Chapter Title: Quantifying vascular remodelling in the mouse spinal cord

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ii. Summary/Abstract

The spinal cord, a compartment of the central nervous system, is made up of a number of architecturally distinct neural centres that influence an array of neurophysiological systems. The primary role of the spinal cord is the modulation of sensory and motor function via acting as a relay station between the periphery and the brain. Inherently these are considered as neural networks, however the functional dynamics of these tissues consist of a heterogenic population of cell types, all working in harmony to maintain physiological function. Part of this cellular diversity comprises of the vascular network that delivers essential nutrients and oxygen to the spinal cord tissue, whilst also protecting it from potentially tissue damaging substances such as foreign entities including toxic pharmacological agents or pathogens. Therefore the viability of the spinal cord is dependent upon the harmonious balance between opposing angiogenic processes; vascular remodelling and vascular regression, tipping the balance to either side contributes to neurodegeneration. Exploring vascular remodelling in the central nervous system requires consideration of the anatomical landscape of the spinal cord and the dynamic nature of the microvasculature. Utilising immunofluorescent staining and 3D image rendering analysis of the endothelium and mural cell population allows for investigation of cellular as well as molecular mediation of vascular remodelling in the spinal cord. This method can be utilised in a range of rodent models (utilising pharmacological, disease models, transgenic and/or viral approaches) offering extensive appreciation of the blood-spinal cord barrier.

iii. Key Words

pain, angiogenesis, endothelial, spinal cord, astrocyte, pericyte

1. Introduction

The dialogue between the vascular and neuronal networks is of fundamental importance to the maintenance of neurophysiological outcomes. The microvessels deliver essential provision (such as nutrients) to neural centres to support healthy neurological function such as those associated with cognition, movement and nociception [1–3], whilst providing a protective barrier from potentially harmful agents[4]. Disturbances in the viability of this system are implicated in disease, whereby an impairment of the microvessel network demonstrated by alterations in vessel architecture, vessel perfusion or vessel leakage within neural tissues, are associated with neurodegenerative disease such as Alzheimers and Stroke [1, 3, 5]. The vascular network consists of a heterogenous population of cell types including endothelial cells, pericytes, smooth muscle cells and astrocytes, which are essential to supporting a healthy organism and contribute to such disturbances in microvessel function. These cellular interactions have been investigated in human tissues, as well in rodent models (disease models and transgenic models) allowing for interrogation of the differing cellular compartments that make up the neurovascular unit. For example, alterations in endothelial cell tight junction maintenance and/or loss of pericytes have been proven to be associated with vascular complications such as in the retina[6] and brain [7].

The role of angiogenesis in tissue development and pathology has been extensively discussed. However, the role of microvessel development and function are increasingly important in relation to those physiological processes associated with the spinal cord and consequent impact of disease [8]. The spinal cord is essential to the processing of incoming sensory information and coordination of motor function in relation to the organisms surrounding environment. Typically disturbances in microvessel function defined as prominent pro-angiogenic actions are associated with exacerbated vascular leakage in the spinal cord [9–12], with a breakdown in the endothelial junctional barrier a causative factor in the development of neurodegenerative disease (e.g. multiple sclerosis [13]) and motor impairment (e.g. spinal cord traumatic injury[14]). This is in addition to disturbances in nociceptive processing and resulting chronic pain manifestation (such as in arthritis, traumatic injury [9, 15]). However, pro-angiogenic activity depicted as heightened function and/or expansion of the vascular network in neural tissues such as highlighted in the spinal cord [9–12], necessitates opposing actions to curtail aberrant vascular remodelling processes such as vessel regression and occlusion [16]. For example, impaired blood flow through vessel constriction and/or occlusion is now regarded as a prominent feature of neurodegenerative processes in relation to cognitive function [1, 5, 17] as well as sensory [18] and motor [14] disturbances in age and disease related neuropathologies [19]. In such instances the

Commented [HR1]: Current approaches to investigate the integrity and functionality of the blood spinal cord barrier have focussed upon primarily histological evaluation of spinal cord tissue from rodents, humans and transgenic/viral models[15, 16].

endothelium degenerates leading to vessel pruning, mediated in part via vascular endothelial growth factor receptor 2 signalling [19].

