

Quantifying hypoxia with diffuse reflectance spectroscopy for advanced prognostication and real-time response monitoring in rectal cancer: an *in vivo* feasibility study

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ABSTRACT

Tumour hypoxia is the inevitable consequence of a tumour's rapid growth and disorganised, inefficient vasculature. The compensatory mechanisms employed by tumours, and indeed the absence of oxygen itself, hinder the ability of all treatment modalities. The scientific community has failed to capitalise on this discovery due to inherent difficulties in measuring hypoxia. However, with the emergence of sensing tools based on diffuse reflectance spectroscopy (DRS), we reach an exciting stage in potentially accurately assessing tumour in a continuous fashion. The aim of this study was to assess the feasibility of sequentially measuring evolving tumour hypoxia over the course of tumour growth using a multi-depth DRS probe. HCT116 colorectal cancer cells were implanted subcutaneously in 5 Balb/C nude mice. Tumour oxygenation measurements were collected every 3 days with the DRS probe under anaesthesia over a 30-day period of tumour growth. There was a high level of hypoxia heterogeneity, both over the period of tumour growth (30 days) and between mice. Tumour hypoxia did not progress in a linear fashion as expected, with significant peaks at days 5-6 and 17-23 in all 3 of the tumours reaching the experimental endpoint. We demonstrated that *in vivo* sequential hypoxia measurements with a multi-depth DRS probe are feasible and reproducible. For further validation of this approach, serial DRS measurements will be collected in artificially hypoxic and super-oxygenated environments and correlated with current gold standard (but invasive) techniques.

Keywords: Hypoxia, diffuse reflectance spectroscopy, colorectal cancer, *in vivo*, prognostication

1. INTRODUCTION

The role of neoadjuvant chemoradiotherapy within rectal cancer is rapidly expanding as we enter the era of organ preservation and deferral of surgery [1]. As such, there is an urgency to predict which tumours are likely to completely respond, and a need for real-time response monitoring to enable precision oncology and personalised treatment. Current prognostic methodologies such as genetic profiling lack both accuracy and clinical translation [2]. Whilst Magnetic Resonance Imaging can provide accurate response assessment, it lacks dynamism as it is performed at a single point in time. The accurate measurement of tumour hypoxia could fill these prognostic and response monitoring voids [3].

Tumour hypoxia is the inevitable consequence of a tumour's rapid growth and disorganised, inefficient vasculature. The compensatory mechanisms employed by tumours, and indeed the absence of oxygen itself, hinder the ability of all treatment modalities. The net consequence of this is poorer therapy response and long-term survival [4]. Unfortunately,

the scientific community has failed to capitalise on this discovery due to inherent difficulties in measuring hypoxia. However, with the emergence of sensing and imaging tools based on diffuse reflectance spectroscopy, we reach an exciting stage in potentially accurately assessing and mapping tumour in a continuous fashion.

Diffuse reflectance spectroscopy (DRS) involves the study of light and its impingement on biological tissues. The study of a tissue's absorptive and scattering properties, and the degree at which individual wavelengths are backscattered across a spectral range, allows the quantification of otherwise invisible cellular properties. This technology is suitable for assessing a tissue's oxygenation profile due to the differing absorption profiles of oxygenated and deoxygenated haemoglobin [5]. There is a wealth of evidence demonstrating how this can be done in a qualitative, quantitative, and reproducible fashion [6].

The aims of this study are threefold: (1) To sequentially measure evolving tumour hypoxia over the course of tumour growth (in mouse models) with a multi-depth DRS probe; (2) To assess HCT116 (human derived) colorectal cancer (CRC) cell line growth in Balb/C nude mice; and (3) To correlate pre-terminal DRS values with immunohistochemical (IHC) carbonic anhydrase (CAIX) staining.

2. METHODS

2.1 Cell culture, implantation and animal models

The HCT116 (human derived CRC) cell line was obtained from the American Type Culture Collection and cultured in McCoy's 5A medium. 4 million cells were implanted subcutaneously in the right flank of each mouse. 5 Balb/C nude female mice aged 6-8 weeks were sourced from Charles River Laboratories and were housed in a single cage at the central biomedical services (CBS) training unit at Imperial College London in a 12-hour dark-light cycle. Food and water were allowed *ad libitum*.

