

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

Application Note

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]. The appropriate experimental model, however, must be used to design accurate and robust assays to assess these issues.

Certain aspects of cancer research rely on animal models but these have issues about feasibility and ethical concerns [3;4]. These issues may be overcome by using *in vitro* models that allow a controlled approach to investigating cancer cell biology, screening compounds and modelling the disease. 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 which cells experience *in vivo*. The morphology of cultured cells as monolayers is not realistic, cell-to-cell contact is limited and the microenvironment 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 proliferate in 3D. Alvetex®Scaffold is a highly porous and inert polystyrene material with large voids designed to create a 3D environment into which cells can grow and occupy, creating 3D structures (www.reinnervate.com).

In this application note, we demonstrate the growth of the popular breast cancer cell line, MCF-7 and show how Alvetex®Scaffold technology can be used to create 3D structures *in vitro*. 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.

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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 confluency 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 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 the quality of the culture was reduced resulting in areas of dead or dying cells.

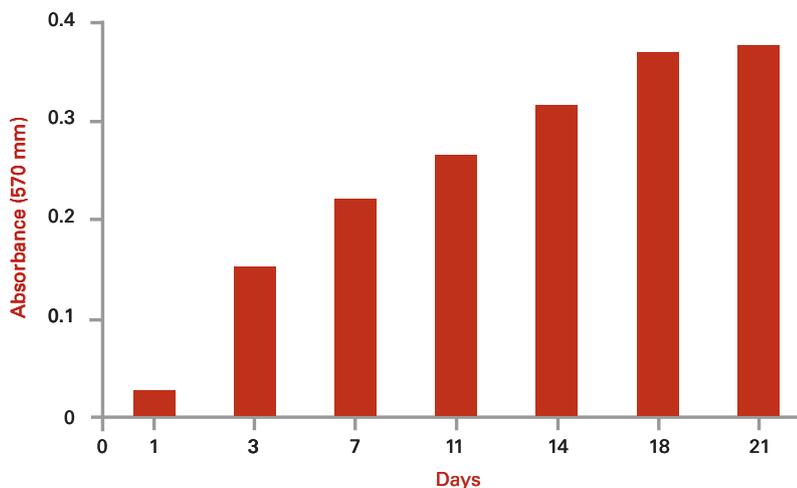


Figure 1. MCF-7 viability of cells grown in Alvetex[®]Scaffold for up to 21 days.

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.

Previous work shows that MCF-7 transplanted into mice results 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.

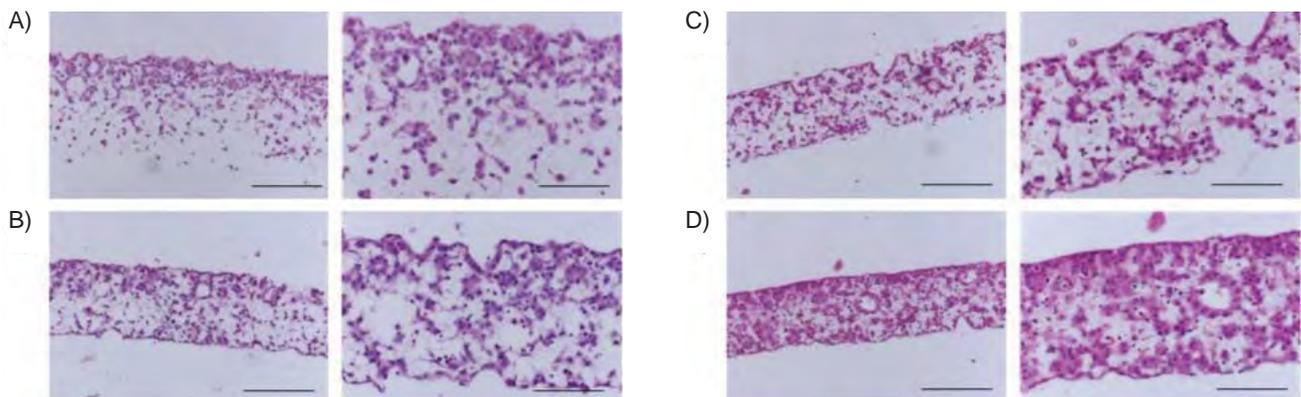


Figure 2. Morphology of MCF-7 cells grown on Alvetex® Scaffold.

Brightfield micrographs show the structure of MCF-7 cells cultured for (A) 3 days, (B) 7 days, (C) 10 days or (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 µm) and counterstained with haematoxylin and eosin. Scale bars: 200 µm left-hand images; 100 µm right-hand images.

