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# Three dimensional reconstruction of ductal carcinoma *in situ* using virtual slides

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## Abstract

*Aims*

This study aimed to assess the feasibility of using virtual slides to create 3D histopathological reconstructions to aid in the study of the biology of DCIS .

*Methods*

4µm thick serial sections of formalin fixed paraffin embedded tissue from three cases were cut and mounted onto glass slides, stained with haematoxylin and eosin, then scanned. The three image stacks comprised 30, 115 and 100 scanned sections creating a similar number of virtual slides. The virtual slides were registered using custom 3D software to create 3D tissue volumes. The volumes were annotated to highlight distinct features and 3D visualisations (segmentations) were created to study these features in 3D.

*Results*

The most time-intensive step was the manual annotation of virtual slides 3D histopathological reconstructions were created of a) DCIS surrounded by adjacent invasion; b) pure DCIS and c) a 'normal' lobule.

*Conclusion*

3D *in silico* reconstructions of DCIS were created and more extensive studies can now be done within a realistic timescale. We have identified structural similarities between a benign lobule and DCIS which support the view that much DCIS, apparently in a 'duct' is contained within and expanded lobule. This method has the potential to provide insights into the biology of DCIS.

Word count: 194

Key Words: 3D, DCIS, Breast cancer, image analysis.

## Introduction

Ductal carcinoma *in situ* accounts for 20% of breast cancers identified through the National Health Service Breast Cancer Screening Programme (NHSBSP) [1] There is a growing awareness that such *in situ* disease is heterogeneous with respect to pathological features, progression and clinical outcome[2-5]. Awareness of the fact that some cases of DCIS, if left alone, would cause no symptoms during a patient's lifestyle, has led to concerns of 'overdiagnosis' and 'overtreatment' [6, 7] Despite investigation of clinical[8-13] histological[5, 14] and molecular features[10, 15-18] it still cannot be predicted which cases of DCIS will progress to the life-threatening invasive carcinoma.

Much current information regarding DCIS, has been discovered through analysis stained slides cut from paraffin blocks[19] with evaluation of two dimensional (2D) images. It is possible that a better appreciation DCIS in three dimensions (3D) might provide additional insights, for example whether the disease is truly continuous in affected areas or where there is co-existent invasive disease, multiple origins to invasion the norm or is this usually from a single point..

Early 3D studies were performed by Wellings et al. in the late 1960s and 1970s[20-22]. Their methodology involved matching subgross images (2.5-10 magnification of 2mm thick sections), with corresponding histological sections (6-8µm thickness). Today the tools available to study 3D histology have improved substantially such that high resolution archivable digital images may permit more elaborate studies. It is now possible to generate image stacks of serially sectioned tissue[23, 24] and most recently, computer-generated 3D reconstructed graphical models of breast carcinoma[25, 26]. Such reconstructions have demonstrated differences between tumours that appear histologically similar in 2D[25]. The Leeds Institute of Cancer and Pathology has previously employed 3D histopathological techniques to reconstruct a variety of tissues, including liver and kidney, providing detailed visualisation of structural features and spatial relationships[27].

The primary aim of this project was to assess the feasibility of the use of whole slide imaging to produce 3D reconstructions of DCIS, *in silico* with a view to increasing understanding of the biology of DCIS in 3D and its relationship to invasive disease. We also made an assessment of the timings of each of these steps to give baseline data upon which we can consider ways of increasing the efficiency of such studies. The ultimate, but not immediate, goal of this programme of research is to gain insights that will help the recognition of 'dangerous' DCIS, which has a high chance of progression, from that which is relatively safe.

## Materials and methods

*Case selection*

Single sections were cut from each of 35 formalin fixed paraffin embedded (FFPE) blocks from 18 breast cancer cases from the Leeds archival breast tissue collection. These were stained with haematoxylin and eosin (H&E), according to standard protocol. From each block, 2 stained slides were reviewed by authors MB and VS and presence of DCIS within each case was confirmed by a specialist breast histopathologist (AH). Tissue quality and quantity (thickness) of remaining tissue and pathology of interest in the block were key selection criteria. From this initial cohort 3 cases, arbitrarily lettered A – C were picked to study. Case A was used to trial the necessary tissue thickness to gain useful information, B and C to gauge the usefulness of the technique for two different scenarios; B to look at the relationship of invasion to DCIS and immediately adjacent invasion and C to compare a benign lobule and nearby region of DCIS. The ethical permissions to use this material were covered under application 06/Q1206/180.All material studied was anonymised in accordance with the Data Protection Act.

