Longitudinal three-photon imaging for tracking amyloid plaques and vascular degeneration in a mouse model of Alzheimer's disease

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Abstract.

Significance

Vascular abnormalities may contribute to amyloid-beta accumulation and neurotoxicity in Alzheimer's disease (AD). The ability to monitor vascular degeneration as AD progresses is essential. Three-photon fluorescence microscopy (3PM) enables high-resolution deep tissue imaging with minimal invasiveness and photodamage.

Aim

This study established a longitudinal 3P imaging pipeline to quantify vascular degeneration and amyloid plaque formation in the $APP^{NL\text{-}G\text{-}F}$ mouse model.

Approach

A cranial window allowed repeated 3P imaging at four-week intervals beginning at five weeks after surgery. Vessels labelled with Texas-Red were segmented using DeepVess, while plaques labelled with methoxy-XO4 were segmented using custom scripts. Quantitative analyses assessed vascular parameters (diameter, density, tortuosity, length, inter-vessel distance) and plaque metrics (radius, nearest plaque-to-vessel distance).

Results

We imaged the same field over 4 weeks quantifying a decrease in vasculature and increase in amyloid plaque formation with age. Significant decreases in vessel diameter, increases in inter-vessel distance, and alterations in vessel length were observed. Changes in vessel tortuosity, plaque radius, and plaque proximity to vessels were not significant.

Conclusions

This pipeline tracks vascular remodeling and amyloid pathology in deep cortical structures. It offers a tool for studying the interplay between vascular and amyloid pathologies in AD, supporting future research into disease mechanisms and therapeutic strategies.

Keywords: three-photon microscope, multicolour, longitudinal, vasculature, amyloid plaques, image segmentation.

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1 Introduction

Alzheimer's disease (AD) is the leading cause of dementia worldwide, impacting the quality of human life and imposing significant healthcare costs.[1] A widely accepted theory, the amyloid hypothesis, suggests that cognitive decline in AD patients is partly due to the accumulation of extracellular amyloid beta (Aβ) peptides in the brain.[2] This accumulation is believed to trigger a cascade of pathological events, including the formation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau and subsequent neuronal loss.[3,4] Nonetheless, while amyloid pathologies have been the primary focus, emerging studies highlight the vascular system as a major factor in the progression of the disease.[5]

To meet its high energy demands, the brain relies on a constant supply of oxygen and energy substrates. As the brain has no long-term energy storage, regional cerebral blood flow is precisely regulated to align with the local energy requirements of nervous tissue. Any disruption in this adaptive process or in the delivery of necessary substrates can lead to imbalances in homeostasis, tissue damage, and functional impairment.[6] These vascular abnormalities have been shown to not only contribute to Aβ accumulation but also exacerbate neurotoxicity in AD.[7–9] It is believed that the interplay between vascular degeneration and Aβ deposition is central to understanding the pathophysiology of AD progression. Brains of human AD patients commonly exhibit distinct vascular anomalies, including significant reductions in vascular diameter,[10] density,[11] and blood flow,[12] while vessel tortuosity and length tend to increase.[13,14] However, the precise mechanisms by which vascular abnormalities contribute to amyloid plaque buildup and subsequent neurotoxicity in AD remain unclear.[15]

Animal models are crucial for elucidating the pathophysiological pathways of AD and de-

veloping preventive, diagnostic, and therapeutic strategies. While wild-type mice do not form senile plaques or neurofibrillary tangles,[16] genetically engineered mice consisting of familial AD (FAD)-linked mutations in genes like *APP*, *PSEN1*, and *PSEN2* are employed to mimic amyloidosis features of FAD-AD.[17] Most AD cases are caused by sporadic AD, however no animal models mimicking this form have been developped yet. Early transgenic models overexpressed amyloid precursor protein (APP), leading to excessive Aβ production and accumulation of APP fragments.[18] However, APP overexpression can introduce artifacts not representative of human pathology.

The $APP^{NL\text{-}G\text{-}F}$ knock-in mouse model, which avoids APP overexpression by introducing specific mutations into the endogenous *APP* gene, provides a more accurate representation of preclinical AD.[19,20] This model incorporates the Swedish (NL), Arctic (G), and Iberian (F) mutations, with homozygous mice exhibiting greater Aβ accumulation starting at 2 months than heterozygous ones starting at 4 months.[21,22] It is considered a standard for studying mechanisms of Aβ amyloidosis.[18] FAD typically manifests in humans in the heterozygous state, therefore the heterozygous $APP^{NL\text{-}G\text{-}F}$ mutation more accurately models human pathology.[23] Although other AD mouse models such as APP/PS1, 5xFAD, and APP23 have been utilized to study changes in vascular morphology and plaque progression,[24–27] chronic imaging of vascular and amyloid interactions in the $APP^{NL\text{-}G\text{-}F}$ knock-in model has not been thoroughly investigated. This gap presents an opportunity to examine the relationship between vascular abnormalities and amyloid plaque formation in a model that more closely replicates human Aβ pathology.

Despite advancements in AD research, the disease's complex pathogenesis remains poorly understood, highlighting the necessity for advanced longitudinal imaging methods to sequentially visualize brain structures and gain further insights into the mechanisms of the disease. Advanced

imaging techniques are essential for studying the dynamics of vascular degeneration and amyloid pathology *in vivo*. Three-photon fluorescence microscopy (3PM) has significantly extended the penetration depth of high-resolution optical imaging, enabling the visualization of individual cells in preclinical rodent models with high spatial and temporal resolution.[28] Compared to conventional imaging modalities like CT, MRI, and PET, 3PM offers cellular-level resolution with minimal tissue damage.[29–32] Additionally, 3PM employs pulsed laser sources that deliver high peak power for excitation at low repetition rates, resulting in low phototoxicity.[33]

Specifically, 3PM enables deep tissue imaging using long excitation wavelengths (1300–1700 nm) and low average power, minimizing photodamage and phototoxicity.[33,34] This approach reduces out-of-focus signal and enhances the signal-to-background ratio (SBR), allowing imaging at depths up to 1.4 mm—surpassing the limitations of two-photon fluorescence microscopy (2PM), which is often restricted to depths of 700 μm.[31,35] Multicolor 3PM, using fluorescent dyes and genetically encoded markers, has successfully imaged structures such as GCaMP6s-labeled neurons, Texas Red–labeled blood vessels, and third-harmonic generation (THG) signals from myelinated axons up to 1.1 mm deep. THG is a nonlinear optical phenomenon that enables label-free brain imaging by generating a signal at three times the original laser frequency, with its efficiency enhanced by structural discontinuities within the focal volume.[36] However, simultaneous multicolour imaging of vasculature and Aβ using 3PM has not been demonstrated, and longitudinal imaging using 2PM has been limited to superficial cortical regions within layer five.[30,37]

