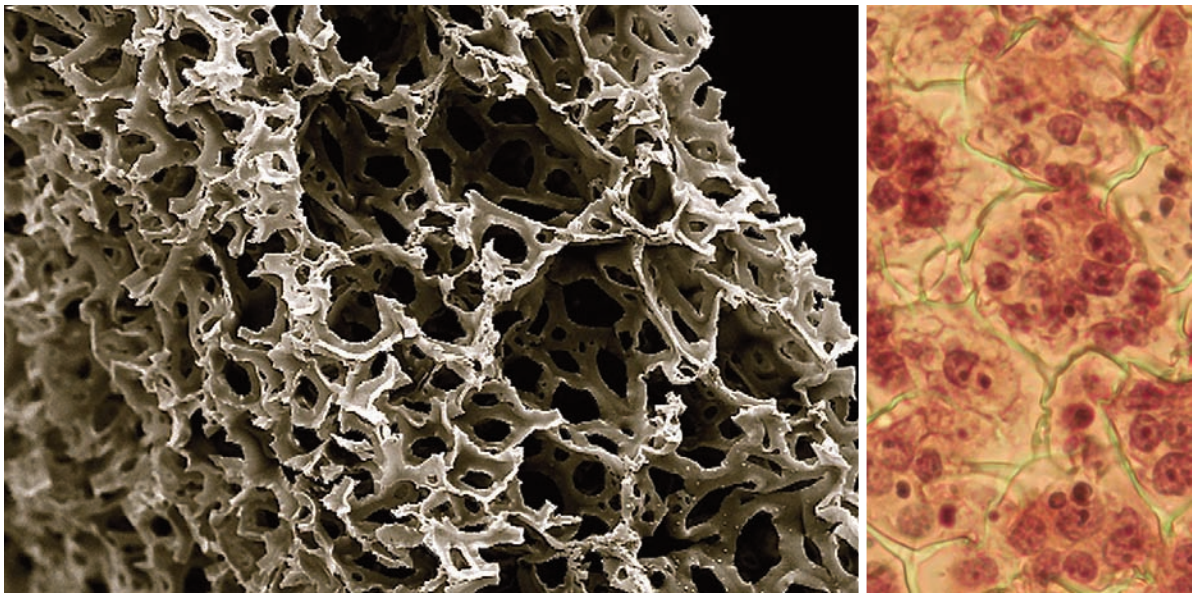
Fisher Scientific

alvetex® scaffold

Alvetex®Scaffold technology represents a novel tool for the scientist working in the cell culture field by offering an opportunity for major advancements in cellular organisation over traditional 2D cultures. Because cells in Alvetex®Scaffold form tissue-like structures, sophisticated techniques traditionally reserved for the analysis of tissues become more appropriate for cell visualisation.



Legend: *Scanning electron microscopy image of the structure Alvetex®Scaffold (main picture) and populated with TERA2.cl.SP12 cells as visualised by histological staining (right)*

A Review of Imaging Techniques Compatible with Three Dimensional Culture of Cells Grown in Alvetex®Scaffold

Introduction:

As scientists better understand the benefits of growing cells in three dimensions (3D) and routinely adopt 3D culture techniques, methods for visualising cells must also be adapted and optimised.

The most common and routinely used technique for tracking two dimensional (2D) cell cultures is light microscopy. Traditional 2D monolayer cultures are highly transparent and within a single optical plane. The minimal light diffraction and diffusion presented by the plastic surface allows the collection of focussed microscopic images. Cells cultured in genuine 3D environments, such as in Alvetex®Scaffold present some of the same constraints as tissue samples or biopsies in that simple, live observation of cultures via phase microscopy is not optimal.

There are however, other techniques that can be implemented which will allow the user to monitor culture progress easily and effectively in 3D; Simple dyes can be used to identify culture confluence and viability. The variety of end-point visualisation techniques available to those culturing cells in 3D is extensive. Options include, but are not limited to, live cell imaging, fluorescent marker analysis, confocal analysis, histology using a range of cytological stains and electron microscopy. All of these techniques have been performed on cultures grown in Alvetex®Scaffold with excellent results. Here we review common imaging methods and outline their use and suitability for cultures grown in 3D within the Alvetex®Scaffold.

Cell visualisation options within Alvetex®Scaffold:

One of the simplest methods to visualise cultured cells is phase contrast microscopy. This technique is routinely used to observe cells grown in 2D. Cells do not have to be sacrificed, therefore cultures can be visualised throughout the experimental time course, allowing the scientist to gauge culture quality and progress. This method of visualisation, however, is not optimum for detailed analysis of 3D cultures or sections of tissue.

Why can't I easily see cells in Alvetex®Scaffold under the light microscope?

