

Routine Assessment of Cancer Cell Cytotoxicity in a Novel Three-Dimensional Culture Assay

Introduction

Cancer is a leading cause of death worldwide, accounting for around 7.6 million deaths in 2008 (World Health Organization, 2012). Over recent years, our knowledge of cancer biology and the development of cancer drugs have contributed toward significant advances in cancer treatment [1]. Nevertheless 13 % of the population still die from this disease. It has been highlighted that cancer treatment could be significantly improved in the future if three key problems were considered: first, treatment should be personalized to each patient; second, mechanisms of drug resistance and relapse should be understood; third, new drugs should be identified, developed and tested [2]. Fundamental research into the underlying causes of cancer is therefore critical to addressing these issues. Experimentation in the laboratory at the cellular level provides key information. However, much depends on the design, development and use of appropriate *in vitro* models and robust assays to deliver accurate and representative biological data.

Certain aspects of cancer research rely on animal models but these have associated feasibility and ethical concerns [3, 4]. These issues may be overcome using *in vitro* models that allow a controlled approach to investigating cancer cell biology, compound screening and disease modelling. The vast majority of such models involve culturing cells on conventional two-dimensional (2D) plastic-ware in which cells adapt to the flat polystyrene substrate, flatten and grow as monolayers. Unfortunately, this approach is a poor surrogate and does not mimic the environment that cells experience *in vivo*. The morphology of cultured cells as monolayers is not realistic, cell-to-cell contact is

limited and the micro-environment generated by the cells, for example via extracellular matrix (ECM) deposition, is reduced and altered [5].

Three-dimensional (3D) *in vitro* models are becoming a popular alternative to bridge and reduce the gap between 2D culture assays and animal models. Such 3D systems represent a more realistic approach enabling cells to retain their native 3D morphology, form interactions with adjacent cells and create more complex structures. Alvetex Scaffold provides the ideal environment for cells to grow and function in 3D creating a more representative tissue model. The material consists of a highly porous and inert polystyrene scaffold with multiple voids and interconnects creating a 3D environment into which cells can freely grow and occupy, creating 3D structures *in vitro* (<https://www.reprocell.com/alvetex>).

In this application note, we demonstrate the growth of the popular breast cancer cell line, MCF-7, using Alvetex Scaffold technology. We show that Alvetex Scaffold can be used to create 3D structures that closely resemble the structure of tumour tissues grown *in vivo*. We compare the viability of these cells with equivalent cultures grown on conventional 2D plastic-ware. The data show that MCF-7 cells cultured on Alvetex Scaffold possess a different sensitivity pattern to certain cytotoxic drugs than their 2D counterparts that is more likely to resemble *in vivo* responses. This underlines the value of Alvetex Scaffold technology by providing a superior culture environment for cancer cell research.

Results

1. Growth and Viability of MCF-7 cells on Alvetex Scaffold

The analysis of the growth and expansion of MCF-7 cells cultured on Alvetex Scaffold has been assessed over 21 days. MCF-7 cell viability was measured by the MTT assay and results showed good linear growth and expansion of the cell population during the first 2 weeks (**Figure 1**). Between 14 and 21 days, cell proliferation had plateaued and cells had reached confluence in the 3D construct. Histological data confirmed the increase of cell density over time (**Figure 2**). The penetration of the cells into the scaffold started from the top of the scaffold membrane where the cells were initially seeded. By day 7 the MCF-7 cells had reached the bottom of the membrane and continued to expand by forming aggregates of cells within the voids of the scaffold. Beyond 14-18 days, the cells had started to become overcrowded and confluent in 3D, and the quality of the culture was reduced resulting in areas of dead or dying cells.