Our study aims to address this gap by establishing a longitudinal imaging methodology for deep cortical structures in the brain, utilizing a 3PM imaging pipeline to investigate vascular and amyloid pathologies in the $APP^{NL\text{-}G\text{-}F}$ knock-in mouse model. By leveraging the deep imaging capabilities of 3PM, we seek to enable future studies to explore the dynamics of vascular degen-

eration and Aβ plaque accumulation. This approach will provide more accurate and translatable insights into the physiological mechanisms underlying AD progression, ultimately supporting the development of effective preventive and therapeutic strategies. Understanding these mechanisms could significantly impact future research and clinical practices in AD, paving the way for improved diagnostic and therapeutic approaches.

2 Methods

2.1 Three-Photon Microscope Setup

The 3P microscope [Fig. 1(a)] consisted of a 40 W fibre laser (Satsuma), an optical parametric amplifier (OPA, Mango) running at a repetition rate of 2 MHz, and a hyperscope (Scientifica). The laser pulses, with a width of 65 fs at 1340 nm, passed through a pulse compressor composed of mirrors (M3, M4, M5) and prisms (P1, P2). The beam was then directed through a series of mirrors (M1-M15), a half-wave plate and a beam-splitter for beam conditioning. The final power under the objective ranged between 165–220 mW. A tiltable objective mount was used, supporting a 25× water-immersion objective (XLPlan NA=1.0, Olympus), with an ultrasound gel as the immersion liquid. The signals were divided into two detection channels using two photomultiplier tubes (PMTs) and dichroic beam splitters with specific wavelength filters (ET440/40 and ET620/60 from Thorlabs). The ET440/40 filter was used to detect amyloid plaques via methoxy-XO4 fluorescence,[38] while the ET620/60 filter was used to detect vessels via Texas-Red fluorescence.[36] Before reaching the objective lens, a beam expander was used to ensure the filling of the back aperture for optimal imaging resolution. The focal spot sizes along the x, y, and z axes were determined using 0.20-μm-diameter fluorescent beads, calculated as the full-width at half-maximum (FWHM) [Fig. 1b]. Lateral resolution was measured to be 0.87 ± 0.12 μm while axial resolution was 4.59 ± 0.48

μm (mean $\pm$ s.e.m, n=5).

2.2 Animals

All experimental procedures complied with the project licence (PPL number: PP6988384), Home Office, and Imperial College London institutional norms. Heterozygous $APP^{NL\text{-}G\text{-}F}$ knock-in mice[19] with one copy of the mutant APP gene were employed as AD models. The humanised Aβ buildup induced by mutations results in similar disease development, making the mouse model suited for studying the link between amyloid plaques and blood vessels. Six mice were used: one female aged 8.9- to 9.9- months and two females aged 6.5- to 7.5- months for chronic imaging, and a 10- and 11-month-old mouse for image analysis pipeline development; a 1-month old wildtype (C57BL/6) mouse was also imaged for pipeline development.

2.3 Cortical Surgery and Viral Injection

Mice were given Carprofen (5 mg/kg) and buprenorphine (0.07 mg/kg) for pre-operative pain management and were operated on under 1.5–3% isoflurane anaesthesia. A heated blanket and rectal thermometer were used to maintain body temperature at 37°C. A circular craniotomy was made over the visual cortex (from bregma, in mm: 2.25 ML, -3 AP; Fig. 1c), outlined with a 3 mm biopsy punch, and the skull piece removed using a dental drill. A cranial window made of a 3 mm coverslip glued to a 5 mm coverslip using optical glue was attached to the craniotomy with dental cement. After surgery, an image was taken as a reference for vascular mapping. Mice were allowed to heal for 35 days before beginning imaging.

For one mouse imaged for pipeline development, 5uL of the virus CAG-NLS-GFP (Addgene 104061, titer 2.5×10^{13} GC/ml) was injected retro-orbitally at P0. The virus contains a green

genetically encoded nuclear fluorescent protein. Imaging was done at P32.

2.4 Chronic Imaging

Mice underwent two imaging sessions with a four-week interval. Mice were anesthetized with 1–1.2% isoflurane, maintained at 37°C, and monitored via an infrared camera. Amyloid plaques were visualized using methoxy-X04 (10% DMSO, 45% propylene glycol, 45% saline), injected intraperitoneally 24 hours before imaging, and vessels were imaged with Texas-Red (50 mg/kg at week 5, 25 mg/kg at week 9), delivered via tail-vein injection 30 minutes before *in vivo* imaging. The mouse headplate was fixed in a holder. During both imaging sessions, the post-surgery vascular map was used to locate the area using a 10× air-immersion objective (TL 10X-2P, Thorlabs). The region with the most distinct vascular pattern was then imaged with a 25× water-immersion objective (XL Plan NA=1.0, Olympus) [Fig. 1(e)]. Image stacks (512×512 pixels, 500×500μm FOV, 5 μm step size along the z-axis) were taken with 5–7 frames per plane, depending on image quality, using ScanImage.[39]