Alvetex®Scaffold is a polystyrene scaffold in which voids have an average diameter of 40µm and interconnects of average diameter 13µm. Alvetex®Scaffold is supplied as a 200µm thick membrane. It is this specific architecture that allows extensive cell-cell contacts throughout the scaffold and the formation of tissue-like structures. Cells within Alvetex®Scaffold retain a more cuboidal morphology and 3D cell structure particularly in the z-plane (Figure 1) and this in turn has a significant impact on cell function.

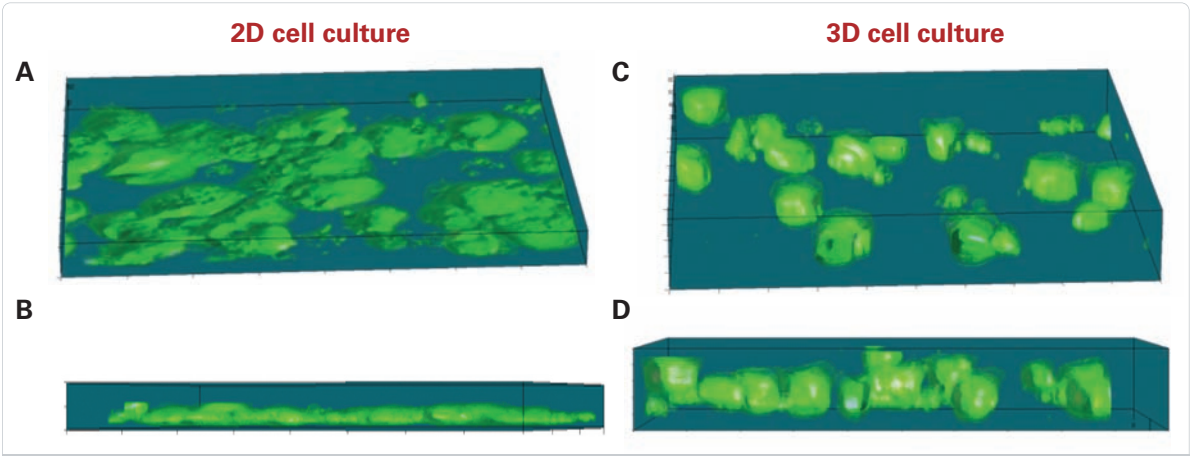


Figure 1. *An example of how cells retain a more in-vivo-like structure when cultured in 3D within Avetex®Scaffold. It is the increased volume of cells in the z plane that hinders optimal in-focus visualisation of 3D cultured cells under standard light micrcoscope.conditions. Data generated as part of a collaborative project between Reinnervate and LGC Standards [1].*

When viewing an unstained, unsectioned Alvetex®Scaffold culture under a standard light microscope, the combined density and thickness of the scaffold and the 3D culture within it prevent the clear visualisation of individual cells, due to light diffraction and its inability to penetrate into the 3D structure. This is to be expected of true 3D cultures and pieces of tissue, and is a well documented fact in recent published reviews, for example see [2,3].

As a result, many groups are focussing on devising alternative methods for cell visualisation within 3D scaffold cultures and tissue-engineered materials (for a recent review see [4]). Methods vary, ranging from the detection of fluorescent labelling (for example see [5, 6]) or autofluorescence [7], to the use of magnetic resonance imaging to locate and track cells within the scaffold architecture [8]. Many of these methods require expensive equipment, although alternative methods exist to track 3D culture progress easily and cheaply as outlined in this document. A quick comparison of common techniques available for imaging cells in 2D versus 3D cultures / tissues is summarised in Table 1 (page 3).

To visualise cells cultured in Alvetex®Scaffold, a range of methods are appropriate and available. These are discussed in detail below.

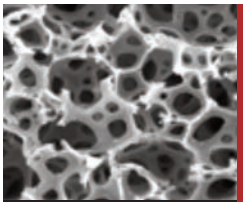
Which techniques can be used to visualise cells in Alvetex®Scaffold?

Choosing the most appropriate technique to visualise cells in Alvetex®Scaffold depends on several factors. The following sections will help in deciding which methods are best adapted to the individual experimental situation.

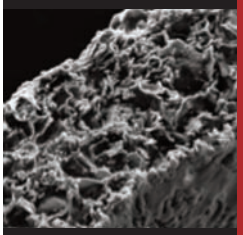
If the aim is to simply check for and monitor the presence of growing cells within Alvetex®Scaffold, then a number of different techniques can be employed. Images obtained via standard light microscopy can be enhanced by the addition of a cellular dye which increases the contrast of cells over the background.