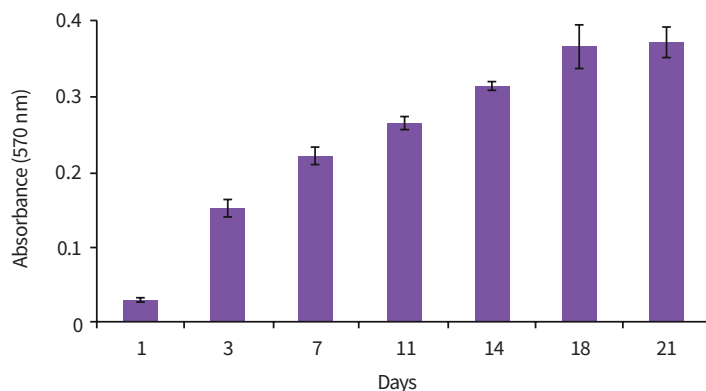


Figure 1: Viability of MCF-7 cells grown in Alvetex Scaffold for up to 21 days. Biochemical analysis of cell viability using a standard MTT assay. Cells were cultured for up to 21 days on 22 mm Alvetex Scaffold discs presented in 6-well inserts in 6-well plates. Data from 3 sample replicates of MCF-7 cells are shown. Data represent mean \pm SD, $n = 3$.

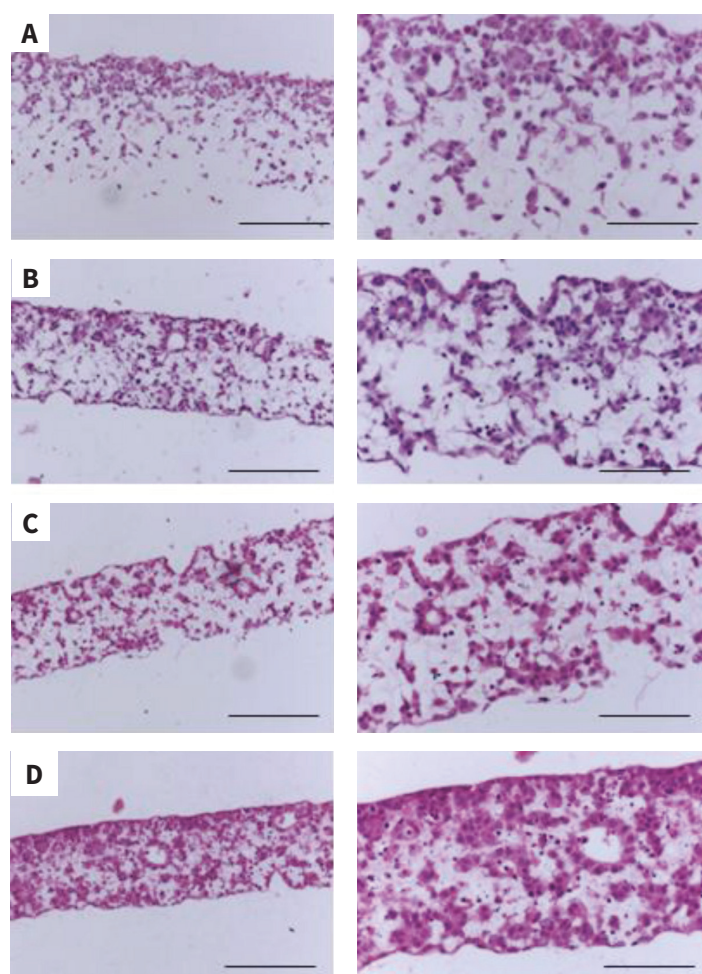


Figure 2: Morphology of MCF-7 cells grown on Alvetex Scaffold. Brightfield micrographs show the structure of MCF-7 cells cultured for (A) 3 days, (B) 7 days, (C) 10 days, and (D) 14 days on 22 mm diameter Alvetex Scaffold discs presented in 6-well inserts in 6-well plates. Cells were fixed, embedded in paraffin wax, sectioned (10 μ m) and counterstained with Haematoxylin and Eosin. Scale bars: 200 μ m left-hand images; 100 μ m right-hand images.