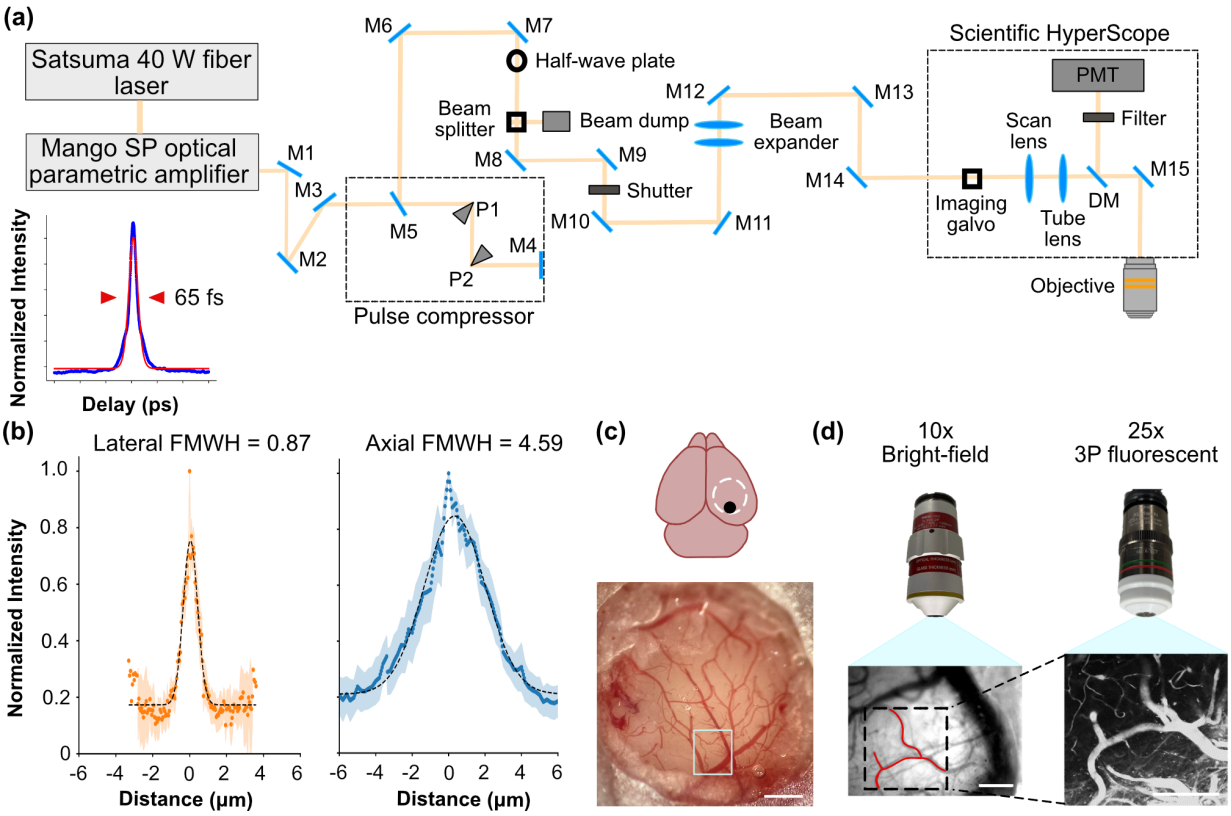

Fig 1 Imaging pipeline using a three-photon fluorescent microscope. (a) Schematic of the three-photon excitation imaging system with a pulse width of 65 fs. The system uses a Satsuma 40 W fiber laser and Mango SP optical parametric amplifier as the excitation sources. Key components include mirrors (M1-M15), a prism pulse compressor (P1-P2), a half-wave plate, a beam splitter, a beam expander, a shutter, a dichroic mirror (DM), scan and tube lenses, and an objective lens. The fluorescence signal is collected using a photomultiplier tube (PMT). (*Lower left*) Measured pulse width shown in blue with sech^2 fit (pulse width 65 fs) shown in red. (b) Intensity profile in the lateral (orange) and axial (blue) distance of 200-nm fluorescent beads (n=5). (c) Cranial window preparation and imaging locations: Diagram and bright-field image of a mouse brain with a cranial window. Imaging area (ML: 2.25 mm, AP: -3 mm) is marked. Scale bar: 1 mm. (d) Imaging procedure and objectives: Bright-field and three-photon fluorescent images are acquired using 10x and 25x objectives to create vascular maps. Scale bars: 200 μm.

2.5 *Image Processing*

Images from both channels were intensity-matched using adaptive histogram equalization, and crosstalk was removed. Preprocessing included dura removal, normalization, noise reduction, and motion correction[25] [Fig. 2(a)]. The pipeline was built using MATLAB R2023b and Python.

2.5.1 *Blood Vessels*

Vessels were segmented using the DeepVess 3D Convolutional Neural Network (CNN) model,[25] which consists of six 3D convolutional layers with ReLU activation and max-pooling, followed by a softmax layer for classification. After segmentation, vessel parameters, including diameter, length, tortuosity, inter-vessel distance, and density, were calculated using MATLAB, based on previous works[40–43] [Fig. 2(b)]. The diameter D of a vessel segment was determined as twice the median radius measured along the vessel's centerline, where the radius r_j at each point was the minimum distance to the nearest background voxel:

$$r_j = \min\left(\text{dist}\left(v_j, \text{background}\right)\right) \tag{1}$$

Here, v_j is the voxel at the jth point along the vessel. The diameter D is then given by:

$$D = 2 \times \text{median}\left(r_j\right) \tag{2}$$

The length L of a vessel segment was calculated by summing the Euclidean distances between consecutive points along the vessel centerline:

$$L = \sum_{i=1}^{N-1} \sqrt{(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2 + (z_{i+1} - z_i)^2} \tag{3}$$

where (x_i, y_i, z_i) are the coordinates of the ith point along the vessel, and N is the total number of points. Tortuosity T was defined as the ratio of the vessel segment's path length L to the straight-line distance between its endpoints:

$$T = \frac{L}{\sqrt{(x_N - x_1)^2 + (y_N - y_1)^2 + (z_N - z_1)^2}} \tag{4}$$

Here, (x_1, y_1, z_1) and (x_N, y_N, z_N) represent the coordinates of the starting and ending points of the vessel segment.

The vascular density ρ was determined as the ratio of the total volume occupied by the vessels V_v to the total volume of the region of interest V_{ROI}:

$$\rho = \frac{V_v}{V_{ROI}} \tag{5}$$

Finally, the inter-vessel distance (IVD) was calculated as the minimum Euclidean distance between the midpoints of two closest vessels:

$$\text{IVD}_{ij} = \min_v \sqrt{(x_{ij} - x_v)^2 + (y_{ij} - y_v)^2 + (z_{ij} - z_v)^2} \tag{6}$$

where (x_{ij}, y_{ij}, z_{ij}) are the coordinates of the midpoint of vessel segment ij, and (x_v, y_v, z_v) are the coordinates of the nearest segment midpoint v.

