Hierarchical bioimaging and quantification of vasculature in disease models using corrosion casts and micro-computed tomography

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ABSTRACT

A wide range of disorders are associated with alterations of the central and peripheral vascular system. Modified vascular corrosion casting using a newly developed polymer, allows for the first time hierarchical assessment of 3D vessel data in animals down to the level of capillaries. Imaging of large volumes of vasculature at intermediate resolution (16 \textmu m) was performed using a desktop micro-computed tomography system. Subsequently regions of interest were identified for additional high resolution imaging (1.4 \textmu m) at the X-ray Tomographic Microscopy (XTM) station of the Swiss Light Source (SLS). A framework for systematic hierarchical imaging and quantification was developed. Issues addressed included enhanced XTM data acquisition, introduction of local tomography, sample navigation, advanced post processing, and data combination. In addition to visual assessment of qualitative changes, morphometrical and architectural indices were determined using direct 3D morphometry software developed in house. Vessel specific parameters included thickness, surface, connectivity, and vessel length. Reconstructions of cerebral vasculature in mutant mice modeling Alzheimer's disease revealed significant changes in vessel architecture and morphology. In the future, a combination of these techniques may support drug discovery. Additionally, future ultra-high-resolution \textit{in vivo} systems may even allow non-invasive tracking of temporal alterations in vascular morphology.

Keywords: hierarchical imaging, vascular corrosion casting, microCT, synchrotron-CT, local tomography

1 INTRODUCTION

There is a wide range of disorders associated with alterations of the central and peripheral vascular system. Angiogenesis plays a fundamental role in many normal physiological processes and in a number of pathological conditions including tumor growth, macular degenerations and chronic asthma. More recently, neurodegenerative diseases other than vascular dementia or stroke, have been associated with vascular alterations. Reduced blood flow has been reported as a consistent physiological deficit in late stages of Alzheimer’s disease (AD).\textsuperscript{2} AD takes an enormous toll on society. The Alzheimer’s Association and National Institute on Aging estimate that current direct and indirect costs of caring for the 4.5 million Americans with AD are at least $100 billion annually.

The typical clinical picture of AD includes a progressive decline of memory function, often accompanied by changes in personality. Nonetheless, as of today brain autopsy is needed to positively confirm the diagnosis. A high density of neuritic plaques, neurofibrillary tangles and vascular amyloid are characteristic neuropathological markers of AD.\textsuperscript{3} Plaques and tangles in the neuropil may affect neuronal function and also contribute to the neuronal damage, however it is unclear whether their incidence correlates with the clinical signs and symptoms of cognitive impairment characteristic of the disease. To find early disease markers is of particular importance to define therapeutic strategies and enable initiation of treatment.

The recent development of mouse models for AD allows the comprehensive study of the temporal progression of the disease. To describe age-dependent alterations in the cerebral vasculature potentially affecting the blood flow in AD, we...
are currently using transgenic mice that overexpress a mutated form of the amyloid precursor protein (APP), and display the pathological hallmarks of this disease. \textit{In vivo} imaging assays with currently available techniques are able to provide information about many aspects of the vasculature including blood flow and functional assessment of the surrounding tissue. However, the temporal and spatial resolution of these techniques is very limited and a sub-regional quantification is almost impossible. We therefore implemented techniques that allow us to study the architecture and morphology of the vasculature, including microvessels and capillaries. Additionally, three-dimensional vascular patterns in normal and pathological tissue and organs can be assessed.

Various methods are available for 2D and 3D quantitative analysis and imaging of biomedical material. Each of them shows its specific characteristics with regard to the type of accessible material, spatial resolution, throughput, maximum sample size, effect on the tissue, availability, ease of use, and cost. With these methods at hand, the technical question is how to combine them to solve the original biological question, i.e. the study of time lapsed structural changes in brain vasculature caused by AD. Since information from each level of resolution helps understanding the pathological processes in the diseased brain, our approach targets a systematic hierarchical investigation of disease models. The aim is to assess corrosion casts qualitatively and quantitatively. Non-destructive treatment allows multiple usages of the specimens, and can help reduce the number of animals needed for each study. On the other hand, we must be aware of the fact that with increasing resolution the amount of data grows even more rapidly, in the case of volumetric methods with the third power. From these considerations, we can formulate three specific challenges regarding hierarchical bioimaging:

(a) Design of a protocol that allows specimen investigation using various imaging methods
(b) Fully exploit capabilities of involved imaging techniques
(c) Handle large-scale data in an efficient manner

In chapter 2 the technique of vascular corrosion casting, the imaging methods involved, and the 3D morphometry software used for quantification will be briefly described. Chapter 3 introduces a framework for hierarchical bioimaging and quantification. Addressed issues are overall workflow, cast material compatibility with all the methods, a common sample interface, sample navigation, and data combination. The next two chapters present innovations that have been implemented at the XTM station\textsuperscript{11,13} at the Materials Science beamline\textsuperscript{10} of the Swiss Light Source (SLS) playing an important role in the hierarchical framework: Chapter 4 introduces a new measurement software, and chapter 5 describes the use of local tomography. Chapter 6 will show preliminary results from the biological application. The paper is concluded with a summary and an outlook on future development.

2 \hspace{1em} \textbf{MATERIAL, IMAGING SYSTEMS AND QUANTIFICATION}

In the following we introduce the technique of corrosion casting. It allows to image vasculature using microscopy and micro-computed tomographic systems. A brief description of each imaging systems involved in the study is given. We finally give a description of the software used for the morphometrical analysis of the acquired 3D image data.

2.1 Vascular corrosion casting

X-ray absorption imaging, as it is used in micro-computed tomographic scans, requires a sufficient difference in the attenuation coefficient of the material to be visualized and its surroundings. Because the contrast behavior of most soft tissues is dominated by water absorption, differential soft tissue imaging requires contrast enhancement using contrast agents, as it is used in cardiovascular imaging. In very small vessels such as capillaries with a diameter of only 5 to 6 $\mu$m, contrast agent concentration is typically too low for accurate assessment of these structures. Fluorescence confocal laser scanning microscopy has been successfully used in this context\textsuperscript{20}, but the technique is limited in terms of the actual tissue depth that can be penetrated with the laser, which lies between 60 and 300 $\mu$m.

Figure 2.1 Left: LM image of an entire mouse brain vascular corrosion cast. Right: High resolution SEM micrograph illustrating imprints of cell nuclei in the cast material (black arrows).
Corrosion casting is a well established technique to produce a replica of vasculature. Animal models, in the present study APP23 transgenic mice (see chapter 6), are deeply anesthetized and subsequently perfused by intracardial injection of a polymer resin. In a second step soft tissue is macerated, followed by decalcification of surrounding bone. The resulting cast exhibits even finest details like imprints of endothelial cells (see Figure 2.1). In order to reach better absorptions in the X-ray scans, corrosion casts were stained using osmium. The method of vascular corrosion casting is unique because the three-dimensional organization is not retained by standard histological techniques.

For visualization of vessel architecture a hierarchical approach was chosen, allowing imaging of the whole organ down to cellular features using one single specimen.

2.2 Light microscopy

A stereo light microscope (Wild, Heerbrugg, Switzerland) equipped with a digital camera (PowerShot G5, Canon) was used to create planar images of corrosion casts. These images allowed inspection of the cast quality and served to generate an overview of the specimen, e.g. for general documentation purposes or for the selection of regions of interest for subsequent SEM-photographs.

2.3 Micro-computed tomography

For full brain 3D imaging, a desktop µCT system (µCT 40, Scanco Medical AG, Basserdorf, Switzerland) was used. The device is equipped with a 5 µm focal spot microfocus X-ray tube as a source. A CCD detector coupled to a thin scintillator permits parallel acquisition of stacks of X-ray images in cone beam mode. An optional oversampling mode allows acquisition of the same stack up to ten times, storing only the averaged image. This results in an excellent signal-to-noise ratio while keeping the amount of produced data constant. For our experiments, the X-ray tube was operated at 50 kVp and 160 mA at an integration time of 200 ms. Two-dimensional CT images were reconstructed in 1024 x 1024 pixel matrices from 1000 projections using a standard convolution-backprojection procedure with a Shepp and Logan filter. The reconstruction is initiated automatically and runs on a 20-node VMS cluster (HP Alpha). Images were stored in 3D index-sequential files with an isotropic voxel size of 16 µm. The excellent signal-to-noise ratio of the scanner allowed direct segmentation of the vessels at a fixed global threshold for all samples without prior noise filtration. The µCT system is comprehensive and easy to use, but can not reach the resolution needed to visualize and quantify capillary structures.

