**A novel method to visualise the three dimensional organization of the human cerebral cortical vasculature**

C.H. Harrison1, G.R. Buckland1, S.E. Brooks2, D.A. Johnston2, D.S. Chatelet2, A.K.L. Liu3, S.M. Gentleman3, D. Boche1, J.A.R. Nicoll1,4

1Clinical Neurosciences, Clinical and Experimental Sciences, University of Southampton, Southampton UK

2Biomedical Imaging Unit, University of Southampton, Southampton General Hospital, Southampton UK

3Neuropathology Unit, Division of Brain Sciences, Department of Medicine, Imperial College London, London UK

4Department of Cellular Pathology, University Hospital Southampton NHS Foundation Trust, Southampton, Southampton UK.

Corresponding author: Charlotte Harrison, Clinical and Experimental Sciences, University of Southampton, email: [chh1g13@soton.ac.uk](mailto:chh1g13@soton.ac.uk)

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**Abstract**

Current tissue clearing protocols for imaging in 3 dimensions (3D) are typically applied to optimally-fixed, small volume rodent brain tissue – which is not representative of the tissue found in diagnostic neuropathology laboratories. We present a method to visualise the cerebral cortical vasculature in 3D in human *post-mortem* brain tissue, which had been preserved in formalin for many years. Tissue blocks of cerebral cortex from 2 control cases, 2 Alzheimer’s brains and 2 cases from Alzheimer’s patients immunised against A42 were stained with fluorescent *Lycopersicon Esculentum agglutinin* (Tomato lectin), dehydrated and cleared using an adapted three-Dimensional Imaging of Solvent Cleared Organs (3DISCO) protocol, to visualise the vascular endothelium. Tissue was imaged using light sheet and confocal microscopy and reconstructed in 3D using Amira software. The method permits visualisation of the arrangement of the parallel penetrating cortical vasculature in the human brain. The presence of four vascular features including anastomosis, U-shaped vessels, spiralling and loops were revealed in all cases. In summary, we present a low cost and simple method to visualise the human cerebral vasculature in 3D compatible with prolonged fixation times (years), allowing study of vascular involvement in a range of normative and pathological states.

**Key words:** vasculature, brain, human, three dimensions, light sheet microscopy

**Introduction**

The vascular supply to the human cerebrum is required for delivering oxygen and glucose, essential for the survival and functioning of neocortical neurons. The continuous and regulated blood flow is derived from paired internal carotid arteries, each dividing into anterior and middle cerebral arteries, and paired vertebral arteries fusing to form the basilar artery and then dividing into paired posterior cerebral arteries with anastomoses occurring at the circle of Willis (Kalaria et al., 2015). Branches of these arteries traverse the surface of the brain within the arachnoid mater then pierce the cortical surface in a perpendicular manner, forming penetrating cortical arteries and arterioles, some of which supply the capillary bed within the cortex and others passing through to the underlying white matter. The capillary bed is drained by veins running back to the cortical surface. However, our current understanding of the detailed configuration of the vasculature within the cortex is limited. As described by Florey in 1925 using animals perfused with Indian ink, the leptomeningeal vessels perpendicularly enter into the parenchyma (Florey, 1925). Several decades later, Duvernoy used the alkaline phosphatase reaction on thick histological sections of human brain to demonstrate a dense distribution of cortical arteries and arterioles running through each neocortical cellular layer, until they dissipated at the grey-white matter boundary (Duvernoy et al., 1981). Using the same methodology on celloidin sections, Fischer demonstrated the presence of “abnormally contoured vessels” in Alzheimer’s disease (AD) brain defined as looping, tortuosities or kinking of vessels (Fischer et al., 1990). Since these studies, as the more “modern” approach to histopathology typically involves thin sectioning of tissues leading to the inability to visualise blood vessels in 3D, there has been limited further progress in our understanding of the configuration of blood vessels within the brain parenchyma. This is an omission considering the increasing interest in the role of the vasculature in a range of disorders, including stroke and dementia.