2.6 Amyloid Plaques

A Python script was developed to segment Amyloid plaques either semi-automatically using the SciPy package[44] with manual correction via Napari,[45] or fully manually [Fig. 2(c)]. Semi-automatic segmentation was used when the data showed more than 45 plaques with good imaging quality;

otherwise, manual annotation was performed. For semi-automatic segmentation, four intensity thresholds were generated using multi-level Otsu thresholding, with plaques identified as regions with the two highest intensities. A binary image was then created, and morphological operations filled small holes and removed small objects. Area-based filtering was applied after 3D-connected component labeling. Regions smaller than 49 μm^2 were excluded.[46] The 500 × 500 labeled areas in 2D planes (XY) with a 0.98 μm pixel size were summed and transformed into 3D using a 5 μm (Z) step size. For each plaque, properties were calculated, with volume determined by the sum of 2D areas and radius estimated by assuming spherical volume.

2.7 Nearest Plaque-to-Vessel Distance

The coordinates of plaque centroids were retrieved from the segmented 3D binary data set. The closest neighbouring vessel of each centroid was identified using 3D Euclidean distances analysis.[47]

2.8 Data Analysis

Python was used for statistical analysis using the SciPy[48] and Scikit-learn[49] packages. The signal-to-background ratio (SBR) of imaging stacks was determined as the average of the brightest 15 and darkest 15 pixels in each profile.[50] The signal strength was quantified by averaging the intensity of the top 0.5% of pixels in each frame.[32] Data was tested for normality before statistical testing of vascular parameters and amyloid plaques between imaging sessions within a mouse. Mann-Whitney U tests were used as statistical testing. We reported distribution means as sample mean ± standard error of the mean unless specified otherwise.

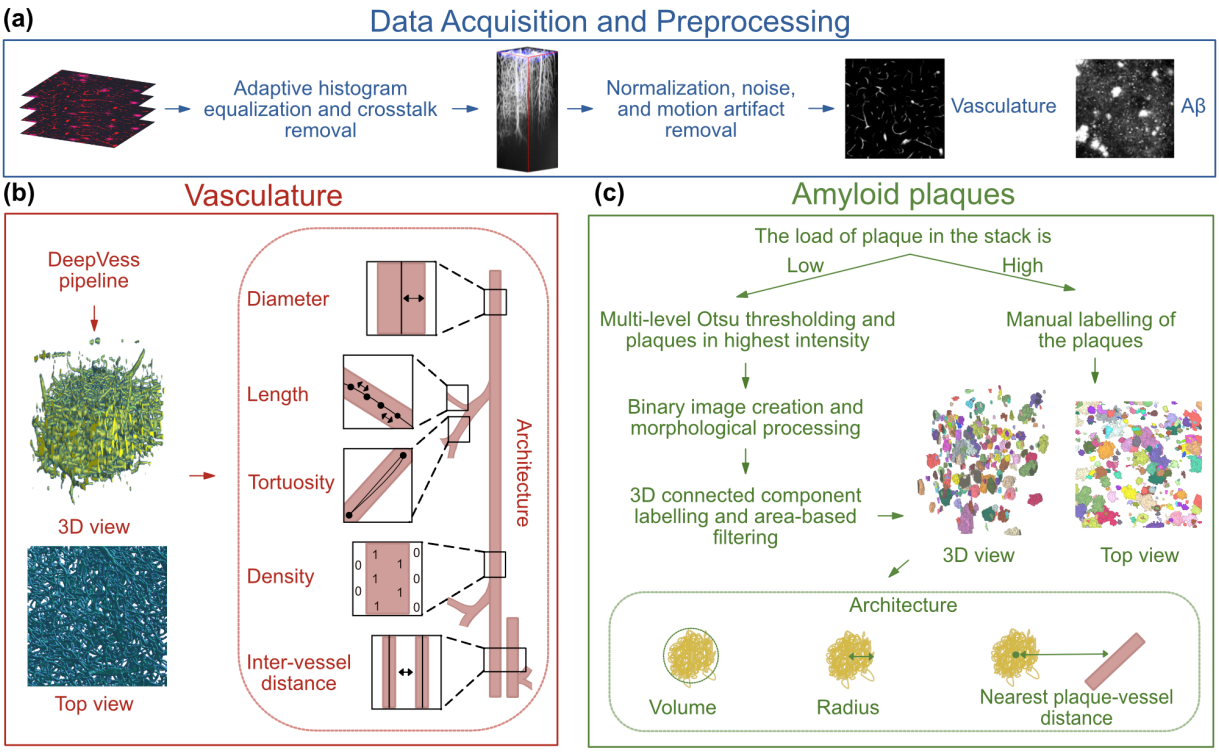

Fig 2 Segmentation and quantification pipeline for blood vessels and amyloid plaques in multiphoton images. (a) Data Acquisition and Preprocessing: adaptive histogram equalization, crosstalk removal, normalization, noise, and motion artifact removal to extract vasculature and amyloid beta images. (b) Vasculature Analysis: segmentation using the DeepVess pipeline, generating 3D and top-view visualizations. Quantification includes measures of (i) diameter, (ii) length, (iii) tortuosity, (iv) density, and (v) inter-vessel distance. (c) Amyloid Plaque Analysis: segmentation through multi-level Otsu thresholding for low plaque load or manual labeling for high plaque load. Quantification includes (i) plaque volume, (ii) plaque radius, and (iii) the nearest plaque-vessel distance.

3 Results

3.1 3PM Imaging: Dual-Channel Visualization of Vessels with Amyloid Plaques or Neurons

Using 1340-nm three-photon (3P) excitation, we acquired deep-tissue fluorescence images of the mouse cortex labeled with multiple fluorescent probes [Fig. 3]. In one configuration, vessels were visualized by Texas Red (magenta), while amyloid plaques were selectively stained with methoxy-XO4 (cyan). An additional signal from third-harmonic generation (THG, shown in yellow) shows the dura matter. In a second configuration, Texas Red (magenta) was used to label the vasculature, while neurons labeled with GFP (green) highlighted neuronal structure. The resulting 3D renderings captured both vascular architecture and either plaque or neuronal features in a single scan, underscoring the utility of 3PM for simultaneous, deep-tissue imaging of multiple tissue components.