2.4 Synchrotron radiation micro-computed tomography

Imaging of vascular structures at capillary level was done at the X-ray Tomographic Microscopy (XTM) station of the Materials Science beamline of the Swiss Light Source (SLS, PSI, Villigen, Switzerland). Synchrotron radiation is extracted at the 4S straight section of the ring through a hybrid minigap wigglerv, providing a very intense, hard X-ray beam. Wavelength selection is performed with a Si(111) double-crystal fixed-exit monochromator with an energy resolution of 10^-4. The monochromatic beam passes the sample and is converted to visible light by a Ce-doped YAG single crystal scintillator of 20 µm thickness (Crismattec Saint-Gobain, Nemours, France). A microscope optic collects the light under 2x, 4x, 10x or 20x fold magnification and projects it onto a CCD camera of 2048 x 2048 pixels (Photonic Science Ltd., East Sussex, UK). In combination with a 2x relay lens the theoretical pixel size varies in this setup between 0.35 µm and 3.5 µm with a corresponding field of view of 715 µm x 715 µm and 7.15 mm x 7.15 mm, respectively.

The camera writes 16-bit gray scale images, which are automatically converted into flat field corrected sinograms during the measurement. Sinograms can be reconstructed to slice images using a 16-node Linux cluster also running a filtered backprojection algorithm. A 3 TB fileserver provides disk space for measurements and subsequent post processing. For local tomographic imaging, a slightly different setup is used (See 5.2).

2.5 Scanning electron microscopy

To analyze corrosion casts with Scanning electron microscopy (SEM) a special treatment was necessary. Casts were fixed to SEM-stubs with double adhesive, conductive tabs (Provac AG, Balzers, Liechtenstein) and subsequently covered
with a 10-20 nm gold layer using a sputtering device (Balzers Union, Liechtenstein). This coating prevents an accumulation of electric charges which could result in artifacts on the photographs of the samples. The stability of the casts was additionally increased using drops of conductive silver paste (Provac AG, Balzers, Liechtenstein). SEM provides 3D views of replicated vessels and microvessels with a very high resolution (up to around 3 nm). Details of the surrounding vascular tissue such as outlines of endothelial cells and nuclear imprints are readily visible. Nevertheless, the SEM method is limited in that 2D photographs are not suitable for 3D quantification. Only pictures from the surface of the brain vasculature can be generated. In order to investigate the interior of the corrosion cast with SEM, the cast would need to be destroyed.

2.6 Image rendering and direct 3D morphometry

For slice image analysis and Z-buffer views, in-house software was used. Ray-traced and surface rendered 3D views of CT data were generated using commercial and in-house products.

In-house software is also available to quantify a variety of morphometric parameters directly from 3D data\(^1^0,1^7\). This software permits calculation of vessel volume density and vessel surface density as well as direct and local measures of vessel thickness, vessel spacing, vessel number, and connectivity density (Euler number). Evaluation runs on the 13-node VMS cluster and is fully automated. On top of these existing systems, a new biomedical information technology platform (BIT) is currently built which allows web-based data processing for enhanced group collaboration.

3 FRAMEWORK FOR HIERARCHICAL BIOIMAGING AND QUANTIFICATION

Hierarchical imaging aims to assess biological materials at different levels of resolution in order to more thoroughly understand a biological phenomenon\(^1^8\). A new hierarchy level is entered if the increase in resolution reveals substantial new details of the investigated specimen. Consider the following example. A µCT scan at 16 µm resolution captures the coarse vascular structure of an entire mouse brain. Going from organ level to tissue level, SRµCT imaging at 1.4 µm resolution reveals the capillary network. SEM imaging with a resolution of a few nanometers advances to the cell level. Imprints of endothelial cells in the cast material become visible.