*Sectioning and staining of selected group*

From each block, sequential 4µm thick serial sections were cut and mounted onto Superfrost Plus glass slides and sequentially numbered in order of cut, with the same orientation throughout. The slides were H&E stained as previously described.

*Scanning*

Stained slides were scanned using Aperio ScanScope slide scanners (Aperio Technologies, Vista, CA) at a 20x objective lens, creating virtual slides.

*Registration*

"Registration" is the process of aligning images in stacks, so that the features in them align to form smooth 3D features. Registration results in a stack of images which have been aligned and can be rendered in 3D as a 3D "volume". This "volume" is a 3D image, which is analogous to a cube of glass containing the H&E stained tissue.

The virtual slides were registered by NR using Slice Registration Application (SliceRegApp) program, (version 11.1.1 [64bit OpenMP build], University of Leeds, UK) as previously described [27, 28]

For this a reference image was selected in the middle of the image stack and was used to align subsequent images proceeding out from the centre aligning all images to their neighbours. This aligned each virtual slide to adjacent slides within the dataset. Subsections were selected and re-registered for two cases B and C, in order to provide a higher resolution volume enabling smaller cellular components to be visualised. A subsection containing DCIS and surrounding invasive carcinoma was selected from case B. One subsection containing a 'normal' lobule and one subsection containing an area of DCIS with no invasive carcinoma were selected from case C.

*Segmentation*

In order to separate different elements of this dense 3D tissue volume, we apply a visualisation technique called "segmentation". This may be manual (as in this work) or automatic . During segmentation, features in every image in the 3D volume/stack are annotated in software. The resulting 2D annotations can be visualised in 3D to see the shape of the object of interest, usually separate from the background 3D volume. Volume Viewer (11.1.1 [64bit OpenMP build], University of Leeds, UK)[28] was used manually segment features chosen to be visualised, as shown also in **Figure 1**. Segmentation was performed using a combination of a variety of methods: manual annotation, using the fill tool and using the 'colour exemplar threshold' method[27], which automatically selects connected pixels of a similar colour. A graphics tablet and stylus were used for all segmentation ( Wacom Cintiq 21ux).

The components segmented from the case B subvolume included DCIS and adjacent invasion (**Figure 1**). These and also lumen contour were segmented in separate layers. Only the invasive tumour immediately adjacent to the tumour was investigated as it was felt this would be that most likely to connect with the DCIS. The overlap of these segmentations was reviewed to look for possible regions of invasive origin form the *in situ* disease. Any necrotic debris within lumina, was included in the segmented area of lumina. Lumina were classified as such if the diameter was greater than double the diameter of adjacent nuclei.

The two subsections from case C were annotated to identify the ductal system. In order to deal with the larger scale of the structure analysed a lower resolution image of the DCIS containing subvolume was produced and therefore, when segmenting the ductal structures from case C, spaces and their contents mapped together.

The Volume Viewer program function 'isosurfacing', which used information created from

segmentation, was used to create 3D colour visualisations of the volumes. "Isosurfacing" is a computer graphics technique to generate a smooth 3D segmentation by joining all the 2D annotations together in 3D with computer-generated surfaces. The isosurfacing algorithm joins the 2D contours in such a way as to generate as smooth a 3D shape as possible.

Multiple layers from case B were viewed simultaneously, to visualise how the different layers related to each other in 3D. The 3D isosurfaced reconstructions of the normal and DCIS containing ductal structures from case C were presented at the same scale and compared morphologically.

The Volume Viewer program function 'volume rendering' allows the original virtual slide images to be superimposed onto the 3D reconstructions. The volume render function was used to superimpose the H&E stained images onto the normal and DCIS containing ductal structures of case C.

*Segmentated volume calculation*

The total volume segmented was calculated for both subsections from case C, using **Equation 1**. The voxels (volumetric pixels) segmented and total number of voxels were found using the Volume Viewer function 'count voxels'. The volume of the selected subvolumes was calculated by multiplying lengths in the x, y and z axes. The x and y lengths were directly measured using ImageScope (version 11.2.3.780, Vista, CA); the z length was calculated by the number of sections by thickness (4µm). The thickness of sections lost due whilst sectioning was assumed negligible and therefore ignored.

$$volume\ of\ segmentation = \frac{number\ of\ voxels\ segmented}{total\ voxels\ in\ subvolume} \times volume\ of\ subvolume$$

## Results

The aggregate timing of each key step involved to produce the 3D reconstructions are shown in **figure 2**. The steps of digital scanning and image registration did not involve human labour for the most-part (i.e. beyond setting up), whereas the other steps did. The most time-intensive step is the manual annotation (segmentation) of virtual slides, whereby details of interest are highlighted on a tablet computer using a stylus.