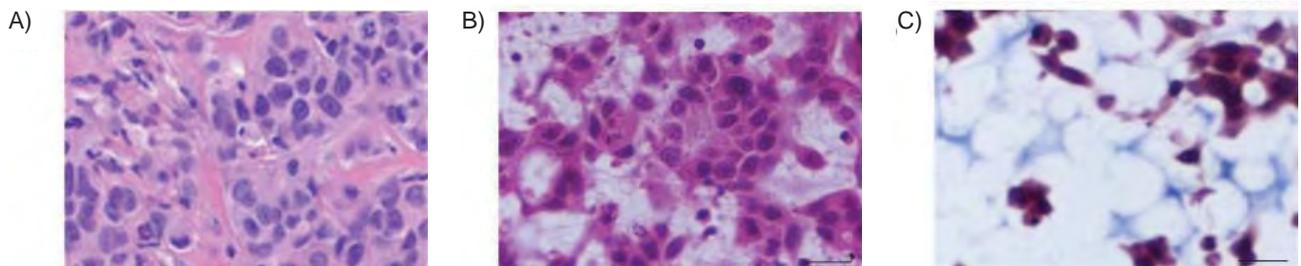


Figure 3. Long-term culture of MCF-7 cells on Alvetex® Scaffold results in xenograft-like structures.

(A) Structure of a xenograft tumour derived from transplanted MCF-7 cells (H&E stained, image source on-line: Premier Laboratory's Gallery of Slides (<http://www.premierlab.com/>)). (B and C) High magnification brightfield 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.

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 plastic-ware. Three cytotoxic drugs have been chosen based on their popularity in peer-reviewed papers. 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 which 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 plastic-ware which are usually exposed to the compound for 24h or 72h 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 this study we have treated MCF-7 cells grown in 2D on conventional plastic-ware or in 3D on Alvetex®Scaffold for 3 days followed by either 24h or 72h drug exposure.

Figure 4 shows the growth curves obtained with Tamoxifen treatments. After both 24h and 72h 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 24h treatments the IC₅₀ values were 12 µM and 32 µM for 2D culture and 3D culture, respectively. As would be anticipated, the effects of the drug were more marked after 72h and IC₅₀ values were 9 µM and 20 µM for 2D culture and 3D culture, respectively. For 2D cultures, these IC₅₀ values are in agreement with those previously reported [9].

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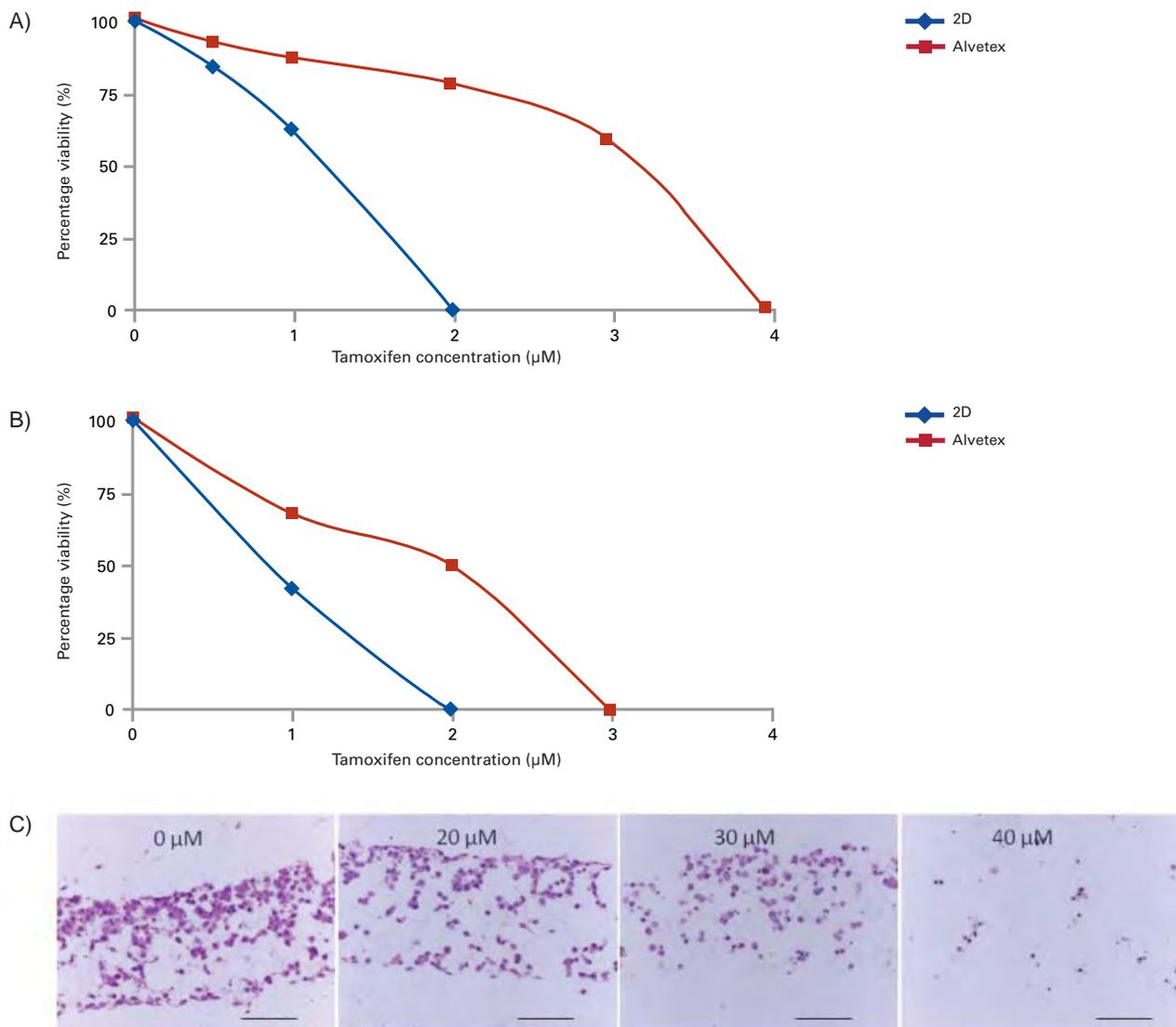


Figure 4. Cytotoxicity of MCF-7 cells to Tamoxifen when grown in 2D and 3D culture.