To understand the dynamics of this vascular remodelling of the blood spinal cord barrier, the resulting impact upon neurophysiological processes and development of pathological states, an appreciation of vessel density or leakage is needed. Typically image acquisition of microvessels in the spinal cord provide a 2D representation of the capillary network in the dorsal-ventral plane of the spinal cord providing an understanding of the angiogenic processes in play. Microvessel adaptation to changes in their environments, as in this instance the spinal cord, do not occur in a single plane as represented by 2D image analysis but in the 3D dimension, with vessels expanding and retracting in a multitude of directions. In the central nervous system the microvessel network also extends medial-laterally as well as in the anterior to posterior axis. Furthermore, the extension and regression of endothelium sprouting is tortuous in nature, potentially moving in out of the chosen visual plane. Utilising fluorescent-based immunohistochemistry in combination with image rendering analysis allows for a greater unbiased appreciation of the microvessel architecture and function within nervous tissues. Image segmentation of the blood vessel networks enables extraction of experimental data including volume of the vascular network in the region of interest, blood length, branch number and vessel diameter.

2. Materials

2.1 Anaesthesia

- Sodium pentobarbital (Euthatal; 200mg/ml)
- 25 Gauge needle and 1ml syringe (all syringes and needles manufactured by Terumo)

2.2 Transcardial Perfusion and Tissue Preparation

- Electric clippers
- All surgical tools from World precision instruments. Surgical tools include Dumont Tweezers, Swiss Curved Tweezers, Haemostats, Vannas scissors, Iris straight scissors, Scalpel handle and blade (#10)
- 21G needle
- 50ml Syringes
- 4% Paraformaldehyde
- Phosphate buffered saline
- (Optional) Dissection microscope and light source
- Sucrose (in PBS)

2.3 Slide Preparation

- Superfrost slides (VWR international or Equivalent)
- Cryostat (Leica CM1860 UV Cryostat or Equivalent)
- Optimal Cutting Tek (O.C.T or equivalent)
- Triton x-100
- Hydrophobic Pen (Imm pen, Vectorlabs)
- Humidified Chamber (Sigma-Aldrich)

2.3 Immunofluorescence and Slide Imaging

- Primary antibody – list of suitable antibodies, concentrations/dilutions and suppliers.
    1. Endothelial Cell marker; biotin conjugated isolectin B_4 (IB_4; 1:100, Cat no. L2140, Sigma-Aldrich);
    2. Endothelial Cell marker; goat anti-CD31 (1 in 200, Cat no. AF3628, R&D systems).
    3. Pericyte Marker; rabbit anti NG2 proteoglycan, Cat no. AB5320, Merck)
    4. Smooth Muscle marker; mouse anti-α smooth muscle Cy3 (1 in 200, Cat no. C6198, Sigma-Aldrich)
    5. Basement Membrane marker; Rabbit anti collagen IV (1:200; Cat no. ab6586, Abcam)
    6. Astrocytic marker; rabbit anti-GFAP (1:500, Abcam),

7. Additional neuronal markers can be used in combination rabbit anti-fos (1:100, Santa Cruz); mouse anti-NeuN (1:200; Millipore, Billerica, MA, USA).

- Secondary fluorescent antibodies - utilise Alexafluor fluorescent dyes (conjugated where appropriate with appropriate species IgG – typically mouse and rabbit or streptavidin) sourced from Thermofisher. Secondary antibodies typically raised in donkey (or chicken) utilising Alexfluor 488, 555 and 647. Consideration made with reference to the imaging capabilities of the microscope in relation to fluorescent dye emission wavelength. Used at 1 in 1000 dilution in PBS/0.2% Triton x-100.
- Nuclear stains (such as DAPI, Hoescht)
- Confocal microscope (Leica SP8 or equivalent)
- Glass coverslips (VWR International)
- Antifade Mounting medium (Vectorshield, Vectorlabs; Fluorosave, Sigma-Aldrich) to preserve fluorescence.

2.4 Software Analysis

- Imaris
- Statistical Package (such as GraphPad Prism v8)

3. Methods

- **Surgical Preparation**

All surgical equipment (e.g. surgical tools) and consumables (e.g. PBS) are to be sterilised and suitable provision for appropriate aseptic surgical practice is needed (e.g. drapes, surgical gloves).

