

Modelling Cancer Cell Migration and Invasion Using Novel Technology for Three Dimensional Cell Culture

Application Note 3

Introduction

Cancer is characterised by unregulated and abnormal cell growth in which cells divide uncontrollably, forming malignant tumours, and invade into adjacent tissues. The ability of malignant tumour cells to spread and invade normal surrounding tissues is the most life threatening aspect of the disease leading to significant morbidity and mortality. In 2008, cancer caused about 13% of all human deaths worldwide (7.6 million) (World Health Organization, 2012). Rates are rising as more people live to an old age and as mass lifestyle changes occur in the developing world.

Over recent years, our understanding of cancer cell biology and the development of pharmaceuticals have contributed toward significant advances in combating the disease [1]. The treatment of cancer, however, can be significantly improved. Much relies on understanding the basic cellular and molecular mechanisms underlying the causes and progression of the disease and the subsequent development of novel therapeutic approaches and the application of anti-cancer drugs [2].

A major aspect of cancer research is the development and application of suitable experimental models to investigate specific elements of the disease process. It is now more important than ever to use *in vitro* models that deliver accurate information about cell function that closely represent the activity of cells in real tissues. This is necessary to advance the science but also there are economical and ethical reasons including increasing the efficiency of the discovery process, reducing research costs and decreasing the numbers of animals used in research.

Reinnervate has developed novel technology that provides researchers with the opportunity to create more physiologically relevant models to investigate the structure and function of mammalian cells in health and disease, including cancer. In this particular application note, we demonstrate the use of this technology to study the migration of cells as models of cancer cell invasion.

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Developing Cell Invasion Models Using Cancer Cell Lines

Cancer cell lines that are derived from tumour tissues explanted into culture are widely used to study the molecular and cellular functions of cells representative of the parent tumour. SW480 and SW620 cells are established and widely recognised examples of colon carcinoma cells frequently used in research. These lineages were originally isolated in the mid 1970s from the same patient representing different stages of disease progression [3]. SW480 cells originated from the primary colon adenocarcinoma, whereas the SW620 lineage was isolated from a lymph node metastasis. Although related, these cell lines differ in terms of their morphology (Figure 1), surface markers [4] and behaviour *in vivo* [5,6]. These characteristics make these cells an ideal subset to study cellular changes from early to late stages during cancer progression.

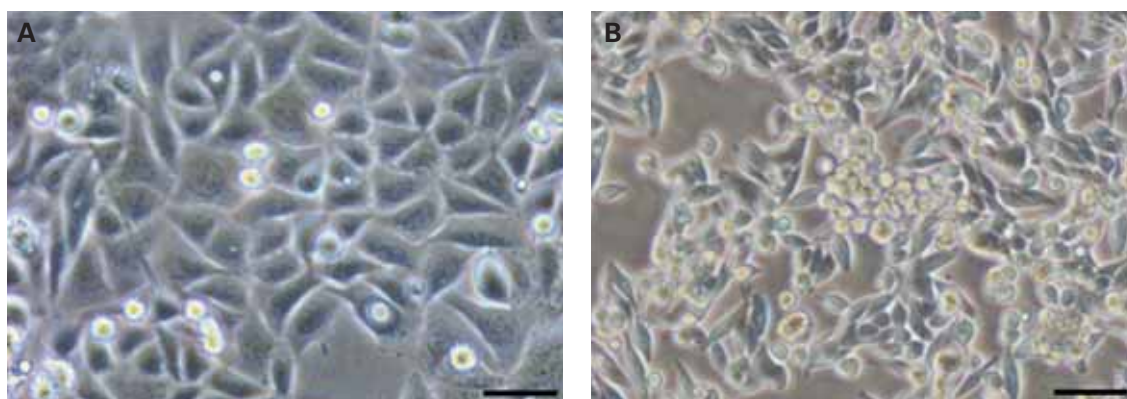



Figure 1. Growth of colon adenocarcinoma cells in conventional 2D culture.

Phase contrast images showing monolayer cultures of (A) SW480 and (B) SW620 cells. SW480 cells are derived from a Dukes' type B colorectal adenocarcinoma, whilst SW620 cells were acquired from the same patient with a Dukes' type C tumour representing a later stage of the disease. More than one cell type may be represented in these lineages. Generally, SW480 cells are slower growing and appear less motile than SW620 cells. Scale bars: 50 μm .

Numerous studies have reported the migration of colon cancer cells *in vitro* using standard scratch assays [7]. However, such models rely on the migration of cells on flat substrates, most often glass or polystyrene surfaces, in which cells have adapted to the 2D environment. The structure and function of cells under such conditions is different and it is well recognised that cells behave in an aberrant manner when grown in 2D culture. Therefore, models of cell function based on 2D culture do not truly represent the migration of cells in real tissues and potentially provide less accurate information about the molecular and cellular mechanisms regulating the movement of cancer cells [8].



Cancer cell biologists have long recognised the values of 3D over 2D *in vitro* models, especially for the retention of *in vivo*-like phenotype and the ability to more closely replicate tissue organisation. Here we demonstrate the application of Alvetex® Scaffold technology to study the growth of cancer tissues *in vitro* and enhance the ability to investigate the cellular and molecular mechanisms that regulate cancer cell migration in more physiologically relevant models. 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 space which cells can grow into and occupy. Cells growing in the scaffold do not adopt flattened morphologies as in conventional 2D cultures and retain a more natural 3D shape and form. Adjacent cells grow in close association with one another, enabling complex 3D cell-to-cell interactions, thus creating tissue-like structures that more closely represent the architecture of tumours and associated tissues *in vivo*. Alvetex® Scaffold is designed for routine 3D cell culture and is straightforward to use. Together with its compatibility with many existing downstream analytical methods, this makes Alvetex® Scaffold immediately accessible to users as an exciting new approach to study the behaviour of cells in the laboratory. Full technical information and supporting literature are available on-line (www.reinnervate.com).

