[†]Combined Multidimensional Microscopy as a Histopathology Imaging Tool

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Abstract

Herein, we present a highly versatile bioimaging workflow for the multidimensional imaging of biological structures across vastly different length scales. Such an approach allows for the optimised preparation of samples in one go for consecutive X-ray micro-computed tomography, bright-field light microscopy and backscattered scanning electron microscopy, thus facilitating the disclosure of combined structural information ranging from the gross tissue or cellular level, down to the nanometre scale. In this current study, we characterise various aspects of the hepatic vasculature, ranging from such large vessels as branches of the hepatic portal vein and hepatic artery, down to the smallest sinusoidal capillaries. By employing high-resolution backscattered scanning electron microscopy, we were able to further characterise the subcellular features of a range of hepatic sinusoidal cells including, liver sinusoidal endothelial cells, pit cells and Kupffer cells. Above all, we demonstrate the capabilities of a specimen manipulation workflow that can be applied and adapted to a plethora of functional and structural investigations and experimental models. Such an approach harnesses the fundamental advantages inherent to the various imaging modalities presented herein, and when combined, offers information not currently available by any single imaging platform.

1. Introduction

Biological organisms and their comprising subcellular constituents exist amongst immensely diverse length scales. Concordantly, the visualisation of such structures demands the utilisation of an extensive array of microscopy techniques, which utilise unique imaging particles and detection systems (e.g. light, electrons, x-rays, deflected cantilever), in order to attain structural information that is not conferred by a single imaging modality. Throughout the literature, it is abundantly evident that no single imaging technique is capable of holistically depicting biological organisms along the various length scales amongst which they exist. In order to overcome such limitations, it is becoming common practice to combine multiple cellular imaging modalities in order to attain complementary structural information that is not conferred by one single microscopy technique (Handschuh et al., 2013; Muller-Reichert et al., 2007; Shami et al., 2014). Herein, we present a sample preparation workflow utilising liver tissue as a sample model, that can be easily applied to any experimental tissue material, which combines X-ray micro-computed tomography, bright-field light microscopy and backscattered scanning electron microscopy in order to characterise various tissue, cellular and ultrastructural features of the hepatic microarchitecture, ranging from the micron to nanometre scale in three-dimensions (3-D). Our approach aims to highlight the

respective advantages conferred by utilising a correlated multimodality imaging approach, in order to generate holistic structural information from a single sample.

Traditionally, bright-field light microscopy (BFLM) has dominated as the premier technique for histological investigation at the tissue and cellular level. The technique offers a number of advantages in that it allows for the visualisation of live or fixed cells, is compatible with an extensive range of histochemical techniques for the differential staining of biological material, and involves relatively simple sample preparation and microscope setup. Moreover, the development of automated tissue processing, staining and embedding devices has facilitated high sample throughput, which has been particularly expedient in clinical settings, where rapid diagnosis is of critical importance. Such aspects of light microscopy are elegantly outlined by Wilson and Bacic (2012).

With regards to the microanatomical structure of the liver, much of the knowledge that we currently possess was revealed by BFLM, dating as far back as the 19th Century. Such notable findings include the parenchymal cell and its core (Dutrochet, 1824), the liver lobule (Kiernan, 1833), the bile canaliculi, Kupffer cells, confirmation of the perisinusoidal space (space of Disse) and demonstration of the dual blood supply of the liver (Kuntz et al., 2008). With regards to the pathobiology of the liver, BFLM has been an instrumental technique in the investigation of such conditions as sinusoidal obstruction

syndrome (Vreuls et al., 2012), primary and metastatic cancer (Terayama et al., 1996), fatty and non-fatty alcoholic liver disease (Lefkowitz, 2005; Rabin, 1989), hepatic cirrhosis (Waldrop and Puchtler, 1982) and hemochromatosis (Hennigar et al., 1979), to mention a few. Despite the plethora of advantages conferred by BFLM, the maximum attainable resolution is limited to ~200 nm in the lateral dimension, and ~500 nm in the axial dimension, owing to the diffraction barrier of visible light (Abbe, 1873; Rayleigh, 1880). Conventional BFLM is further limited to the generation of two-dimensional (2-D) data, however this may be overcome by means of serial sectioning or array tomography (Micheva et al., 2010; Micheva and Smith, 2007).

