3-D Tissue Modelling and Virtual Pathology as New Approaches to Study Ductal Carcinoma *In Situ*

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Summary — Widespread screening mammography programmes mean that ductal carcinoma *in situ* (DCIS), a pre-invasive breast lesion, is now more frequently diagnosed. However, not all diagnosed DCIS lesions progress to invasive breast cancer, which presents a dilemma for clinicians. As such, there is much interest in studying DCIS in the laboratory, in order to help understand more about its biology and determine the characteristics of those that progress to invasion. Greater knowledge would lead to targeted and better DCIS treatment. Here, we outline some of the models available to study DCIS, with a particular focus on animal-free systems.

Key words: *3-D, cell culture, DCIS, virtual pathology.*

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Introduction

Ductal carcinoma *in situ* (DCIS), a non-invasive stage of breast cancer, often precedes the development of invasive breast carcinoma. Currently, DCIS accounts for 20% of the breast cancers identified through the UK National Health Service Breast Cancer Screening Programme (NHSBSP; http://www.cancerscreening.nhs.uk/breastscreen/). The existence of cancer screening programmes, such as the NHSBSP, means that DCIS is now more frequently diagnosed. However, the evidence suggests that not all DCIS will progress to invasive breast cancer, and currently there is no robust way of knowing which DCIS lesions will become invasive. Left untreated, half of DCIS lesions do not progress to invasive disease (1, 2), but clinicians cannot identify which ones these are at the time of diagnosis. This leads, at least theoretically, to the unnecessary overtreatment of some patients. Considering that progression can occur — up to 20% of women diagnosed with DCIS will experience recurrence within 10 years, even after breast-conserving surgery and radiotherapy, with around half of these recurrences manifesting as invasive disease (3) — this presents a dilemma for clinicians involved in breast cancer management. As a result, there is considerable interest from the research community in the study of DCIS, in an effort to understand its biology and translate these findings into clinical management. These include programmes to audit and characterise the manifestation, treatment and natural history of DCIS, as well as to identify its molecular characteristics, e.g. the Sloane Project (http://www.sloaneproject.co.uk/). The LOw RISk (LORIS) study (4) is taking a more conservative approach to low-risk DCIS management, by comparing surgery with active monitoring by yearly mammograms.

In addition to these important clinical programmes, there is an urgent requirement to be able to model DCIS in the laboratory. Much of what we know about DCIS has been gained from the 2-D analysis of tissue sections cut from paraffin blocks, supplemented with histochemical or immunohistochemical staining (5). A recent notable immunohistochemical analysis of a large cohort of DCIS lesions showed that expression of αvβ6 integrin was significantly associated with progression to invasive cancer (6). However, limitations of this type of approach include the 2-D evaluation of what is, in reality, a 3-D structure. Better appreciation of this might be obtained by evaluating tissues in three dimensions. Furthermore, with researchers having to consider ways of fulfilling Government priorities in the *replacement, refinement* and *reduction* of animal use, while, at the same time, addressing public con-

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cerns about the use of animals in research, animal-free alternative models are required. Examples of approaches to DCIS modelling are outlined below.

Existing Laboratory Models of DCIS

In order to understand the events leading to DCIS development, a robust model of normal human mammary gland is required. While some effort has been made to model this *in vitro* in 2-D and 3-D culture systems, the current models of the normal human breast have significant drawbacks. These include: a) employing only a single epithelial cell line in 3-D culture, commonly MCF-10A cells (7, 8), when the normal mammary duct is more complex and multicellular; b) growing cells in non-physiological matrices, such Matrigel™, which is a solubilised basement membrane matrix extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, or using the EHS matrix itself, prepared from EHS tumours grown as xenografts in mice (7–10).

Matrigel or EHS-type matrices, while convenient to use in a practical sense, are not physiologically appropriate. We, and others, have demonstrated that, unlike Matrigel, collagen I is the principal basement membrane protein found in human breast tissue (11). Matrigel is heterogeneous in composition, containing collagen IV, laminin, entactin, heparan sulphate proteoglycans and growth factors (notably TGF-β and EGF), in amounts and ratios that can vary hugely between batches (12). Furthermore, existing models of DCIS typically include non-invasive cell lines, such as DCIS.com and SUM225, grown in 3-D culture in Matrigel (13), or, more typically, transplanted as subcutaneous xenografts into nude mice (14, 15). Moreover, none of these models mimic the microenvironment and heterogeneity observed in human disease.

