

# Investigating human neocortical architecture in 3D

Citation for published version (APA):

Hildebrand, S. (2021). *Investigating human neocortical architecture in 3D: new approaches for clearing, labelling and imaging large samples*. [Doctoral Thesis, Maastricht University]. Maastricht University. <https://doi.org/10.26481/dis.20211214sh>

## Document status and date:

Published: 01/01/2021

## DOI:

[10.26481/dis.20211214sh](https://doi.org/10.26481/dis.20211214sh)

## Document Version:

Publisher's PDF, also known as Version of record

## Please check the document version of this publication:

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- The final published version features the final layout of the paper including the volume, issue and page numbers.

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## Impact Paragraph

The main objective of this thesis was the development of methods for 3D imaging in the field of histology (the investigation of tissues), with large intact tissue blocks of the human brain being the main investigative focus. In traditional histological methods, 2D images of thin slices are either individually interpreted, or retrospectively reconstructed in 3D. In contrast, the nascent field of 3D histology focuses on the immediate acquisition of 3D information over large fields of view. The aim is to slice the tissue as little as possible and in this way preserve the structures of interest within it. This thesis focusses mainly on the primary steps necessary for such an endeavour: 1) optical tissue clearing, i.e. the transformation of the opaque tissue into a transparent histological sample that can be microscopically imaged in 3D, 2) the reliable labelling of thick, large pieces of tissue, and 3) the volumetric microscopy necessary to image these transparent samples over large fields of views. This thesis provides several improvements towards such 3D histological investigations in the human brain. In this impact paragraph, the dissemination, scientific and commercial applications, and wider academic and clinical translation potential is briefly set out. This is split into MASH, ct-dSPIM, and hFRUIT, as these are the three central methodological developments of this thesis.

## MASH

The MASH (Multiscale Architectonic Staining of Human cortex) clearing and labelling method has been primarily deployed in this thesis to visualize cytoarchitecture (the organisation of cell bodies; Chapters 2, 4, and 5) as well as angioarchitecture (the organisation of blood vessels; Chapter 3) in the healthy human brain. As such, MASH is a useful tool for fundamental neuroscientific investigations of brain architecture and brain mapping. In particular, the combination of a high-throughput MASH pipeline for very large samples (Chapter 4) with the mesoscopic imaging capacities of the ct-dSPIM microscope set-up (Chapter 5) is well suited for human brain mapping (and the brains of other large mammals).

### *Dissemination and current applications*

The original MASH protocol was first disseminated at the Society for Neuroscience (SfN) and Light Sheet Fluorescence Microscopy (LSFM) conferences in 2017 and is widely available as an open access publication<sup>1</sup>. The high quality of tissue clearing achievable with this method has generated a lot of attention within the tissue clearing community and helped to initiate the ongoing collaboration with the Helmchen laboratory in Zürich (results of which are reported in Chapter 3 and in the recent publication on the mesoSPIM microscope system<sup>2</sup>). Furthermore, it has caught the attention of both academic as well as industrial light-sheet microscope developers. Large MASH-processed samples have been provided to Dr. Fabian Voigt (former

member of the Helmchen laboratory) and Björn Eismann (Luxendo GmbH, Heidelberg, Germany) for tests on proof-of-principle light-sheet set-ups, optimized for very large cleared samples, demonstrating the commercial interest in such large-scale systems. The angioMASH protocol has been presented as a selected talk at the LFSM conference 2020 and the detailed protocol will be made available as an open access publication. Likewise, it is planned that all data obtained in this study will be made available on online repositories. Similarly, the high-throughput MASH protocol has been presented in poster form at the Swiss Light-Sheet Microscopy Workshop 2019 as well as at the SfN and LFSM conference of the preceding year. Protocols as well as files for the 3D printable hardware will be made openly available both upon publication and in online repositories. Additionally, MASH has been presented repeatedly locally at Maastricht University and its clearing capacity was explored on highly myelinated, white matter rich samples. Specifically, rat and human spinal cord samples (in collaboration with the School for Mental Health and Neuroscience (MHeNs) and the Department of Urology, respectively) as well as peripheral human nerve samples (in collaboration with the Department of Anatomy and Embryology) have been successfully cleared with MASH in several pilots.