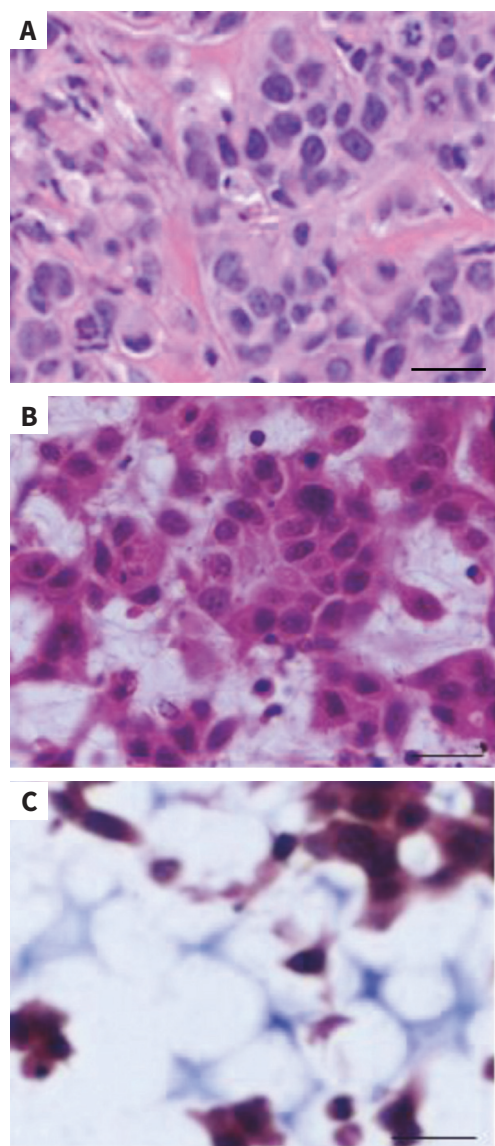


Figure 3: Long-term culture of MCF-7 cells on Alvetex Scaffold results in xenograft-like structures. (A) Structure of a xenograft tumour derived from transplanted MCF-7 cells (H&E stained, image source online: Premier Laboratory's Gallery of Slides (<http://www.premierlab.com/>)). (B and C) High magnification bright-field micrographs showing the morphology of MCF-7 cells cultured for 14 days (B) or for 21 days (C) on Alvetex Scaffold discs. Cells were fixed, embedded in paraffin wax, sectioned (10 µm) and counterstained with H&E (B) or Masson's Trichrome (C). In Masson's Trichrome staining, collagen deposits are stained in blue. Scale bars are 20 µm.

Previous work has shown that MCF-7 cells transplanted into mice resulted in a xenograft tumour (**Figure 3A**). In the xenograft, MCF-7 cells grow as aggregates amongst a network of collagen fibres and diffuse connective tissues. Similarly, MCF-7 cells cultured in Alvetex Scaffold for 14 days formed aggregates as seen at higher magnification (**Figure 3B**). Furthermore, Masson's Trichrome staining showed collagen deposits secreted by the cells indicating the production of extracellular matrix in the culture (**Figure 3C**). Taken together these data indicate that MCF-7 cells grown on Alvetex Scaffold produce tissue-like morphologies resembling the structure of MCF-7 derived xenograft tissues.

2. Assessment of MCF-7 cytotoxicity to known compounds in 2D and 3D culture

Cytotoxicity assays have been performed to compare the behaviour of MCF-7 cells grown either in 3D on Alvetex Scaffold or 2D on conventional plasticware. Three cytotoxic drugs have been chosen based on their popularity in published toxicity studies. These drugs have been routinely used to study cancer cells *in vitro* and in animal models *in vivo*, and have human therapeutic applications. Tamoxifen is used in the treatment of estrogen receptor (ER)-positive breast cancer patients and as chemoprevention in high-risk women [6]. Tamoxifen is an antagonist of the estrogen receptor in breast tissue that decreases DNA synthesis. Paclitaxel is considered a highly active chemotherapeutic agent in various cancers including breast cancer [7]. Paclitaxel is a mitotic inhibitor that blocks the progression of mitosis. Doxorubicin is frequently used as a chemotherapeutic agent against metastatic breast cancers [8]. Doxorubicin is an anthracycline antibiotic, which works by intercalating DNA and thereby stopping the process of replication.