The aim of the work presented in this chapter was to unify LM, SEM, µCT, and SRµCT in a framework which facilitates hierarchical investigation of cerebral vascular corrosion casts. We were interested in how synergies between different methods can be used to make investigations more efficient and how they can contribute to answer a specific biological question. Missing links and restrictions had to be identified. The resulting framework and specific solutions are discussed below.

3.1 The framework

A systematic workflow builds the backbone of the framework (see Figure 3.1). It describes the interconnection of individual processing steps that make up a study. In a sample preparation phase, corrosion casts of the brain vasculature of animal disease models are harvested. In a second phase, sample data is scanned and quantified using the various imaging systems of the framework. The workflow is concluded by a result analysis phase, which tries to answer the biological question based on image and morphometry data acquired in this process.

From the above workflow, missing links and restrictions could be identified. Within the framework, the first processing step aims to interface specimens to all the imaging systems (see 3.2). Subsequent 2D imaging could be done using LM and SEM according to the protocol described in \(^1^6\). Similarly, 3D scans at the highest hierarchical level were acquired with

![Figure 3.1](https://www.spiedigitallibrary.org/conference-proceedings-of-spie)
the desktop µCT system (see 2.3) in a straightforward manner. A technique to select regions of interest for later high-resolution scanning had to be developed and is presented in 3.3. Most work was done for high-resolution image acquisition using SRµCT. For non-destructive sample treatment, local tomography had to be established (see chapter 5). A new measurement software was developed to enhance efficiency and ease of use of the SRµCT facility (see chapter 4). The output of all imaging devices was fed to the BIT platform (see 2.6), which provides automated post-processing, visualization, and morphometric quantification of the image data.

3.2 Common sample interface

An important part of the framework consists in interfacing specimens to all of the imaging systems. Addressed issues include compatible sample material, reproducible measurements, and provision of a coordinate system attached to the sample for all subsequent measurements.

Sample casting with polyurethane and additional staining with osmium (see 2.1) provides sufficient absorption contrast for µCT and SRµCT measurements. The same casts can easily be imaged in the light microscope. SEM imaging, however, necessitates additional coating of the samples with a gold layer. This would lead to heavy scattering artifacts in the CT systems. Thus SEM imaging somewhat violates our goal of full sample preservation within the framework and therefore was done with a restricted number of samples only.

For easy transition between the imaging systems, brain samples are glued onto custom made plexiglas tables. Two stainless steel pins are inserted at the bottom of the sample. They are used as landmarks defining origin and angular orientation of a coordinate system attached to the sample. A sample remains on its holder during the entire study, granting preservation of sample alignment and coordinate system. A measurement \( M \) can easily be reproduced by doing a second measurement \( M' \) of the same sample, followed by an alignment of the landmarks in \( M \) and \( M' \).

3.3 Sample navigation and data combination

An inherent problem using high resolution systems consists in controlled navigation within a specimen. Consider a corrosion cast of a mouse brain with a size of 16 mm x 10 mm x 8 mm. Seen through the CCD camera of the SRµCT system, only a portion of 1.4 mm x 1.4 mm is visible. How can now a specific brain region be imaged without cutting the sample into pieces? How can further data from the high-resolution system be combined with data from the low-resolution system?

![Flow diagram illustrating the process of region of interest selection, data acquisition in the high resolution system, and back transformation to the low resolution coordinate system for data combination.](image)

The internal structure of a sample can be revealed using a low-resolution 3D imaging system. It serves as a map of the sample using the coordinates of the low-resolution system. From this map, regions of interest can be selected. Doing the transition to the high-resolution system, theses regions of interest must be projected to the coordinates of the high-resolution system. Through location of the landmarks (see 3.2), origin \( O \) and angular orientation \( \alpha \) of the sample can be determined in both high- and low-resolution coordinate systems. The projection \( T \) is then given by the translation \( \Delta O \).
$O_{\text{high}} - O_{\text{low}}$ and subsequent rotation by the angle $\Delta \alpha = \alpha_{\text{high}} - \alpha_{\text{low}}$. Upon imaging and reconstruction in the high-resolution system, the inverse application of the projection $T$ brings the high-resolution data back to the low-resolution system, where both data sets can be visualized. This procedure is illustrated in Figure 3.2.