*Wider scientific and academic potential*

Beyond the reported cytoarchitecture and angioarchitecture mapping in the human brain and their further development, other

areas of fundamental neuroscience could also benefit from MASH and 3D histology in general. Tracer studies, in which live animals (or more rarely *post mortem* human tissue, see Chapter 6) are injected with a tracer to label sparse axon populations, either originating or terminating at a specific site of interest, are one example. With classical 2D histology, the axon segments contained in each thin tissue slice have to be reconstructed in 3D. This is a very labour intensive and error prone method, as individual axon segments have to be identified and the likelihood of losing traced axons over the consecutive sections is high, especially when they are very sparse. Optical clearing in general offers a way to visualise entire brains of smaller mammals such as mice<sup>3-5</sup>, rats<sup>6,7</sup>, and even marmoset monkeys<sup>8</sup>. Since there is no need for sectioning, the traced connections can be identified more robustly<sup>9,10</sup>. MASH offers the additional advantage of providing several dyes to counterstain the brain's cytoarchitecture and hence offers more anatomical context, given that the tracers can be retained in the tissue in an organic-solvent-compatible manner.

Volumetric imaging of large samples cleared with MASH provides a promising way for the validation of other imaging methodologies such as magnetic resonance imaging (MRI)<sup>11</sup> and near infra-red spectroscopy (NIRS)<sup>12,13</sup>. In the case of MRI, especially acquisitions at ultra-high field (UHF; field strengths of 7T and more)<sup>14</sup> and applied to *post mortem* tissue<sup>15</sup>, provides good overlap with MASH cleared samples, with resolutions in the range of 10s of micrometres and field

of views in the centimetre range. Therefore, the comparison of structural information gained with *post mortem* UHF MRI, with more specific labels introduced in the cleared samples becomes feasible. In turn, *post mortem* MRI information can be translated to non-invasive *in vivo* imaging with the same structural MRI techniques. Studies using such non-invasive methods in animal models, particularly for functional imaging, such as fMRI and fNIRS, would provide an interesting opportunity to deepen our insights into the nature of the blood vessel dependent signals in these functional imaging techniques. These studies would offer the unique opportunity to compare the same specimen after functional imaging with higher resolution vasculature maps obtained with e.g. the angioMASH protocol (Chapter 3). Taken together, the MASH technique in its different variations has the potential to become a standard clearing method in many laboratories, both in the field of neurosciences as well as in other fields.

*Clinical translation potential*

Aside from fundamental neuroscientific applications, MASH has already shown its potential to clear FFPE (Formalin-Fixed Paraffin-Embedded) specimen on other tissue types and diseased samples, such as prostatectomy samples (Chapter 5). Hence, it could become relevant for the clinical investigation of pathological specimen, for which the FFPE procedure is a standard preservation protocol. In the field of pathology, the visualisation of whole biopsies or ectomy

samples would be particularly useful, as it would provide a more sensitive and complete sampling of potential disease marker over the entire extent of the available tissue. In cancer biopsy samples for instance, the categorisation of different cancer stages depends on the visualisation of abnormal cells and structures. Especially at earlier stages, undercounting of these cells is more likely with classical 2D histology which samples only a few out the hundreds or thousands of (potential) slices of an entire biopsy or ectomy. This could lead to a (partial) misclassification and hence in a less suitable treatment plan for patients<sup>16</sup>. 3D histology (or more succinctly: 3D pathology) could therefore help patients receive a more accurate diagnosis and more suitable treatment, through the time- and cost-efficient sampling of entire biopsies. The histochemical dyes neutral red and methylene blue already provided a simple way for a general overview staining on prostate tissue and human endometrium biopsies (the latter processed in a pilot together with the Department for Anatomy and Embryology). For a staining pattern more similar to the traditional Haematoxylin and Eosin (H&E) staining, the fluorescent properties of eosin<sup>17</sup>, in combination with methyl green as a marker for DNA could be exploited. One further aspect that has been addressed recently<sup>18</sup>, is the development of faster clearing for a timely diagnosis of biopsy samples. This new dehydration method is potentially compatible with MASH and therefore a promising method for a future pathoMASH pipeline.

It has also been shown in samples with Alzheimer's disease that the visualisation of disease biomarkers in a 3D volume can reveal features of the disease that would be very difficult to detect in 2D slices<sup>19</sup>. For instance, iDISCO+-cleared human hippocampal samples revealed so called Three dimensional Amyloid Patterns (TAPs) forming layered, sheet-like structures in some patients. In other patients, the patterns formed gradients or complex 3D shapes such as ribbons. The high number of amyloid plaques sampled in these volumes also enabled a quantification of the large variability of plaques in terms of their morphology, volume, and inter-plaque distance. The investigation of other brain diseases, such as epilepsy, Parkinson's disease or vascular dementia, with 3D histology could lead to the discovery of similar spatial relations of biomarkers or pathological brain architecture, which have so far been overlooked.

