3D Nanoanalysis of the Tumour Microenvironment: Subcellular Ultrastructure of Model Systems

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Many cancer tumours have a hypoxic core, I wanted to determine ultrastructural differences of the tumour regions using a 3D cell culture model. When cells lack oxygen, they metabolise differently via *hypoxia inducible factors (HIF)*. I therefore compared non-hypoxic outer regions and hypoxic core regions of the spheroid culture model of HIF-1 or HIF-2 knockouts using resin embedded sections and transmission electron microscopy.

Aim

The aim of my project was to look at the subcellular structural changes in different hypoxia inducible factor (HIF) knockouts.

Addressing the aim

I used resin-embedded spheroid knockouts that were stained with osmium and uranyl acetate, they were grown by Luke Thornton and sectioned by Denise McLean. Using these spheroids, I used a transmission electron microscope (TEM; Tecnai-12), to image cross-sections through the core of the spheroids.

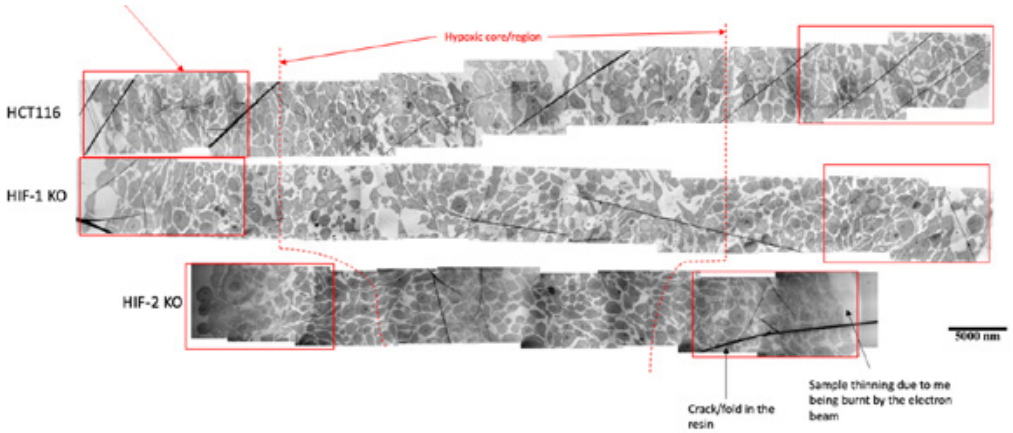


Figure 1. Transmission electron microscopy overviews of the control, HIF-1 and HIF-2 HCT116 spheroid knockouts. I have marked regions considered the hypoxic core and outer regions used for the pilot analysis.

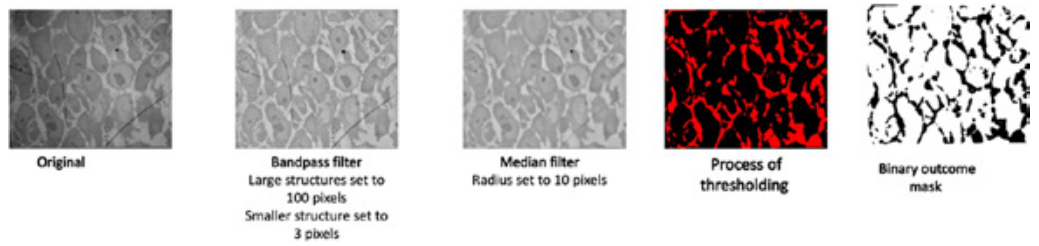


Figure 2. Shows the process of taking a micrograph and converting it to a binary mask. The mask is used to work out the percentage of area that is cells using thresholding. Sample demonstrated is from the outer region of the control spheroid.

Firstly, I imaged the full diameter of the spheroids, at a microscope nominal magnification of 1050x. Once I had the images, usually seven at this magnification, I used Fiji software to edit the images creating a stack allowing me to piece them into one montage on MosaicJ, a plugin for Fiji (Figure 1). I also took images at higher magnification at the spheroids to allow me to observe and quantify changes in the mitochondria and other organelles.