Neutral Red Staining of Cultures for Light Microscopy

Neutral Red is a common histological dye used for staining cell nuclei (Figure 2.) It is also used widely as a cell vitality stain. Results can be qualitative or quantitative depending upon the method of analysis implemented.



SEM image of the structure of Alvetex®Scaffold (high magnification).



SEM image showing cells growing through Alvetex®Scaffold so that the scaffold itself is no longer clearly visible.

Alvetex®Scaffold is a 200µm thick highly porous, inert polystyrene scaffold that provides cultured cells with an optimal environment to grow in 3D. This allows for the formation of 3D niche microenvironments in which cell-cell interaction and communication networks occur.

The geometry and dimensions of Alvetex®Scaffold have been specifically designed to mimic the *in vivo* cellular environment: no cell is more than 100 µm from a source of nutrients and gasses. This compares favourably to the typical *in vivo* arrangement where cells are generally no more than 150-200 µm away from a capillary. Once seeded onto the Alvetex®Scaffold, typically cells easily invade the scaffold and start to produce genuine, homogeneous 3D cellular structures that resemble micro-slabs of tissue.

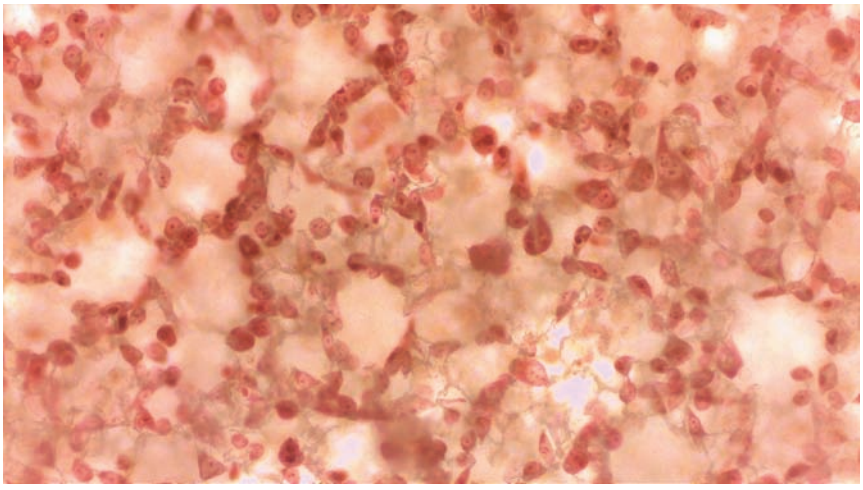
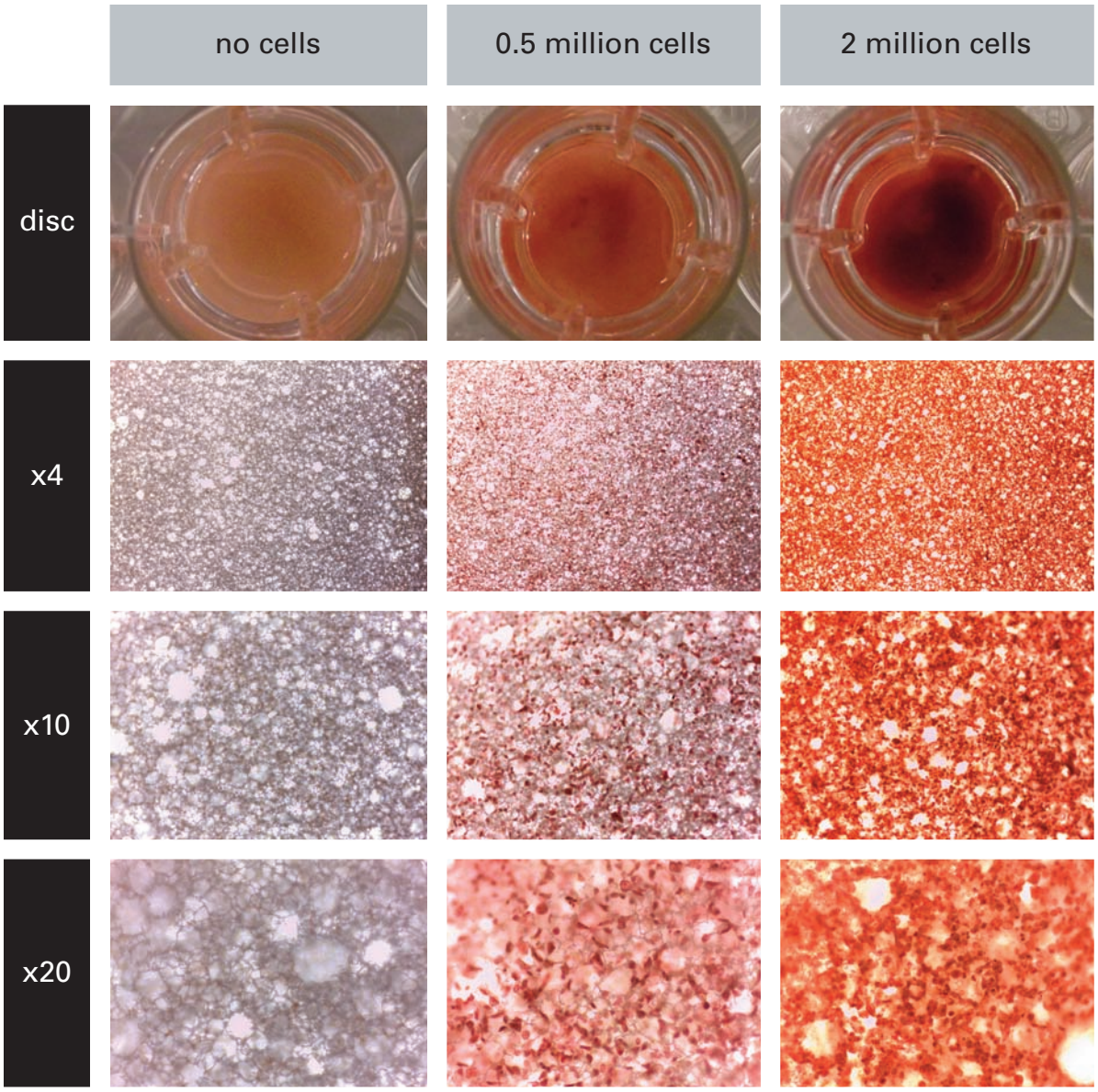