## ct-dSPIM

The ct-dSPIM (cleared-tissue dual view Selective Plane Illumination Microscopy) set-up introduced in Chapter 5 is an oblique light-sheet system optimized for very large cleared samples, which was co-developed with Applied Scientific Instrumentation (ASI Inc., Eugene, US). Together with the MFS (Mesoscopic Fast Scan) imaging method, mesoscopic overview scans of large regions or entire samples can be acquired at a fast rate and with adjustable resolution without the need to change the optics of the microscope.



*Dissemination and current applications*

The full details of the set-up including all components and the MFS imaging method will be made openly available upon publication. The design of this system has already been presented at SfN and LSFM in 2018 and has been well received within the light-sheet microscopy community. Furthermore, the group in which the work for this thesis has been conducted was recently awarded a VENI grant (awarded to Dr. Anna Schueth) to advance the hardware of the system, to further utilize its potential for neuroscientific and clinical 3D imaging. In this context, the set-up at Maastricht University is embedded at MHeNS and is already used in collaborative projects on prostate cancer imaging, together with the Department of Pathology. Further collaborative projects are already scheduled with both the Cardiovascular Research Institute Maastricht (CARIM) and Division 3 (Translational Neuroscience) of MHeNs at Maastricht University involving whole brain imaging of mouse and rat brains. The large imaging chambers of the ct-dSPIM (allowed by the oblique setup and large translation stage) in principle allow for the rapid high-throughput acquisitions of multiple rodent brains in parallel.

The imaging of more diverse tissue types from biopsy, ectomy, or *post mortem* samples is already planned together with both, the Department of Anatomy and Embryology and the Department of Pathology. Future adjustments to the ct-dSPIM system could allow for higher resolution imaging than currently possible and hence make it

an even more attractive imaging solution for other groups at Maastricht University and outside. To achieve this, prospective hardware updates with higher magnification objectives and the installation of a static light-sheet generated by a cylindrical lens, in order to allow for axial scanning at near isotropic resolution without the need for multi view deconvolution, is necessary.

*Wider scientific and academic potential*

This system is not only relevant for neuroscience, but potentially for all areas of structural/anatomical investigations of human organs and those of other species, for questions in which a large field of view with relatively low microscopic resolution is required. Therefore, the current system has a wide applicability in many fields of fundamental and clinical research. To further expand the potential range of applications to even more diverse fields and research questions, the effective resolution of the system could be increased not only by the hardware updates indicated above, but also in combination with new histological methods such as expansion microscopy (ExM)<sup>20,21</sup>. ExM effectively increases the resolution, by physically separating structures of interest while maintaining their original spatial relationship<sup>22-25</sup>. This would allow for the investigation of structures which are impossible to resolve on the current system.

*Clinical translation potential*

As mentioned above, the set-up at Maastricht University is already used in a collaborative project on prostate cancer imaging, together with the Department of Pathology. In this project, the potential of the ct-dSPIM in combination with MASH optimised for FFPE tissue is explored for the fast 3D visualisation and classification of these prostate cancer samples. The large imaging chamber of the ct-dSPIM allows for the parallel mounting and rapid imaging of many clinical specimen in parallel, which makes the ct-dSPIM particularly interesting for diagnostic high-throughput imaging. Instead of slicing multiple thin sections out of e.g. a core needle prostate biopsy, many such biopsies from the same patient or from several patients could be quickly imaged together in a single acquisition at a mesoscopic resolution. Diagnosis could either be performed on site during live imaging by the pathologist or at a later point after inspection of the 3D rendered dataset. In the latter case, the imaging would be decoupled from the diagnosis and, therefore, the microscopist would be able to focus exclusively on data acquisition, further increasing the throughput speed of the pipeline. This potential for massive parallelizing of the imaging could potentially outweigh the comparatively longer processing times of the current pipeline as compared to standard thin sections stained with the H&E staining (however see comment above on potential decrease in processing time in the MASH section). It should also be considered that processing entire biopsies into hundreds to thousands of classical

sections and the inspection of these would take much longer than MASH processing followed by 3D inspection. It is assumed, however, that while the 3D imaging of whole clinical specimens is ultimately superior to standard 2D histology, it will take some time for this method to become an accepted, let alone a standard tool in diagnostics. 3D histological pipelines need to be made highly robust and reliable to work in a variety of sample types while providing a similar visual appearance as H&E stained sections, as discussed above in the MASH section. The coordination of the image acquisition and the diagnostic inspection of acquired data is another step in the pipeline which needs to become more standardized. As the widespread implementation of light-sheet systems on the pathologist's desk is considered unlikely, better ways to quickly share data or live stream acquisitions would be highly useful. Despite these significant current limitations, the long-term clinical impact of fast volumetric microscopy methods is assumed to be considerable and its routine implementation on pathology labs ultimately only a question of time.