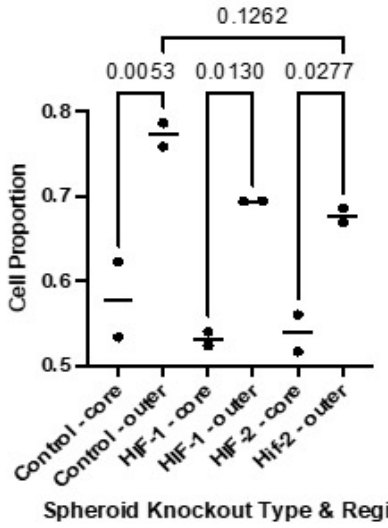


Figure 3. Proportion of spheroid cellular content is higher in the outer region. The area that is cell as a proportion of the total area for the different spheroid groups.

With these micrographs I was able to perform analysis on the spheroids structure between the regions. Firstly, to determine the ratio of cells to total micrograph area I used both filtering then thresholding to segment the cells and the intercellular space (Figure 2). I expected in the hypoxic core of the spheroid there to be a smaller area taken of cells, from many dying due to hypoxia, than the outer regions.

Next, I worked out the proportion of cells in the different regions of the spheroids. I used the cell counter plugin in Fiji. I determined if cells were alive, at time of fixation, depending on if their overall appearance had the characteristics of alive cells or necrotic cells. I used the results to analyse the proportion of cells in the spheroids and the ratio of proportion of cells in the core compared to the outer regions.

Finally, I analysed the length of the mitochondria using the high magnification images taken at different regions of the spheroids. I worked out the length of a large majority of the mitochondria in each of the knockouts to see if this influenced the sectioned size of mitochondria as an indicator of preferred orientation of the cells.

What did I find out?

I used the areas marked 'outer regions' and 'hypoxic core' in figure 2 to perform regional analysis on the necrotic proportion of cells and the spatial organisation of the core.

There is consistent difference by observation, even in this n=2 pilot, in the number of non-necrotic cells in the core compared to the outer regions (Data not shown). The non-cellular space was significantly less in the core for all groups (Figure 3) and indication of changes in adhesion. Further, there was a trend that in the knockouts there was more adhesion between the cells in the outer regions compared to cells in the hypoxic core. As a significance is seen we will continue to look at this with a larger sample size. I also tried normalising to the outer region (figure 4), which may with

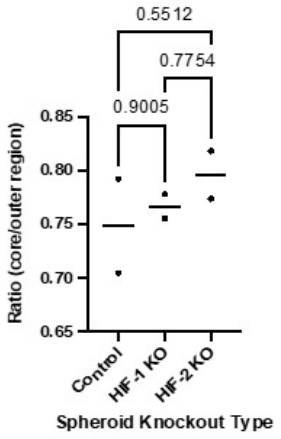


Figure 4. Portion of hypoxic core cellular content normalised to the outer region shows no significance with only 2 spheroids per group.

increased sample size turn out to be a more robust measure, but here there was no significance.

Using the higher magnification micrographs, I also compared the length of the mitochondria in each of the spheroid knockout types (Figure 5). As expected, (as the mitochondria are sectioned tubes) I did not see any significance between spheroids or groups indicating there was no difference in cellular orientation/alignment induced by knockout.

From this initial pilot study, I can continue the analysis on a complete data set and look specifically for intercellular connections, quantify cristae and likely changes in the ER. Further, we also high pressure froze the samples for mass spectrometry imagining (3D-OrbiSIMS) so watch this space!

What did I learn?

I learnt to resin embed my spheroids (post osmium steps). I was trained (using autophagosomes for a

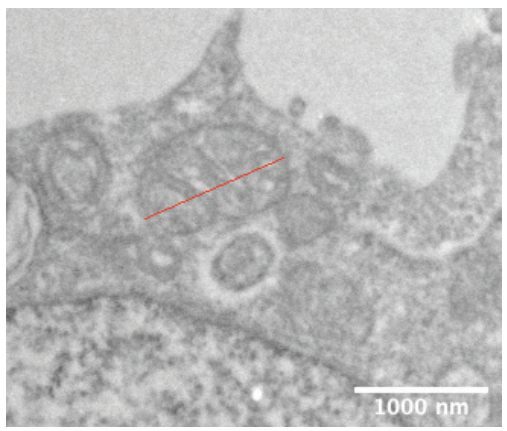


Figure 5. A high magnification image of a mitochondrion. The red line shows how I measured each single mitochondrion for analysis.