Figure 2. *Cell cultures can be visualised on Alvetex®Scaffold by staining with the non-toxic dye Neutral Red. CHO-K1 cells were seeded onto Alvetex®Scaffold at a density of 0.5 million cells per scaffold. After 2 days cells were exposed to 0.5 % Neutral red dye solution (Sigma, N6264-50ML). For full experimental details refer to Neutral red staining protocol located at* ***www.reinnervate.com/alvetex/protocols***

Method	Comments	2D samples	3D & tissue samples
Brightfield / Phase Contrast Microscopy	Commonly available No cost Easy-to-use Routine Best suited for 2D culture and tissue sections	Unstained or stained samples Live or fixed samples	Stained samples only (brightfield) Fixed and sectioned samples preferably Live or un-sectioned samples may be visible if the cells are at a relatively low density (phase)
Standard Fluorescence Microscopy	Generally available Expensive Moderate training required Routine Best suited for 2D culture and tissue sections	Stained samples only Live or fixed samples	Stained samples only Fixed and sectioned samples preferably Not recommended for un-sectioned samples
Confocal Laser Scanning Microscopy	Less available Expensive Training required Less routine Suited for both 2D and 3D cultures and tissues	Stained samples only Live or fixed samples	Stained samples only Fixed and sectioned samples obtain higher resolution images Live or un-sectioned samples can be visible (using reporters) Reconstruction of 3D architecture of thicker samples is feasible
Histology	Generally available Inexpensive Moderate training required Routine Ideally suited for 3D cultures and tissues	Fixed and Stained samples only Fixed samples only	Stained samples only Fixed and sectioned samples only
Transmission Electron Microscopy (TEM)	Less available Expensive Substantial training required Less routine Suited for both 2D and 3D cultures and tissues	Fixed samples only	Fixed and sectioned samples only
Scanning Electron Microscopy (SEM)	Less available Expensive Substantial training required Less routine Suited for both 2D and 3D cultures and tissues	Fixed samples only	Fixed and un-sectioned samples only

Table 1. *Comparison of common techniques available for imaging cells in 2D versus 3D cultures/tissues.*

alvetex scaffold



Visualisation of cells growing in 3D is enhanced by reagents which produce a colour contrast between the cells and the scaffold: Light Microscopy is thus compatible with Alvetex®Scaffold by staining the cells. Cell staining as the by-product of a colorimetric assay or fixation procedure can also be exploited for cell visualisation and monitoring the progress of cultures.