## hFRUIT

Chapter 6 presents a clearing approach fundamentally different from MASH. hFRUIT is an adapted version of the aqueous clearing protocol FRUIT, optimized for human brain tissue. Both the original FRUIT and hFRUIT are simple to perform and do not require any specialised equipment. Since hFRUIT does not remove the brain's lipids, it is

uniquely suited for the combination with lipophilic dyes to trace intrinsic cortical connections in human brain tissue. This however, comes with the trade-off of a reduced clearing capacity.

*Dissemination and current applications*

The hFRUIT protocol is available as an open access publication<sup>26</sup> and was presented at both the SfN and LSFM conferences in 2016. The hFRUT protocol is has an important niche application, as the injection of lipophilic dye crystals is the only method to study the interareal connectivity in the human brain by directly visualising specific axonal connections down to the single fibres and retrogradely labelled neuron populations. With hFRUIT it is now possible to view these labelled connections directly in 3D, at a microscopic resolution and yet over mesoscopic distances (tracer diffusion is limited to about 1-2 cm). Given the highly specialised character of this clearing protocol, the future application of this method is seen in fundamental neuroscientific research, with potential for further academic rather than clinical or commercial impact. For instance, the application of hFRUIT to existing as well as future material from the collection of the Systems Neurophysiology group of the Technische Universität Darmstadt, with injection sites in different brain areas, is planned in connection with our ongoing collaboration.

*Wider scientific and academic potential*

The virtual dissection of cortical microcircuitry, in combination with the cytoarchitecture stainings developed in the MASH protocol, will hopefully broaden our understanding of the minute differences in that circuitry between different brain areas. The combination of labels for cell bodies used in MASH could provide further anatomical information regarding the exact location of intrinsic connections by revealing the layered cortical architecture. The unique compatibility of lipophilic dyes with 3D imaging makes aqueous non-delipidating clearing protocols such as hFRUIT highly interesting tools in the field of human brain connectomics. Aside from the knowledge on intrinsic connectivity gained directly by this method, it could be used in combination with *post mortem* diffusion-weighted MRI (dMRI) to validate the latter method<sup>27</sup>. The combination of lipophilic tracers with dMRI has been used by the lab hosting this thesis work in the past<sup>28</sup>, however the histological analysis in that dMRI validation work was limited on 2D sections. With hFRUIT, these validation experiments could now be performed in 3D to further optimize e.g. dMRI tractography and connectomics methods<sup>29,30</sup> in order to derive a more precise representation of human brain connectivity. Additionally, the discovery of the positive effect of the PLP (periodate-lysine-paraformaldehyde) fixation<sup>31</sup> on the clearing capacity is of interest to the wider clearing community. The tissue fixation itself has not received much attention as a factor influencing the performance of clearing protocols and different fixation methods could further

improve other clearing methods as well. It could therefore be possible to produce even more highly transparent samples and enabling even deeper imaging with less light scattering, when combining more potent clearing methods such as MASH with these beneficial fixation methods.

## Conclusion

Taken together, the work presented in this thesis introduces a set of techniques and devices to bring human tissue clearing to a different scale as well as to more diverse fields of research. While this thesis does not address every aspect necessary for the 3D histological investigation of human brain tissue, it demonstrates substantial progress on the tissue processing and imaging aspects of such a pipeline. Scalable labelling strategies for brain angio- and cytoarchitecture have been developed together with a clearing pipeline that can process large parts of the human brain, or other organs such as the prostate. The further upscaling of these methods to whole human brain slices of up to 5 mm thickness is feasible and the microscope platform presented in this thesis would allow for the fast mesoscopic imaging of samples this size. A more specialised clearing protocol for the investigation of intrinsic human connectivity could greatly improve our insights into cortical circuitry and its variations between areas. The techniques presented in this thesis were primarily designed for their use in fundamental neuroscience. However, they are potentially interesting for many academic

disciplines including clinical fields, such as pathology or clinical neuroscience, and the ct-dSPIM developments were performed in collaboration with a commercial company, showing further potential for commercial valorisation. Therefore, the developments reported in this thesis, have the long-term potential to benefit not only the fundamental understanding of the human brain, but also to improve diagnostics and hence eventually benefit clinicians and patients.



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