SEARCHBreast Workshop Proceedings: 3D Modelling of Breast Cancer

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Summary — SEARCHBreast, a UK initiative supported by the NC3Rs, organised a workshop entitled *3D Modelling of Breast Cancer*. The workshop focused on providing researchers with solutions to overcome some of the perceived barriers to working with human-derived tumour cells, cell lines and tissues, namely: a) the limited access to human-derived material; and b) the difficulty in working with these samples. The workshop presentations provided constructive advice and information on how to best prepare human cells or tissues for further downstream applications. Techniques in developing primary cultures from patient samples, and considerations when preserving tissue slices, were discussed. A common theme throughout the workshop was the importance of ensuring that the cells are grown in conditions as similar to the *in vivo* microenvironment as possible. Comparisons of the advantages of several *in vitro* options, such as primary cell cultures, cell line cultures, explants or tissue slices, suggest that all offer great potential applications for breast cancer research, and highlight that it need not be a case of choosing one over the other. The workshop also offered cutting-edge examples of on-chip technologies and 3-D tumour modelling by using virtual pathology, which can contribute to clinically relevant studies and provide insights into breast cancer metastatic mechanisms.

Keywords: *3-D models, animal replacement, breast cancer, humanised breast cancer models.*

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Introduction

SEARCHBreast (Sharing Experimental Animal Resources: Coordinating Holdings in Breast Cancer; https://searchbreast.org) was developed, with support from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs; https://www.nc3rs.org.uk), as a means of connecting breast cancer researchers with expertise spanning *in vivo*, *in vitro* and *in silico* models of breast cancer, and aims to create a portal to facilitate the sharing of archival material derived from *in vivo* studies. This multilateral approach aims to deliver a greater understanding of breast cancer biology, whilst addressing each of the Three Rs.

A Workshop on *3D Modelling of Breast Cancer*

One of the aims of SEARCHBreast is to promote the use of humanised breast tissue models as *replacement* alternatives to animal experimentation, as a means to actively address one of the Three Rs. On 21 May 2015, SEARCHBreast organised a workshop entitled *3D Modelling of Breast Cancer*, at Barts Cancer Institute, London, UK. The goal of the workshop was to demonstrate to participants that replacing animals with human-derived tissues or cell lines does not diminish the quality or applicability of the research, but can often be more relevant to the human disease. The models discussed included:

— primary cultures;

— 3-D co-cultures;

— precision-cut tissue slices;

— organ-on-a-chip models; and

— *in silico* 3-D models.

The workshop focused on three core principles: a) practical considerations of handling humanised cell and tissue models; b) the importance of recapitulating the *in vivo* tumour microenvironment; and c) examples of how these samples are used in the laboratory. To maximise audience engagement and knowledge exchange, the format comprised eight short presentations split over two themed sessions, each culminating in a panel discussion with interjections from both speakers and the audience. In addition, a plenary speaker, Dr Anja

van de Stolpe, working at Philips Research in Eindhoven, The Netherlands, and the representative of the recently-founded Institute for Human Organ and Disease Model Technologies (hDMT; http://hdmt.technology/) in The Netherlands, presented an overview of the emerging 'organ-on-a-chip' technologies. Finally, there were opportunities for a number of early-career researchers to present their research, selected from the submitted abstracts. Recipients of the early-career travel bursaries presented posters during the networking session, and gave a 1 minute/1 slide taster of their posters. These presentations were very well received, as they provided all the workshop participants with a snapshot of a range of research projects. The poster presentations included:

— The role of Bag-1 in acinar morphogenesis and breast cancer (Caroline Barker, University of Southampton);

— Targeting endocrine-resistant breast cancer stem cells with the novel protein, FKBPL, and its peptide derivatives (Stephanie Annett, Queen's University Belfast);

— Generation and characterisation of primary lobular breast cancer cells and fibroblasts (Laura Gomez Cuadrado, University of Edinburgh);

— Human breast cancer bone metastasis — a novel 3-D model system for studies of tumour cell–bone cell interactions (Penny Ottewell, University of Sheffield);

— Modelling tumour invasiveness using engineered scaffolds (Robert D. Hume, University of Cambridge);

— Developing and utilising a 3-D mammary organoid model for the study of the Wnt pathway in cancer (Mairian Thomas, Cardiff University); and

— Effect of ionising radiation on 3-D models of normal breast tissue (Emma Waring, Queen's University Belfast).