- **Animal Anaesthesia and Transcardial Perfusion**

1. Terminally anaesthetise mice (adult 25-30g male C57bl6) via intraperitoneal injection of Euthatal (10mg/kg). Anaesthetic maintenance should be regularly checked and anaesthetic depth monitored e.g. foot and corneal reflex.
2. Additional anaesthetic maybe provided via intraperitoneal injection of Euthatal where appropriate if a sufficient depth of anaesthesia hasn't been reached
3. Once the mouse has reached sufficient depth of anaesthesia (no corneal reflex, no righting reflex, no pedal response to pinch) it should be placed on its back in a stainless steel tray.
4. Using dissection scissors and forceps, an incision is made across the base of the rib cage. Starting midline at the base of the sternum through the abdominal wall terminating at each lateral aspect of the mouse. This incision should be approximately 4-6cm in length.
5. Using forceps secure the sternum and the dissection scissors should be used to cut along the rib cage penetrating the diaphragm exposing the heart and lungs.
6. Dissection scissors should be used to cut through the rib cage from the base up towards the mouse shoulder. This should be repeated on both sides. Using haemostats secure the base of the sternum and move the rib cage to position towards the head exposing the heart and lungs.
7. A small incision is made in the right atrium. Fill a 50ml syringe with ringers or PBS and secure a 21g needle. Prepare an additional 50ml syringe and needle. Blunt forceps should be used to secure the left ventricle and the 50ml syringe is inserted into the left ventricle. The forceps (or alternatively haemostats) should be used to secure the needle in the ventricle. Ensure the needle tip does not penetrate other chambers of the heart as it will compromise blood circulatory system preventing efficient fixation.
8. Introduce upto 50ml of ringers or PBS (or until the blood has been removed) and at this stage remove the syringe containing ringers and insert the syringe containing 4% paraformaldehyde and repeat the process.

- **Tissue Extraction and Preparation**

1. A laminectomy is performed to expose the spinal cord. Make an insertion along the vertebral column on the dorsal surface using a scalpel blade. This would be approximately ~5cm in length from the base of the tail to the neck.
2. Separate skin from underlying tissue using blunt dissection scissors exposing the muscle and connective tissue.
3. Use haemostats to secure the skin to allow access to the dorsal surface of the vertebral column.
4. Use sharp dissection scissors and the scalpel blade to remove the muscle and connective tissue and expose the vertebral processes. Furthermore, remove any attached muscle and connective tissue from lateral aspects of the vertebrae T12-L1.
5. Perform a laminectomy using vannas scissors between T12-L1 to allow exposure of lumbar segment L3-L5 of the spinal cord. Insert a single arm of the vannas scissors between vertebra T12-T13 into the epidural space. With careful movements cut the bone away on both distal sides. Be careful to minimise injury to the spinal cord ie pressure applied or scissors penetrating the spinal cord. Peel the bone off gently. Once the vertebra are removed the L3-L4 segment can be visualised.
6. Using a scalpel blade and forceps the lumbar spinal cord can be isolated by cutting either side of the lumbar enlargement. Carefully remove the lumbar region of the spinal cord and submerge in 4% paraformaldehyde for 24 hours at 4^{o}C.
7. Replace the paraformaldehyde with 30% sucrose solution for cryoprotection and leave for 24 hours at 4^{o}C.
8. Remove the spinal cords using forceps considering how to handle the tissue to minimise damage to the tissue and dry the tissue by placing on tissue paper.
9. Submerge spinal cord tissue in optimal cutting temperature compound and freeze. Tissue is ready for cryosectioning or store at -80oC.

- **Immunofluorescence**

1. Cryosection the spinal cords at a minimum of 40µm thickness and mount onto superfrost slides. These can be used immediately or stored in a slide box at -80^{o}C until needed.
2. Using a hydrophobic pen draw a hydrophobic barrier around the tissue sections and place in a humidified chamber.
3. Wash the slides for 5 minutes three times with PBS at room temperature. Subsequently, wash the slides with PBS/0.2% Triton x-100 at room temperature.
4. Incubate for 60min in blocking solution (5% bovine serum albumin, 10% fetal calf serum PBS/0.2% Triton) at room temperature.

5. Incubate slides in primary antibody (or combination) in blocking solution (5% bovine serum albumin, 10% fetal calf serum) overnight at 4°C for 72 hours. Endothelial Cell marker goat anti-CD31 to be used at 1 in 200 dilution.