The assessment of drug cytotoxicity is normally performed on cells seeded on 2D plasticware that are usually exposed to the compound for 24 h or 72 h at a range of increasing concentrations in order to create a dose-response curve and to determine the IC₅₀ value. The IC₅₀ is the concentration of the compound required to kill 50 % of the cells. In an initial short term study, MCF-7 cells were grown for 3 days in 2D culture using conventional plastic-

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ware or in 3D culture using Alvetex Scaffold. The cultures were subsequently treated with test compounds for either 24 h or 72 h.

Figure 4 shows the growth curves obtained with Tamoxifen treatments. After both 24 h and 72 h treatments the viability of the cells was reduced to 100 % mortality as the drug concentration increased in both 2D and 3D culture. However, the profiles of the responses were not the same

for 2D and 3D cultures and the IC₅₀ values were different. For 24 h treatments the IC₅₀ values were 12 μ M and 32 μ M for 2D culture and 3D culture, respectively. As would be anticipated, the effects of the drug were more marked after 72 h incubation and IC₅₀ values were 9 μ M and 20 μ M for 2D culture and 3D culture, respectively. For 2D cultures, these IC₅₀ values are in agreement with those previously reported [9].

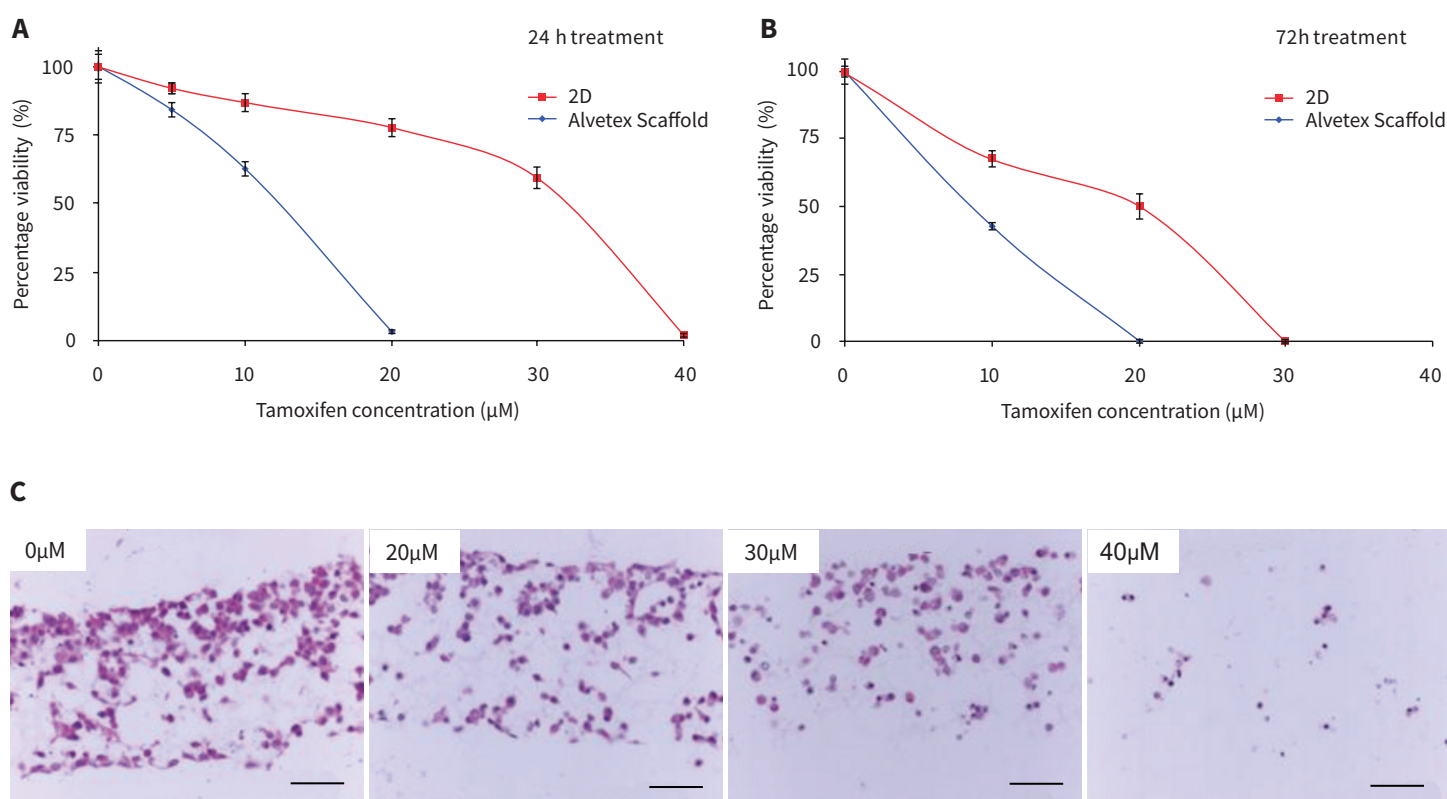


Figure 4: Cytotoxicity of MCF-7 cells to Tamoxifen when grown in 2D and 3D culture. The effect of Tamoxifen on MCF-7 cancer cell viability was determined using an MTT assay. Cells were cultured in 2D on conventional plastic-ware (blue) or in 3D on Alvetex Scaffold (red) for 3 days prior to either (A) 24 h or (B) 72 h treatment with Tamoxifen over a concentration range of 5-40 μ M. Values are shown as percentage viability expressed relative to untreated cells. Data from 3 sample replicates are shown (mean \pm SD). (C) Brightfield micrographs showing the morphology of MCF-7 cells grown for 3 days in 3D on Alvetex Scaffold prior to treatment for 24 h with increasing concentrations of Tamoxifen. After treatment, cells were fixed, embedded in paraffin wax, sectioned (10 μ m) and counterstained with H&E. Scale bars: 100 μ m.