In this application note, we validate the use of Alvetex® Scaffold to study the migration of colon carcinoma cells through a complex 3D environment. We show how cells respond to chemical stimuli that interact with signalling pathways known to play a role in cancer cell invasion. Furthermore, we demonstrate the potential of creating complex co-culture models to study the interaction of cancerous epithelial cells with underlying stromal tissues. Collectively, this underlines the value of Alvetex® Scaffold by providing a practical, versatile and superior culture environment for cancer cell research.

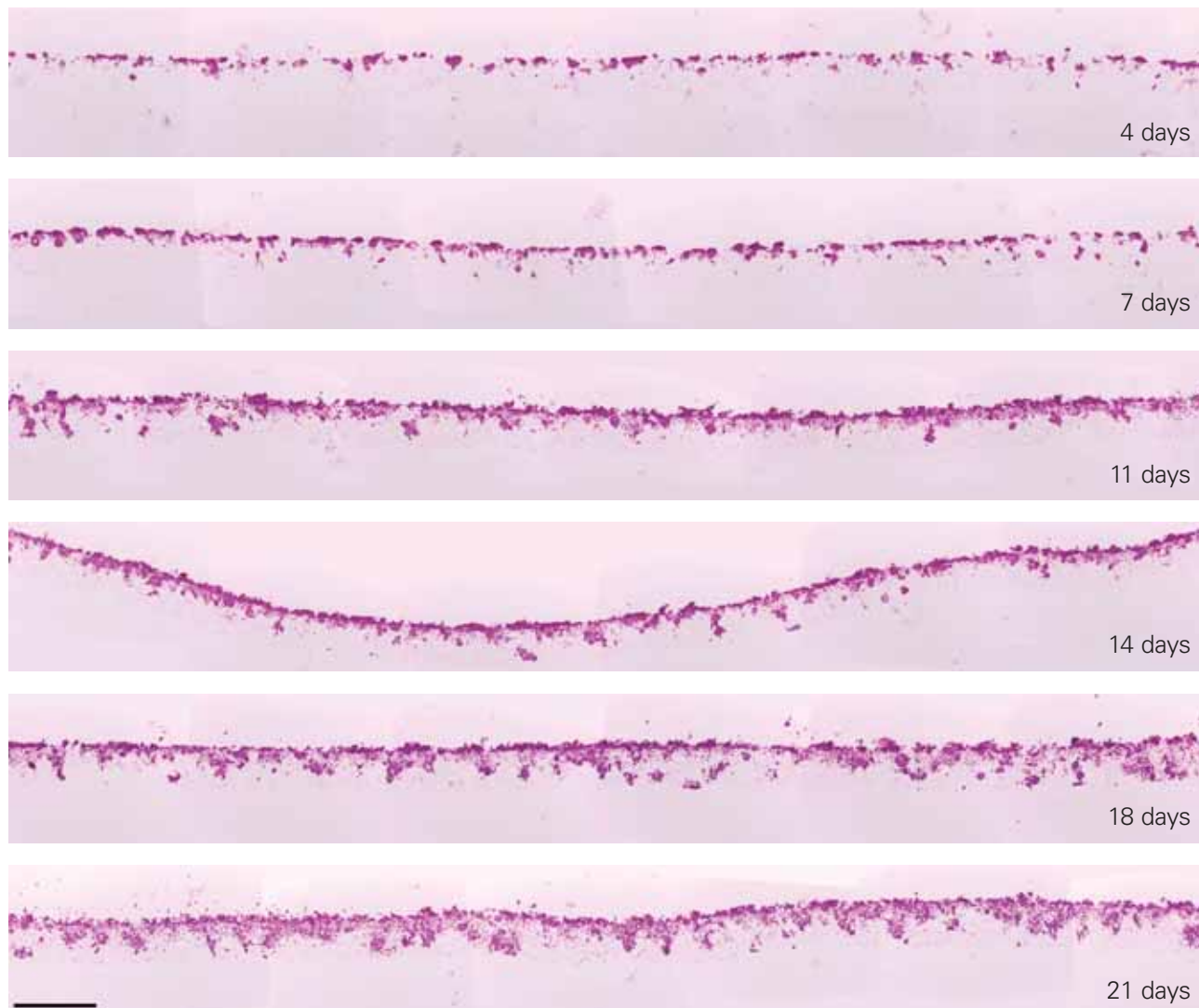
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Results

1. Growth and Viability of Colon Carcinoma Cell Lines in 3D Culture

SW480 and SW620 cells were cultured on Alvetex[®]Scaffold for up to 21 days. Both cell types proliferated on the material and cells penetrated inside the scaffold forming 3D cultures. Figure 2A shows the outcome of histological analyses demonstrating the formation of the culture over 3 weeks. Cells penetrated deeper into the scaffold over time and also spread laterally forming a uniform 3D structure.

