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Detecting microvascular changes in the mouse spleen using optical computed tomography

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ABSTRACT

Methods of monitoring drug toxicity in off-target organs are very important in the development of effective and safe drugs. Standard 2-D techniques, such as histology, are prone to sampling errors and can miss important information. We demonstrate a novel application of optical computed tomography (CT) imaging to quantitatively assess, in 3-D, the response of adult murine spleen to off-target drug toxicity induced by treatment with the vascular disrupting agent ZD6126. Reconstructed images from optical CT scans sensitive to haemoglobin absorption reveal detailed, high-contrast 3-D maps of splenic structure and microvasculature. A significant difference in total splenic volume was found between vehicle and ZD6126-treated cohorts, with mean volumes of 61 ± 3 mm^3 and 44 ± 3 mm^3 respectively (both $n = 3$, $p = 0.05$). Textural statistics for each sample were calculated using grey-level co-occurrence matrices (GLCMs). Standard 2-D GLCM analysis was found to be slice-dependent while 3-D GLCM contrast and homogeneity analysis resulted in separation of the vehicle and ZD6126-treated cohorts over a range of length scales.

Introduction

Toxicity is an important consideration in drug development and off-target effects are common, with potentially significant adverse side-effects. Methods that provide informative, accurate and rapid assessment of drug-induced toxicity to major target organs, such as the liver, kidneys and spleen, can either positively impact and accelerate the development of promising new agents, or force early closure of a programme that ultimately will not deliver a safe drug. They also inform design of subsequent clinical trials, ensuring that imaging protocols are optimised to include potentially critical normal tissues.

In the treatment of cancer, a number of agents designed to specifically target the tumour vasculature have been and continue to be developed. Given the hypervascular nature of the spleen, its essential role as a blood filter and the unique presence of acute endothelial contractility (Ragan et al., 1988), the effects of these vascular targeting agents on splenic perfusion are also commonly assessed. Typically, this has been achieved pre-clinically through the use of histological markers of perfused vasculature or tissue uptake of radiolabelled blood flow tracers (Cullis et al., 2006; Horsman and Murata, 2003). Splenic perfusion has also been assessed as part of clinical trials of vascular targeting agents by incorporating functional imaging, where the spleen is included in the imaging field-of-view (FOV) (Anderson et al., 2003; Evelhoch et al., 2004).

The spleen has a complex 3-D structure made up of several distinct tissue types. Extensive interconnections between red pulp, white pulp and intermediary marginal zones are integral to the organ's function (Groom, 1987). Traditional 2-D histology methods are not well suited for the examination of these 3-D features and they are often subject to sampling error due to the limitations of the physical sectioning techniques used. In addition, it is time-consuming to match information between adjacent slices, which may be distorted by the histological preparation process. A number of imaging approaches have been applied to the spleen, including microvascular-corrosion casting, scanning and transmission electron microscopy (SEM, TEM), and *in vivo* microscopy (Groom, 1987). These can have excellent resolution, but are often complex to perform and expensive.

Abbreviations: BABB, benzyl benzoate:benzyl alcohol; BV, blood vessel; CMOS, complementary metal-oxide semiconductor; CT, computed tomography; FOV, field-of-view; GLCM, grey-level co-occurrence matrix; LN, lymph node; LV, lymph vessel; MZ, marginal zone; OPT, Optical Projection Tomography; PBS, phosphate-buffered saline; RBC, red blood cell; ROI, region-of-interest; RP, red pulp; SEM, scanning electron microscope; SNR, signal-to-noise ratio; TEM, transmission electron microscope; VDA, vascular disrupting agent; WP, white pulp.

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Optical computed tomography (CT) is an emerging imaging modality. Although initially introduced in 1996 to solve problems in the physical sciences (Gore et al., 1996; Winfree et al., 1996), the first microimaging measurements on tissue were presented somewhat later, in 2002, using the Optical Projection Tomography (OPT) variant of the scanner geometry (Sharpe et al., 2002). This technique has subsequently been applied to the imaging of whole rodent organs and embryos in a variety of contexts (Oldham et al., 2007; Sharpe, 2003). The FOV size sits in the "imaging gap" between confocal microscopy and magnetic resonance imaging (MRI), ranging from a single cell to several centimetres, with resolution generally proportional to the FOV.

Prior to optical CT imaging, tissue samples must, in general, undergo a process of optical clearing to render them transparent at optical wavelengths (Oldham et al., 2008; Zhu et al., 2013). Optical CT images can be acquired in absorption or fluorescence modes, and the contrast observed can be either *endogenous* (related to the remnant optical absorption of tissue after the clearing process, or the tissue autofluorescence), or *exogenous* in the form of optical stains which are well characterised in the field of histopathology. Previously OPT has been used to probe developing spleens in embryos (Asayesh et al., 2006; Hecksher-Sørensen et al., 2004), but the technique has not been applied to adult mouse spleen in which the 3-D features are fully developed.

In this study, the ability of endogenous optical CT contrast to detect and to assess quantitatively the microstructure of normal adult murine spleen was investigated. In addition, structural changes were also characterised in spleens excised from mice following treatment with the vascular disrupting agent (VDA) ZD6126. A previous 2-D assessment, using fluorescence microscopy of Hoechst 33342 uptake (Cullis et al., 2006), demonstrated a significant reduction in splenic perfusion with this agent, making it a highly relevant case study.