Histological analysis of MCF-7 cells treated for 24 h with Tamoxifen confirmed the viability assay results.

As the drug concentration increased, the cell morphology changed and the quantity of cells within the scaffold decreased. In control (0 μM Tamoxifen) cultures, cells possessed a large clear cytoplasm and close inter-cellular connections with adjacent cells. As the concentration of Tamoxifen increased, the cells became rounded, smaller and died, and some were lost floating into the medium.

These results indicate that MCF-7 cells cultured on Alvetex Scaffold are more robust and that they can tolerate a higher concentration of Tamoxifen. In contrast, cells grown in 2D culture are placed under greater stress due to their abnormal structure and are therefore likely to be more sensitive when challenged by this compound.

Similar results were obtained with Paclitaxel treatment (**Figure 5A**). After 24 h, 40 % of the cells grown in 2D showed cytotoxicity toward this compound whereas only 20 % of cells grown on Alvetex Scaffold were no longer viable. A similar profile was observed after 72 h treatment (data not shown). It is known that MCF-7 cells from ECACC are partially resistant to Paclitaxel [10] and such resistance appears to be greater in 3D culture compared to 2D culture.

Responses to Doxorubicin after 72 h exposure were similar between cells cultured in 2D on conventional plasticware and in 3D on Alvetex Scaffold (**Figure 5B**). The IC_{50} values were 1 μM and 0.7 μM for 2D culture and 3D culture, respectively. The 2D IC_{50} value is in agreement with previous work [9]. Interestingly, a small proportion of cells grown on Alvetex Scaffold in 3D culture appear to be resistant at the higher drug concentrations. Whilst 100% cell mortality can be achieved with 4 μM Doxorubicin in 2D culture, between 5-10 % of the cell population remains viable when grown on Alvetex Scaffold. This example demonstrates how resistance to a compound drug could be overlooked if the cells were responding in an aberrant way due to growth in 2D culture.