The advent of fluorescence microscopy (FM) throughout the early 20th Century provided the ability to selectively observe structures of interest; either those that possess intrinsic autofluorescent properties, or via the induction of fluorescent probes or markers (Monici, 2005). Advances within FM techniques gave rise to confocal laser scanning microscopy (CLSM), which is capable of generating 3-D structural information via the superimposition of multiple 2-D optical sections; a feat achieved by means of the exclusion of out-of-focus light by virtue of an adjustable pinhole. With regards to the investigation of the hepatic architecture, FM has been a particularly expedient technique in quantifying hepatic stellate cells – also known as fat storing cells – based on the

intrinsic autofluorescence of their intracellular vitamin A stores (Zhang et al., 2000), selectively observing cellular interactions between fluorescently labelled colorectal cancer cells with the hepatic sinusoids (Vekemans et al., 2004), and reconstructing and quantifying key features of the hepatic microarchitecture (Hammad et al., 2014). Whilst CLSM provides a slight improvement in resolution relative to BFLM – between a factor of 1.08 (Sheppard and Choudhury, 1977) – 1.31 (Cox and Sheppard, 2004) – the technique often provides insufficient detail for disclosing fine-structure detail below the optical diffraction limit of the light microscope (i.e. ≤ 200 nm).

To-date, electron microscopy (EM) modalities including both scanning and transmission variants prevail as the most superior technique for the high-resolution visualisation of cellular structures at the nanometre scale. The application of EM within the fields of gastroenterology and hepatology has been particularly well documented throughout the second half of the twentieth century, providing valuable insight into the hepatic architecture (Brooks and Haggis, 1973; Burt et al., 1993; Pieri et al., 1975; Rieder et al., 1992). Specifically, these include the ultrastructural characterisation of both the parenchymal and non-parenchymal cells (i.e., Kupffer cells, liver-associated natural killer cells, stellate cells and sinusoidal endothelial cells) of the liver, as well as the differentiation of the various cell types comprising the biliary and lymphatic systems.

More specifically, such fine-structure details as the presence and characteristics of liver sinusoidal endothelial cell fenestrae were visualised for the first time, a feat previously unattainable using conventional BFLM (Wisse, 1970). EM has also contributed a great deal to morphometric studies, in providing a quantitative basis for understanding both the normal and diseased liver. Such studies have included the distribution and measurement of subcellular structures in stereological studies (Blouin et al., 1977), the regulatory role of the thymus in the aging liver (Giuli et al., 1980) and the effect of ursodeoxycholic acid in primary biliary cirrhosis (Neuman et al., 2002).

Despite the expedience of diagnostic EM in a limited number of applications such as determining vascular changes in different hepatic diseases (e.g. sinusoidal stenosis and sinusoidal capillarisation), diagnosing cholestasis, establishing the origin of liver tumours and demonstrating sinusoidal haemangioendothelioma (Biel and Gelderblom, 1999; Vonnahme, 1984), its use has not been as extensively employed in diagnostics as has light microscopy. This is attributable to a number of factors including, the immense cost of establishing EM facilities, the requirement of specialist expertise and learning, low throughput and typically long sample preparation times, all of which conflict with the requirements of diagnostic medicine.

As a relatively contemporary technique that has only commercially emerged at the commencement of the 21st Century, X-ray micro-computed tomography (Micro-CT) has facilitated the non-destructive 3-D reconstruction of small, X-ray dense biological organisms down to the micron scale. With regards to the liver, Micro-CT has been applied to investigate the hepatic vascular network (Wan et al., 2000), liver lesions in small animal models (Martiniova et al., 2010) and selective imaging of liver tumours using a hepatocyte-selective contrast agent (Weber et al., 2004).