6. Wash the slides for 5 minutes three times with PBS at room temperature.

7. Secondary antibodies (1 in 1000 dilution) to be made up in PBS + 0.2% Triton X-100. Add secondary antibody (or combination) to the slides and incubate for 2 hours at room temperature.

8. Wash the slides for 5 minutes three times with PBS at room temperature. Add a stain in the final wash step (ie DAPI 1µg/ml) to identify cellular nuclei.

9. Remove excess fluid from the slides and mount glass coverslips with antifade medium. Store at 4^{o}C until needed

- **Image Analysis**

1. Slides are imaged on a confocal microscope (Leica SP8 or equivalent (Fig.1A-E) to generate Z stacks of region of interest. Vessel structures and cellular makeup should be visible through fluorescent staining with a random selection acquired focussing upon neural centre of interest i.e. dorsal horn for nociception. Consideration regarding controls needs to be made with reference to omission of primary antibodies, blocking peptides or accessing knockout animal tissue of your protein target.

2. Z stacks are processed using 3D image rendering software such as Bitplane Imaris. The software allows you to visualise and perform analyses. Images are opened in surpass view. The filament tracer tool is used to trace microvessels (Fig.2A-B). Paths are traced manually using autopath with diameter correction. This allows segmentation of the individual microvessels and extraction of a multitude of datapoints including length and diameter of vessel, number of branch points and volume of microvessels as well as interaction with mural cells (Fig. 3A-B).

3. Notes (can be short just few bullet points 100 words)

- Depending on species of animal consideration of differing blocking agents is required such as species specific serum.
- Concentration of primary antibody should be determined from manufacturers guidance and/or reputable literature in line with inhouse validation of optimal antibody concentration. In certain instances amplification steps maybe required through employing biotin-avidin binding to enhance image acquisition.
- Measurement of vascular leakage can also be determined. Quantification of vascular permeability following infusion of a fluorescent tracer (e.g. tritc dextran, evan blue[9]) or fluorescently labelled cell types (e.g. monocyte[20]) administered either via intravenous or systemic approaches can be evaluated [9, 21]. Introduction of fluorescent dye during the perfusion step allows vessel leakage to acquired inline with 3D architectural datapoints.
- If rats are utilised, a larger cardiac perfusate volume is required. Peristaltic pump can be used to perfuse the animal.
- Spinal cord tissue should not be submerged in 4% paraformaldehyde for greater than 24hrs as it can hinder antibody binding.

Commented [HR2]: As we all know, even the simplest techniques go wrong from time to time. Would you therefore indicate any major problems or faults that can occur with your technique? Try to indicate the major sources of problems and how they can be identified and overcome. With reference to related techniques, any variations of the technique that you have described should also be made in this section, as well as--where relevant--an indication of the sensitivity of the method, timescale for the singled technique, etc. This "Notes" section is a hallmark of this series and has been singled out for praise by a number of reviewers. Please try and make this section as extensive as possible by putting on paper all of your various experiences with the technique. Each 'Note' should be cross-referenced with the 'Materials' and 'Methods' sections, e.g. (*see* **Note 1**).

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RPH, MD and LW performed the experimental work. RPH, MD and LW contributed to the conception or design of the work in addition to acquisition, analysis or interpretation of data for the work. RPH, MD and LW drafted the work or revised it critically for important intellectual content. RPH drafted the manuscript with contributions from all authors. All authors approved the final version of the manuscript, agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure Legends

Figure 1 - Spinal cord microvessel identification

Blood vessel endothelium is identified following fluorescent labelling of the spinal cord. Lumbar spinal cord sections from C57.bl6 adult mice are cryosectioned (40µm thickness) following cardiac perfusion with paraformaldehyde. Cryosections are incubated with endothelium marker [A-E] CD31 as well as with [B & D] DAPI (nuclear label) allow identification of the spinal cord vasculature [A; scale bar = 300µm & C; scale bar = 200µm]. [E] High magnification images of spinal cord microvessels identified with CD31 and IB_4 (scale bar=30µm).

Figure 2 – Image rendering.

Cryosectioned lumbar spinal cord sections (50µm thickness) prepared from adult C57.bl6 mice were incubated with endothelial marker [A] CD31 allowing visualisation of the microvasculature (scale bar = 50µm). Using Imaris a surface rendering of the microvessel can be produced to produce a 3D digital representation of the section (scale bar = 50µm).