2.2 Data acquisition

DRS measurements were performed prior to tumour implantation, and then, once the tumours became palpable (6 days post implantation), every 3 days until the maximum permitted tumour size (18 mm in a single diameter) was reached. All measurements were performed under inhaled isoflurane anaesthesia. At each measurement, a multi-depth compact spectrometer (Flame, Ocean Insight) probe was placed in direct contact with the centre point of the tumour and secured with a clamp stand (**Figure 1, I&II**). At each time point, 3 readings were captured at 5 different depths (approx. 0.1–2mm) using OceanView Spectroscopy software. The acquired spectra were normalised by dividing each spectral data point by the value at 800 nm. Subsequently, the resulting normalised data was divided by the normalised diffuse reflectance spectrum obtained from a 99% reflectance standard (USRS-99-010; Labsphere, Inc, North Sutton, NH). Oxygenation Ratio Metrics (ORM's) were calculated (by dividing the peak value at 560nm by the mean of dual trough values (α and β bands) at 542nm and 576nm) to reflect changing oxygenation trends. Therefore, a higher ORM value represents a greater degree of haemoglobin oxygenation saturation.

2.3 Tumour preservation and IHC analysis

Tumours were harvested immediately after termination, fixed in formalin (18 hrs) and then washed and preserved in PBS. Central tumour sections were cut and stained for CAIX IHC (**Figure 1, V**). QuPath software was used to calculate viable tumour percentage and hypoxic percentages of viable tumour.

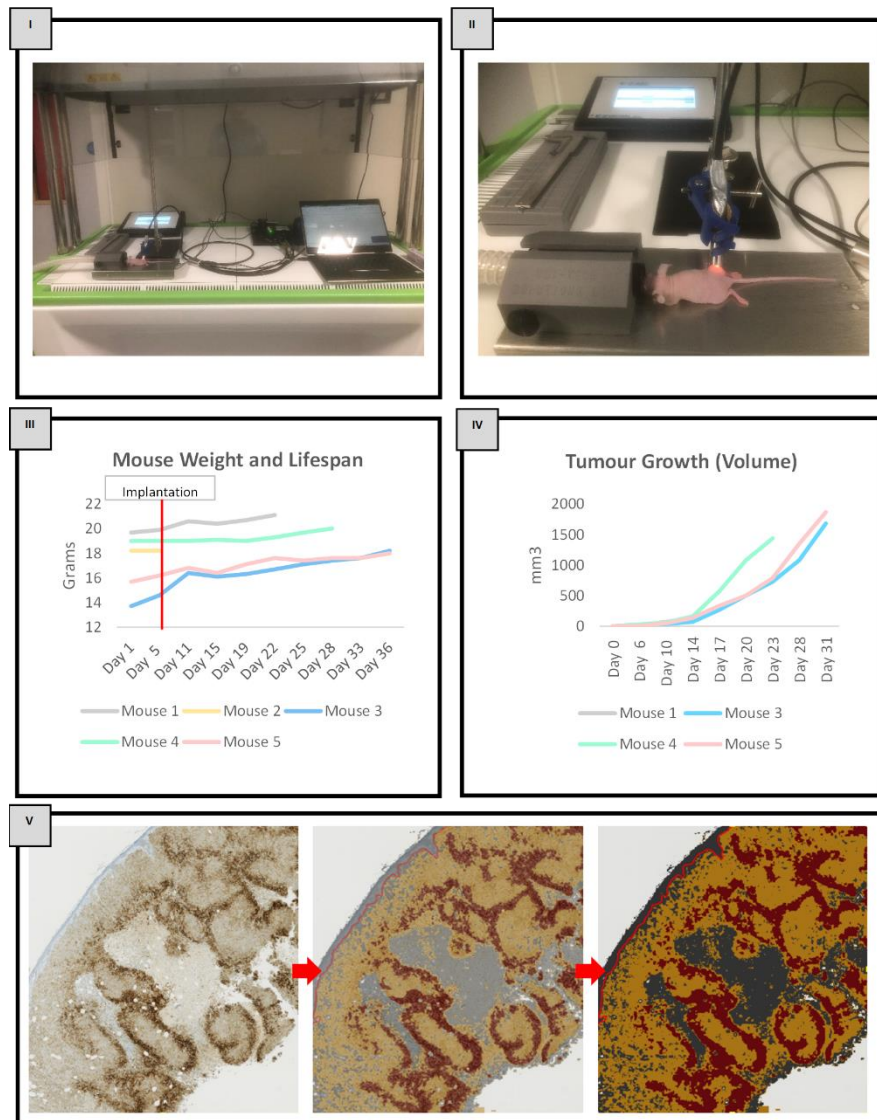


Figure 1: **Experimental set-up, tumour growth and CAIX staining:** (I) and (II) Experimental set-up, comprising a multi-depth DRS probe (feeding into a spectrometer, in turn feeding into a laptop) held in contact with the mouse tumour by a clamp stand, with the mouse under isoflurane anaesthesia on a heat pad; (III) Mouse weight and longevity; (IV) Exponential tumour growth; (V) IHC CAIX staining. Black represents necrotic non-viable tumour, and red represents areas strongly positive for hypoxia