Taking the latest advances of each of the above discussed microscopy methodologies together, we herein present a relatively straightforward combinatorial sample preparation workflow followed by a swift imaging workflow, facilitating the easy collection of cross-correlative structure-function information on the same sample across different volumes and length scales.

2. Materials and methods

2.1. Sample preparation

Wistar rats (10–12 weeks of age) were housed in plastic cages at 21°C with a 12 h light–dark cycle and were fed and watered *ad libitum*. All procedures were conducted in accordance with the guidelines and approval of the Animal Care and Ethics Committee of the University of Sydney. A strip of liver tissue measuring ~1 mm x 1 mm x 15 mm was fixed by means of “jet-fixation” as described by Vreuls et al. (2014) in a solution containing 1.5% glutaraldehyde in 0.067 M sodium cacodylate buffer, 1% sucrose and 2 mM calcium chloride pH 7.4 at 37°C. Following jet-fixation, the tissue was cut into ~1 mm³ blocks and allowed to react in the primary fixative for 1 h at room temperature. Samples were prepared following a modification of a protocol developed by Deerinck et al. (2010) involving the following steps: (1) 2% osmium tetroxide + 1.5% potassium ferrocyanide for 1 h, (2) 1% thiocarbohydrazide for 20 min, (3) 2% osmium tetroxide for 30 min in darkness, (4) 1% aqueous uranyl acetate overnight at 4°C and (5) Walton’s lead aspartate staining for 30 min at 60°C. Samples were dehydrated in a graded ethanol series, and finally infiltrated and embedded in hard-grade Epon resin.

2.2. X-ray micro-Computed Tomography (Micro-CT)

Micro-CT is a non-destructive 3-D imaging technique that involves generating cross-sectional 2-D projections of physical objects, via the differential attenuation of X-rays based upon variations in sample radiodensity. Images are typically acquired over a rotation axis of 180° and subsequently “back-projected” or “stacked” in order to generate a 3-D reconstruction of the sample. In this manner it is possible to generate large volume, high resolution 3-D data within the micron range (Ananda et al., 2006; Cheng et al., 2016). For an in-depth review see Ritman (2011).

Resin-embedded rat liver samples were imaged using a Skyskan 1172 system (Bruker microCT, UK), operating at 40 kV and over a 180° rotation angle. A total of 1775 images were acquired with an exposure time of 1.18 s and at an angular increment of 0.110° using the microCTs’ computer-controlled specimen rotation stage. The acquired individual image projection data were subsequently assembled using the NRecon reconstruction software program (version 1.5.25, Bruker microCT, UK). Next, 3-D modelling was performed by means of “isosurface” modelling within 3dmod, a graphical user interface application that is bundled with the IMOD shareware software package (version 4.5.8, Boulder, Colorado, USA) allowing the operator to contour, and hence render 3-D models of structural features of interest (Kremer et al., 1996).

2.3. Serial sectioning and bright-field light microscopy (BFLM)

Proceeding Micro-CT imaging, excess resin surrounding the embedded tissue was trimmed using an injector blade, forming a trapezoid, and the block was carefully faced up to expose the underlying tissue. A sparring mixture of Welwood® glue and xylene (1:2) was applied to the long edge of the trapezoid following the methods developed by Blumer et al. (2002) and Micheva and Smith (2007). A series of 250 consecutive sections (~300 nm thick) were generated with a Leica EM UC7 ultramicrotome (Leica, Heerbrugg, Switzerland) equipped with a histo-jumbo knife (Diatome, USA) and collected on a glass slide. The slide was dried on a heating block and stained with filtered 0.5% toluidine blue in 1% aqueous sodium tetraborate for 30 s. Furthermore, in order to demonstrate the versatility of the histopathology imaging approach, a number of alternative and commonly used histological dyes compatible with resin-embedded tissues are proceedingly outlined. These include methylene blue-azure II-basic fuchsin, paragon stain, malachite green and Richardson's stain.

BFLM images were generated of each serial section using a Leica DM600 light microscope (Leica, Germany), with 10 x and 40 x objectives. Images were automatically

aligned using the *StackReg* plugin for ImageJ (Thevenaz et al., 1998). The hepatic structures of interest were segmented as described under section 2.2.