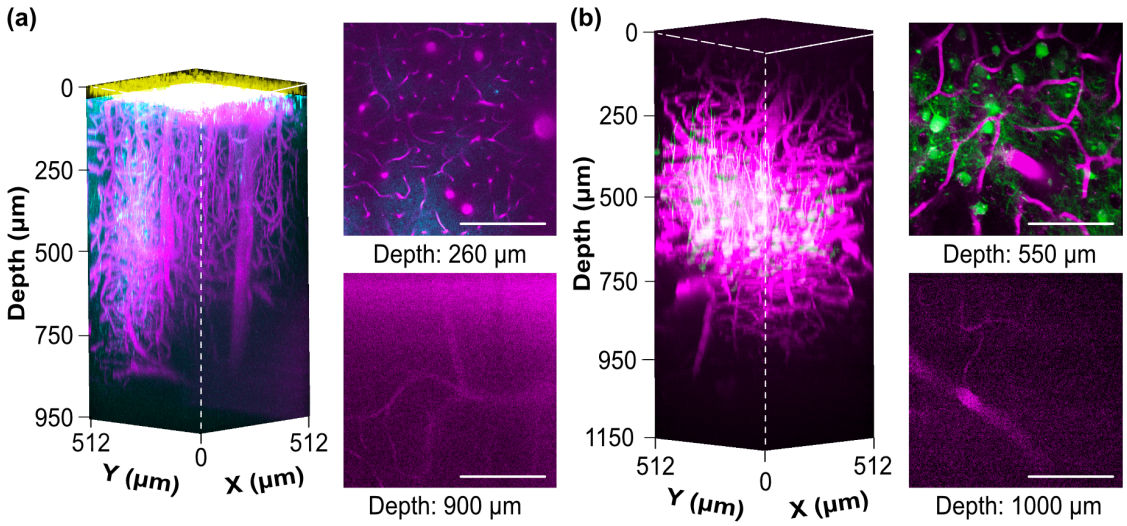

Fig 3 Multicolour 3P fluorescence images using a 1340-nm excitation and the maximum laser power was 182 mW under the objective lens. 3D reconstruction of (a) vessels dyed with Texas-Red (magenta), plaques with methoxy-XO4 (cyan) and THG (yellow) image stack taken with 5 μm z-steps and (b) vessels dyed with Texas-Red (magenta) and neurons labeled with GFP (green) taken with 10 μm z-steps. Stack are normalized by adjusting the intensity of each slices based on the intensity range of the last non-overexposed slice (95% of the brightness) to ensure consistent brightness across the stack. Scale bars are 200 μm.

3.2 Consistent Field of View Tracking Across Two Imaging Sessions

We conducted longitudinal imaging of the same FOV in APP$^{\text{NL-G-F}}$ knock-in mice at weeks 5 and 9 after surgery to assess the progression of vascular and amyloid pathologies. Using vascular landmarks identified in postoperative bright-field images [Fig. 4(a)], we localized the FOV with a 10× air-immersion objective. High-resolution imaging was performed with a 25× water-immersion objective, enabling 3PM of cortical depths ranging from 45 to 795 μm.

To evaluate the reproducibility and depth-resolved imaging capabilities of our 3PM setup, we analyzed intensity profiles of the brightest vessels at depths of 405 and 795 μm across both imaging sessions [Fig. 4(b)]. The brightest vessels were identified by averaging the intensity of the top 0.5% of pixels in each image slice. Normalized line profiles demonstrated consistent peak intensities at the same spatial locations between sessions, confirming reliable re-identification of vascular

structures over time.

We quantified signal intensity and SBR for both vascular and amyloid signals as a function of imaging depth [Figs. 4(c) and 4(d)]. Vessel signals exhibited consistently higher SBR values compared to amyloid plaques at all depths, indicating superior contrast and detectability for vascular structures during deep imaging. Both signals displayed an initial increase near the cortical surface, attributed to higher tissue density in the dura mater, followed by a decline with depth due to light scattering and absorption. The dura mater location was determined using the THG signal at the top of the stack. Variations in signal strength and SBR between sessions were primarily attributable to differences in laser power settings and dye concentrations (Texas Red for vessels and methoxy-XO4 for amyloid plaques). To minimize these effects, raw data were processed using adaptive histogram normalization, and the dura mater was excluded during segmentation to avoid artifacts.