Figure 3. *Cell cultures can be visualised on Alvetex®Scaffold by staining with the non-toxic dye Neutral Red. CHO-K1 cells were seeded onto Alvetex®Scaffold at a density of 0, 0.5 million and 2 million cells per scaffold. After 24 hours cells were exposed to 0.5 % Neutral Red dye solution (Sigma, N6264-50ML). Scaffolds were then transferred to a glass microscopic slide, kept wet by adding a drop of PBS and imaged under an ICC50HD Leica microscope with LAS EZ software (brightfield setting). For full experimental details refer to 'Simple staining methods for viewing cells on Alvetex®Scaffold by light microscopy' protocol located at* ***www.reinnervate.com/alvetex/protocols****.*

The benefit of using non-toxic dyes are that they can be administered at a range of time points throughout the experiment, washed off and the scaffolds re-incubated with culture media for further cell growth. (Please note; it is recommended that if using dyes for the first time their effect on culture growth and cell survival is checked for each cell type used. Reinnervate recommends setting up extra scaffolds for dye analysis). This allows users to monitor cell survival and proliferation within the Alvetex®Scaffold over a time course.

In this context, Neutral Red solution can be used as a very quick and simple staining technique to follow culture growth and survival within Alvetex®Scaffold. Note with increased cell densities the staining intensity also increased both macroscopically and microscopically (Figure 3).

Methylene Blue Staining of Cultures for Light Microscopy

Methylene Blue is a heterocyclic aromatic chemical compound with the molecular formula $C_{16}H_{18}N_3SCl$. Following a very quick and easy staining procedure, the dye stains cell nuclei a blue colour which are visible under standard light microscopy (Figure 4). Methylene Blue dye is toxic to cells, therefore once cells have been stained, the culture is sacrificed.

Visualisation as a By-product of an Assay

Cultures can also be visualised as an extra step during routine chromogenic cell viability assays such as MTT. This assay involves the conversion of a yellow, water soluble compound, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a tetrazole, to a purple, insoluble formazan, which remains within the cell until the membranes are lysed, releasing the dye for assay detection. At the point before cell membranes are usually lysed, images of the purple-stained cells can be obtained (Figure 5). While this method provides a means for imaging cells, the MTT reagent is cytotoxic and therefore can only be used at the experiment end point, or on surplus scaffolds.

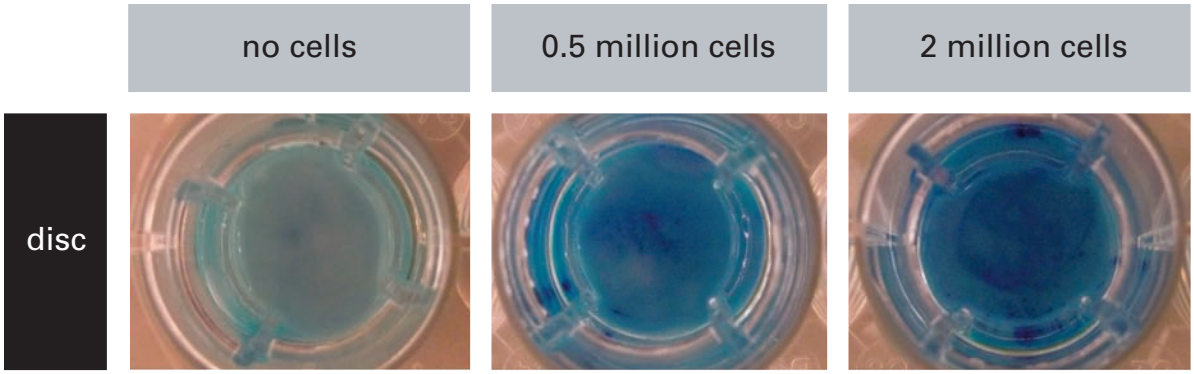


Figure 4. *Visualisation of cells on Alvetex®Scaffold with Methylene Blue solution. HepG2 cells were seeded onto Alvetex®Scaffold at a density of 0, 0.5 million and 2 million cells per scaffold. After 24 hours cells were exposed to 0.5 % Methylene Blue dye solution (Sigma, 03978-250ML). For full experimental details refer to 'Simple staining methods for viewing cells on Alvetex®Scaffold by light microscopy' protocol located at* ***www.reinnervate.com/alvetex/protocols****.*