Materials and methods

Tissue collection

ZD6126 (N-acetylcolchinol-*O*-phosphate, Angiogene Pharmaceuticals Ltd.) was formulated in 20% of 5% sodium carbonate and 80% phosphate-buffered saline (PBS).

All *in vivo* experiments were performed in accordance with the local ethical review panel, the UK Home Office Animals (Scientific Procedures) Act 1986, the United Kingdom National Cancer Research Institute guidelines for the welfare of animals in cancer research (Workman et al., 2010) and the ARRIVE (animal research: reporting in vivo experiments) guidelines (Kilkenny et al., 2010). Six-week-old female Balb/c mice were randomised to be treated with either vehicle alone ($n = 3$) or 200 mg/kg ZD6126 i.p. ($n = 3$). The small sample size was chosen to minimise animal use in this initial proof-of-concept study. No adverse effects were observed in any mice and after 24 h the mice were killed by cervical dislocation and the spleens excised and placed in 70% ethanol in PBS overnight at 4 °C.

Optical CT imaging

Spleens were first embedded in 0.75% agarose (Sigma-Aldrich, Gillingham, UK) and kept in 70% ethanol in PBS overnight at room temperature. The samples were then dehydrated with three washes of 100% ethanol over three days. Optical clearing was achieved with a graded series of ethanol and 1:2 benzyl benzoate:benzyl alcohol (BABB) solutions (Sigma-Aldrich, Gillingham, UK), with washes of 30% and 70% 1:2 BABB in ethanol, each for one day, followed by two washes of 100% 1:2 BABB over a one week period.

Imaging was performed using an in-house optical CT system, shown in Fig. 1, which was previously developed and well characterised for microbeam radiotherapy applications (Doran et al., 2013). Optical CT image reconstruction is similar to X-ray CT in that a series of 'projection' images are acquired, recording photon attenuation at different angles. Each sample was suspended from a sample holder and rotated 180° from above in a matching tank containing 1:2 BABB fluid, which has the same refractive index as the optically cleared samples. In order to achieve high resolution, a microscope zoom lens (Z16 APO zoom system, Leica Microsystems GmbH, Wetzlar, Germany) was used to focus each projection image onto a complementary metal-oxide semiconductor (CMOS) camera (Zyla sCMOS, Andor Technology PLC, Belfast, UK). Filtered backprojection was used to reconstruct axial slices through each sample from the many 2-D projection images.

Two datasets were acquired for each optically cleared spleen, each based on a raw dataset of 1000 projection images of 512×512 pixels, acquired over 180° rotation and reconstructed to a 512^3-voxel volume. Dataset 1 had a FOV of $(13.3\ \text{mm})^3$ and isotropic voxels of size $(26\ \mu\text{m})^3$, while Dataset 2 had a FOV of $(5.3\ \text{mm})^3$ and isotropic voxels of size $(10.4\ \mu\text{m})^3$.

The first scan encompassed the entire spleen height, enabling accurate total volume measurements. The second scan had a reduced FOV to enable acquisition of higher resolution data over a limited height,

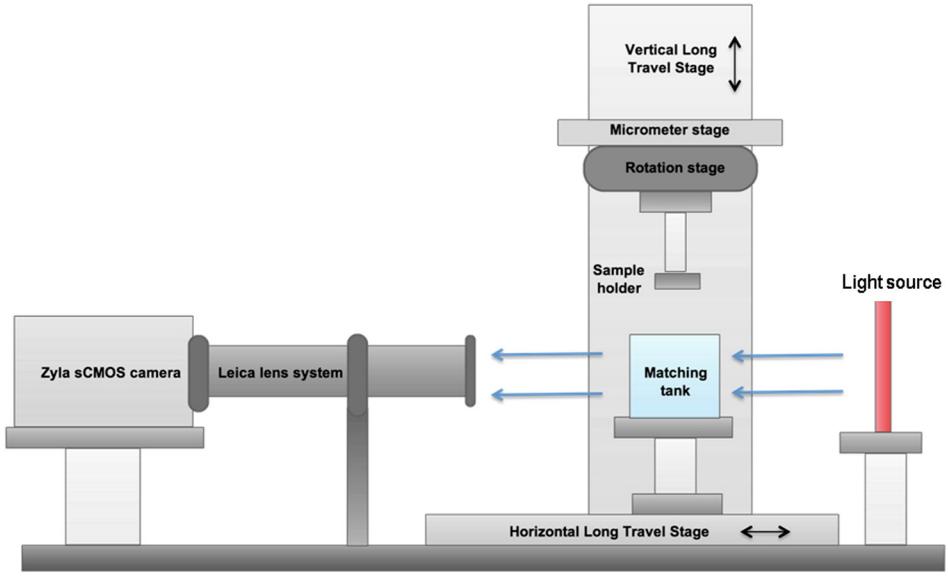

Fig. 1. Diagram of the optical CT system used for imaging optically cleared spleen samples. Light passing through the sample was focused by a microscope lens system onto a camera chip and recorded in a 'projection' image for each rotation angle. See Doran et al. (2013) for details of individual components.