The posters were of considerable quality, and were delivered by enthusiastic early-career researchers. We look forward to the publication in due course of the work presented by these researchers.

The workshop proved to be of high interest, with over 130 delegates from across the UK, Ireland, The Netherlands and the USA. The broad geographical distribution of UK participants (Figure 1a) will help achieve the goal of widespread dissemination of the message across the country. Importantly, many of the delegates who attended the workshop were early-career researchers (Figure 1b), meeting one of the aims of introducing the next generation of scientists to alternative experimental methods that avoid the use of animals. The themes covered in the workshop are described below.

Practical complexities associated with the use of human tissue and cells

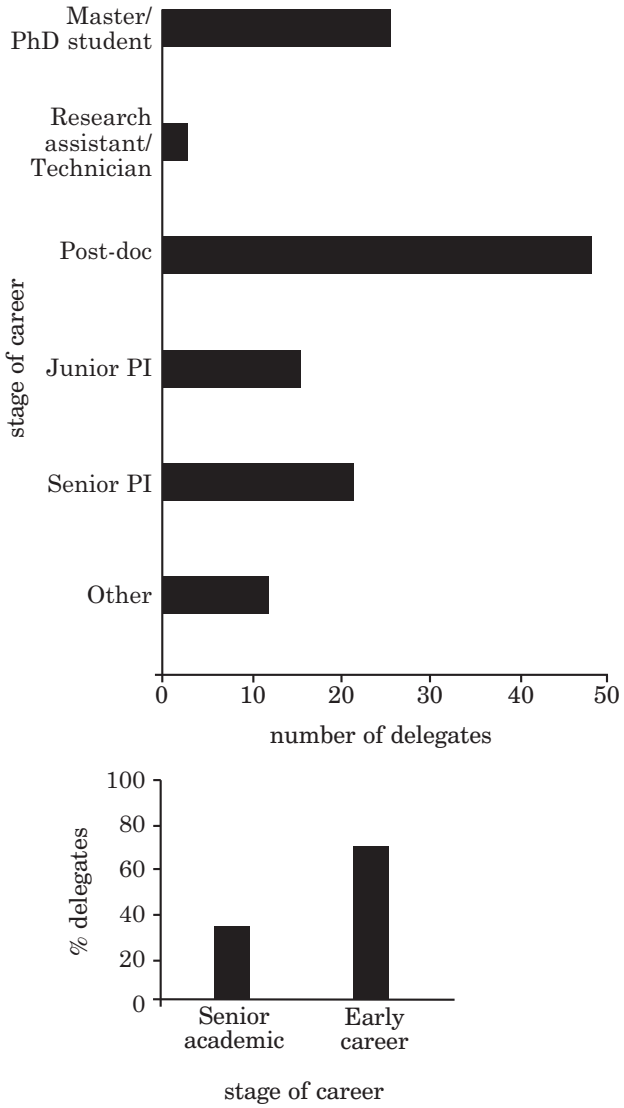
In order to develop human models, a source of high-quality material is needed. It was fitting then that the workshop started with a presentation by Dr Jenny Gomm, from the Barts Cancer Institute, on behalf of the Breast Cancer Now Tissue Bank (BCNTB; http://www.breastcancertissuebank.org/). The multi-site BCNTB, is the UK's first national breast cancer tissue and cell bank, and currently has close to 32,000 samples, collected from around 8,000 patients. In addition to formalin-fixed human breast tissue samples, the BCNTB offers a bespoke cell culture programme that prepares primary human breast cells and viable tissue explants to meet the individual requirements of a particular researcher's needs. Cells can be isolated and purified from tumour and uninvolved surrounding tissues, normal tissues, reduction mammoplasties, and prophylactic mastectomies (Figure 2a). The primary culture material currently available to researchers includes purified epithelial and myoepithelial cells, fibroblasts and tissue explants, which can include fibroblasts, epithelial cells, malignant epithelial cells and myoepithelial cells. Dr Gomm presented a number of protocols established by BCNTB, emphasising that, whilst working with human-derived breast cancer cells is regarded as being notoriously difficult, with the support of the BCNTB cell culture programme, researchers can now have access to this material. This is significant progress toward overcoming one of the perceived barriers to the use of human tissue.