2.4. Backscattered scanning electron microscopy (BSEM)

Following BFLM imaging, the glass slide was next carbon coated in order to render the glass slide conductive, and mounted on a stub in preparation for backscattered electron microscopy (BSEM). Two thick lines of silver paint were applied from the top surface of the slide, to the underlying stub to further improve conductivity of the sample. Backscattered electron images were acquired using a variable pressure, field emission scanning electron microscope (Sigma VP, Carl Zeiss, Australia) operating at 4 kV with a working distance of 7.6 mm. Consecutive sections were aligned as described under section 2.3., and 3-D modelling of cells of interest was performed by means of computer-assisted tracing of high contrast lines within IMOD (Kremer et al., 1996).

3. Results and Discussion

In this communication, we present a practicable workflow facilitating the large volume, 3-D reconstruction of the microanatomy of biological samples, ranging from the gross tissue level down to the subcellular scale (Fig. 1). Such an approach facilitated the collection of holistic morphological information of the murine hepatic microvascular network utilising an array of different microscopy techniques including, X-ray micro-computed tomography (Micro-CT), bright-field light microscopy (BFLM) and backscattered scanning electron microscopy (BSEM) (Fig. 2), which respectively confer specific advantages for the collection of combined structural information. Furthermore, the approach can be combined with resin-compatible histological dyes, allowing the swift histochemical characterisation of supramolecular structures of interest, thereby providing the researcher with an arsenal of light microscopy staining tools to aid rapid bench identification (Fig. 3).

Micro-CT imaging facilitated the large volume, non-destructive collection of 3-D data of the murine vascular network, based on the differential X-ray density, and scattering of biological structures. The primary jet-fixation method used in this investigation rendered the hepatic vascular network patent and clear of blood – due to the forcible spraying of fixative throughout the thin piece of liver tissue. The subsequent concomitant application of heavy metal stains and mordanting agents rendered the

various cellular components of the liver highly X-ray dense (e.g. parenchyma, endothelium and blood components), whilst the lumen of the vascular network remained radiolucent. As such, it was possible to easily segment and model the 3-D arrangement of the hepatic vasculature, which appeared as a labyrinthine network of channels interspersed between the anastomosing liver plates (Fig 2. A and B). The major advantages inherent to Micro-CT include the relatively short imaging times – approximately 3 h per sample – the high degree of automation and the non-destructive generation of data. Moreover, the high-contrast data generated facilitated the quick and easy delineation of the vascular network by means of thresholding, or “isosurface” segmentation. Whilst our study only investigated a small segment of liver tissue measuring $\sim 1 \text{ mm}^3$, the technique can be applied to image volumes as large as entire experimental animal models (e.g. mice and zebrafish (Cheng et al., 2016)) or resected organs (e.g. rat liver (Ananda et al., 2006)). Furthermore, Micro-CT can be performed in *in vivo* studies in order to determine such functional pathologies as pulmonary fibrosis (Johnson, 2007), or the metastatic pathways of cancer cells to specific organs (Geffre et al., 2015; Namati et al., 2010). The current limitations of the technique include the lack of specific stains available for the selective visualisation of particular cellular features, as well

as the limited spatial limited resolution, which in the dataset presented was calculated to be an isotropic voxel dimension of 2.3 μm .

Because of the non-destructive nature of Micro-CT, it was next possible to generate ribbons of serial tissue sections for BFLM investigation. The application of toluidine blue, as is routinely applied in diagnostic laboratories (Allegra et al., 2009), allowed for the quick and simple staining of resin-embedded tissue sections. The polychromatic nature and convenience of the stain additionally allowed for the observation of an extensive array of subcellular structures such as nuclei, mitochondria and endoplasmic reticulum, including cell inclusions ranging from lipids to polysaccharides and nucleic acids (Fig. 2 C). A variety of additional histological stains compatible with our specimen preparation workflow and imaging set-up were also explored, given the affinity of particular stains for specific tissue or subcellular features (Fig. 3). Relative to the Micro-CT data, BFLM allowed for the definitive characterisation of key tissue and cellular features of the liver including, the portal tract – which is composed of branches of the hepatic portal vein, hepatic artery and bile duct – parenchymal cells and the hepatic sinusoids, which are formed via a simple layer of liver sinusoidal endothelial cells (LSECs) (Fig 2. C - Inset). Given the limited resolving power inherent to BFLM (i.e., $\sim 0.22 \mu\text{m}$), it was demanding to conclusively identify particular leukocytes