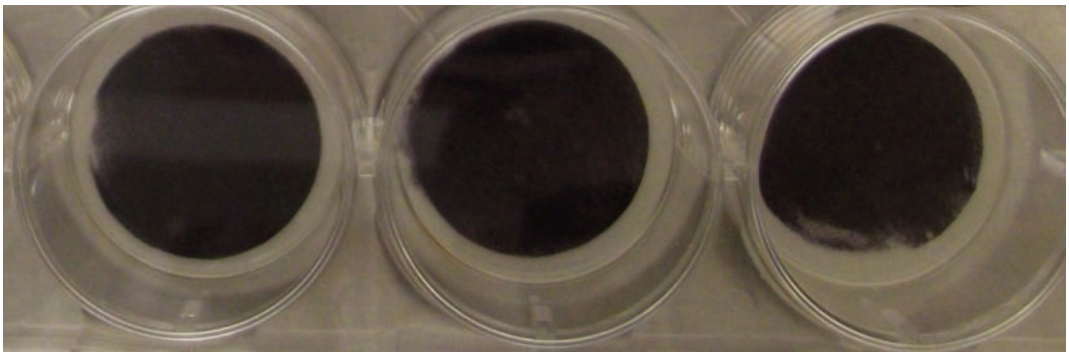


Figure 5: *The gross location of viable cells is clearly visible on the Alvetex®Scaffold disc after staining with MTT cell viability reagent. HaCat cells were cultured on Alvetex®Scaffold in the 12-well plate format for 4 days prior to analysis (for full experimental details see separate MTT protocol available at* ***www.reinnervate.com/alvetex/protocols****.*

Visualisation as a by-product of Cell Fixation

Cell fixation methods which employ coloured fixatives are also able to provide a visual estimation of cell growth in Alvetex®Scaffold. Bouin's reagent colours areas of cell growth yellow during fixation, which enables visual comparison of cell growth between samples and also cellular distribution within a single sample (Figure 6A). The Bouin's stain remains visible through wax embedding and is only lost during subsequent histological staining, for example using haematoxylin and eosin.

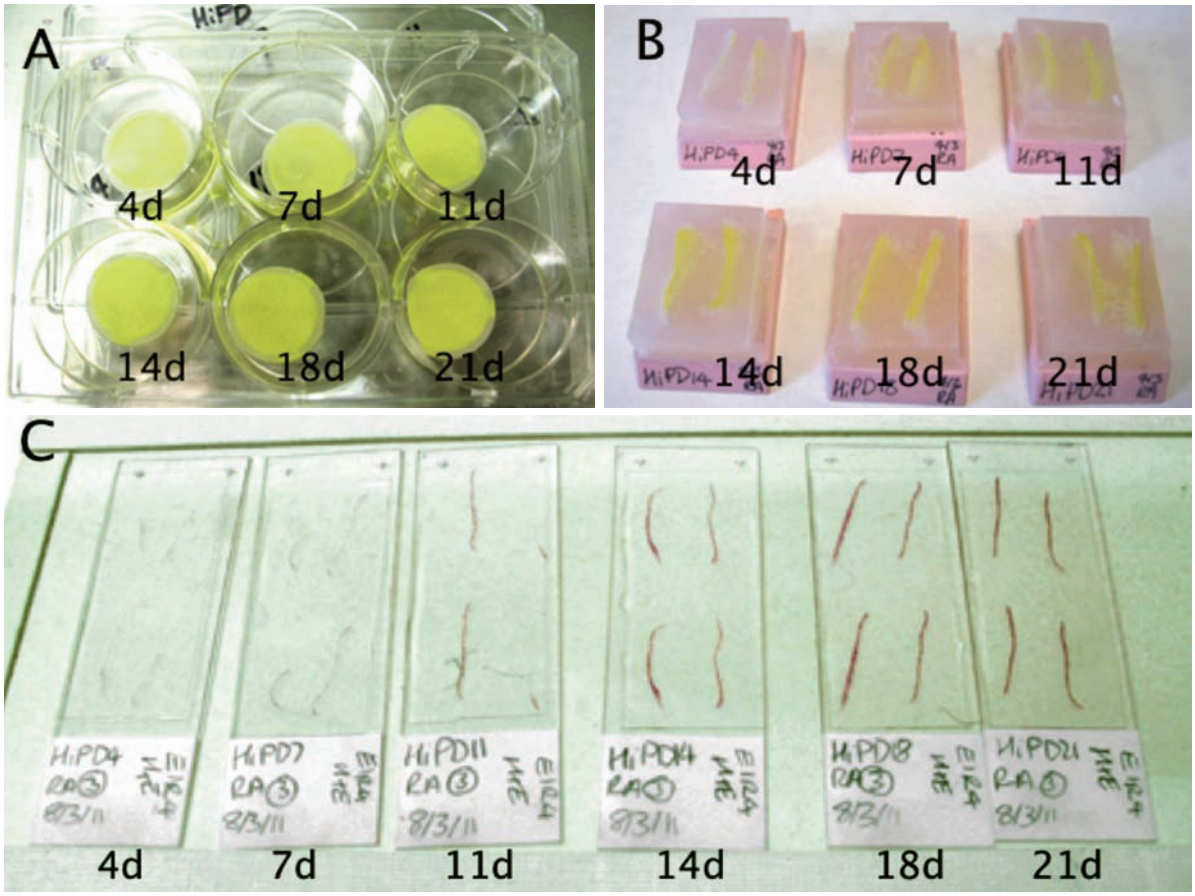


***Figure 6**. Cells can be visualised on Alvetex®Scaffold following fixation with the yellow coloured Bouin's fixative. SW480 colon carcinoma cells were cultured on Alvetex®Scaffold and removed for fixation at 4, 7, 11, 14, 18, and 21 days after seeding. The increasing number of cells over time is reflected in increasing staining intensity. **A.** SW480 cultures in Alvetex®Scaffold discs following Bouin's fixation and ethanol dehydration; **B.** Bouin's fixed and wax embedded SW480 Alvetex®Scaffold cultures; **C.** Slide mounted cross sections (10µm slices) of Bouin's fixed and wax embedded SW480 Alvetex®Scaffold cultures stained with haematoxylin and eosin.*