A practical workflow on how to best handle tumour tissue after removal from the patient, in order to ensure its viability and maintain its physiological relevance in further downstream applications in tissue slice cultures, was eloquently presented by Dr Emma Davies, AstraZeneca, Macclesfield, UK, on behalf of the Innovative Medicines Initiative (IMI) project, PREDECT (www.predect.eu). In the first stage of the process, fresh tumour tissue is sliced on a vibrating microtome. Analysis of samples generated by this procedure has confirmed that minimal stress is imposed on the cells at this stage, and that the cells represent a close *in vivo* approximation. After testing several conditions, it was found that the most appropriate way to preserve tissue architecture is under atmospheric oxygen conditions, with the tissue placed on an Alvetex® Polaris filter support (1). The choice of filter is important: over time, the part of the tissue on the filter undergoes stress-related changes, which vary with filter type; the face of the tissue exposed to the atmosphere undergoes less of a physiological change than the side attached to the filter. These tissue slices can be used to determine cellular responses to different drugs, includ-

Figure 1: The geographical distribution of workshop delegates and their career-stage demographics

a) Delegate distribution across the UK and Ireland

b) The career-stage demographics of the workshop attendees



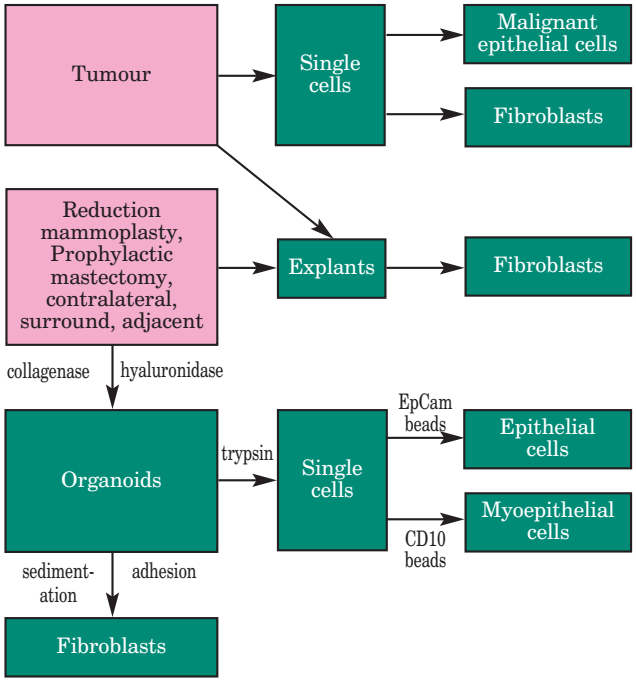
ing drug uptake, proliferation and induction of cell death (Figure 2b), and can also be used for biomarker discovery. Such models have the potential to offer an excellent platform for animal-free drug screening assays as preclinical screens for testing therapeutics (1).

The importance of recapitulating the tumour microenvironment

More than 90% of anti-cancer drugs in development fail during clinical trials and never reach the clinic. Dr Anna Grabowska, from the University of Nottingham, UK, suggested that more clinically-relevant preclinical models would help reduce this failure rate. During her presentation, Dr Grabowska explained that several factors, missing in the current preclinical assays, are contributing to the failure rate of drugs in trials. Two of them — the source of the cancer cells used and the environment in which they are grown — were discussed in her presentation. Currently, monolayer cancer cell lines are grown on plastic, with little consideration of the tumour microenvironment found *in vivo*. A drawback of more-established cell lines is the propensity for genetic drift, which means that the molecular profile of the cells can be

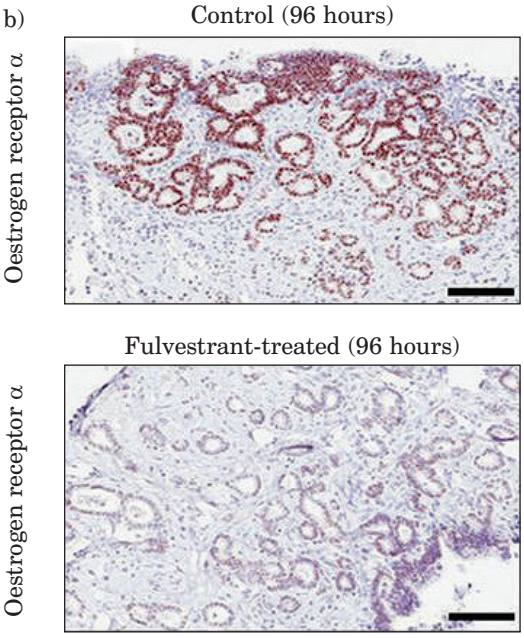
Figure 2: Examples of some of the technologies described at the workshop

a)