In an effort to address this, the mouse intraductal (MIND) xenograft model has been developed, which involves intraductal transplantation of either DCIS-like cell lines (e.g. DCIS.com), or fragments of xenograft developed from a primary human DCIS sample, into immunocompromised mice (16). However, the development of DCIS-like structures is inconsistent in the MIND model, and can depend on the type of mouse strain used: for example, DCIS-like structures failed to develop in SCID beige mice, while in NSG mice DCIS-like structures only developed in around 50% of cases (17). Moreover, the mouse mammary gland is not a faithful model for the human mammary gland (18). Despite these shortfalls, the MIND model is being promoted by some experts as a suitable model to functionally test molecular events occurring in the initial changes in premalignant progression (19).

Multicellular 3-D *In Vitro* Models of DCIS

Recognition of the need for better models has resulted in the development of heterotypic co-culture models, which more-closely represent the histology of human breast tissue (20). At a very basic level, these comprise breast epithelial cells in co-culture with fibroblasts (21–24), extending to more-complex models with myoepithelial cells, monocytes, endothelial cells and adipocytes (25–30). The incorporation of lumenal and myoepithelial cells with mammary fibroblasts in collagen, means that the cells can organise themselves into structures recapitulating normal breast tissue and DCIS, with homing of myoepithelial cells around the luminal population (11, 31), which is a characteristic that is observed in human breast tissue. When morphologically and immunohistochemically compared with human breast tissue, lumen formation was observed in an *in vitro* model of normal breast tissue, with the correct spatial expression of biomarkers known to be present in normal human breast tissue (11). Furthermore, in this model, the overexpression of HER2 (also known as *ERBB2*, a recognised proto-oncogene) in breast epithelial cells resulted in the development of a DCIS-like phenotype, consisting of the loss of the ordered lumenal structures seen in the normal model, with cells growing into the centre of the lumen, expanding its size. These multicellular 3-D models have been shown to be potentially useful in cancer initiation studies (11).

Scientists have also used fragments of human DCIS to develop 3-D *in vitro* DCIS models. Farnie *et al.* (32) used the mammosphere culture system (33) to grow DCIS derived from human clinical samples. With a culture success rate of around 70%, they explored the role of Notch and epidermal growth factor receptor signalling pathways in DCIS (32). This approach is attractive, as the tissue is retained in its entirety, so the cell–cell interactions that exist *in vivo* are maintained. The human breast tissue required to develop these models is available from the Breast Cancer Now Tissue Bank (http://www.breastcancertissuebank.org/). As well as supplying tissue samples, the bank offers a cell culture programme that provides scientists with the specialised individual cell types necessary to build multicellular models. These 3-D *in vitro* models have the potential to replace animals as preclinical screens in the therapeutic discovery pipeline, exemplified by a recent report of the use of 3-D co-cultures as a high-content phenotypic screening system for fibrotic invasion (34), and the growing recognition of their promise as high-throughput preclinical screening tools (35, 36).

Microfluidics is emerging as a discipline in its own right, with several recent excellent reviews on the

subject (37, 38). Bespoke microfluidic devices (commonly termed 'lab-on-a-chip') have been developed to study various disease processes. Microfluidics approaches have advantages, in that they can improve the physiological relevance of 3-D cell cultures by introducing perfusion flow (akin to blood flow), and permitting the spatial control of the cocultures and signalling gradients, such as added growth factors or drugs which can be temporally modulated and delivered in a controlled manner. However, unlike the 3-D multicellular models described above, the validation of these models against clinical material remains limited (39). Nevertheless, these systems are starting to be developed for the study of DCIS (40–42). In contrast with the 3-D models, where different cell types are mixed together from the outset, cells of interest are typically added to different compartments of the microfluidic device, and then exposed to various gradients of, for example, growth factors or drugs. A further advantage of microfluidics is the requirement for much less input material, compared to that required for the multicellular 3-D models described above. This could be of benefit in breast cancer research, because tumour size at diagnosis is reducing as a result of better screening programmes and awareness, thus impacting on the amount of tissue that is available for research after a histopathological diagnosis is made. Microfluidics systems also lend themselves more easily to the addition of vasculature and immune components (39, 43), which is much more challenging in 3-D heterotypic models. However, microfluidics technology is not yet routine in research laboratories, although it is gaining traction as an emerging field.