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) 24h or (B) 72h treatment with Tamoxifen over a concentration range of 5-40 μM . 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 24h with increasing concentrations of Tamoxifen. After treatment, cells were fixed, embedded in paraffin wax, sectioned (10 μm) and counterstained with H&E. Scale bars: 100 μm .

Histological analysis of MCF-7 cells treated for 24h with Tamoxifen confirmed the viability assay results. As the drug concentration increased, the quantity of cells within the scaffold decreased. Cell morphology also changed as concentrations of the drug increased. 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 24h, 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 72h treatment (data not shown). It is known that MCF-7 cells available 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 72h exposure were similar between cells cultured in 2D on conventional plastic-ware and in 3D on Alvetex[®]Scaffold (Figure 5B). The IC_{50} values were 1 μM and 0.7 μM for 3D culture and 2D 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.

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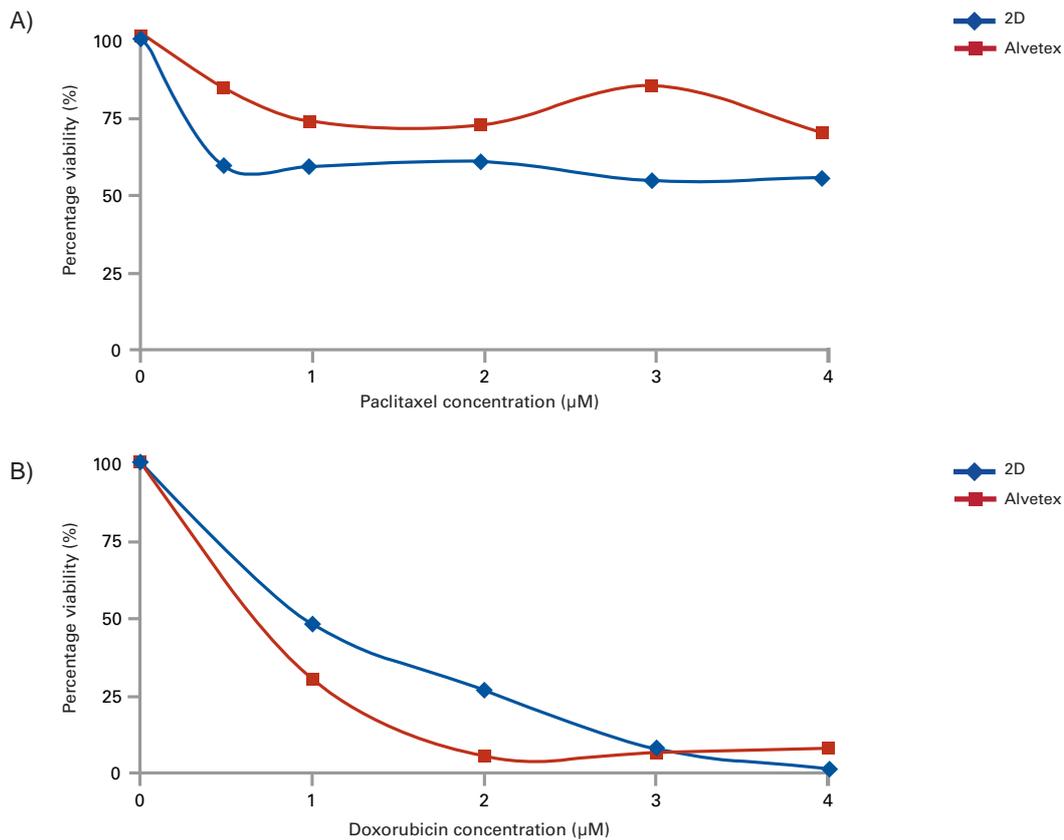


Figure 5. Cytotoxicity of MCF-7 cells to Paclitaxel and Doxorubicin when grown in 2D and 3D culture.

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) 24h treatment with Paclitaxel or (B) 72h 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).

Conclusions

This study demonstrates that human MCF-7 breast cancer cells grown in Alvetex®Scaffold provide a realistic *in vivo*-like model for cancer research. 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 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 3D culture models to more accurately study the action of test compounds in cancer research.

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References

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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 plates 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. Plates 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 plastic-ware 6 well-plates.

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 72H before MTT viability assay and histological staining.

For histology and MTT assay protocols refer to: www.reinnervate.com/alvetex/protocols