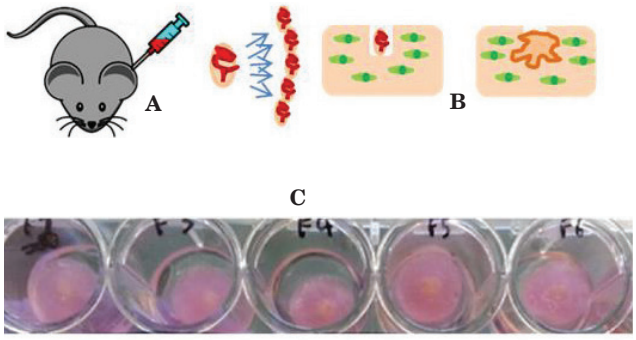
Workflow for the isolation and purification of cells from human breast tissue, as undertaken by the Breast Cancer Now Tissue Bank *(Dr J. Gomm).*

b)



Examples of tissue slice technology: Tissue slices (250μm) of a hormone receptor-positive primary human breast tumour were cultured on filter supports and treated with 100nM fulvestrant or DMSO control. After 96 hours of treatment, a pharmacodynamic response to drug treatment was observed in primary patient tumour cells, indicated by a reduction in oestrogen receptor α staining. Scale bars represent 100μm. (Dr E. Davies).

c)



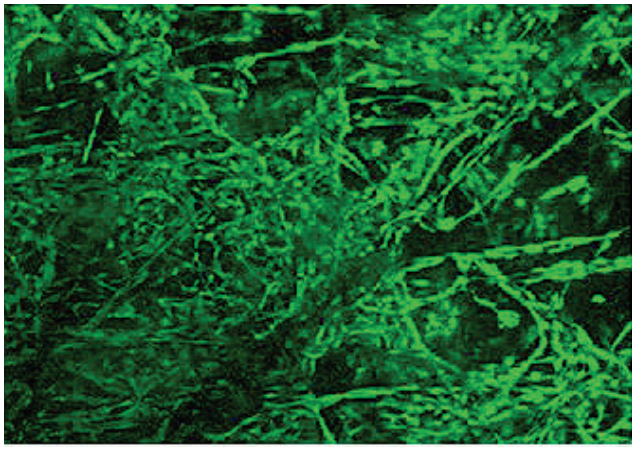
Scheme for tumour propagation, harvesting, seeding and culture in engineered collagen scaffolds:

***A** Balb/C mouse mammary gland was injected with 10,000 Her2 over-expressing syngeneic cells;*

***B** four weeks later, the tumours were collected and mechanically digested into smaller fragments; and*

***C** the fragments were placed into collagen scaffolds pre-seeded with 3T3-L1 cells that had been induced to undergo adipogenesis. Tumour cells were allowed to grow and invade into the collagen scaffold before being imaged. This can be adapted for human breast tissue biopsy fragments (Professor C. Watson).*

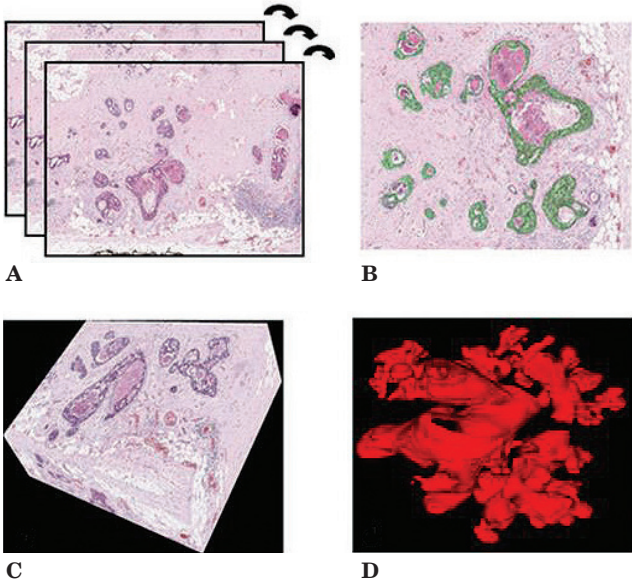
d)



Ten day-cultured, E18-harvested, primary cortical neurons in 7.5mg/ml protein Matrigel™ extracellular matrix in SeedEZ. This image shows Calcein AM/Propidium Iodide staining of live cells and dead cell nuclei at 10× magnification. The field of view is 1.181mm × 0.886mm. The overall culture thickness is 400μm (Ms J. Vukasinovic).