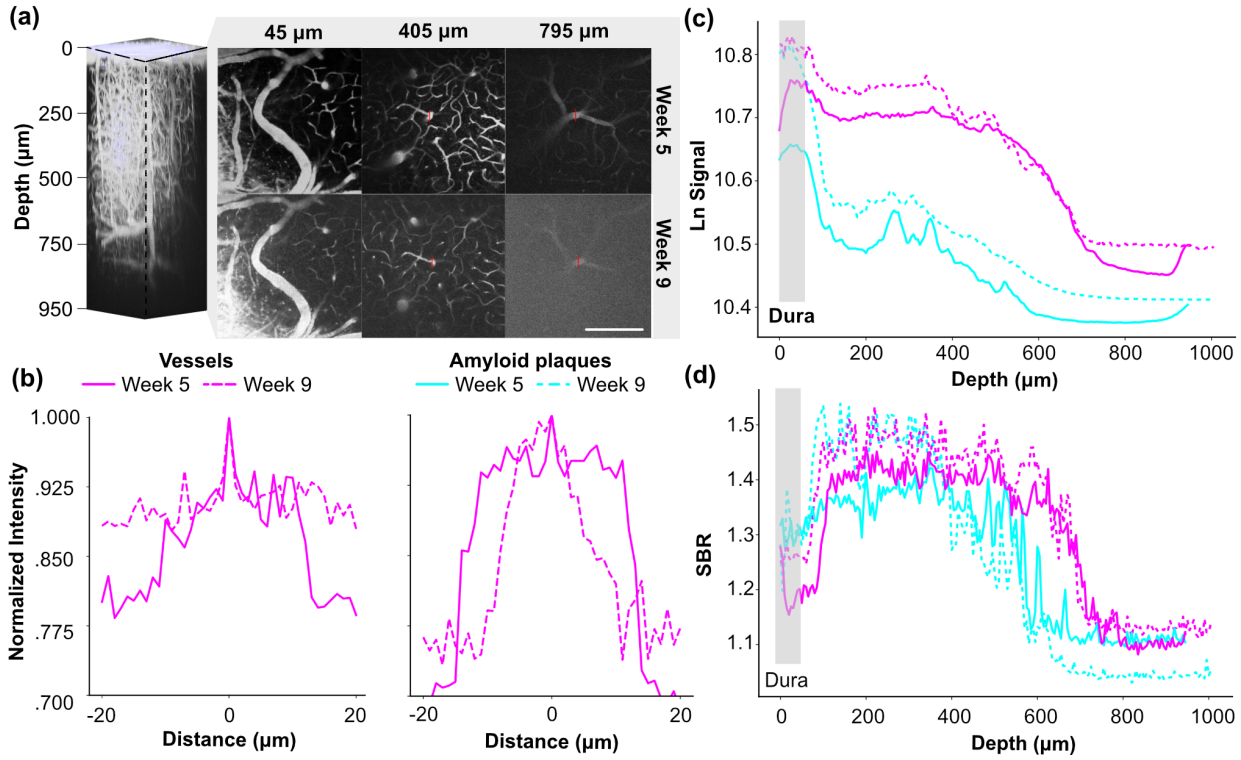

Fig 4 Signal-to-background ratio (SBR) and signal strength analysis over depth. (a) 3D reconstruction of vessels and plaque image stack. The amyloid plaques are labelled with methoxy-XO4 (blue), and the vessels are labelled with Texas Red (grey). The excitation wavelength is 1340 nm, the maximum laser power was 182 mW under the objective lens, and images were taken with 5 μm z-steps. Shown are images taken with 25x objective lens at different depths (45, 405, 795 μm). The brightest vessel at 405 and 795 μm are marked in red. (b) Line profiles of the vessels highlighted with the brightest markers in red at depths 405 and 795 μm. Scale bars is equal to 200 μm. (c) The signal strength of the vessels was quantified by averaging the intensity of the top 0.5% of pixels in each frame.[32] (d) The SBR was determined as the average of the brightest 15 and darkest 15 pixels in each profile. The SBR decreased with increasing depth, and there was an overall higher SBR in the first imaging session.[50]

3.3 *Vessel density decreases and amyloid plaques increases with age*

After segmentation, the vascular and amyloid architectures were quantified across two imaging sessions in three mice aged 6.5 to 9.9 months. A overall reduction in vessel density (R^2 = 0.55) was observed, indicating potential vessel pruning or capillary loss over time [Fig. 5(a)]. In contrast, total plaque volume showed a general increase (R^2 = 0.56) in all time points [Fig. 5(b)]. These results suggest an age-associated decline in vessel density and an accumulation of amyloid plaques in the mice observed.

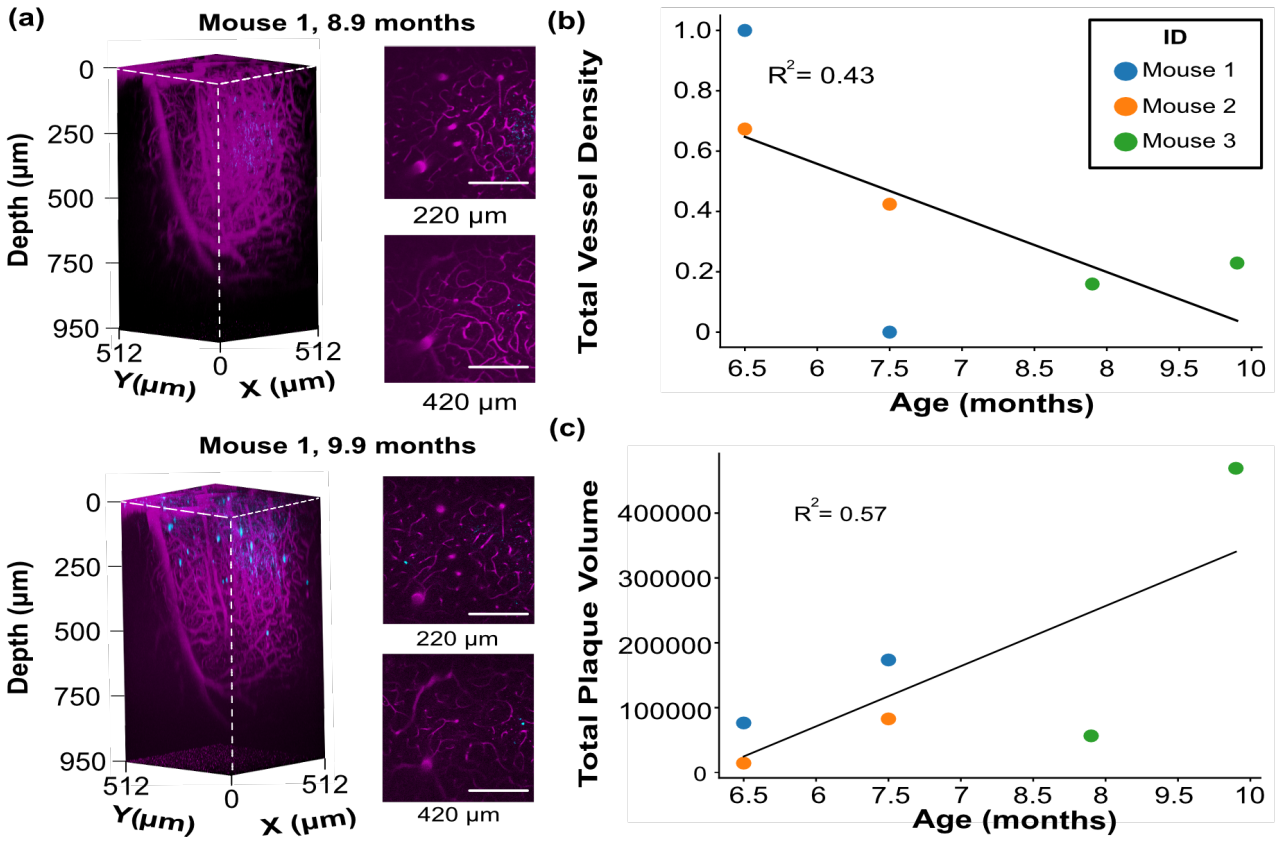

Fig 5 Vessel density decreases and amyloid plaques increases with age. (a) Representative three-photon fluorescence volume rendering of the vasculature (magenta) in Mouse 1 at 8.9 and 9.9 months and slices at 220 and 420 μm. (b) Total vessel density (magenta) versus age for sessions at 6.5, 7.5, 8.9, and 9.9 months, with a linear best-fit line indicating a decrease over time (R^2 = 0.55). (c) The total vessel density measured at each time point is plotted (cyan) with a linear best-fit line highlighting the upward trend (R^2 = 0.56). Scale bars are 200 μm.