4 IMPROVED XTM MEASUREMENT SOFTWARE

The XTM station at the SLS facilitates synchrotron radiation micro-computed tomography (SRµCT) with resolution levels at the micron level and below (see 2.4). Besides complex beam conditioning through mirrors and a monochromator, sample positioning, projection image acquisition and data reconstruction are essential elements of the tomographic measurement process. The complexity of these processing steps and their interplay make measurements laborious and error prone, in a highly experimental environment such as a synchrotron facility. The amount of data produced when measuring at highest resolutions is enormous. Only strict automation, optimized dataflow, and well-established quality control can guarantee high throughput and by that the success of the measurements in the long run.

Within the framework of this project, a new measurement software was developed, unifying measurement control in a centralized system. Three aims served as a guideline to the whole software development process:

- Unify experiment setup and control elements in a single user interface
- Reduce dataflow and enhance cluster based processing
- Provide an intuitive workflow on a step by step basis

The resulting system design as well as two noteworthy implementation details regarding reconfigurable, extensible workflows and improved data flow are discussed in the following.

4.1 System design

A thorough analysis in collaboration with the beamline scientist and beamline users led to the following functional requirements: The new system should facilitate specimen data management, logging of measurement and reconstruction parameters, automatic determination of important parameters like camera focus or rotation center, automatic acquisition of multiple scans per specimen, measurement and reconstruction control, automatic and interactive quality control, data backup and data export.

The system design (see Figure 4.1) exhibits a multi-tier architecture. Most of the well-proven existing beamline software tools have been incorporated seamlessly facilitating rapid development which was restricted to either building gluing functionality to interconnect these tools, or else to build entirely new functionality, i.e. the central user interface or database integration. One exception to this practical design issue consisted in the conception of a new camera server. In the former solution, projection data was acquired via commercial imaging software. Preservation of this software would have made it impossible to control
the system from a single user interface, where as a remotely controllable camera server overcomes this problem. Third party products have been integrated in the system where appropriate.

4.2 Implementation

Since the XTM station at SLS is a facility with high potential for scientific development, it was an important requirement to keep the XTM manager software extensible. Another goal was to guide the users through the experimental process on a step by step basis. From these two aims, the notions of reconfigurable processing units and compilation of workflows have been developed. A processing unit is a small entity of activity, which results form breaking down the overall process into manageable components. Examples are sample data registration, beam profile setup, rotation axis calibration, measurement region selection, and so on. Dependencies between processing units may exist in that a unit cannot execute prior to successful termination of one or more other ones. The performance of a specific type of experiment, e.g. global tomography or phase contrast imaging, may then be modeled as an assembly of interdependent processing units, the so called workflow. A workflow manager component in the system allows an administrator to configure workflows tailored according to the needs of the beamline users. Once the experiment has started, the user simply follows the instructions given by the workflow manager. The object model which implements this idea is illustrated in Figure 4.2.

![Figure 4.2 Object model facilitating reconfigurable workflows assembled from individual processing units.](image1)

More sensitive CCD detectors, higher beam flux, and faster sample stages will allow increased speed of tomographic experiments in the near future. The data produced when acquiring a single SRµCT scan is considerable. 1000 projections with a CCD detector size of 2048 x 2048 and a pixel depth of 16 bit result in 8 GB of raw data and additional 16 GB of reconstructed slice data if stored as 32 bit float values. In the new software, measurement and reconstruction efficiency is optimized by the introduction of raw sinograms. The idea consists of writing projection data directly to raw sinogram files, a method that has been implemented in other systems before. Each file stores data associated with one row of the CCD detector, including rows from dark and flat field images. Flat field correction can be done at reconstruction time in memory, feeding the result directly to the reconstruction algorithm without further accessing the file server (see Figure 4.3). Compared to the former solution, the amount of data produced is reduced by 40%.

5 LOCAL TOMOGRAPHY AT THE SWISS LIGHT SOURCE

Non-destructive hierarchical imaging as introduced in chapter 3 necessitates acquisition of small portions of a specimen when resolution exceeds certain boundaries. Theoretical papers on local tomography have been known in the literature for two decades, but only few practical applications have been reported. Recognizing the high potential of this method for hierarchical bioimaging, local tomography has now been successfully implemented at the X-ray tomographic microscopy (XTM) station of the SLS.