located within the sinusoidal lumen, on the basis of their morphological appearance (see, *vide infra*). Having aligned and stacked consecutive BFLM images, it was next possible to segment and model the 3-D arrangement of the hepatic microvasculature, as was done for the Micro-CT dataset. Relative to the former, the 3-D BFLM series revealed superior lateral and axial resolution (i.e., $\sim 0.36\ \mu\text{m}$ XY and $\sim 0.3\ \mu\text{m}$ Z), with such structures as the branching points of individual sinusoids being visible (Fig 2. D). Whilst the image acquisition of BFLM datasets was relatively fast, the generation of serial sections was a relatively time consuming process, and certainly a hold-up in the swift processing of samples for combined multidimensional imaging. Nevertheless, the approach ensures the retention of sections for future BFLM and EM analysis.

Due to the limited resolving power of BFLM, we next employed EM in order to further characterise features of the hepatic microarchitecture at the nanometre scale. By easily relocating and correlating the same areas of interest that were previously captured using Micro-CT and BFLM, it was possible to gain increasingly detailed morphological and structural information using BSEM not previously attainable under the two former techniques. Such a combined bioimaging approach allowed us to clearly classify and visualise various cells located within the lumen of a hepatic sinusoidal vessel. These included two liver-associated natural killer cells – also known as pit cells – which

displayed a clear polarisation of their organelles at one side of their nucleus, and the presence of characteristic electron dense granules distributed throughout the cytoplasm (Fig 2. F-J). Indeed, the characterisation (Wisse et al., 1976) and later designation (Kaneda et al., 1983) of the pit cell as being one of the four cell types comprising the hepatic sinusoids, was confirmed by means of transmission electron microscopy, highlighting the importance of EM for high-resolution ultrastructural analysis. BSEM further revealed a large elongated Kupffer cell interacting with both the sinusoidal endothelium, and two adjacent pit cells by means of well-developed pseudopodia. Of particular note, the Kupffer cell cytoplasm contained an extensive endoplasmic reticulum network, a well-developed multilaminar body, an indented nucleus and a considerable amount of electron dense lysosomes, as is commensurate with their phagocytic function. High-magnification BSEM also revealed such intricate details as the hepatocytic microvilli projecting into the perisinusoidal space, which significantly increases the surface area for exchange to take place. BSEM allowed the capturing of high resolution/pixel array images (XY pixel size = 7 nm), allowing the extraction of high-magnification data from intermediate-magnification images. Whilst this was certainly advantageous, it significantly increased imaging time, and given the sheer number of 2-D images comprising a 3-D dataset, represented a second major bottleneck in the generation of a holistic and

combined multidimensional imaging dataset. This issue was further compounded by the need to manually segment individual cells – which was not possible using thresholding or “isosurface” segmentation – in order to delineate, model and study them in 3-D.

In conclusion, (1) a straightforward and universally applicable sample preparation protocol, that can be applied irrespective of tissue origin (Cheng et al., 2016) is outlined; (2) allowing the collection of image data in 3-D over different length and resolution scales; and (3) by doing so, simplifies the disclosure of holistic fine structural information in large volumes (Fig. 4). Hence, we put forward that this contemporary bioimaging tool allows one to investigate rare events, and as such can be used as a screening method. In addition, this workflow can be applied to quantify events in a large volume first and subsequently dial-in into the micro- and/or nanometre scale, thereby permitting the structural or physiological investigation of biological organisms at the nanometre scale.

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6.

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