3.4 Vessel diameter decreases while inter-vessel distance and vessel length increase

As shown in Figure 6, all three mice exhibited significant vascular remodeling between the first and second imaging sessions. Specifically, vessel diameter decreased for each mouse (Mann–Whitney U tests: all $p < 0.001$). In contrast, vessel length increased in the second session for Mouse 1 ($p < 0.001$) and Mouse 3 ($p = 0.035$), but did not change significantly for Mouse 2 ($p = 0.060$). Vessel tortuosity did not differ across the two sessions for any mouse (all $p > 0.050$).

By comparison, inter-vessel distance increased at the second time point for all three mice (Mouse 1 and 2, $p < 0.001$; Mouse 3, $p = 0.002$). Plaque radius remained unchanged for each mouse. Collectively, these findings indicate a decrease in vessel diameter, as well as increases in vessel length (for two of the mice) and inter-vessel distance, supporting the presence of ongoing vascular remodeling.

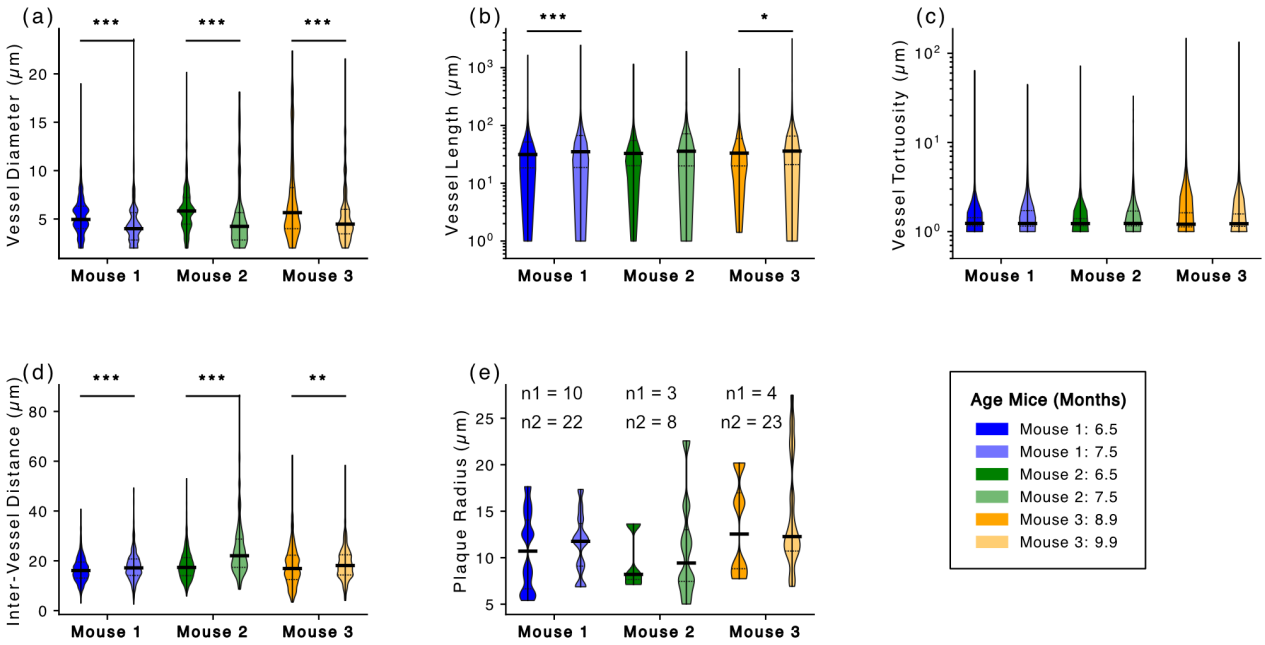

Fig 6 Vessel diameter decreases while inter-vessel distance and vessel length increase. Subplots show (a) vessel diameter, (b) vessel length, (c) vessel tortuosity, (d) inter-vessel distance, and (e) plaque radius. Data was tested for normalization and statistical analysis was done using the Mann-Whitney U test. p values are indicated by stars, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4 Discussion

In this study, we established a longitudinal 3PM imaging approach to investigate vascular and amyloid pathologies in deep cortical structures of $APP^{NL\text{-}G\text{-}F}$ knock-in mice, a model of AD. This methodology overcomes depth limitations of 2PM and enables in vivo imaging of structural changes over time at high resolution. Despite a small sample size ($n = 3$ animals) and relatively short monitoring periods, our results underscore the utility of this approach for studying the interplay between vascular degeneration and amyloid pathology in AD.

4.1 Longitudinal Three-Photon Imaging of Cortical Structures

Our imaging methodology enabled longitudinal visualization of amyloid plaques at cortical depths of up to 420 μm, together with vascular structures observable to depths of up to 920 μm [Fig. 3(a)], which exceeds the previously reported range of 150 to 200 μm achieved in *in vivo* studies of amyloid plaques using two-photon microscopy (2PM).[30,37] While longitudinal studies using MRI and PET have achieved greater imaging depths,[51,52] they do so at a significantly lower resolution. 3PM, with its cellular resolution, bridges the gap between superficial multiphoton imaging and macroscopic modalities like MRI and PET, and has the capability to analyze neuronal circuit activity in addition to amyloid plaque load and vasculature. In this paper, we achieved a maximum imaging depth of 1.09 mm [Fig. 3(b)] in a 5-week old mouse, which is consistent with the previously reported depth of 1.1 mm by Ouzounov et al.,[31] using the same excitation wavelength of 1340 nm. Shallower imaging depths were achieved with chronic imaging in much older animals (6.5-9.9 months old; Fig. 5(b)) because of increased scattering in older brains. Improvements can be expected with the use of longer wavelengths or optimized immersion media such as deuterium oxide (D_2O).[36] Field-of-view consistency across imaging sessions was ensured using vascular landmarks and precise mounting techniques [Fig. 4(a)], with normalized line profiles demonstrating reproducibility [Fig. 4(b)]. This highlights the feasibility of 3PM for longitudinal studies, offering high resolution with minimal photodamage due to longer excitation wavelengths and low average laser power.[33]

4.2 Vascular Degeneration and Amyloid Accumulation

Although the small sample size limits statistical power, there was a decrease in vessel density in mice 1 and 2, and an overall increase in plaque load [Fig.5] with significant vascular changes

[Fig.6]. There was a significant reduction in vessel diameter and increases in inter-vessel distance across all three mice. The vascular alterations align with observations in other AD models.[53–64]

Such changes have implications for cerebral blood flow and neuronal function, potentially exacerbating AD progression. These comparisons suggest that vascular remodeling is a shared feature across models, though its severity varies with genetic background and disease progression. No significant trends were observed in amyloid plaque radius or plaque-to-vessel distances. This could be due to the slower plaque progression reported in heterozygous $APP^{NL\text{-}G\text{-}F}$ mice compared to the homozygous model and other more aggressive AD models.[22] Since FAD typically manifests in humans in the heterozygous state, the heterozygous $APP^{NL\text{-}G\text{-}F}$ mutation more accurately models human pathology.[23] The lack of significant plaque-related findings underscores the need for longer observation periods and larger cohorts to detect subtle changes or spatial interactions in this mouse model.