In the following we summarize the mathematical basics of local tomography. We then give a description of the measurement setup which made it possible to visualize also the smallest vascular structures without additional sample
preparation. Part of it is a newly developed motorized sample stage. It allows controlled sample navigation, which is crucial using the method. The chapter ends describing the result of using a global reconstruction algorithm to reconstruct local data.

5.1 Theoretical background

In computed tomography, a 3D object is projected at different angles onto a plane. Mathematically, the rows in the projection images represent line integrals over the attenuation of the object. In order to reconstruct a certain point \( p \), ordinary tomography requires integrals over lines far from \( p \). Local tomography, on the contrary, uses only integrals over lines close to \( p \), reconstructing a related function that is an approximation of the attenuation of the real object. Images resulting from this approach preserve boundaries as well as the direction of density jumps across boundaries, but they are cupped in regions where the real object is constant. Thus local tomography can be used to accurately reconstruct the geometry of an object, while it cannot preserve its quantitative material properties.

5.2 Measurement setup and motorized sample stage

Vascular corrosion casts of entire mouse brains (see 2.1) were glued on custom made plexiglas tables (see 3.3) without further cutting of the specimen. Sample alignment and region selection was performed using a newly developed motorized sample stage (see Figure 5.1). Beam energy was increased to 17.5 keV in order to provide sufficient photon flux to penetrate the large sample. The sample detector distance (SDD) was experimentally optimized to make smallest vascular structures visible using the effect of edge enhancement. A 20 \( \mu \)m YAG scintillator transformed X-rays into visible light. 20x fold optical magnification and on-chip binning provided isotropic voxels of 1.4 \( \mu \)m. Pre selected volumes of 1 mm\(^3\) were measured by acquiring 1001 projections and a number of periodic dark and flat field images at an integration time of 2 sec each. Scanning time for one block summed up to 88 minutes.

In order to assess small selected regions of interest (ROI) within the full specimen, it was necessary to develop a sample aligning device. For a first test we used a manually adjustable sample stage allowing sample displacement in the plane perpendicular to the rotation axis with an accuracy of 10 \( \mu \)m. Assuming that this axis is aligned to the center column of the CCD, this setup facilitates imaging of a distinct ROI by aligning its center with the rotation axis. The success achieved with the manual device motivated the development and integration of a motorized sample stage. An existing sample stage was equipped with two step motors with a precision of 1 \( \mu \)m, allowing fully automated sample positioning within a displacement range of \( +/\sim 15 \) mm in both horizontal directions. The original top was replaced by a custom made elongated adapter piece in order to increase stability and to prevent collisions of the stage motors with the microscope. The resulting setup is illustrated in Figure 5.1.

5.3 Local tomographic image data

In a first attempt local projection data was reconstructed using a conventional filtered back projection algorithm. Although this clearly violates the mathematical requirements for local reconstruction (see 5.1), meaningful slice data could be obtained. Smallest vessels like capillaries were surprisingly well displayed. Thicker vessels showed saturated vessel walls, where as their interior exhibited decreased intensity. This effect has been reported as a result of local tomography. This effect is further enhanced by the measurement setup, which provides a sample-detector distance...
(SDD) of 30 mm, resulting in slight edge enhancement. A strongly expressed buckling pattern has been observed in all images, which is most probably a result of the improper global reconstruction algorithm. Prior to segmentation using global thresholding, data was smoothed with a constraint Gaussian filter employing a filter width $\sigma$ of 1.0 and a filter support of 2 pixels. Finding an appropriate threshold was difficult due to bad signal-to-noise ratio resulting from a relatively low flux during the measurements. Threshold selection was therefore performed on a specimen by specimen basis. As expected, capillaries could be nicely reconstructed, where as thick vessels showed a cupped interior (see Figure 5.2).

A successful first empiric verification of the resulting 3D image data has been achieved by fitting reconstructed 3D data obtained from the cast surface to a stereo light microscopy photograph of the same specimen (see Figure 3.2).