To more closely mimic the treatment and response of tumour tissues to cytotoxic compounds, experiments were performed on more established 3D cultures of MCF-7 cells. MCF-7 3D cultures were left to grow for 10 days prior to drug treatment. Under these conditions, the MCF-7

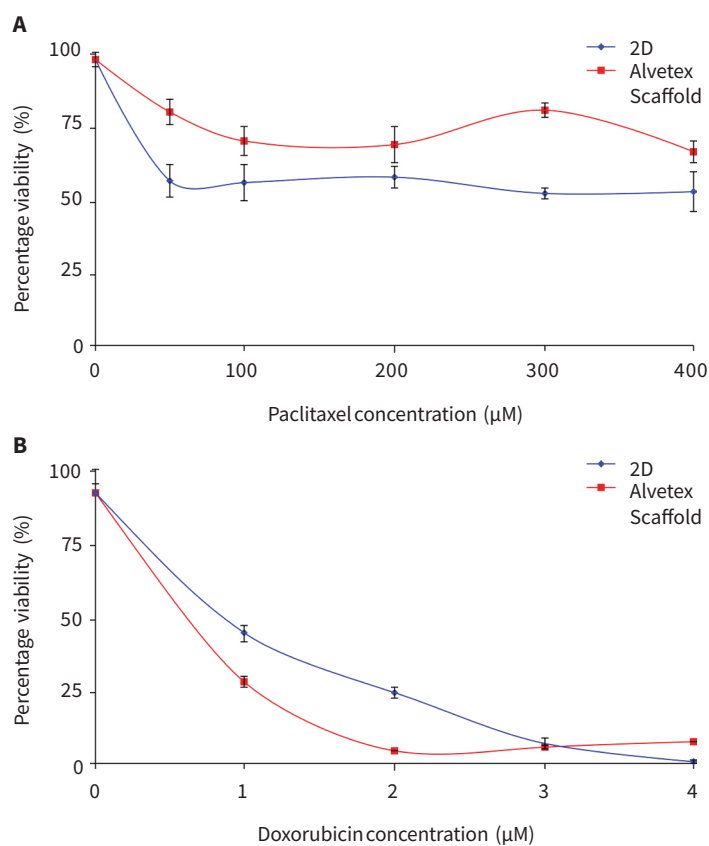


Figure 5: Cytotoxicity of MCF-7 cells to Paclitaxel and Doxorubicin when grown in 2D and 3D culture. The effect of Paclitaxel and Doxorubicin on MCF-7 cancer cell viability was determined using an MTT assay. Cells were cultured in 2D on conventional plastic-ware (blue) or in 3D on Alvetex Scaffold (red) for 3 days prior to either (A) 24 h treatment with Paclitaxel or (B) 72 h treatment with Doxorubicin over a range of concentrations. Values are shown as percentage viability expressed relative to untreated cells. Data from 3 sample replicates are shown (mean \pm SD).

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cells form much denser 3D tissue-like masses that resemble the structure of xenograft tumours (**Figure 3**). MCF-7 10 day 3D cultures were then exposed to Tamoxifen or Doxorubicin at concentrations based approximately on their IC₅₀ values determined from 2D cultures after 72 h treatment (10 μ M and 1 μ M, respectively). **Figure 6** shows data relating to the cytotoxicity of these compounds on MCF-7 cells grown in 3D culture for 10 days followed by drug exposure for up to 18 days.

During the first five days of treatment with either Tamoxifen or Doxorubicin a significant delay in the response of MCF-7 cells grown in Alvetex Scaffold was identified compared to conventional 2D cultures. Indeed for the cells grown in 2D plasticware the cytotoxicity of the compounds was effective from the first days of treatment. After 10 days of exposure, the proportion of cells responding to either compound was similar for cells grown in 2D or 3D culture. However, although no viable cells were detected in 2D cultures after 10-14 days, a small proportion of the 3D MCF-7 cultures remained viable for up to 18 days of treatment with either Tamoxifen or Doxorubicin. It is possible that a fraction of the cell population in 3D culture was resistant to the compounds under these test conditions but no such cells were found in 2D culture. Taken together, these results further indicate that MCF-7 cells cultured in 3D on Alvetex Scaffold are more robust and that resistance to candidate molecules is observed which could be overlooked if the cells were grown in 2D culture.