Live Cell Imaging in Alvetex®Scaffold Using Confocal Microscopy

For a more in-depth, single cell analysis, live cell imaging, and other more involved techniques can be implemented. Traditionally, imaging of live cells allows migrating cells to mimic wound healing or substrate invasion in vitro. In more recent years this technique has been implemented to follow live cell cultures grown in 3D scaffolds [9], and is providing information regarding how cell cultures interact within the 3D niche microenvironment.

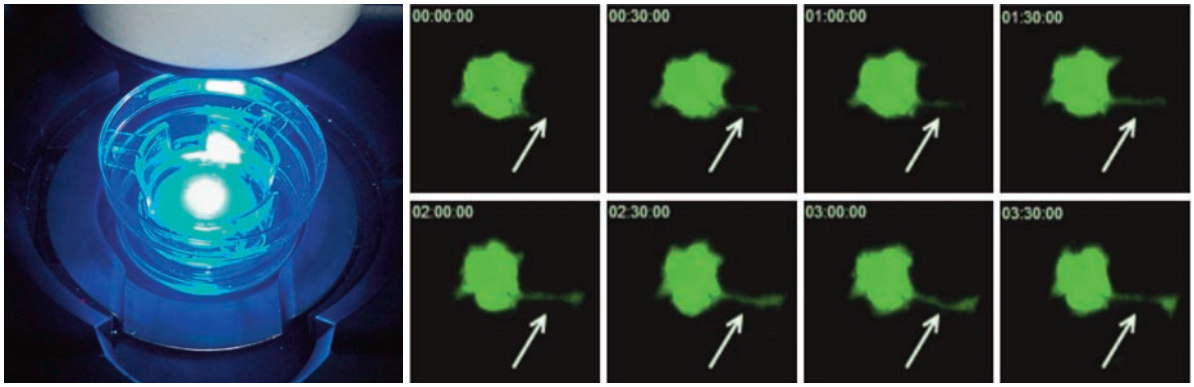


***Figure 7:** GFP-transfected CHO-K1 cells were cultured on Alvetex®Scaffold 6 well inserts for 6 days. Captured images were obtained every 30 minutes using a Zeiss LSM 510 confocal microscope with heated stage (for full experimental details refer to 'live-cell imaging protocol' located at www.reinnervate.com). In this example, a series of integrated z-stacks is presented which shows the behaviour of a single transfected CHO-K1 cell over a period of 3 hours.*

Fixation of Cells Within Alvetex®Scaffold and Subsequent Downstream Analysis

All of the techniques discussed below require fixation of cells within the Alvetex®Scaffold. Once cells are fixed numerous downstream analytical techniques can be performed. The choice of the method will depend on the specific data required. Fixation is achieved either by chemical means or rapid freezing with the use of a tissue support solution such as the cryoprotective embedding solution OCT to keep the membrane in place. For full details of Alvetex®Scaffold -compatible fixation protocols refer to 'Histology Series Part 1. Choosing the Right Fixative to Preserve 3D Cell Cultures' found at **www.reinnervate.com/alvetex/protocols**. Given the relatively thin nature of Alvetex®Scaffold compared with typical tissue samples, fixation of cells is rapid, uniform and efficient, preserving the 3D culture in a life-like condition.

Fluorescence Microscopy:

Immunofluorescence uses the recognition of cellular targets by fluorescent dyes or antigen-specific antibodies coupled to fluorophores. Depending on the antibody or dye used, proteins, lipids and DNA can be visualised within individual cells and tissues. Alvetex®Scaffold can easily be processed like a standard tissue sample, allowing established immunocytochemical methods to be followed with excellent results (Figure 8).

Alvetex®Scaffold cell cultures are amenable to highly sophisticated cell imaging techniques such as confocal imaging. Confocal microscopy can be used to visualise fixed cells or to follow living cultures in real time.

Alvetex®Scaffold cultures can be regarded as in vitro miniature tissues. As such standard histological techniques can be applied to their analysis. These methods include immunohistochemistry, histological staining, confocal microscopy, and electron microscopy.