A



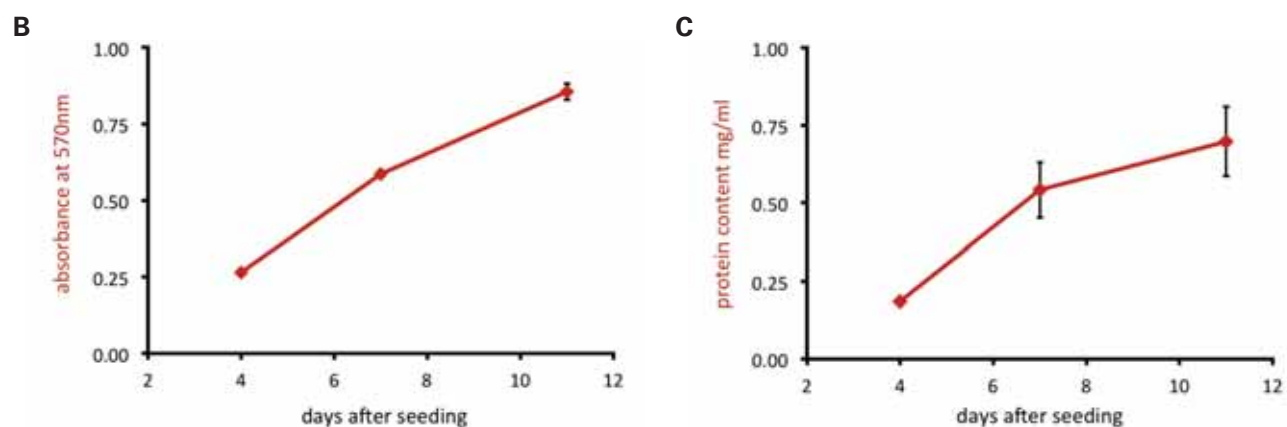
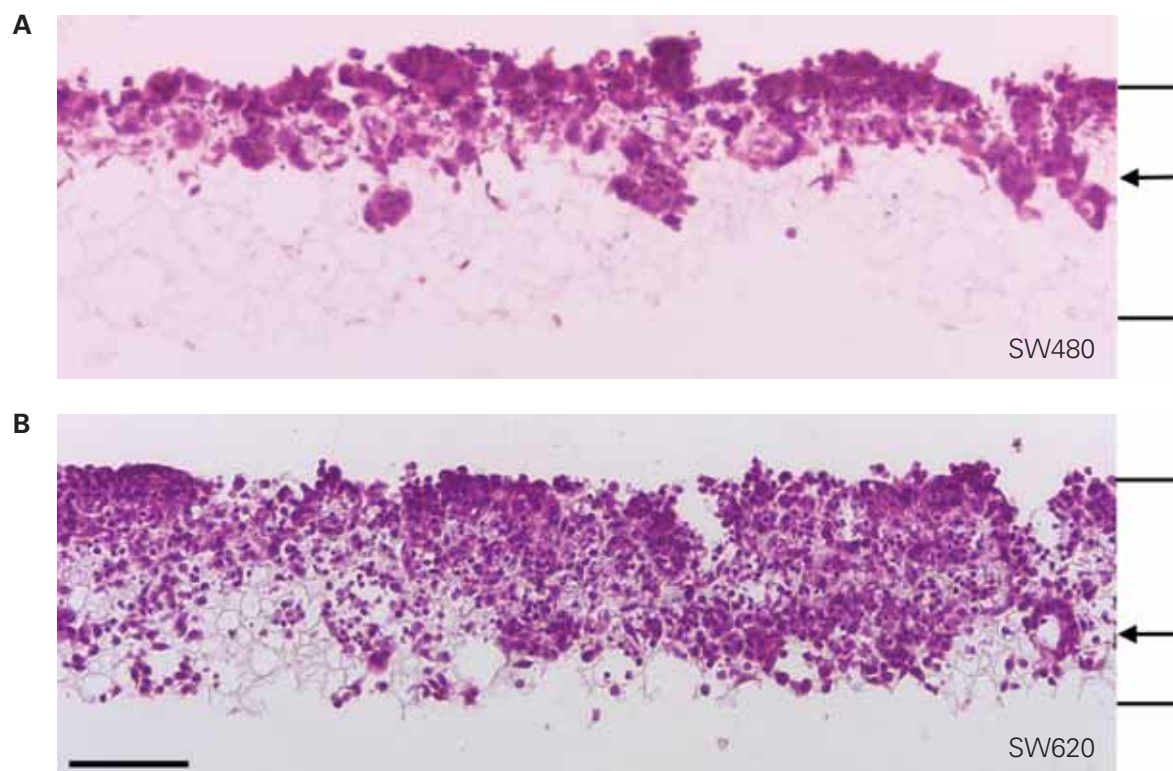


Figure 2. Growth of colon adenocarcinoma cells on Alvetex® Scaffold.

Both SW480 and SW620 cells proliferated and remained viable on Alvetex® Scaffold, and formed substantial 3D cultures. Examples of data for each cell type are shown: (A) Histological analysis of SW480 cells growing in 3D culture for up to 21 days. Cells initially populated the upper levels of the scaffold and subsequently migrated, penetrating the full thickness of the scaffold in parts after approximately 18-21 days. Samples counter-stained with Haematoxylin and Eosin. Scale bar: 500 μ m; (B) Viability of SW620 cells over 11 days in 3D culture. Data determined by MTT cell viability assay. Data points represent average MTT absorbance at 570nm, $n=3$, \pm SEM; (C) Total protein produced by cultures of SW620 cells over 11 days in 3D culture. Protein levels were determined by a standard Bradford assay on cell lysates prepared directly from the 3D cultures. Data points represent mean protein concentration, $n=3$, \pm SEM.