The recent development of tissue clearing techniques, combined with the use of light sheet and confocal microscopy, has enabled tissue blocks to be stained and cleared for visualisation in three dimensions (3D). Current methods include CLARITY (Chung et al., 2013) which uses hydrogel scaffolds to remove lipid whilst preserving proteins (Erturk et al., 2012, Erturk and Bradke, 2013, Erturk et al., 2014, Renier et al., 2014) and immunolabelling-enabled three-Dimensional Imaging of Solvent-Cleared Organs (iDISCO) (Reiner et al 2014) which involves the dehydration and clearing of tissue blocks using organic solvents. Such methods can be expensive and labour-intensive and have typically been applied to rodent or fresh tissue with very short fixation times. In addition, immunolabelling in tissue clearing remains challenging, particularly in human tissues where antibodies penetration is limited possibly due to the high density of antigen expressed (Liu et al., 2017; Marx, 2016). Therefore, a non-immunohistochemical approach applicable to formalin-fixed human brain tissue, using volumes large enough to sample the full depth of the cerebral neocortex (or comparably sized subcortical structures) whilst using widely available reagents will be useful. Here we present a methodology employing *Lycopersicon Esculentum agglutinin*, known as Tomato lectin (Nachbar et al., 1980), to label vascular endothelial cells (Nag, 1985, Jilani et al., 2003, Robertson et al., 2015), in order to gain a novel perspective on the 3D arrangement of the vasculature in the human cerebral cortex. As we have specific interests in the vascular alterations associated with AD and its therapy with immunization with amyloid-β peptide (Boche et al., 2008), our investigation included AD cases and immunised AD (iAD) cases in addition to aged-matched non-demented subjects.

**Methods**

*Samples description*

Archival formalin-fixed brain tissue which had been in formalin for >2 years was employed in this study. Cuboidal blocks of approximately 8x4x4mm were taken from the cortex of the superior/middle temporal gyrus (Brodmann area 21/22) from each case. Cases included the brains of 2 age-matched, disease-free control subjects, 2 subjects with AD and 2 subjects who had received Aβ42 immunotherapy in life (iAD cases) (Boche et al., 2008). Details of the cases are shown in table 1. Methodology and analysis were performed blind to case status.

*Ethics*

The study was performed under ethical approval from Southampton and South West Hampshire Local Research Ethics Committees reference LRC 075/03/w; and UK Brain Archive Information Network (BRAIN UK) Research Ethics Committees reference 14/SC/0098.

*Tissue preparation and staining*

*Permeabilisation: The first step* included 1h incubation in a solution of 0.2%Triton-X 100 and 4% sodium dodecyl sulphate (SDS), followed by a second incubation in the same solution heated at 50°C for 1h.. The samples were then placed in 20% dimethyl sulphoxide (DMSO), 0.2% Triton-X 100, 4% SDS solution or 6 hrs, before being left overnight in a solution composed of 0.1% Tween, 0.1% Triton-X 100, 0.1% sodium deoxycholate, 0.1% NP40, 4% SDS and 20% DMSO. All solutions were prepared in phosphate buffered saline (PBS) and incubations from the second step were performed at 50°C.

*Staining*. Samples were washed for 1hr in 1% Triton-X 100 solution before being stained overnight at 4oC with a 5% tomato lectin solution to image the vasculature (DyLight 649 Tomato lectin, Vector Laboratories). The far red fluorophore 649 was chosen as longer wavelengths are known to generate less autofluorescence and scattering (Davidson and Campbell, 2009). Following staining, samples were washed in 0.2% Tween-20 solution.

*Tissue clearing.* Dehydration was achieved by incubation for 8 hours each in increasing concentrations of tetrahydrofuran (THF) as follows: 70% THF x3, 80% THF x2, 95% THF x2, 100% THF x2. Samples were then placed in dichloromethane (DCM) solution followed by a di-benzyl ether solution (DBE) for 24 hrs before being imaged. The total duration of the protocol was 9 days.

*Image acquisition and 3D reconstruction*

*Image acquisition*. To visualise the penetrating cortical vasculature, the 6 sample blocks were imaged in their entirety with the LaVision light sheet Ultramicroscope at x6.4 magnification using the filters: 640nm corresponding to the excitation peak for the tomato lectin fluorophore, and 480nm to image the autofluorescence signal. For each sample, stacks of approximatively 250 images separated by 5µm were generated for subsequent 3D reconstruction and analysis (Figure 1A).

The capillaries were visualised using the Leica TCS-SP8 Scanning Confocal Microscope in regions of interest identified with the light sheet microscopy. Typically 2x3x3mm cubes were dissected from the same tissue block and observed with a x63 objective and 0.75 zoom using the excitation laser frequency of 633nm and 488nm for the vasculature and autofluorescence respectively. For each sample, z-stacks of approximatively 100 images separated by 1µm were generated for subsequent 3D reconstruction and analysis.

*3D reconstruction:* All acquired stacks of images from the light sheet and confocal microscopes were imported into Amira software (FEI v6.0) to undergo 3D visualisation using the Multi-Channel Field (MCF) and Volume Rendering modules (Figure 1B). MCF is used to group separate channels into one object, allowing all channels to be operated on simultaneously, as well as mapping the data of each channel to black (lower data) and a chosen colour (upper data). Accordingly, we adjusted the data window of each channel to reduce the autofluorescence and noise in order to optimize the 3D visualisation of the vessels. The Volume Rendering module is used to visualize the dataset in 3D by calculating the intensity and transparency of each point of the dataset and projecting them in space.

