image of about 350 MB. A SR $\mu$ CT system operated at 1.4- $\mu$ m voxel size that scans a cylinder of 1.4-mm diameter and 1.4-mm height, on the other hand, produces already an image of 2 GB. In this paper, we present a new and unique approach to systematic imaging of large volumes of vasculature at any depth of the vascular network. The method is based on the technique of modified vascular corrosion casting, desktop  $\mu$ CT, local SR $\mu$ CT, and scanning electron microscopy (SEM) imaging, following a hierarchical and strictly non-destructive approach. Hierarchical imaging is the ability to resolve anatomical features at a variety of resolution and size scales using several complementary imaging modalities and ideally covering a few orders of magnitude in resolution (Müller and van Lenthe, 2004). Precise ROI positioning was realized through a common sample interface, a land-marking mechanism, and dedicated ROI selection software. The resulting imaging framework was optimized for high-throughput measurements and was successfully applied to scan a total of 120 regions in 30 mouse brains, resolving vascular networks, ranging from cerebral and pial over intra-cerebral vessels down to arterioles, venules, and capillaries. Our results, which outline basic visual and quantitative evaluations on the hierarchical image data, demonstrate that the method is a powerful tool for assessing vascular architecture in animal disease models, also referred to as structural phenomics.

## Materials and Methods used

### *Sample preparation and land-marking*

Standard methods were used for vascular corrosion casting (Beckmann et al., 2003). APP23 transgenic mice (SturchlerPierrat et al., 1997) were deeply anesthetized and perfused by intra-cardial injection with a polymer resin (PU4ii, vasQtec, Zürich, Switzerland) (Krucker et al., 2006). After curing, soft tissue was macerated, followed by decalcification of surrounding bone. The cerebral vasculature, including cerebellum and olfactory bulb, was dissected from the remaining vasculature, resulting in full brain casts with a size of about 16x10x8 mm. Casts exhibit even finest details like imprints of endothelial cells (Krucker et al., 2004, 2006; Reina- La Torre et al., 1998). In order to reach better absorptions in the X-ray scans, corrosion casts were stained using osmium tetroxide (Riew and Smith, 1971).

### *Desktop $\mu$ CT data acquisition*

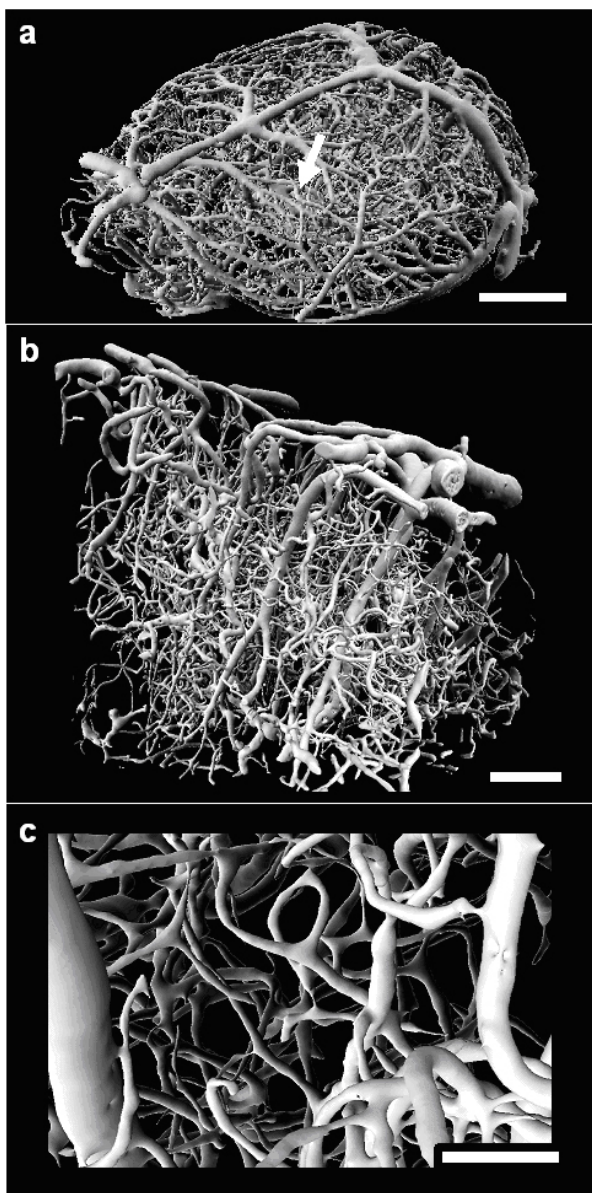
A desktop  $\mu$ CT system ( $\mu$ CT 40, Scanco Medical AG, Switzerland) was used for the acquisition of whole brain samples with a nominal isotropic voxel size of 16  $\mu$ m, referred to as intermediate resolution. The X-ray tube was operated at 50 kVp and 160 mA with a focal spot size of 5  $\mu$ m. Per scan, 1000 projection images were acquired with an integration time of 200 ms and additional three times frame averaging for improved signal-to-noise ratio (SNR). Detailed information on the experimental setup is available elsewhere (Heinzer et al., 2004; Rügsegger et al., 1996). Tomographic images were reconstructed on a VMS cluster (HP Alpha, HP, Palo Alto, USA) in 1024x1024 pixel matrices using a conebeam back projection procedure. Because of the frame averaging, noise filtration could be omitted, and data were segmented directly under application of a global threshold. A bounding box was applied in order to reduce the empty space surrounding the effective brain vasculature, resulting in images of approximately 600x900x300 voxels.