*Specimens*

Both blocks from case A and case B contained areas of high nuclear DCIS and surrounding grade 3 invasive carcinoma. The block from case C contained pure high nuclear grade DCIS and several adjacent benign lobules.

*Registration*

An image stack was successfully created from case A. This use of 30 slides, resulted in a thin stack. This was a valuable exercise as it was apparent this depth was sub-optimal to appreciate the 3D architecture and that to appreciate this larger stack sizes are necessary, guiding block selection for additional and future studies.

The 115 H&E stained slides from case B were reviewed before reconstruction. Ninety were suitable and therefore included in the registration. A subvolume containing a focus of DCIS was selected and re-registered. **Figure 3a** shows the successful image stack created from this subvolume.

On review of the H&E stained slides from case C, all 100 were suitable for and therefore included in registration. Two subvolumes containing i) a benign lobule and ii) an area of DCIS were selected for re-registration The lobule was present in 79 of the 100 slides; 79 slides were therefore included in this subvolume. The DCIS was continuous throughout the depth of the sampled tissue and therefore all 100 slides were included in this subvolume. **Figures 3b** and **c** show the image stacks created these subvolumes.

*Segmentation*

Due to the insufficient depth of the stack, no segmentation of case A was performed.

A 3D reconstruction of the subvolume of case B containing DCIS and surrounding invasion was created (**Figure 4**). **Figures 4a** and **b** show the DCIS and the surrounding invasion and **c**, the DCIS alone The surrounding invasion was extensive. No continuity between *in situ* and invasive disease was identified. **Figure 4d** shows the lumina within the DCIS. The lumina were mostly continuous throughout the ductal structure, with additional multiple small, unconnected lumina. These 3D images can be rotated on the computer screen.

**Figure 5** shows the 3D reconstructions of the normal and DCIS containing duct-like structures from case C at the same scale. The DCIS containing structures were clearly much larger than the lobule in the same case. The volume of the segmentations of the normal ductal structure and DCIS were 0.00231mm$^3$ and 0.312mm$^3$ respectively. The DCIS segmented was therefore 135 times the volume of the lobule.

The isosurfaced visualisation of the lobule showed a complexity was of a higher order than was apparent from any one section in 2D and indeed of that commonly portrayed in histology and histopathology texts. (**Figure 5b)** The DCIS containing structures were less complex i.e. there were fewer branches than in the normal lobule but are more complex than normal true ducts.

## Discussion

In this project, we set out to assess the do-ability and practical feasibility of generating 3-D reconstructions *in–silico* of DCIS, associated invasion and normal tissue.

We demonstrated the creation of high quality 3D reconstructions of DCIS, providing scope for further, more detailed analysis of DCIS in 3D.

This methodology, we believe, has great potential to generate biological insights into the biology of DCIS. For example this approach would be a good way to ascertain whether the origin of invasion from DCIS is generally widespread in invasive cancers or more commonally a focal phenomenon. If the latter were true, investigations into progression would need to take into account the local nature of the phenomenon.

To have necessary power to test hypotheses generated by this pilot study such we plan to study larger numbers of samples and would seek to improve the efficiency of the methodology in order to do this. Other researchers have studied the use of automated sectioning[23] and staining[23, 25] with comparable results. Whilst the scanning and image registration take a considerable amount of time, much of this does not involve human labour. The time taken to segment is, however, a major factor contribution to the total time, but can be considerably reduced by efficient working. During this study, it was noted that fine detail included in the segmentation may be unnecessary, thus reducing the resolution the time taken doing this task can be reduced. The accuracy of segmentation was discussed by Norton[26], who stated that 90% accuracy was not necessary to determine the 3D structure.

Norton[26] developed an algorithm that enabled successful automatic segmentation of cribra of DCIS, analogous to the lumina segmented in our study. However, such automatic segmentation is likely to be more difficult when the segmentation is more subtle than the stark contract between white spaces and the purple shades of H&E stained tumour cells. For example with regards to discriminating between DCIS and adjacent invasive disease.