Each lineage exhibited different growth patterns that reflect their behaviour *in vivo*. The viability and total protein content of SW480 cultures increased slightly and remained relatively constant in older cultures (data not shown). In contrast, SW620 cells showed a large linear increase in population growth that did not plateau until later time points (Figure 2B). This difference reflects the mitotic potential of the two cell types and also indicates that SW620 cells avoid cell-cell contact inhibition during 3D growth for at least 11 days in culture. Such differences between SW480 and SW620 cells agree favourably with previous studies performed in both chick [5] and rodent [6] models.

Distinct differences were also observed between SW480 and SW620 cells after examining the colonisation of Alvetex[®]Scaffold and the formation of the 3D cultures. Alvetex[®]Scaffold allows for cell movement and migration to be monitored in 3D. Lateral colonisation across the surface and within the substrate from a concentrated seeding area can be visualised using simple techniques such as Neutral Red staining (see www.reinnervate.com/alvetex/workflow for details). Vertical colonisation and downward cell invasion can be visualised microscopically and subsequently measured using either histological- or confocal-based methods. Figure 3 shows data concerning the vertical movement of colon carcinoma cells and their invasion into Alvetex[®]Scaffold. The average depth of cell penetration from the surface of the scaffold can readily determine the leading edge of the culture. The data suggest that SW620 cells migrate more rapidly into the scaffold and achieve greater depths of cell penetration within the same period compared to the migration of the SW480 lineage. It is however also important to take into account any differences due to cell proliferation as demonstrated in the next section.



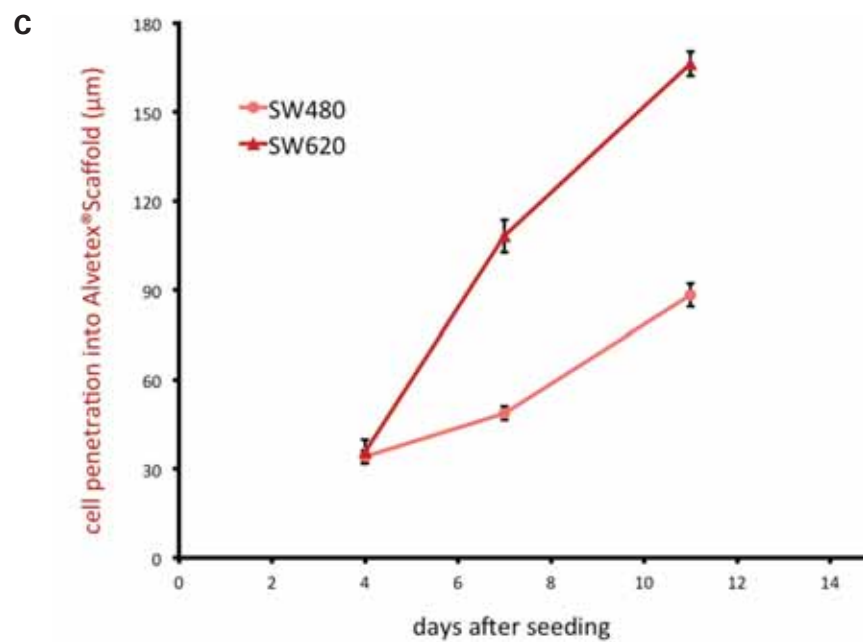


Figure 3. Differential Migration of SW480 and SW620 cells on Alvetex® Scaffold.

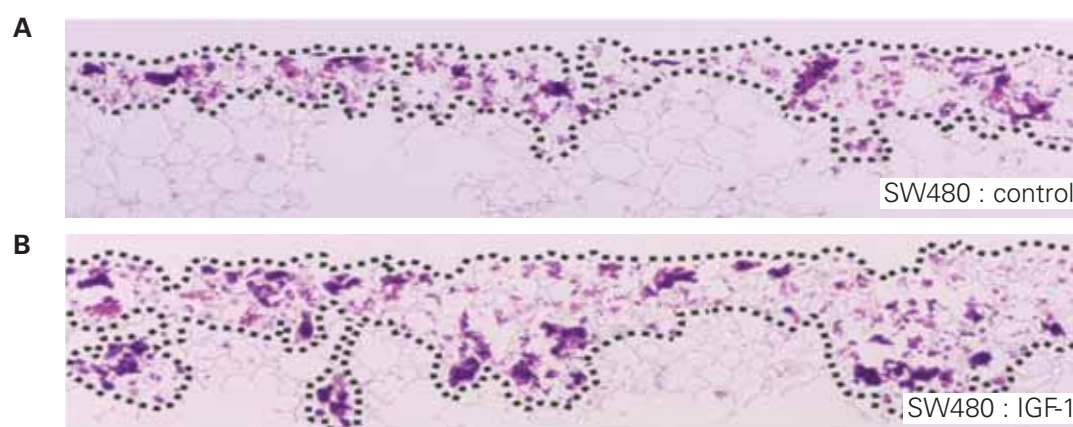
Assessment of cell penetration depth was performed by histological analysis and measurement of the cultures' leading edge over 11 days in 3D culture. Brightfield micrographs of Alvetex® Scaffold are shown in transverse section containing (A) SW480, and (B) SW620 cells counter-stained with Haematoxylin and Eosin. Arrows indicate the average cell penetration depth for the examples shown. Scale bar: 100 µm. Quantification of the differences between these two cell lines is shown in plot (C). Data represent mean cell penetration depth, $n=3$, \pm SEM. It is clear that SW620 cells invade and penetrate to significantly greater depths inside Alvetex® Scaffold compared to the SW480 lineage.