In silico modelling of DCIS

Digital pathology is an expanding discipline that uses high-resolution scanning to create virtual slides. With appropriate software, these can be used to create 3-D reconstructions of tissues. Until now, this has been technically difficult and, consequently, not routinely available to the general research community, but it is starting to be applied more widely. Examination of tissue in 3-D can potentially enhance the study of disease processes. Technological advances in 3-D reconstruction, together with whole-slide imaging, have permitted the creation of image stacks of serially sectioned tissue (44, 45) and, most recently, computer-generated 3-D reconstructed graphical models of breast carcinoma (46). Our group has employed novel whole-slide high-resolution 3-D virtual microscopy to reconstruct a variety of tissues, including liver and kidney, providing detailed visualisation of structural features and spatial relationships (47, 48). Such reconstructions have demonstrated differences between tumours that appear histologically similar in 2-D.

More recently, we have applied this to the study of breast cancer (49). The workflow (shown schematically in Figure 1) involves the generation of multiple serial sections of tissue (ideally around 100 × 4μm sections). Following haematoxylin and eosin (H&E) staining, these are scanned to create a digital image. At this point, it is also possible to incorporate immunohistochemical stains into the model, as shown for colorectal cancer (50), or to apply this to radiological images (47). Thereafter, the virtual slides are aligned to adjacent slides throughout the depth of the image stack (a process called 'registration'), and features of interest are highlighted on each 2-D virtual slide (a process called 'segmentation'). Images are then reconstructed in 3-D, by using software developed by the University of Leeds, UK. The 3-D reconstructions can be rotated in all planes, permitting the model to be viewed from the desired angle, and allowing a perception of depth that is unique to 3-D visualisation. Although some of these processes can be automated, a major limitation of this method of 3-D reconstruction, currently, is the time taken to produce reconstructions, particularly the segmentation process. This is because many features of interest on H&E staining can only be accurately detected by the human eye. Nevertheless, this technique has enabled the successful creation of 3-D reconstructions of normal breast ducts and DCIS. Same-scale images have shown that the latter is much larger and less complex than the complex branching structure seen in the healthy tissue (49). While still in its infancy, 3-D tissue modelling of DCIS by using virtual pathology may help scientists recognise features of 'dangerous' DCIS (i.e. those lesions with a high risk of progression), as compared to those of lesions not in need of immediate surgery.

Conclusions

The animal-free *in vitro* and *in silico* models that we have described provide scope for understanding how DCIS develops, and may be useful test systems for examining the progression from DCIS to invasive disease, or for studying inhibitors that may prevent DCIS recurrence. Their pros and cons are illustrated schematically in Figure 2, which was compiled from recent publications (38, 47, 49, 51–52). Significantly, such *in vitro* and *in silico* systems offer robust alternatives to animal models, and can assist in addressing an important problem, by facilitating the uptake of scientific advances by clinicians.