Figure 2: continued

e)



Workflow for the 3-D creation of human breast tissue by using virtual slides:

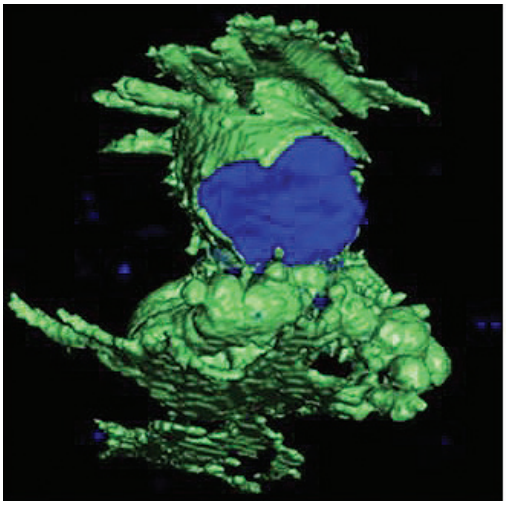
***A** multiple serial sections are prepared and H&E stained;*

***B** areas of interest are identified by segmentation; and*

***C** registration and colour correction are then applied before application of software to reconstruct the tissue in 3-D; and*

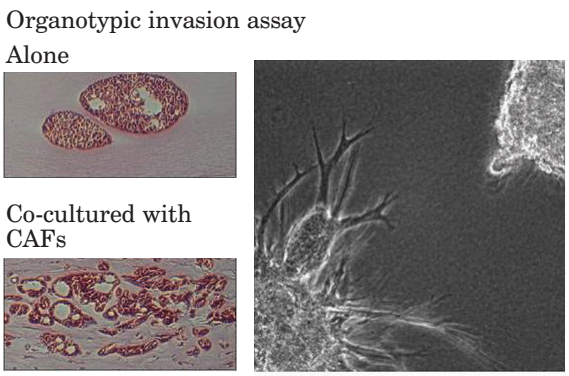
***D** virtual 3-D images can be visualised and analysed (Professor V. Speirs).*

f)



Optical Projection Tomography was utilised to reconstruct patient samples and measure the volume of residual epithelium (green = CK 8/18) in patient samples following exposure to tamoxifen, in order to determine tamoxifen sensitivity (Dr A. Leeper).

g)

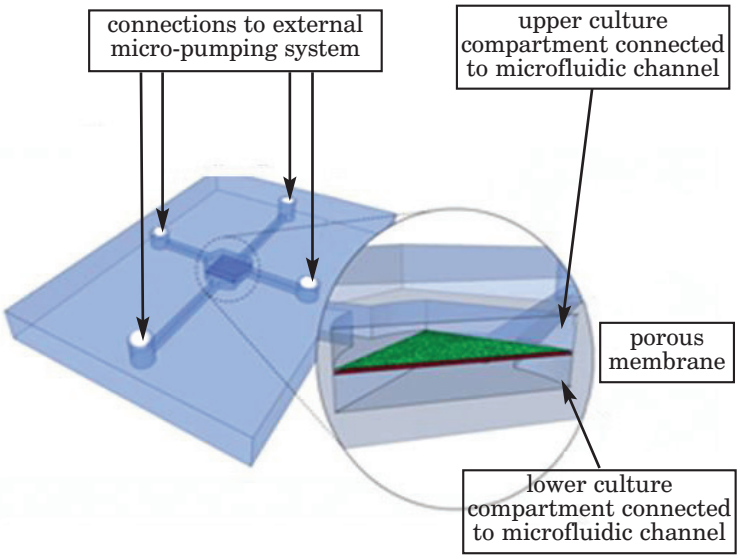


Co-culture with cancer-associated fibroblasts (CAFs) promotes breast cancer invasion.

The top left panel shows 410.4 breast cancer cells cultured alone in collagen-rich ECM; the bottom left panel shows the same 410.4 cells co-cultured with CAFs. Both panels show formalin-fixed and H&E-stained tissue.