2. Regulation of Cancer Cell Invasion by IGF-1 in 3D Culture

The generation of 3D cell invasion models using Alvetex®Scaffold opens up new opportunities to study the molecular mechanisms by which cancer cells migrate. Cell movement in 3D is very different to conventional 2D migration models such as the scratch wound assay. In this example, we demonstrate the sensitivity of colon carcinoma cells to insulin-like growth factor (IGF-1) as they migrate through a complex 3D environment. It has previously been shown that IGF-1 plays an important role in the regulation of cell proliferation, attachment, migration and cell death [9]. For example, IGF-1 functions to modulate cell migration in breast tumours, often involving cell-adhesion receptors and extracellular proteins [10-12].

In this application we examine the rate of cancer cell migration in response to a gradient of IGF-1 exposure across the Alvetex®Scaffold membrane suspended in a well insert. Human adenocarcinoma SW480 and SW620 cells were initially grown in Alvetex®Scaffold for 5 days to establish the 3D culture. IGF-1 was added to the lower chamber beneath the well insert and the cells were incubated for a further 5 days. After histological examination and measurement of cell penetration into the scaffold, it was recorded that SW480 cells responded to IGF-1 whereas SW620 cell invasiveness appeared unchanged (Figure 4). Data showing that the number of cells in the culture was not affected by IGF-1 demonstrated that this difference in cell penetration depth was unlikely to be due to changes in cell proliferation or cell death but rather that SW480 cells showed greater ability to migrate further into the scaffold.

Previous experiments have implicated the role of IGF-1 signalling in mediating the migration and invasion of colorectal carcinoma cells [13,14]. The data presented herein support the application of 3D cell culture technology to further study the role of IGF-1 in the migration of colon tumour cells representative of different stages of cancer progression.



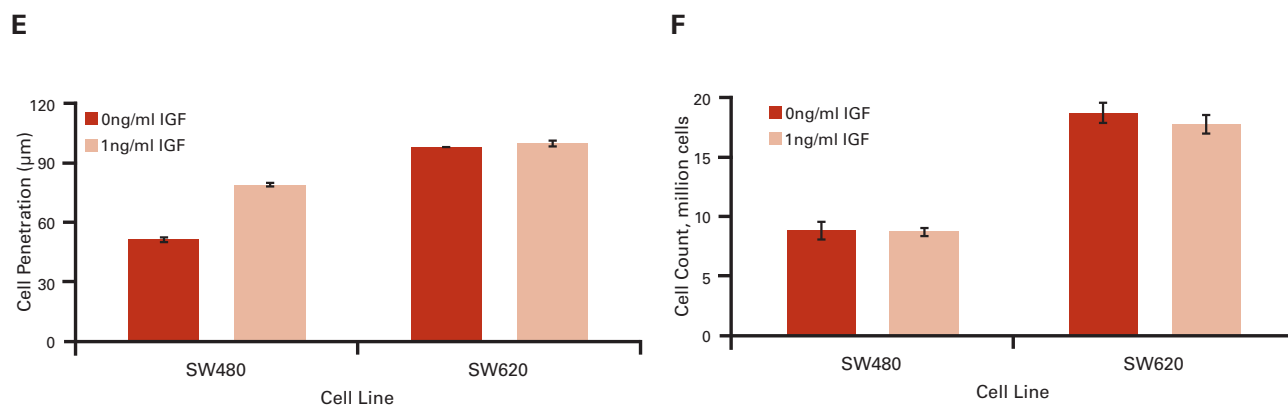
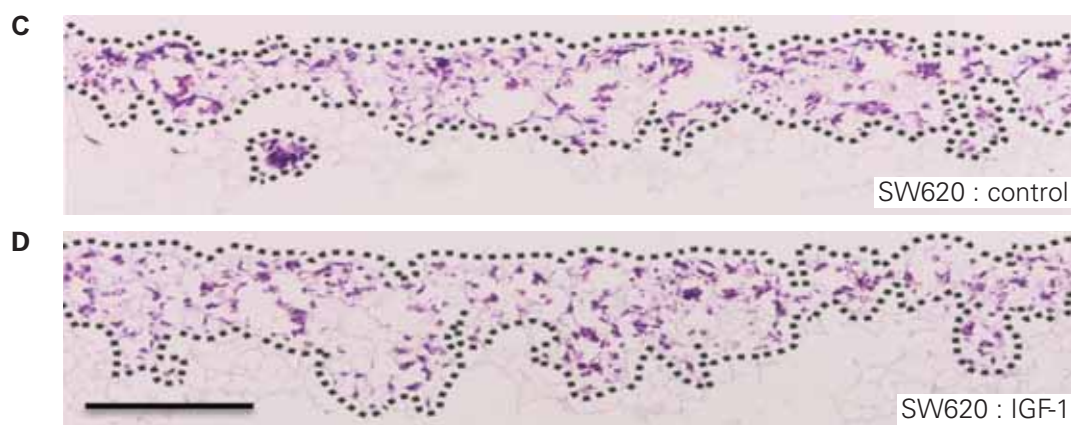


Figure 4. Sensitivity of Colon Carcinoma Cells grown in 3D Culture to IGF-1.