5 Conclusion

We developed a longitudinal 3PM imaging methodology for studying vascular and amyloid pathologies in deep cortical structures of $APP^{NL\text{-}G\text{-}F}$ mice. This approach provides detailed insights into AD progression and surpasses traditional depth limitations of 2PM. Despite sample size limitations, our findings highlight significant vascular remodeling in AD, including reductions in vessel density and diameter, as well as increases in inter-vessel distance, demonstrating the utility of 3PM for longitudinal studies. Future research should leverage this methodology with larger cohorts, extended monitoring periods, and advanced analytical tools, such as machine learning algorithms, to further elucidate the interplay between vascular and amyloid pathologies in AD and evaluate potential therapeutic strategies.

Disclosures

The authors declare no conflicts of interest.

Code, Data, and Materials Availability

Code and data used in this paper can be obtained from the corresponding author upon reasonable request.

Ethics Statement

The animal study was reviewed and approved by Imperial College Animal Welfare Ethical Review Board and authorized under Home Office Project License with PPL number PP6988384.

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Author Contributions

ES, MY, MA and SS designed the experiments. ES and MY worked out the technical details of the experiments. MY performed the surgeries. ES and MG performed the experiments. ES analysed the data with support from MY, MG, and SS. ES wrote the manuscript. ES, MY, MA and SS supervised the project. All authors discussed the results and commented on the manuscript.

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List of Figures

1 Imaging pipeline using a three-photon fluorescent microscope. (a) Schematic of the three-photon excitation imaging system with a pulse width of 65 fs. The system uses a Satsuma 40 W fiber laser and Mango SP optical parametric amplifier as the excitation sources. Key components include mirrors (M1-M15), a prism pulse compressor (P1-P2), a half-wave plate, a beam splitter, a beam expander, a shutter, a dichroic mirror (DM), scan and tube lenses, and an objective lens. The fluorescence signal is collected using a photomultiplier tube (PMT). (*Lower left*) Measured pulse width shown in blue with sech^2 fit (pulse width 65 fs) shown in red. (b) Intensity profile in the lateral (orange) and axial (blue) distance of 200-nm fluorescent beads (n=5). (c) Cranial window preparation and imaging locations: Diagram and bright-field image of a mouse brain with a cranial window. Imaging area (ML: 2.25 mm, AP: -3 mm) is marked. Scale bar: 1 mm. (d) Imaging procedure and objectives: Bright-field and three-photon fluorescent images are acquired using 10x and 25x objectives to create vascular maps. Scale bars: 200 µm.

2 Segmentation and quantification pipeline for blood vessels and amyloid plaques in multiphoton images. (a) Data Acquisition and Preprocessing: adaptive histogram equalization, crosstalk removal, normalization, noise, and motion artifact removal to extract vasculature and amyloid beta images. (b) Vasculature Analysis: segmentation using the DeepVess pipeline, generating 3D and top-view visualizations. Quantification includes measures of (i) diameter, (ii) length, (iii) tortuosity, (iv) density, and (v) inter-vessel distance. (c) Amyloid Plaque Analysis: segmentation through multi-level Otsu thresholding for low plaque load or manual labeling for high plaque load. Quantification includes (i) plaque volume, (ii) plaque radius, and (iii) the nearest plaque-vessel distance.

3 Multicolour 3P fluorescence images using a 1340-nm excitation and the maximum laser power was 182 mW under the objective lens. 3D reconstruction of (a) vessels dyed with Texas-Red (magenta), plaques with methoxy-XO4 (cyan) and THG (yellow) image stack taken with 5 μm z-steps and (b) vessels dyed with Texas-Red (magenta) and neurons labeled with GFP (green) taken with 10 μm z-steps. Stack are normalized by adjusting the intensity of each slices based on the intensity range of the last non-overexposed slice (95% of the brightness) to ensure consistent brightness across the stack. Scale bars are 200 μm.

4 Signal-to-background ratio (SBR) and signal strength analysis over depth. (a) 3D reconstruction of vessels and plaque image stack. The amyloid plaques are labelled with methoxy-XO4 (blue), and the vessels are labelled with Texas Red (grey). The excitation wavelength is 1340 nm, the maximum laser power was 182 mW under the objective lens, and images were taken with 5 μm z-steps. Shown are images taken with 25x objective lens at different depths (45, 405, 795 μm). The brightest vessel at 405 and 795 μm are marked in red. (b) Line profiles of the vessels highlighted with the brightest markers in red at depths 405 and 795 μm. Scale bars is equal to 200 μm. (c) The signal strength of the vessels was quantified by averaging the intensity of the top 0.5% of pixels in each frame.[32] (d) The SBR was determined as the average of the brightest 15 and darkest 15 pixels in each profile. The SBR decreased with increasing depth, and there was an overall higher SBR in the first imaging session.[50]

5 Vessel density decreases and amyloid plaques increases with age. (a) Representative three-photon fluorescence volume rendering of the vasculature (magenta) in Mouse 1 at 8.9 and 9.9 months and slices at 220 and 420 μm. (b) Total vessel density (magenta) versus age for sessions at 6.5, 7.5, 8.9, and 9.9 months, with a linear best-fit line indicating a decrease over time (R^2 = 0.55). (c) The total vessel density measured at each time point is plotted (cyan) with a linear best-fit line highlighting the upward trend (R^2 = 0.56). Scale bars are 200 μm.

6 Vessel diameter decreases while inter-vessel distance and vessel length increase. Subplots show (a) vessel diameter, (b) vessel length, (c) vessel tortuosity, (d) inter-vessel distance, and (e) plaque radius. Data was tested for normalization and statistical analysis was done using the Mann-Whitney U test. p values are indicated by stars, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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