To isolate the vessels from small non-vascular objects, images acquired on the light sheet microscope were processed using Fiji software (version 1.51o NIH, US) (Schindelin et al., 2012) to remove the non-specific autofluorescence by subtracting the images acquired at 488nm from those acquired at 640nm. The resulting images were then re-imported in the Amira software (FEI v6.0) for 3D analysis. Firstly, the images were thresholded to reduce the non-specific staining. To isolate the vessels from small non-vascular objects, the thresholded dataset was further filtered with the Volume3d parameter to identify vessels based on size and number (Figure 1C, Figure 2). Individual vessels were assigned different colours to facilitate their visualisation. Videos produced using the animation command of the Amira software show: video 1 the “raw dataset” obtained prior to thresholding and autofluorescence removal, and video 2 an example of the reconstruction of the raw dataset.

*Qualitative assessment*

The analysis filter command on the Amira software permits extraction of individual vessels for assessment (Figure 2). Morphological features of the vasculature were qualitatively assessed on the 3D reconstructed images as present or absent using the following structured criteria defined as: spiral/tortuous vessel morphology; anastomoses between vessels; beading for irregularities in vessel calibre; and a “loop-the-loop” appearance, with the vessel curving back on itself before continuing on through the cortex.

**Results/Discussion**

Our novel clearing/staining methodology associated with software-mediated 3D reconstruction allowed visualisation of the human cerebral cortical vasculature in formalin-fixed brain tissue at a high level of resolution. From the light sheet microscopy reconstructions, we confirmed that the vessels travel over the surface of the brain before descending perpendicularly into the grey matter (Video 1). Within the grey matter, the vessels run parallel to one other, clustering together until they reach the grey/white matter boundary where they dissipate (Video 2). On the confocal imaging, the capillary endothelium showed an intricate network (Video 3; Figure 3E, 3F), the arrangement of which could only be fully appreciated on the 3D reconstructions. A single capillary can branch several times without appearing to change in diameter, unlike the typical pattern of artery bifurcations or venous tributaries. Of note, using the cell nuclei for reference on the 3D images, the capillaries appear to represent a higher volume of the sample than one would appreciate from a 2D histological section. The 3D arrangement of the vessels is consistent with the 2D depiction of the cortical vasculature previously described (Duvernoy et al., 1981). However, the 3D image allows for a more comprehensive appreciation of the dense nature of the cortical vasculature, with the complementarity of the use of the light sheet (larger vessels) and confocal (capillaries) microscopy allowing the investigator to appreciate the entire course of the vessel from the cortical surface to the capillary bed.

Specific features of the cortical vasculature were observed, such as a U-shaped profile that reflects a change of direction of the vessel near the interface between the grey and white matter (Figure 3A). Interestingly, several vascular anomalies previously reported in thick histological sections were identified (Fischer et al., 1990) including anastomoses between nearby vessels (Figure 3B), spiralling of vessels running perpendicular to the pial surface (Figure 3C), and a looping appearance of the vessel (Figure 3D). The spiralling of vessels seems likely to be consequence of cortical atrophy, with thinning of the cortex without concomitant shortening of the vessels passing through it. Cortical atrophy is associated with ageing and Alzheimer’s disease and spiralling of vessels was detected in all cases examined. Longstanding hypertension also induces changes in vascular morphology and may be relevant. These findings support and extend the identification of comparable morphological features in images previously obtained from thick histological sections imaged in image 2 (Fischer et al., 1990). It was not possible in this pilot study to comment on whether or not a certain anomaly was more (or less) common in Alzheimer’s disease. However, our ability to identify these anomalies signify that the method is suitable for analysis of the vasculature comparing health and disease in larger cohorts.

The methodology is being expanded to open new areas of exploration, such as the use of fluorescently tagged antibodies permitting a wide range of targets to be visualised in 3D, and derive quantitative data regarding volumes and spatial relationships. Use of antibodies in small volumes of fresh and fixed human brain tissue has been reported (Liu et al., 2015), although antibody penetration in large volumes of tissue appears to be challenging. Recent work has focussed on the development of methods that allow for repeated rounds of tissue labelling in human and rodent tissue (Murray et al., 2015). One of the main advantages of 3D analysis compared to 2D histology is the potential for ascertaining accurate data, such as volumes and distances, that are more representative of the architecture of the human brain and conditions of interest. For example, in the context of ageing and dementia, quantitative estimates of the various forms of vascular pathology and their relationships to vascular risk factors, abnormally aggregating proteins such as amyloid-β and tau, and neuronal and synaptic loss would be of importance.