The response of SW480 and SW620 cells to 1 ng/ml IGF-1 was assessed in 3D culture using Alvetex® Scaffold. Cells were cultured in well inserts for 5 days initially followed by subsequent addition of the growth factor to medium beneath the insert for a further 5 days. Brightfield micrographs of Alvetex® Scaffold shown in transverse section containing either SW480 cells (A,B) or (C,D) SW620 cells in the absence (control, A,C) or presence (B,D) of IGF-1. Slides have been counter-stained with Haematoxylin and Eosin and the outline of the culture area indicated by the dotted line. Scale bar: 200 µm. Quantification of cell penetration depth and observed differences due to IGF-1 treatment is shown in plot (E). Data represent mean cell penetration depth, n=3, ±SEM. It is evident that SW480 cells invade and penetrate to significantly ($p < 0.05$) greater depths inside Alvetex® Scaffold in the presence of IGF-1. SW620 cells did not appear to be affected by the growth factor at this concentration. (F) Determination of total cell number in relation to concentration of double stranded DNA using Picogreen® assay. Analysis shows no significant effect of IGF-1 on cell number for either the SW480 or SW620 lineage, indicating that differences in cell invasion are not due to cell proliferation. Data represent mean, n=3, ±SEM.

3. Studying the Role of Stromal Fibroblasts in Cancer Cell Invasion Using 3D Culture

In most routine cell and molecular biology experiments, conventional 2D cell culture approaches and cell lines are used as models for tissues. In the case of tumour biology, this often means just the mono-culture of the tumour cells in isolation, ignoring the fact that tumours are complex tissues consisting of cancer cells, stromal cells, endothelial cells, to name but a few. There is therefore a chasm in the complexity of existing cell-based *in vitro* assays compared to the *in vivo* situation. This impacts directly on the quality of information derived from such models. Now there is significant demand to reduce the gap between simple monoculture models and *in vivo* animal studies for improved, physiologically relevant approaches that take into account tissue complexity and strive to recreate more *in vivo*-like conditions.

Understanding how cancer cells move and invade within the surrounding tissue is a key issue and main cause of mortality in cancer patients. Stromal fibroblasts within a tumour play an important role in cancer cell proliferation, survival and migration [15]. They can produce growth factors and cytokines and influence tumour growth rate and invasion [16,17]. In squamous cell carcinomas, fibroblasts have been shown to act by leading cohorts of invading carcinoma cells through the stromal tissues [18]. Cell invasion therefore depends on multiple factors and interactions between epithelial and stromal cells and re-modelling the extracellular matrix (ECM) [19].

In this application we demonstrate the potential of Alvetex®Scaffold for the co-culture of alternative cell types. We simply show how colon carcinoma cells can be grown adjacent to established 3D cultures of fibroblasts – a model that is designed to mimic the invasion of colorectal tumours into their surrounding stromal tissues (Figure 5). These data merely show proof of concept at this stage but clearly illustrate how layers of alternative cell types can be constructed to study cell-to-cell interactions in a more physiologically relevant manner. Such an approach is not feasible using existing Transwell®-type technologies that do not allow creation of multiple 3D cultures adjacent to one another.

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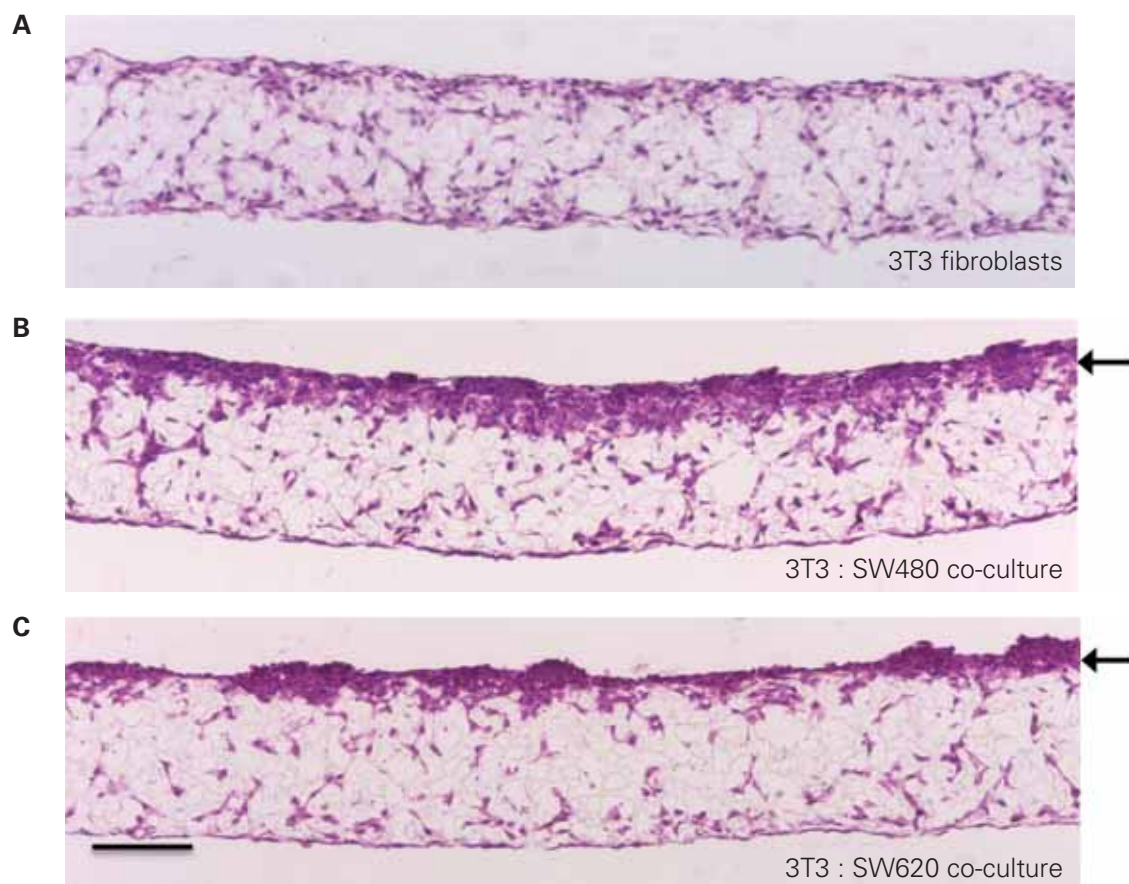


Figure 5: Development of 3D Co-culture Cell Invasion Models.