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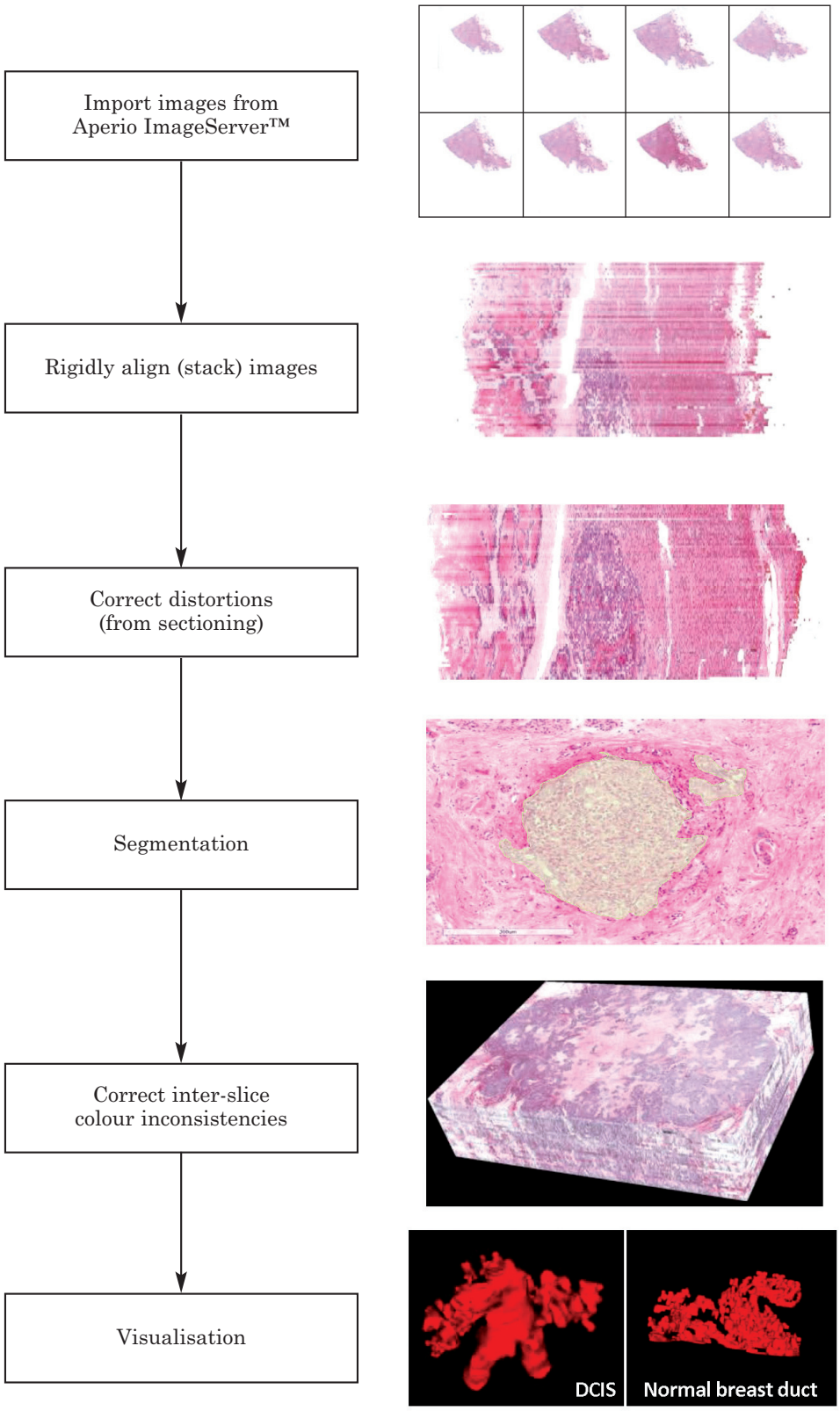
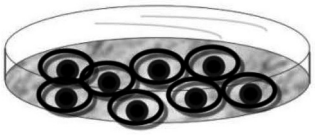
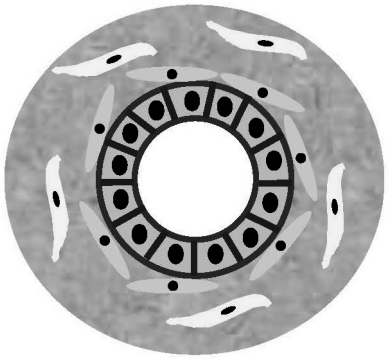
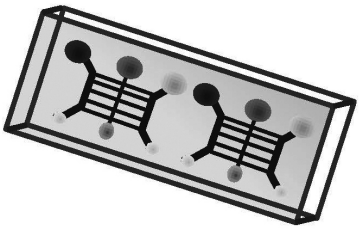
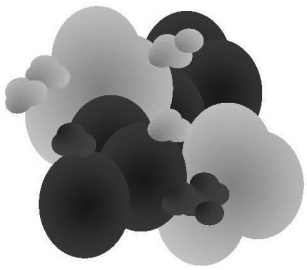
Figure 1: Experimental workflow for the generation of 3-D *in silico* models of breast tissue



Multiple serial tissue sections are prepared, stained and scanned to create virtual slides. Images are aligned, then the features of interest are highlighted on each virtual slide (a process called 'segmentation'). Following colour correction, images are reconstructed in 3-D by using proprietary software. The 3-D reconstructions can be rotated in all planes, allowing a perception of depth that is unique to 3-D visualisation.

Figure 2: Schematic representation of the various ways in which tissue can be modelled, and their advantages and disadvantages

Advantages		Disadvantages
Widely used Easy to propagate Suitable for multiple cell panels Amenable to multiple analyses High-throughput	 **2-D cell culture**	Polarity is lost Lacks extracellular matrix (but can be added) Lacks intercellular communication Lacks vasculature and immune system
Polarity and architecture maintained Multicellular Nutrient and redox gradients present	 **3-D cell culture**	Costly; low-throughput Limited ability to quantify Advanced imaging often required Large amounts of tissue required
Good physiological relevance Perfusion flow Spatial control of signalling gradients Real-time analysis Requires less tissue	 **Microfluidics**	Non-standard cell culture Complex operational control Requires validation against clinical material Not yet routinely used
High-quality 3-D tissue visualisation Polarity and architecture maintained Insights into invasive cancer development	 ***In silico* modelling**	Time-consuming Labour-intensive Requires specialised computing

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