3. Compatibility of Alvetex Scaffold formats for cancer cell cytotoxicity assays

Alvetex Scaffold is available in well insert and multi-well plate formats. The well insert format is ideal for long-term cultures whereby cells receive nutrient support from above and below the 3D culture resulting in the formation of tissue-like structures. For example, in this study we have shown the formation of MCF-7-derived xenograft-like tumour masses in Alvetex Scaffold presented in the 6-well insert format. Such growth is also possible in the smaller 12-well insert format. However, the use of well inserts and the need for long-term cultures may not be appropriate for higher throughput, short-term culture studies.

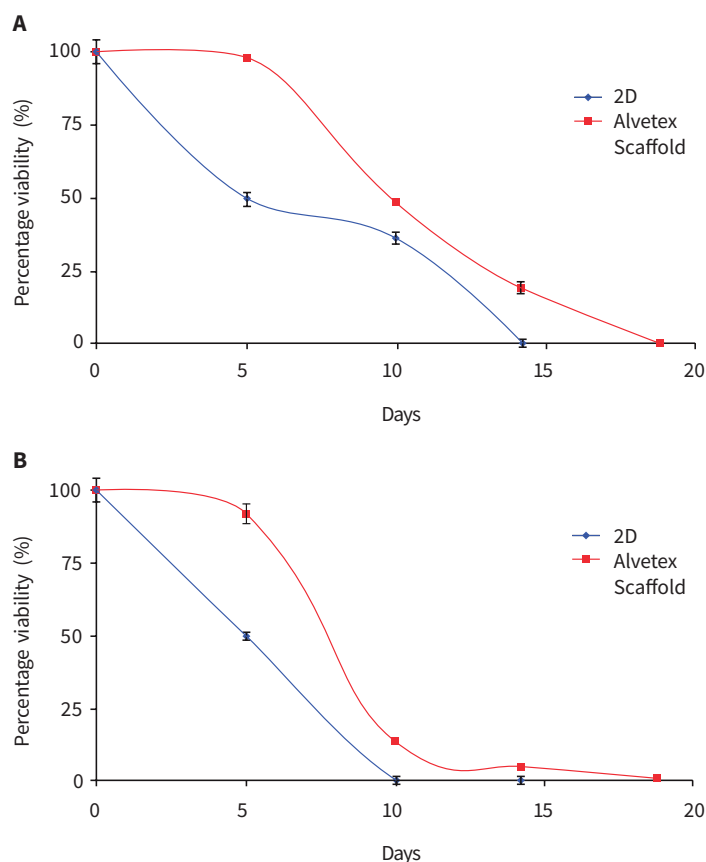


Figure 6: Sensitivity of MCF-7-derived xenograft-like structures to Tamoxifen and Doxorubicin toxicity in comparison with 2D culture. The effect of Tamoxifen and Doxorubicin on MCF-7 cancer cell viability was determined using an MTT assay. Cells were cultured in 2D on conventional plastic-ware (blue) following standard methods, or in 3D on Alvetex Scaffold (red) for 10 days to establish xenograft-like structures, prior to either (A) 10 μ M Tamoxifen treatment or (B) 1 μ M Doxorubicin treatment. Fresh treatments were added every 2-3 days. Values represent percentage viability expressed relative to the quantity of cells before treatment (day 0). Data from 3 sample replicates are shown (mean \pm SD).