Alvetex® Scaffold provides an ideal platform to create layers of alternative cell types and develop co-culture models. In this example, we show how fibroblasts can be used to mimic the stromal tissues found adjacent to a tumour to study cancer cell invasion through associated tissues. Bright field micrographs show: (A) 3D culture of 3T3 fibroblasts alone; (B) co-culture of 3T3 fibroblasts and SW480 cells; (C) co-culture of 3T3 fibroblasts and SW620 cells. Fibroblasts were cultured on Alvetex® Scaffold for 7 days prior to seeding adenocarcinoma cells on the surface and maintained for a further 7 days. Slides have been counter-stained with Haematoxylin and Eosin. Arrows indicate approximate location of co-cultured adenocarcinoma cells on the surface with the 3D fibroblast culture beneath. Scale bar: 100 μ m.

4. Alternative Methods to Assess Cell Migration in 3D Culture

Growing cells in Alvetex[®]Scaffolds enables scientists to create complex 3D tissue models *in vitro*. Reinnervate understands that it is important for users to be able to see and monitor their cells. Alvetex[®]Scaffold is a very versatile technology and visualisation of cells in 3D culture can be readily achieved in a number of ways using a combination of conventional methods and optimised protocols (see 'Review of Imaging Techniques Compatible with 3D Culture of Cells in Alvetex[®]Scaffold' at www.reinnervate.com). For this application, we have demonstrated that cells migrating through Alvetex[®]Scaffold can be followed using conventional histological approaches. Such methods are commonplace in histopathology departments and are routinely used for diagnostic and prognostic purposes in oncology. Like tissue sections, preparation and sectioning of Alvetex[®]Scaffold 3D tissue cultures provide the opportunity for staining and high resolution imaging of cells. These samples can also be further probed for protein and nucleic acid expression using immunocytochemistry or *in situ* hybridisation, respectively.

Alternative approaches using modern fluorescence-based techniques can also be employed which are perhaps more amenable for rapid readout and quantification through digital imaging and automated measurement tools. Confocal microscopy optically sections through 3D biological samples and enables digital reconstruction of tissue structures. In this example, the outcome of an experiment designed to assess the migration of carcinoma cells carrying a mutant p53 protein is shown (Figure 6). Expression of a mutant p53 protein has previously been shown to drive invasion of cells and experiments in Matrigel[™] showed a clear increase in cell invasiveness when carrying the mutant p53 protein compared to control cells [20]. In comparison, the data generated using Alvetex[®]Scaffold (Figure 6) produced identical conclusions to those previously obtained with Matrigel[™]. The use of Alvetex[®]Scaffold for measuring cell migration presents some clear advantages over the use of hydrogels such as Matrigel[™]. Users of Alvetex[®]Scaffold report clearer images and better resolution from confocal microscopy. The use of Alvetex[®]Scaffold can also significantly reduce batch-to-batch variability, since it is totally inert and contains no animal-derived products.

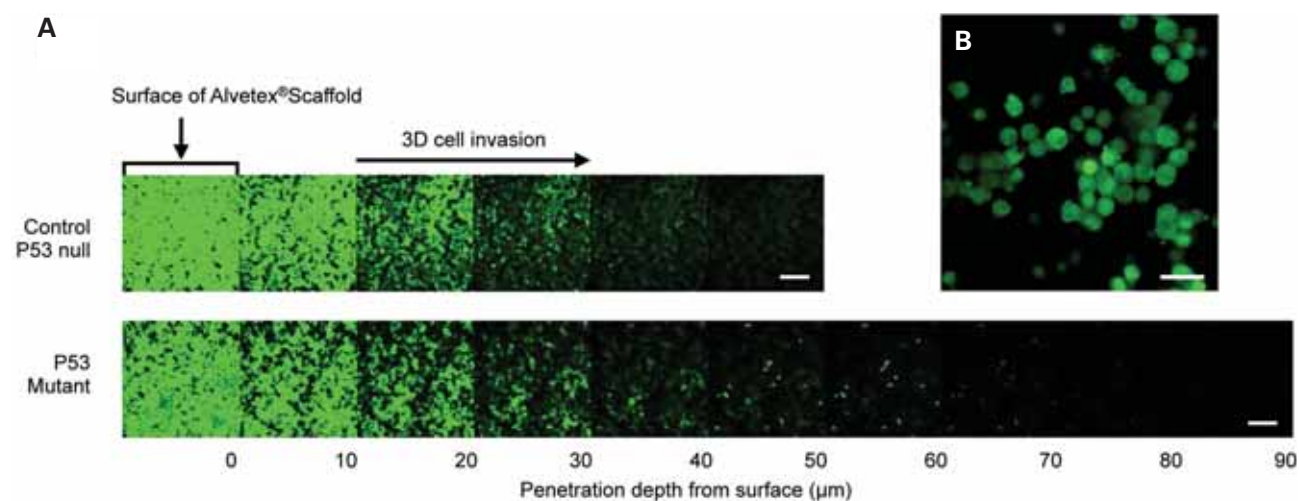


Figure 6: Imaging Invasion of Cells in 3D Culture Using Confocal Microscopy

This example shows the application of fluorescence confocal microscopy to follow the migration of H1299 human non-small cell lung carcinoma cells in the presence or absence of mutant p53 protein. Cells were imaged from the top surface of the Alvetex® Scaffold after 7 days migration. (A) Serial confocal images of migrating control (top) and mutant (bottom) p53 cells taken at 10 µm intervals as the cells moved through the Alvetex® Scaffold. From these images it is clear that migration is enhanced in mutant p53 expressing cells. Scale bar: 250 µm. (B) Confocal image of migrating mutant p53 cells taken using x60 objective lens with oil immersion at a distance of 40 µm below the top surface of the Alvetex® Scaffold. Scale bar: 40 µm. (Data courtesy of Dr P.A.J.Muller, The Beatson Institute for Cancer research, Glasgow).