This methodology presents the advantages of a low cost and high scalability. Indeed, it is possible to run the protocol on up to 25 samples simultaneously, increasing the size of study as required to achieve statistical power for case/control comparison. This tissue preparation method employs readily available reagents, has no need for special equipment and can be applied to large volume samples of long-term archival formalin-fixed human tissue, compared with other 3D protocols (Erturk et al., 2012, Erturk and Bradke, 2013, Erturk et al., 2014, Chung et al., 2013, Renier et al., 2014). The original descriptions of CLARITY and 3DISCO used endogenously fluorescent mouse tissue that had shorter protocols as they did not require a staining step (Erturk et al., 2012, Erturk and Bradke, 2013, Chung et al., 2013); both procedures were of 4 days to a week in length, comparable to the permeabilisation and clearing steps in our human protocol. As with other protocols using fluorescent labels, the stained specimens need to be kept in the dark prior to imaging.

Currently, the methodology includes a degree of subjectivity in the image processing. Indeed, some vessel qualities seen in the raw data set, such as beading (data not shown), were lost with processing. In addition, 3D imaging methods as described are relatively new and evolving rapidly. The images presented here give a subjective insight into the benefits and show the potential for quantification of aspects of microanatomy, such as vascular density per unit volume. However, currently, suitable software is not yet available, but is clearly a priority for future development.

To conclude, this method is a practical cost-effective and simple way to investigate vascular morphology in 3D, adding to the current interest in 3D clearing protocols. In particular, we have demonstrated that this protocol can be successfully applied to tissue volumes that can encompass the whole thickness of the human cerebral cortex in human *post-mortem* brain samples that have been preserved in formalin for prolonged periods, and thus open new areas of investigation using tissue available in the brain banks.

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**Author contributions**

CH Harrison: light sheet tissue preparation, images acquisition and manuscript writing. GR Buckland: confocal tissue preparation, image acquisition, manuscript review. SE Brooks: light sheet microscopy expertise and support. DA Johnston; confocal microscopy expertise and support. DS Chatelet: image analyse software expertise and support. AKL Liu and SM Gentleman: tissue clearing protocols expertise. D Boche and JAR Nicoll: conception and design of the study, sample selection, manuscript writing. All co-authors have reviewed the manuscript.

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Table 1: Description of the cases and identification of the vascular anomalies

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Cases** | **Age at death** | **Sex** | **Spiral vessels** | **Anastomoses** | **Beading** | **Loops** |
| Control | 78 | M | - | - | + | - |
| Control | 88 | F | + | + | - | - |
| AD | 74 | F | + | - | + | - |
| AD | 81 | M | - | - | - | - |
| iAD | 82 | M | + | - | + | + |
| iAD | 87 | F | + | - | + | - |

AD = Alzheimer’s disease

iAD = Alzheimer’s disease after immunotherapy against amyloid-β

**Legends**

**Figure 1**: **Illustration of the light sheet microscopy 3D visualisation (video 2)**

Tissue blocks were visualised on a light sheet microscope to provide a comprehensive overview of the spatial arrangement of larger vessels in the human cerebral cortex. (A) Raw dataset obtained on the light sheet microscopy showing the first, middle and last images of a stack. (B) 3D visualisation of the raw stack using VolRen and MCF modules. (C) Thresholded and filtered stack to identify individual vessels. (D) 3D reconstruction illustrating the parallelism of the vessels descending perpendicularly through the grey matter. Control case.

**Figure 2: Illustration of vessel extraction**

The filter command on the Amira software permitting the isolation of individual vessels based here on size. AD case.

**Figure 3: Illustration of vascular anomalies identified in 3D and of the capillaries network**

Light sheet microscope images: (A) U-shape (control case), (B) anastomosis (control case), (C) spiralling (AD) and (D) loop (iAD). (E (iAD), F (control case)) Capillaries observed on 3D confocal microscope image showing the intricate network.

**Video 1: 3D reconstruction of neocortical vasculature, raw dataset.**

The cortical vessels (blue) can be seen to penetrate the cortex and travel the depth of the neocortex to the grey-white matter boundary, where the vessels dissipate. Residual autofluorescence is seen (green). Control case.

**Video 2: 3D reconstruction of neocortical vasculature, with autofluorescence removal and thresholding.**

The process of 3D reconstruction from the image stack is demonstrated, followed by the appearance of the vasculature once thresholding and removal of autofluorescence has been performed. Control case.

**Video 3: 3D reconstruction of the capillary meshwork.**

The intricate 3D arrangement of the capillary endothelium forming a meshwork like pattern can be seen. Control case.