The right-hand side panel shows phase-contrast microscopy of two 410.4 breast cancer spheroids (Dr E. Sahai).

h)



Design of a dual-chamber microfluidic device, in which a tissue sample can be cultured in the upper chamber and, separated by a porous membrane, an endothelial layer can be cultured in the lower chamber which simulates the blood flow compartment. (Dr A. van de Stolpe).

far from that of the original sample. Dr Grabowska presented 3-D models developed from 'close-to-patient' cell lines, thereby avoiding prolonged *in vitro* passaging. Developing the stroma concurrently with the cancer cells, and incorporating stromal cells (for example, mesenchymal cells) into cancer models, allow 3-D tumour models to grow in a more physiologically-relevant manner, as a result of the presence of paracrine signals involved in promoting epithelial cell growth and the development of a metastatic phenotype. These signals are missing in models lacking these important supporting cells. The use of such models is critical for reducing the rates of both false-positive and false-negative results in drug screens.

Professor Christine Watson, from the University of Cambridge, UK, also discussed ways in which the tumour microenvironment can be mirrored in the laboratory. As an example, Professor Watson demonstrated how engineered collagen scaffolds can be used to develop 3-D breast cancer models that provide many advantages in downstream analyses. In collaboration with materials scientists with expertise in generating scaffold structures for medical applications, novel, stable freeze-dried porous collagen structures have been developed. Importantly, these structures can be manipulated to include various components and conditions, as required. For example, tumour stroma is stiffer than normal breast stroma, and the stiffness of the collagen used in the models can be altered accordingly (3, 4). In addition, the scaffold can be adapted to make it more physiologically-relevant — for instance, preadipocytes can be added to the scaffold, in order to mimic a mammary fat pad to which mammary epithelial cells can then be added (Figure 2c). These synthetic fat pads elegantly recapitulate important characteristics of the glands found *in vivo*, including ductal-like structures and polarised bi-layered alveoli that functionally differentiate to produce milk proteins. Importantly, these structures are stable, and further cell types or therapeutic drugs can be added in a sequential manner, enabling the effects of drug treatment on individual cells to be monitored by immunostaining and imaging. Finally, the scaffold itself can be further used to measure the invasiveness of different breast tumour cell lines, or to monitor the penetrance of cells from tumour fragments into the 3-D model scaffolds (Figure 2c).

A final example was given of a tailored environment for long-term cell maintenance and drug testing in 3-D culture and co-culture models with primary cells, secondary cells, and cell lines. SeedEZ (http://www.lenabio.com/products/seedez/) is a simple 3-D cell culture system that enables researchers to develop complex, yet consistent and reproducible, 3-D tissue models with heterotypic cell populations, immune and inflammatory cells, and exogenous extracellular matrices. As shown in Figure 2d, SeedEZ encourages cell–cell and cell–matrix interactions, and robust 3-D cell network formation, all of which are highly important for drug testing. Ms Jelena Vukasinovic from the Georgia Institute of Technology, Atlanta (GA), USA, introduced the workshop delegates to this technology, outlining its potential use with human breast cancer cell lines, for the development of disease state-relevant breast cancer models, and for the testing of slow-clearing drugs and drug combination strategies. Ms Vukasinovic explained that the SeedEZ system is ideal for oncology research, as it permits the formation of reproducible, therapeutically translatable phenotypes, and for drug testing, because it permits long-term studies by stabilising cell viability in culture. Thus, any increase in cell death after a particular treatment can be attributed to the test compounds added to the cultures themselves, rather than to failings of the cell culture and 3-D model systems *per se*. The SeedEZ system also facilitates the handling of 3-D cell cultures in extended studies, because the cells cannot be accidentally aspirated into the pipette tip or peeled off the culture dish, therefore permitting extended drug exposure and clearance studies, repeat-dose efficacy testing, and the assessment of delayed functional outcomes following drug withdrawal.

Applications of human-derived tissue samples

Professor Valerie Speirs from the University of Leeds, UK, explained that, due to the development of modern-day digital scanners, which produce 'virtual slides', researchers now have many more capabilities at their disposal for histopathology studies. One of these is the ability to generate virtual 3-D models of tumours from tissue slides (5). Digital images can be generated from multiple serial sections of 4μm formalin-fixed, paraffin-embedded tumour tissues by using an automated system. Tissue reconstructions can be then created from as little as 30 serial sections, although the quality greatly improves with more sections, ideally around 100. These 2-D images, following standard reconstruction processing — including aligning, and correcting for distortions and other inconsistencies involved with colour staining and sectioning — can be combined to generate virtual 3-D images with a tailor-made computer program developed by the University of Leeds (5–7).