Accordingly, we have also demonstrated the application of MCF-7 3D culture and analysis of Tamoxifen cytotoxicity using 15 mm diameter Alvetex Scaffold discs presented in the base of conventional 24-well plates. The appropriate cell seeding density and ratio of medium volumes have been calculated to enable comparison of cell growth and cytotoxic responses with the well insert format. Cells were grown for 3 days as before, prior to treatment with Tamoxifen (10-40 μ M) for 24 h or 72 h. Experiments show that similar results can be obtained for short-term 3D culture of MCF-7 cells in either insert or plate formats of Alvetex Scaffold. Consistent with earlier data performed using well inserts (**Figure 4**), treatment of MCF-7 cells in 3D culture in 24-well plates with Tamoxifen for either 24 h or 72 h reduced cell viability in a concentration dependent manner. The IC50 values generated from using either inserts or plates were highly comparable: 24 h treatment — 32 μ M and 30 μ M; 72 h treatment — 20 μ M and 24 μ M, respectively. These data indicate the compatibility of alternative presentations of Alvetex Scaffold technology which is valuable for comparative studies in alternative formats and extrapolation into high throughput studies when using the 96-well plate presentation.

Conclusions

This study demonstrates that human MCF-7 breast cancer cells grown in Alvetex Scaffold provide a realistic model that is a step closer to replicating the *in vivo*-like conditions of tumour growth. MCF-7 cells show a linear growth profile on Alvetex Scaffold for 2 weeks before they reach confluency and plateau. The 3D morphology of the cells, the opportunity for close interactions between adjacent 3D cells, and the production of extracellular matrix within Alvetex Scaffold contribute toward creating tissue-like structures that closely resemble the morphology of xenograft tumours. We demonstrate that cytotoxicity assays can be performed on these 3D cultures to generate valuable data concerning the sensitivity and toxicity of cells to specific compounds. By avoiding the limitations of conventional 2D culture, Alvetex Scaffold technology provides new opportunities to study the effectiveness and mode of action of test compounds on cancer cells. Creation and evaluation of xenograft-like

structures *in vitro* is likely to generate more appropriate data concerning the predictive accuracy of a compound test assay compared to cells compromised by their growth conditions in 2D culture. Furthermore, such 3D models may partially substitute the need for animal experimentation hence reducing animal usage and associated costs. Overall, Alvetex Scaffold provides a simple and robust approach for the routine generation of 3D culture models to more accurately study the action of test compounds in cancer research.

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Methods

Human breast cancer epithelial MCF-7 cells were obtained from the European Collection of Cell Cultures (Sigma). MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) FBS, 2 mM L-glutamine and 100 U/ml Penicillin/Streptomycin in a humidified incubator gassed with 5 % CO₂ (37°C) until reaching 80 % confluency.

Before use, Alvetex Scaffold 6-well inserts were pre-treated for incubation in aqueous solution by dipping in 70 % ethanol and washing twice with culture medium. Cells were then seeded to the centre of the Alvetex Scaffold (1 × 10⁶ cells in 100 µL per disc). The inserts were incubated for 60 minutes at 37°C with 5 % CO₂ to allow the cells to settle into the scaffold before adding 10 ml of

media per well. For cytotoxicity assays in response to compounds using the 24-well plate format, the cells were seeded to the centre of the Alvetex Scaffold (0.2 × 10⁶ cells in 50 µL per disc) and 2 ml of medium was used per well. Samples were re-incubated and maintained by complete media exchange every 2-3 days. For 2D cultures, cells were seeded at the same density in conventional 2D plasticware.

For cell growth and proliferation analysis, cells were maintained in culture for up to 21 days. At specific time points cultures were harvested and analysed by histological staining (H&E and Masson's Trichrome), and by a standard MTT cell viability assay.

For cytotoxicity assays in response to compounds, MCF-7 cells were cultured for 3 days prior to drug treatment. Cultures were treated with Tamoxifen (Sigma, Ref: T5648, 0-50 µM), Doxorubicin (Sigma, Ref: 44583, 0-5 µM) and Paclitaxel (Sigma, Ref: T7402, 0-500 nM) for 24h or 72 h before MTT viability assay and histological staining.

For histology and MTT assay protocols refer to <https://www.reprocell.com/resources/protocols>.

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