It is conceivable that one future additional solution might be via a crowd sourcing/citizen science initiative such as has already been launched to aid the interpretation of immunohistochemistry (IHC) for large tissue-microarray(TMA) [29]. This would not be a trivial task, but if successful could speed up the most time intensive step of this process.

With regards to biological insights from this feasibly study, extreme caution has to be exercised due to the limited size of the cohort, nevertheless some of the observations inspire hypotheses on which to base future, larger scale research.

We observed that the DCIS segmentation was less complex, i.e. contained less branching, than the normal structure. One plausible explanation is that the tumour growth within the duct- like structure caused it to expand and merge with adjacent ductal structures, creating fewer, larger ducts. The DCIS structures were also much larger that the comparator lobule, whilst this study was not adequately powered to draw any conclusions with statistical significance, the fact that the largest benign lobule was chosen would suggest that this volume difference is more likely to be a conservative estimate in volume increase between typical normal and DCIS containing ductal structures. In order to really lay this to rest a reproducible way of defining the boundaries of these structures is necessary.

The morphological observations are fully supportive of the work of Wellings[21] who suggested the TDLU ‘unfolding‘ concept as an explanation of the large size and reduced branching observed in his subgross images of DCIS. The hyperbranching nature observed in our 3D reconstructions of both DCIS subvolumes (cases B and C) and the normal ductal structure (case C) are also in line with these earlier subgross 3D reconstruction studies [20, 21, 30, 31] and support the view that that most cases of DCIS actually originate from TDLUs, and not the larger true ducts[20]. TDLUs are surrounded by a layer of elastin, which is absent in the straighter, less frequently branching mammary ducts. In future work, to verify whether the reconstructed DCIS structures are lobules, an elastin histochemical marker will be incorporated.

As indicated above, the most important avenue in which we believe 3D visualisation may provide insight, is the *in situ* to invasive progression. Whilst the present study revealed an intimate relationship between DCIS and invasive carcinoma, nowhere could a definite *in situ* to invasive origin be found, despite a detailed reconstruction of a large DCIS structure. The fact that no such point could be identified raises the possibility that such this might be a rare event, possibly occurring infrequently throughout the whole tumour.

In conclusion, we have created 3D *in silico* reconstructions of DCIS and believe that more extensive studies can now be done within a realistic timescale. We have identified structural similarities between a benign lobule and DCIS containing 'ductal structures and demonstrated the ability to quantify the their volume. Our results support the view that much DCIS, apparently in a 'duct' is contained within and expanded lobule. No single local region of *in situ* to invasive progression was identified, suggesting that such a localised region was not a frequently occurring event in the case analysed. This method has the potential to provide key insights into the biology of DCIS and ultimately aid in the hunt for features of DCIS at higher risk of progression to invasive carcinoma.

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Booth M E, Treanor D, Roberts N, Magee D, Speirs V, Hanby AM

All the above contributed towards the drafting of the manuscript and gave intellectual imput.

Speirs V, Hanby AM, Treanor D contrived and initiated the study

Treanor D, Roberts N, Magee D developed and tested the technology used in the in silico aspects of the study

Booth M E did much of the 3D reconstruction *in silico* aided by Roberts N

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**Figure legends**

**Figure 1** shows breast cancer in a digitalised image in which the DCIS has been highlighted in green ( a) or the invasive disease in closest proximity to the DCIS in (b). This process is done throughout the whole stack of virtual slides.

**Figure 2** this schematic sumarises the timings in hours for all the major steps taken to generate a 3-D image in aggregate for all the three cases, within the brackets against each step. The key observation is that slide annotation is the most labour intensive, however we believe these timings can be considerably reduced. Volume rendering and isosurfacing can be generated automatically after the steps above and therefore no time is given.

**Figure 3** Shows the successful image stacks created from from the subvolumes of case B-DCIS and co-existent invasion (a) and case C, a benign lobule (b) from a case with DCIS (c) in the same block.

**Figure 4** Shows the selected 3D reconstruction of the subvolume in case B containing DCIS(yellow) and surrounding invasion(orange). **a)** and **b)** show the DCIS and the surrounding invasion and **c**, the DCIS alone. No continuity between *in situ* and invasive disease was identified. **d)** shows the lumina(green) within the DCIS. The lumina were mostly continuous throughout the ductal structure, with additional multiple small, unconnected lumina.

**Figure 5** shows the 3D reconstructions of the lobule (a) and DCIS from case C *at the same scale.* The DCIS containing structures are much larger ( b). The DCIS containing structures are less complex i.e. there were fewer branches than in the normal lobule .