There are many advantages of using virtual slides, including high-resolution cellular image reconstruction, easy handling, and a streamlined high-throughput workflow (8). Once a 3-D image has been developed, this image can be visualised and analysed (Figure 2e). Analysis of the 3-D structure can include, for example, size of structures of interest, position and intensity of biomarkers. In the case of breast cancer, Professor Speirs and her colleagues

have used this system to segment and analyse ductal carcinoma *in situ* (DCIS), invasive carcinoma, and the lumina within a 3-D tissue sample (5). This technique has also been extended to studies on metastatic colon cancer, liver cirrhosis, and the kidney glomerulus (6). In addition, multi-stain biomarker analysis is also possible, permitting 3-D visualisation of the intricate interactions between different parts of the tissue, including, for example, blood vessels, lymphatics and follicles. Such an exciting *in silico* disease modelling technique has the potential to replace current animal models, such as the mouse intraductal xenograft (MIND) model, which is currently promoted as the 'gold standard' for analysing DCIS as a non-obligate precursor to invasive breast cancer (9, 10). The MIND model involves the implantation of human cancer cells directly into the primary mammary ducts of immunocompromised mice. As this is similar to the human DCIS environment, this technique can help researchers to understand the biological mechanisms which occur during the progression from non-invasive to invasive DCIS (10). However, as with most mouse models, interactions between the human and mouse stroma are not well characterised, nor is the effect of the missing immune cells in the immunocompromised mice (10). Therefore, any technique that replaces the use of mice, such as this *in silico* modelling, is advantageous in the development of a more-representative human disease model.

Clinical applications of *ex vivo* breast cancer culture

Dr Alexander Leeper from the Edinburgh Cancer Research Centre, UK, presented an example of how 3-D cell cultures can be used to help derive personalised medicine regimes for breast cancer patients. Dr Leeper and his colleagues have developed a simple, inexpensive assay that involves placing small pieces of breast tumour core biopsy fragments into liquid collagen, which, when exposed to room temperature, solidifies, suspending the biopsy at the centre of the gel. Media is then poured over the gel, and, after three weeks, the samples can be analysed for the expression of markers (e.g. Ki67) or receptors (e.g. ER, HER-2). The effects of anti-cancer drugs (e.g. Tamoxifen) on individual biopsies can be monitored by the addition of the agent to the media covering the gel, permitting qualitative and quantitative analyses (e.g. number of proliferating cells, 3-D measurement of tumour volume) of treated *versus* untreated samples (Figure 2f; 11, 12).

Metastatic modelling

Dr Erik Sahai from the Francis Crick Institute, London, UK, described the work he and his colleagues are undertaking to create a model of breast cancer metastases. Specifically, they aim to model the processes by which cancer cells move away from their primary site to the surrounding tissue, and what happens once the cells arrive at the secondary sites to lead to colonisation. This process can be visualised in real-time in mouse models of metastases by using live intra-vital imaging, in which the simultaneous movement of different cells, e.g. breast cancer cells, fibroblasts, and macrophages, can be tracked. A more-reductionist approach is required to fully understand the molecular processes involved, and as mentioned throughout the symposium, 3-D spheroid breast cancer models could help uncover this information. Dr Sahai *et al.* have achieved this by the *ex vivo* analysis of human tumour cells in co-culture with cancer-associated fibroblasts (CAFs) in a 3-D collagen culture system. The fibroblasts were specifically isolated from human tumours at different stages of tumourigenesis, including normal, hyperplastic, adenomatous and breast carcinoma. He explained that, if these spheroids were grown with no other cells present, they resembled the carcinoma *in situ* state. However, if grown in the presence of CAFs, these spheroids took on a more invasive state. The extent of invasiveness from the 3-D spheroids was directly proportional to the fibroblast phenotype, with CAFs supporting the most aggressive phenotype (Figure 2g). Global mRNA analysis of these CAFs showed changes in many signalling pathways, permitting the dissection of the mechanisms involved in tumour development (13). Dr Sahai also noted the importance of the extra-cellular matrix in these experiments, and specifically, the effects of matrix stiffness. His group is currently validating this model by using human breast tissue and human squamous cancer cells sourced from the BCNTB.