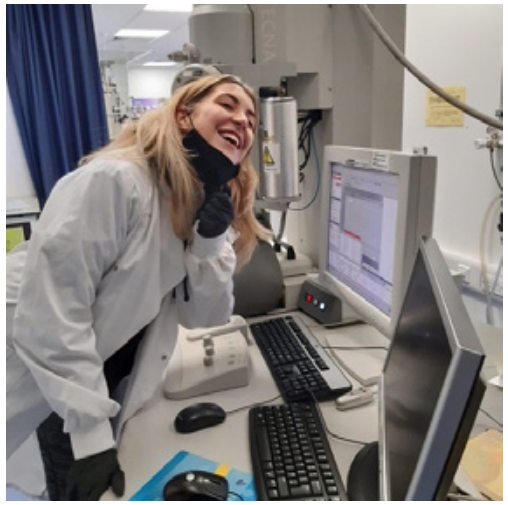


Figure 6. Me next to the Tecnai-12, which I used for my imaging.

related project) to use the Tecnai-12 independently by Dr Julie Watts. I also was able to spend some time on a scanning electron microscope (Zeiss 550 crossbeam) in transmission mode, with Dr Jacqueline Hicks. From this I was able to use the images for comparative microscopy, looking especially at the resolution (or not) of double membranes in the nucleus. I had a great time and managed to join in with many of the general processes within the *nanoscale and microscale research centre*.

I also had an opportunity to present my work to others at different stages of the studentship. This allowed me to grow my confidence in presenting, particularly with this type of data.

How has this affected my long-term goals

From undertaking this summer studentship, I was able to confirm that I would like to have a future career which includes imaging on a nanoscale. Halfway through this placement I decided to transfer from BSc to MSci version of my degree, This summer studentship was key in securing me a place on the MSci, as I gained both lab experience and became confident that I would enjoy the extra year of the course. The MSci includes a nine-month placement in either academia or industry, I am hoping to have a placement which has multiple microscopy elements.

Synchrotron X-ray Nanoprobe Analysis of Archaeological Samples: A Feasibility Study

Student: Jack Pearce
Supervisor: Julia Parker (Diamond Light Source) with Anita Radini (University of York)
Project location: Diamond Light Source and University of York

The summer studentship carried out jointly with Diamond Light Source and the University of York aimed to explore and test the use of synchrotron X-ray Nanoprobe analyses for the study of archaeological remains.

Introduction

Diamond Light Source is the UK's national synchrotron facility. Synchrotrons accelerate electrons in a storage ring where they emit radiation (from UV to X-rays wavelengths). Each beamline uses a specialised setup, energy range, resolution and technique to analyse the internal makeup of a sample, for example X-ray diffraction (XRD) or spectroscopy. Beamline I14 is a Hard X-ray Nanoprobe beamline using X-ray fluorescence (XRF), diffraction and imaging techniques for mapping elemental composition, and structural variations with 50nm resolution [3].

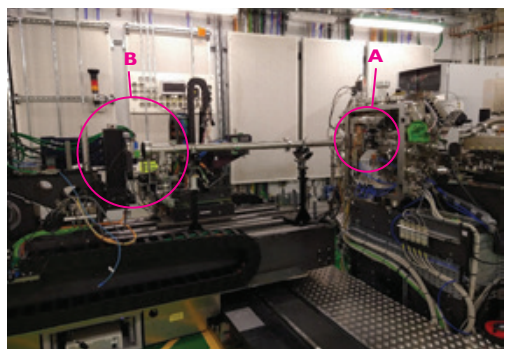


Figure 1. The I14 beamline experimental hutch showing the endstation where the sample was mounted (A) and detector (B).

Analytical techniques used in archaeology and bioarchaeology include XRD, Raman spectroscopy and electron microscopy to study various aspects of historical samples. XRF has been used in various other studies in archaeology such as by analysing pottery fragments to study their composition [1], artefacts such as historic astrolabe [2] and to assist in criminal investigations through the analysis of dental resins [4]. Each of these techniques has its own unique set of advantages and disadvantages, including ease of sample preparation, achievable resolution, and field of view. In this work we have used nano- XRF in order to give a high resolution map of the elemental composition of the samples, and aimed to test the feasibility of preparing suitable samples, their integrity during X-ray exposure and develop knowledge of methods for subsequent data processing and analysis.

Methods

The samples were prepared for analysis using a gallium FIB, milled to a size of 20 x 10 x 0.5 microns mounted onto Copper Onmiprobe grids. The thickness of the samples was chosen to give a good balance between penetration of the X-rays, signal strength and not to compromise the resolution of the X-ray probe.