*For specific ROI selection methods, SR $\mu$ CT data acquisition and SEM verification measurements, see the original paper: Neuro Image 32, pp 626-636*

## Results

In order to provide maximum flexibility regarding reuse of valuable cast material and repeated measurements in different imaging modalities, we sought for a sample treatment that conforms to  $\mu$ CT, SR $\mu$ CT, and SEM imaging. We chose the newly developed PU4ii as a casting resin because it produces high-quality corrosion casts and eliminates some of the casting artefacts previously encountered with other commercially available casting materials. Staining with osmium tetroxide provided good absorption contrast using  $\mu$ CT and SR $\mu$ CT. Regarding SEM, earlier experiments required gold sputtering of the cast surface (Krucker et al., 2004). The heavy metal coating however led to artefacts in tomographic scans and disqualified the sample from further  $\mu$ CT and SR $\mu$ CT experiments. As an interesting result, experiments with low vacuum SEM could now visualize the cast surface without further treatment. This makes osmium tetroxide stained vascular corrosion casts a suitable medium for multimodal hierarchical imaging with  $\mu$ CT and SEM technology. Using this material, structures from all levels of the vessel hierarchy were visualized in detail (Fig. 1).

The problem of repositioning a sample when measuring it with different imaging modalities was solved by means of a custom-made sample holder. Once mounted, a sample was never removed from its sample holder, thus providing a firm interface for repeated, multimodal imaging. In addition, we prepared sample holders with two stainless steel pins that served as landmarks for repositioning the sample at the synchrotron facility. In order to verify that samples were correctly positioned, checks were performed routinely before each measurement, but also after data had been acquired. Z-buffer views in dorsal, sagittal, and coronal direction were used to compare the correspondence of large vessels captured by both  $\mu$ CT and SR $\mu$ CT. The dorsal view allowed assessing rotational errors, whereas translational errors were checked in coronal and sagittal views. We found that with our landmark guided measurement protocol, targeted ROIs and measured regions matched with a precision of about 20–50  $\mu$ m. We believe that this error is small enough for an image registration algorithm to map the datasets on a  $\mu$ m scale if needed.



*Fig. 1: Hierarchical imaging of a selected ROI in a vascular cast from a mouse brain (a) brain overview from  $\mu$ CT, scale bar: 2 mm; (b) ROI indicated in (a) by an arrow acquired with SR $\mu$ CT, scale bar: 100  $\mu$ m; (c) detail from (b) showing the smallest vascular structures resolved at 1.4  $\mu$ m, scale bar: 50  $\mu$ m*

**Framework for high-throughput measurements**

Studies that use SR $\mu$ CT to visualize microvasculature typically are limited to assess a small number of samples. Reasons for this are the limited availability of beam-time at the only few synchrotron facilities worldwide and the relatively complex setup involved in such experiments. Furthermore, high-resolution imaging produces very large 3D image files, typically ranging from 300 MB to 16 GB. Only rigorous automation and streamlining of the imaging process can lead

to the throughput, i.e., the number of scans, needed for biological studies. One key element was to keep samples on individual sample holders (see above), allowing for quick sample positioning in all imaging systems involved. Each sample was then scanned in a “one-click” desktop  $\mu$ CT system ( $\mu$ CT 40, Scanco Medical, Switzerland), automatically reconstructed and pre-processed on a VMS cluster for ROI selection in custom-made ROI picking software. Using scrollable stacks of Z-buffer images, this software allowed for quick browsing of anatomical brain regions and precise selection of ROIs for high-resolution measurements. The software was extended to produce measurement scripts for automated SR $\mu$ CT scans of an arbitrary number of ROIs within one sample, including automated tomographic reconstruction on a Linux PC farm. This setup would permit to assemble any volume of brain vasculature by subsequent scanning of adjacent regions. With our measurement protocol, sample positioning for synchrotron measurements could be reduced to less than 20 min, making it an ideal basis for upcoming fast tomography. Data segmentation and analysis were again performed on the in-house VMS cluster in highly automated fashion.