Local tomography and automated sample alignment have been employed in the AD project (see chapter 6) to acquire four predefined regions of a sample with a single setup. After a setup and verification procedure requiring 30 min to 1 h, the four scans could run 6 h without requiring further user interaction.

Figure 5.2 Cortical vasculature acquired from a mouse brain corrosion cast using local tomography at a resolution of 1.4 $\mu$m. Smallest vessels become visible in impressing detail. Bigger vessels exhibit cavities resulting from improper use of the global reconstruction algorithm for local data and suboptimal signal to noise ratio.

6 BIOMEDICAL APPLICATION

6.1 Background

Neurodegenerative diseases such as Alzheimer’s disease and dementia have been associated with vascular alterations\(^1\). The combined use of modified vascular corrosion casting, electron microscopy and micro-computed tomography enables us to evaluate the vascular pattern in normal and pathological tissue from transgenic animals developed as disease models\(^4,11,15\). The direct value of the combination of these technologies will be the understanding of disease progression which consecutively will help develop and test new therapies.

Figure 6.1 Left: 3D reconstruction of a $\mu$CT scan of the vasculature of an entire mouse brain (lateral view), using a resolution of 16 $\mu$m. Right: Selected blocks of cortical vasculature (white cubes marked in the full brain image on the left) scanned using SR$\mu$CT and local tomography at the SLS, resolution 1.4 $\mu$m.

Reduced blood flow has been reported as one of the most consistent physiological deficits in AD, and overt vascular irregularities have been found in biopsy material from pathologically confirmed AD cases\(^1\). However, it remains unclear whether the reduced cerebral blood flow and possible presence of regional microvascular abnormalities are a response to...
neuronal damage or factors initiating the characteristic neuropathology. The study of microvascular changes in models for AD is shedding light on the progression of vascular pathology and its role during the early phases of preclinical AD.

Based on a preliminary study using vascular corrosion casting and EM, we found that already at young ages, when typically parenchymal amyloid plaques are not yet present, APP tg mice have significant alterations of the microvasculature. Capillaries, the location of the blood-brain barrier, had diameters ranging from 5 to 6 µm. In normal animals they formed a dense array often spaced less than 30 µm apart. These patterns seem to be altered in younger APP23 tg animals. Older animals developed changes in morphology and the three-dimensional architecture of the cerebral vasculature. The location and distribution of these microinfarcts imply that they are associated with parenchymal plaques. In very old mice we also found that main arteries of the Circle of Willis were eliminated completely and cerebral arteries were significantly thinner as confirmed by magnetic resonance angiography (MRA)\(^4,12,15\). Our results suggest that there is an early vascular component associated with the development of amyloid deposition and vascular degeneration that potentially contributes to the cognitive impairment in AD.

Although scanning electron microscopy creates remarkable three-dimensional impressions of typical features of normal and pathological morphology, in reality it reproduces casts only in two-dimensions, which limits accurate morphometric measurements to vessels at the surface of the cast. Despite several attempts using approaches to circumvent these limitations, valid three-dimensional information can only be obtained with techniques such as micro-computed tomography and other truly volumetric imaging modalities.

### 6.2 Results

Three-dimensional vessel morphometry has been assessed using direct quantitative morphometry\(^10\). This software permits calculation of vessel volume density and vessel surface density as well as direct and local measures of vessel thickness, vessel spacing, vessel number, and connectivity density (Euler number) (see Table 6.1).

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Table 6.1 Quantification of morphometry: comparison of 12 month-old APP23 transgenic (tg) and wild-type (wt) animals.

These first results indicate that there is no significant difference in any of the parameters between animals of the two phenotypes (N=6) at 12 months of age, except for connectivity density (+54%). However, there were trends for increased volume (+21%) and surface area (+36%) in the transgenic group. At first, it might be counter intuitive that with progressive destruction and a higher incidence of infarcts (depicted as holes in the dense network of capillaries, see Figure 6.2 Sequential slices from the cortex of a 12 month old APP23 tg mouse. The missing microvasculature is forming holes in the dense vascular network. In a surface view (left) such holes cannot be detected. It is only by making use of the three-dimensional nature of the CT images that allows selecting digitally the appropriate field of view to “zoom in” on such infarcts.)
vessels are more and better connected. But most likely, this might be explained by an angiogenic response as a compensational effect attempting to form more vessels around the infarct regions. Nevertheless, more samples and time points will have to be investigated to make final conclusions.