3. RESULTS

3.1 Tumour growth

The tumours demonstrated uniform growth, reaching the maximum permitted diameter 31 days after implantation (**Figure 1, IV**). However, only 2 mice reached the maximum permitted diameter: 1 was culled at a tumour diameter of 15 mm due to tumour ulceration, and 2 failed to recover from anaesthesia (pre-implantation and day 17) (**Figure 1, III**).

3.2 DRS and tumour oxygenation trends

Oxygenation trends at each of the fibre depths are shown (**Figure 2, I**). There was a high level of hypoxia heterogeneity, both over the period of tumour growth and between mice. Tumour hypoxia did not progress in a linear fashion, as demonstrated in a previous finger clamping experiment (**Figure 2, II & IV**). There were significant peaks at days 6-7 and 17-23. However, there was an overall downtrend towards hypoxia in fibres 4, 5, and 6 (measuring relatively deeper portions of the tumour).

3.3 Carbonic anhydrase immunohistochemistry (CAIX IHC)

CAIX staining of 3 tumours (**Figure 1, V**) revealed that the mean percentage of viable tumour was 57.4 % (STDEV +/- 11.4). The percentage of viable cells strongly positive for hypoxia was 23.9% (STDEV +/- 1.2), demonstrating expected hypoxia homogeneity in tumours of the same size.