Conclusions

The examples presented in this application note demonstrate the utility of Alvetex® Scaffold to study the migration and invasion of cancer cells *in vitro*. This technology provides a compromise between conventional 2D cultures of isolated tumour cells and the manufactured complexity of xenografts of human cancers in immune-suppressed animal hosts. 3D models can be tailored to be biomimetic and accurately recapitulate the native scenario in which they are found to provide an important alternative to both complex whole organism approaches and 2D cultures with their spatial limitations.

Alvetex® Scaffold supports the 3D culture of cells capable of movement and is therefore particularly valuable to study cancer cell migration and invasion. We show differences in the migratory ability of colon adenocarcinoma cells representative of alternative stages of colorectal cancer. We demonstrate how such cells are differentially responsive to a signalling molecule known to play an important role in cancer cell migration. The opportunity is presented to create more complex co-culture models of cell invasion, to study cell-to-cell interactions between epithelial and stromal cells. This has significant advantages over existing co-culture systems such as Transwell® technology. Alvetex® Scaffold is a versatile technology and we also show its compatibility with modern analytical and imaging techniques to monitor the progress of cancer cell migration. Generally, Alvetex® Scaffold provides a simple and robust approach to create more sophisticated 3D culture models that will produce more relevant and accurate data to study cancer cell biology.

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Methods

Analytical methods:

Detailed protocols for MTT viability assay, protein extraction, Picogreen® assay for cell number determination, histology and H&E staining are available on www.reinnervate.com/alvetex/workflow. The website also provides a wealth of additional information concerning the use and application of Alvetex® Scaffold technology.

Cell culture:

Human colorectal adenocarcinoma SW480 and SW620 cell lines were obtained from ATCC (CCL-228 and CCL-227, respectively) and routinely grown in 2D plastic ware in growth medium (DMEM supplemented with 10% heat-inactivated FCS, 2 µM L-glutamine and 100 U/ml penicillin).

Alvetex® Scaffold 6-well inserts (product code AVP004-12S) presented in 6-well plates or deep Petri holders (AVP015-2S) were prepared according to manufacturers' instructions. Cell suspensions were prepared enzymatically using standard methods, cell number determined and re-suspended at a density of 1 million cells per 100 µl.

Cells were inoculated onto prepared Alvetex® Scaffold discs at a density of 1 million cells per scaffold. For concentrated cell seeding, cells were dispensed as a droplet in the centre of each scaffold, which was then placed in a cell culture incubator (5% CO₂, 37°C) for 15 minutes to allow the cells to begin to attach to the scaffold. Each well was subsequently filled with 10 ml of growth medium. For diffuse cell seeding, wells were filled from the outside of the insert with enough medium to allow the medium to rise inside the insert and cover the scaffold substrate but not to go over the sides of the inserts (i.e. inside and outside volumes of medium were not connected). Cells were then dispensed as multiple droplets over the surface of the insert and subsequently placed in the incubator (5% CO₂, 37°C) overnight to allow the cells to attach to the Alvetex® Scaffold. The next day, the wells were filled with the complement of medium for a final volume of 10 ml per well. Full medium changes were made every 2-3 days.

Assessment of IGF-1 on cell migration:

Human colorectal adenocarcinoma cell lines, SW480 and SW620, were seeded following the diffuse method and cultured in Alvetex® Scaffold for 5 days in 6-well inserts placed in deep Petri dish holders. At 5 days the culture medium was changed to contain human recombinant IGF-1 (1.0 ng/ml, Sigma-Aldrich) in the outer chamber, i.e. the medium bathing the underside of the 3D culture. Media levels were controlled such that the volumes inside and outside of the well insert were not in contact through the window in the wall of the well insert. The culture media were changed every 2 days. After 5 days culture, cells were processed for MTT viability, Picogreen® assay, histology and H&E staining.

Co-culture:

3T3 fibroblasts (ATCC, CCL-92) were seeded at a density of 0.5 million cells per insert following the concentrated cell seeding method in 6-well inserts and maintained in 6-well plates. After 7 days, fibroblast 3D cultures were transferred to deep Petri dish holders and either SW480 or SW620 cells were seeded at a density of 1 million cells per insert following the diffuse cell seeding method. Co-cultures were maintained for a further 7 days and the culture medium changed every 2-3 days. Cultures were subsequently fixed and processed for histology and H&E counter-staining.

Mutant p53 cell migration assay:

Migration of control H1299 cells (null) was compared to H1299 cells that were engineered to stably express mutant p53 protein (as described previously [20]). Control or mutant cells were seeded into Alvetex® Scaffold 6-well inserts (0.5 million cells in 125 µl) maintained at the air-liquid interface and allowed to migrate for 7 days. Subsequently, cells were stained with calcein and live cell imaging was performed using confocal microscopy (Leica 2). A series of confocal optical sections were generated at 10 µm intervals.



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