*Hierarchical imaging as a tool for structural phenomics*

The proposed hierarchical imaging method was successfully used to visualize large full brain vasculature in more than 30 mice, complemented by 120 micro-vascular high-resolution measurements of selected ROIs. Visual analysis of high-resolution images allowed for assessment of morphological characteristics of microvasculature that did not become evident from medium resolution data. In an example of age-matched APP23 transgenic and wild-type animals, we visually observed architectural differences such as vessel spacing of the capillary bed. Using quantitative morphometry, also quantitative indices were retrieved indicating, for example, a 34% decrease in vessel spacing in the transgenic animal. Vascular features, e.g., spherical regions containing no microvasculature, could not only be identified and visualized in great detail, but also precisely localized within the context of the intact vascular entity (Fig. 2). This example illustrates how vasculature in the same sample can be assessed in top-down manner, starting at large cerebral arteries,

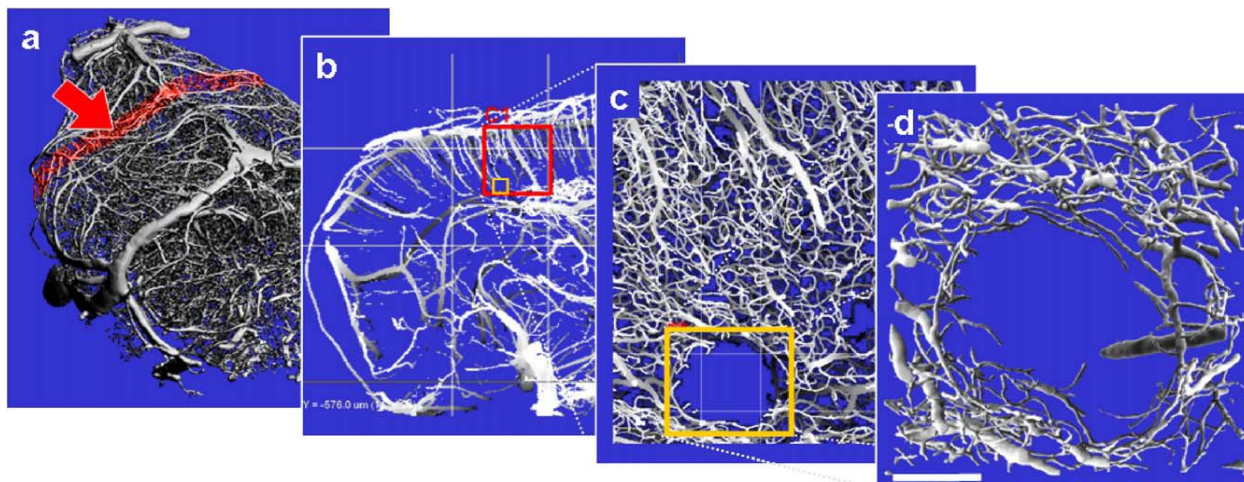


Fig. 2: Hierarchical investigation of vascular architecture: (a) Intact mouse brain vasculature scanned at medium resolution (16-µm voxel size) using a desktop µCT system. (b) Z-buffer slice of the section marked red in panel (a), revealing the frontal cortex. The red square labelled "C1" denotes a region of interest (ROI) which was measured using SRµCT. (c) Z-buffer slice of SRµCT high-resolution data visualizing intra-cerebral arteries and the capillary network. (d) Surface rendering of the infarct region marked in panel (c) suitable for detailed and high-quality 3D analysis.

following the transition from pial to intra-cerebral arteries, finally revealing the dense network of arterioles, venules, and the capillary network. The wealth of information provided in the digitized vessel data was further exploited by colouring vessels according to their thickness (Fig. 3). In good agreement with literature (Reina-De La Torre et al., 1998; Zlokovic, 2005), micro-vasculature observed in the left frontal cortex was organized in vertically penetrating intra-cerebral arteries and horizontally layer-wise branching arterioles and capillaries.

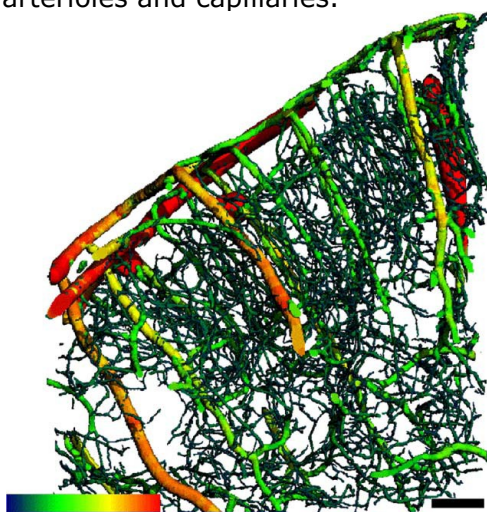


Fig. 3: Portion of microvasculature from the left frontal cortex. The colour coding reflects vessel thickness, ranging in the current example from about 5 µm (dark green) to 30 µm (red).

### Conclusion

We have introduced a novel approach to systematic assessment of brain vasculature in fully non-destructive fashion. Through combination of desktop µCT, SRµCT, and SEM imaging, the method reveals vascular features at a wide range of scales with unprecedented resolution and size, thereby never losing track of the structure in its entirety. Using this approach, it would in principle be possible to successively subsample the whole brain micro-vasculature at 1-µm resolution. With its capability to elucidate vascular network structure at all levels from cerebral arteries down to the capillary bed, the proposed hierarchical method could open new avenues on how to assess vascular architecture in small animals, with impact on drug detection and drug monitoring.

### References

For an extensive list of literature references, see the original paper: *Neuro Image* 32, p 636