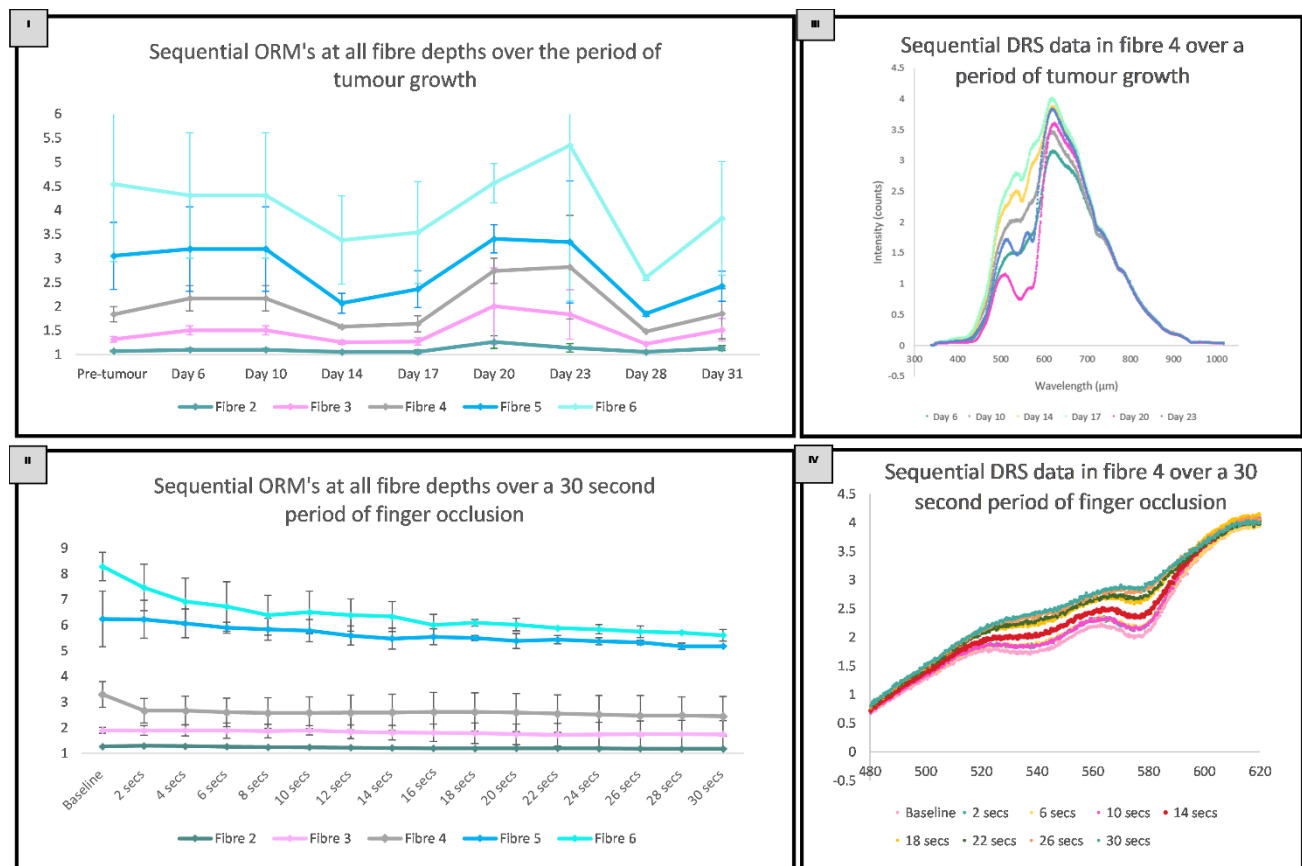


Figure 2: DRS output: (I) Each data point reflects the ratio of a single peak at 560 nm and the mean of 2 troughs at 542 nm and 576 nm, thereby reflecting heterogeneous oxygenation trends over the period of tumour growth – in contrast with (II), which demonstrates a linear downwards trend (reflecting increasing hypoxia) over a 30 second period of finger clamping. (III) Heterogeneous DRS waveforms over a 23 day period of tumour growth in fibre 4, in contrast with the gradual loss of peak and trough wave form over a 30 second period of finger clamping in (IV).

4. DISCUSSION

We have demonstrated that it is feasible to use DRS to dynamically track HCT116 tumour oxygen trends in Balb/C mice over an extended period of subcutaneous tumour growth. We have demonstrated that, although there was a significant

degree of intra- and inter- tumoral heterogeneity, there was a progression towards hypoxia in fibres 4, 5 and 6 – which represent the relatively deeper tumour depths. It is hypothesised that this pattern was not replicated in fibres 2 and 3 since their depths (0.1-0.4 mm) correlate to overlying non-malignant skin, and hence no hypoxic progression can be expected. DRS is particularly well suited to the measurement of a tumours oxygen profile due to the differing absorption profiles of oxygenated haemoglobin (HbO₂) and deoxygenated haemoglobin (dHb), which are the dominant absorbers. dHb exhibits a single peak at 550 nm, whereas Hb expresses peaks (α and β bands) at 542 nm and 576 nm. To provide a fast, uncomplicated, and model-free method for the assessment of oxygen trends, we calculated the ORM (described in the methods). We have demonstrated that this approach, whilst not providing actual tissue saturations, is an efficient and uncomplicated means by which to reflect evolving oxygenation trends.

The CAIX IHC analysis of the excised tumours (at the experimental end point) provided a valuable picture of hypoxia homogeneity in tumours of the same volume. However, a direct comparison with the DRS data readout was impractical for two key reasons: (1) A percentage of severely hypoxic cells (information provided from the CAIX IHC) cannot be accurately correlated with haemoglobin saturation (provided by DRS); and (2) CAIX IHC can only be measured at a single time point, once the animal has been culled and the tumour harvested – and hence cannot be used to verify changes in hypoxic trends.

The limitations of this study were twofold. First, the investigators performed the DRS reading with the animal under isoflurane anaesthesia. However, oxygen is used as a carrier gas for isoflurane delivery, and as such is likely to have affected the DRS readout, albeit uniformly across all measurements. As such, the investigators will perform future experiments under intra-peritoneal anaesthesia without supplemental oxygen. Second, the investigators tracked ORM trends over the period of tumour growth (i.e., over time). Therefore, in this scenario time acts as a surrogate marker for tumour volume. Although tumour growth was uniform (**Figure 1, IV**), future work will examine the correlation between hypoxia and tumour volume directly.

5. CONCLUSIONS

In vivo sequential hypoxia measurements with a multi-depth DRS probe are feasible and reproducible. For further validation of this approach, this experiment will be repeated under intra-peritoneal anaesthesia with a focussed examination of the relationship of tumour volume and hypoxia. Further to this, serial DRS measurements will be collected in artificially hypoxic and super-oxygenated environments and correlated with current gold standard (but invasive) techniques. Future work will involve the application of a course of fractionated radiotherapy to hypoxic and super-oxygenated mouse groups in order to assess whether: (a) hypoxia (as assessed by the DRS probe), can be used to predict response to radiotherapy; and (b) whether diminishing levels of hypoxia (as measured by DRS) reflect tumour response to treatment.

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