7 SUMMARY AND OUTLOOK

A framework for hierarchical imaging and quantification of vascular corrosion casts was established. Incorporated are the 2D methods LM and SEM and the 3D imaging systems μCT and SRμCT, which allow access to the three hierarchical levels that we are interested in: entire brain (LM, μCT), capillary network (SRμCT), and cell (SEM). Creation of a systematic workflow helped to identify missing elements and restrictions in a framework designed for high throughput. Cast material and a custom made sample holder were adjusted to allow reproducible measurements with all imaging systems. Landmarks were used for sample alignment and sample navigation, which was essential for controlled navigation in the high resolution system and for subsequent data recombination.

The sample handler of the XTM station has been improved in order to locally acquire selected regions of interest of a sample in a non-destructive manner at a resolution of one micron and below. The fact that samples do not need to be cut into sections solved previously encountered problems such as the viscoelastic properties of the cast material, leading to motion artifacts during measurements. Notably, a standard global backprojection algorithm was used to obtain meaningful slice data. Artifacts were found inside thicker vessels, resulting in holes which could not be filled using global threshold segmentation. The effect was enhanced by local tomography resulting in lower saturation towards the center of bigger structures. In the future, local reconstruction algorithm will be investigated. If segmentation problems persist, more sophisticated segmentation methods like local thresholding and image closing operators will be needed.

A motorized sample stage was developed and integrated at the XTM station allowing precise and automated sample positioning. With this extension, sample sub-volumes of arbitrary size may be acquired at highest resolutions by subsequent scanning of neighboring blocks and later reassembly using image registration software. Thus, using this method, the entire vasculature of a mouse brain could be digitized at a resolution of one micron.

The high resolution achieved using local tomography poses at the same time a significant limitation. The CCD detector provides a dynamic range of 16 bits per pixel. Therefore a block of 1024^3 pixels results in 2 GB of raw data, and 4 GB of reconstructed gray value slice data. With un-binned data (2048^3) data size is multiplied by a factor of 8. Such large data files are difficult to handle with respect to disk space, network capacity, main memory, and processor speed. A newly developed measurement software alleviates this problem using an optimized dataflow to directly write raw sinogram images to the file server instead of projection images. The software makes measurements easier because it provides the user with a step-by-step workflow, increasing the overall fidelity of the system. It unifies all control elements in a single user interface and automates the measurement process considerably.

Future steps in high throughput, highest resolution tomography at the SLS will focus on improving computing hardware, particularly cluster computing and algorithm optimization. Additionally, it needs to be investigated how the relatively weak signal-to-noise ratio in local tomography can be further improved. Thorough validation of the accuracy of the structure acquired using local tomography must be performed using phantom structures of known sizes.

In general, advanced post-processing methods must be developed for both visual and quantitative assessment of vascular corrosion casts. The main challenge consists in the size of the data retrieved by the 3D imaging systems. We currently investigate methods to compile a vectorized representation of vessel structures, which considerably reduces the data size by retaining only the information of interest. Fast visualization is needed to qualitatively inspect 3D data in real time. The BIT platform is the tool of choice for web-based, group oriented data post processing, visualization, and quantification. Further work will be necessary to seamlessly connect it to the hierarchical framework and to port well proven legacy systems to the new platform.

The experiments carried out so far for the AD research project helped to exploit the possibilities and pitfalls when producing three-dimensional images at highest resolution, using corrosion casts in conjunction with synchrotron X-ray tomography. The good results obtained from local tomography revealed this non-destructive technique as the method of choice for future measurements. Fine-tuning and automation of the experimental setup will provide both larger volumes and completion of age-grouped animal data allowing investigation of temporal alterations in vascular morphology related to AD in a quantitative fashion.
Because of the uniqueness of the combination of technologies in the hierarchical framework, the interest has been growing quickly. We are currently studying other models for neurodegenerative diseases and applying slightly modified versions to study tumor growth and arthritis.

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