Figure 3 – Mural cell-endothelial cell interaction

Lumbar spinal cord cryosections prepared from adult C57.bl6 mice incubated with [A] astrocyte marker (GFAP) and [B] endothelial marker CD31 utilised to identify the proximity of astrocytes and the endothelium ([C] merge) scale bar = 15µm). [D] Imaris is used to 3D render the endothelial-astrocyte interaction (scale bar = 8µm).

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Figure 1

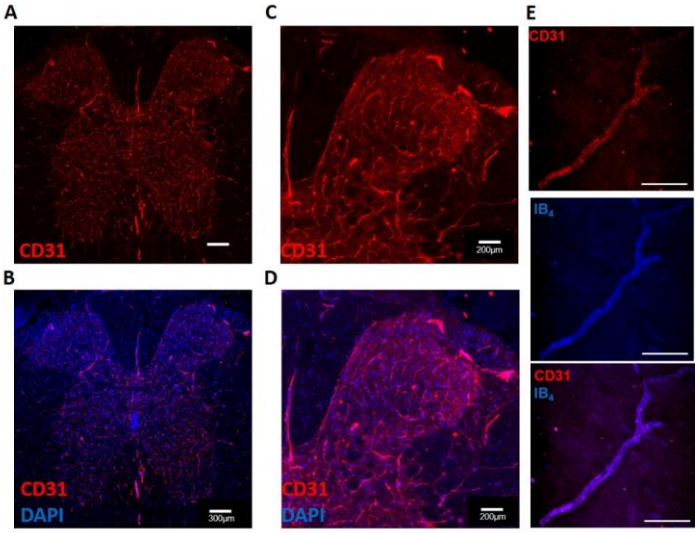
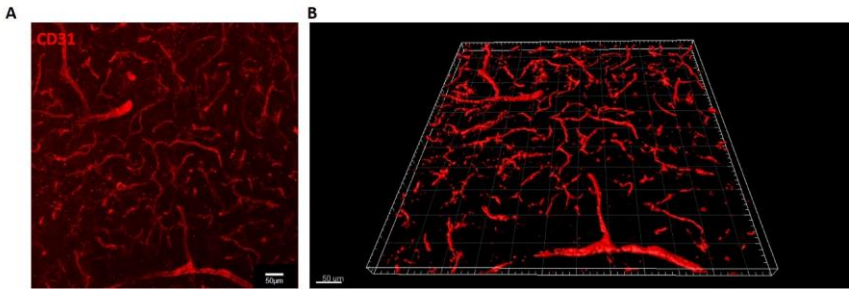
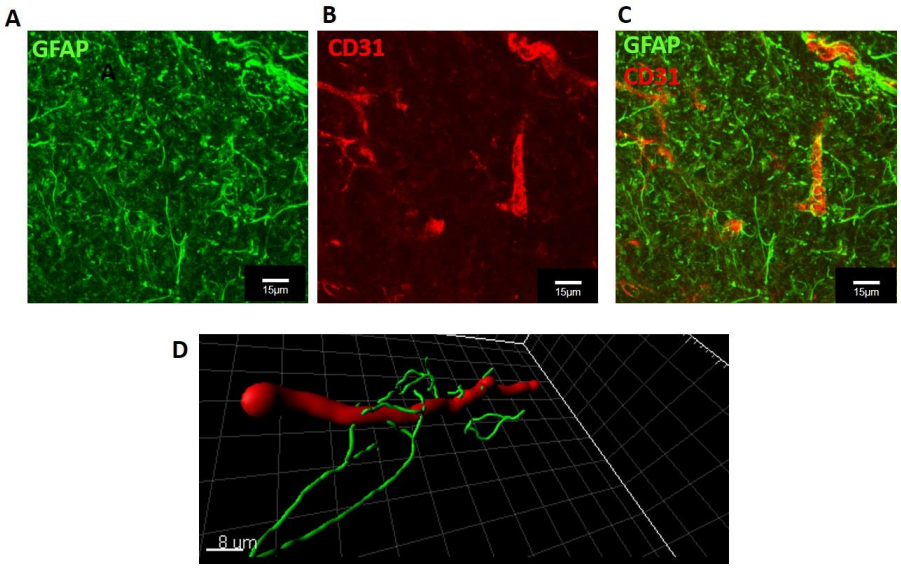


Figure 2

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GFAP
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