Organ-on-a-chip technologies

The plenary speaker was Dr Anja van de Stolpe, from the recently-founded Dutch Institute for Human Organ and Disease Model Technologies, The Netherlands (hDMT; http://hdmt.technology/). The aim of hDMT is to build *in vitro* human organ and disease models, with emphasis on the use of induced pluripotent stem cells (iPSCs), complemented by *in silico* computational models. Dr van de Stolpe spoke about the usefulness of organ-on-a-chip technology (Figure 2h) for the development of personalised treatments, including those for cancer. At its core, this technology involves the culture of the smallest functional modules of healthy or diseased tissues on a microfluidic chip. What this means is that the entire organ is not mimicked on the chip, but rather the individual subunit(s). For example, for the lung, the module would be an

alveolar structure modelled on the chip, rather than the entire lung. By their very nature, 'on-chip' technologies are multidisciplinary, involving biologists, engineers, microfluidics experts and clinicians. The benefits of the organ-on-a-chip over traditional cell culture techniques include the ability to grow the 3-D cultures in a less rigid, more *in vivo*-like environment, and to perform real-time monitoring. Extended cultures, maintained from weeks to months, are possible with the chip system, permitting longer disease progression analysis. The hDMT partners are working toward the creation of immunocompetent primary tissue organoid culture-based cancer-on-chip models. Examples of systems currently being developed within the hDMT programme, include: a) the human blood vessel-on-a-chip, where, for example, atherosclerosis with associated coagulation activation can be modelled; b) human heart-during-exercise on a 'stretchable' chip containing electrodes; and c) human neuronal network-on-a-chip model, with the possibility of single neuron electrical activation readout. As with the general theme of the day, it was emphasised that the type of cells, media, and cell environment, are all critical to physiological evaluation. Human primary cancer-on-a-chip involves incorporating not only the microfluidic device with cancer tissue cells, but also an endothelial layer, and ultimately, also the immune system (lymph node-on-a-chip). Importantly, Dr van de Stolpe indicated how it is crucial to build stepwise toward increasing the complexity of this system, so the effects of each change/treatment could be modelled and monitored. The first generation system is expected to have the potential to model the primary cancer graft innate immune response, metastasis, and metastasis colonisation. The cancer-on-a-chip system can be integrated in the drug/treatment development phase at the target validation, lead optimisation, candidate drug, and phase II trials, in the form of a 'clinical trial-on-a-chip'.

Conclusions

The workshop provided delegates with the opportunity to see high-quality presentations of a number of state-of-the-art and emerging alternative approaches to animal-based experiments. The presentations offered a wide scope of topics, including: solutions for sourcing and maintaining human-derived material; protocols and directions for handling such material in a laboratory setting; relevant information for downstream uses and applications (for example, how to establish and maintain patient-derived cells); an overview of current and emerging technologies and applications, along with their relevance for biomedical research; and database information and resource-sharing opportunities. The combined scope of the workshop aimed to provide a range of constructive strategies for overcoming some of the barriers to the widespread adoption of these technologies. Of particular importance was the interactive discussion during the panel debates, where presenters spoke openly about remaining challenges and limitations in the methods and technologies, and offered practical solutions and helpful advice on getting these methods to work. Poster viewings, informal discussions and networking opportunities took place during refreshment breaks. This workshop was oversubscribed and, together with the extensive audience participation in the debates, shows that there is a strong demand for the adoption of these alternative models by the research community. Furthermore, as the delegates were predominantly early-career researchers, this should encourage the consideration and implementation of these cutting-edge alternative approaches to established animal-based protocols, therefore realising one of the goals of the SEARCHBreast initiative. Feedback from the workshop suggests that the delegates found the workshop to be of an exceptionally high standard, and felt that it was highly relevant and permitted an open and honest environment with networking, funding and collaborative opportunities. Quotations included:

— *"The topics were quite specific, and it's helped to find out new techniques and advice that will be useful to our research."*

— *"Opening my eyes to all the 3-D options that are out there! Bringing together so much expertise which will facilitate new collaborations."*

— *"Highlighted parameters that I will now consider in the development and improvement of* in vitro *models to study breast cancer."*

Going forward, SEARCHBreast will build on the enthusiasm of the workshop participants, to ensure that the momentum of exploring alternatives to animal use in breast cancer research is maintained. SEARCHBreast will do this by hosting additional workshops and other networking and collaborative events. Finally, SEARCHBreast has uploaded approved presentations from the workshop onto the resource section of its database (https://searchbreast.org), which will enable not only the delegates, but also other interested researchers, to have free access to this information.

Acknowledgements

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and Professor Christine Watson for their presentations and for providing the figures which comprise Figure 2.

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