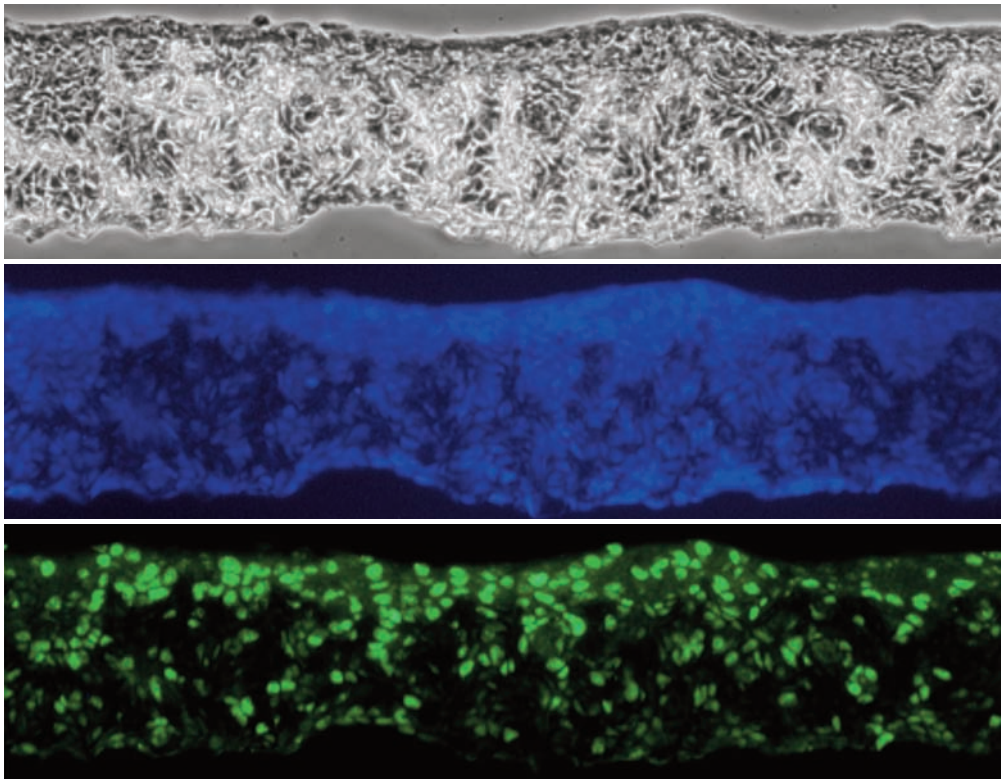


Figure 8. *Human keratinocyte cell line (HaCaT) grown in Alvetex®Scaffold (7 days air exposure). The culture was fixed and processed for paraffin wax embedding and immunohistochemical analysis by fluorescent microscopy. The three images from the same region show; phase contrast (top), blue fluorescent Hoescht 33258 nuclei stain (middle) and Ki67 staining (bottom). For the full experimental details refer to 'Immunocytochemistry protocol' located at* ***www.reinnervate.com/alvetex/protocols****.*

alvetex scaffold

Confocal Laser Scanning Microscopy:

Confocal microscopy relies on the combination of point illumination and a pinhole to eliminate most of the out-of-focus light signal and allows for reconstruction of 3D volumes, making it ideal to image cultures grown in full-thickness Alvetex®Scaffold. It should be noted that lipophilic dyes, such as Nile Red (Figure 9), will bind strongly to the Alvetex®Scaffold. However, this feature can be used to conveniently visualise the scaffold within the cell culture. As high-density cell cultures grown in Alvetex®Scaffold approximate the complexity and structure of in vivo tissues, fluorophores specifically developed for *in vivo* deep imaging can be used to improve performance if needed.

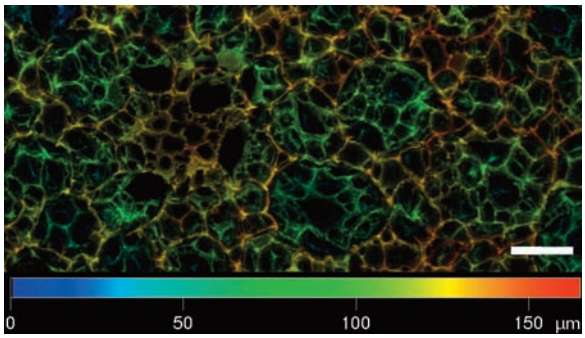


Figure 9. *Depth colour-coded Z stack of cell-free Alvetex®Scaffold stained with Nile Red. Picture taken on a Zeiss LSM 510 confocal microscope. Note the depth of the Z-stack exceeds 150µm. Scale bar 50µm.*

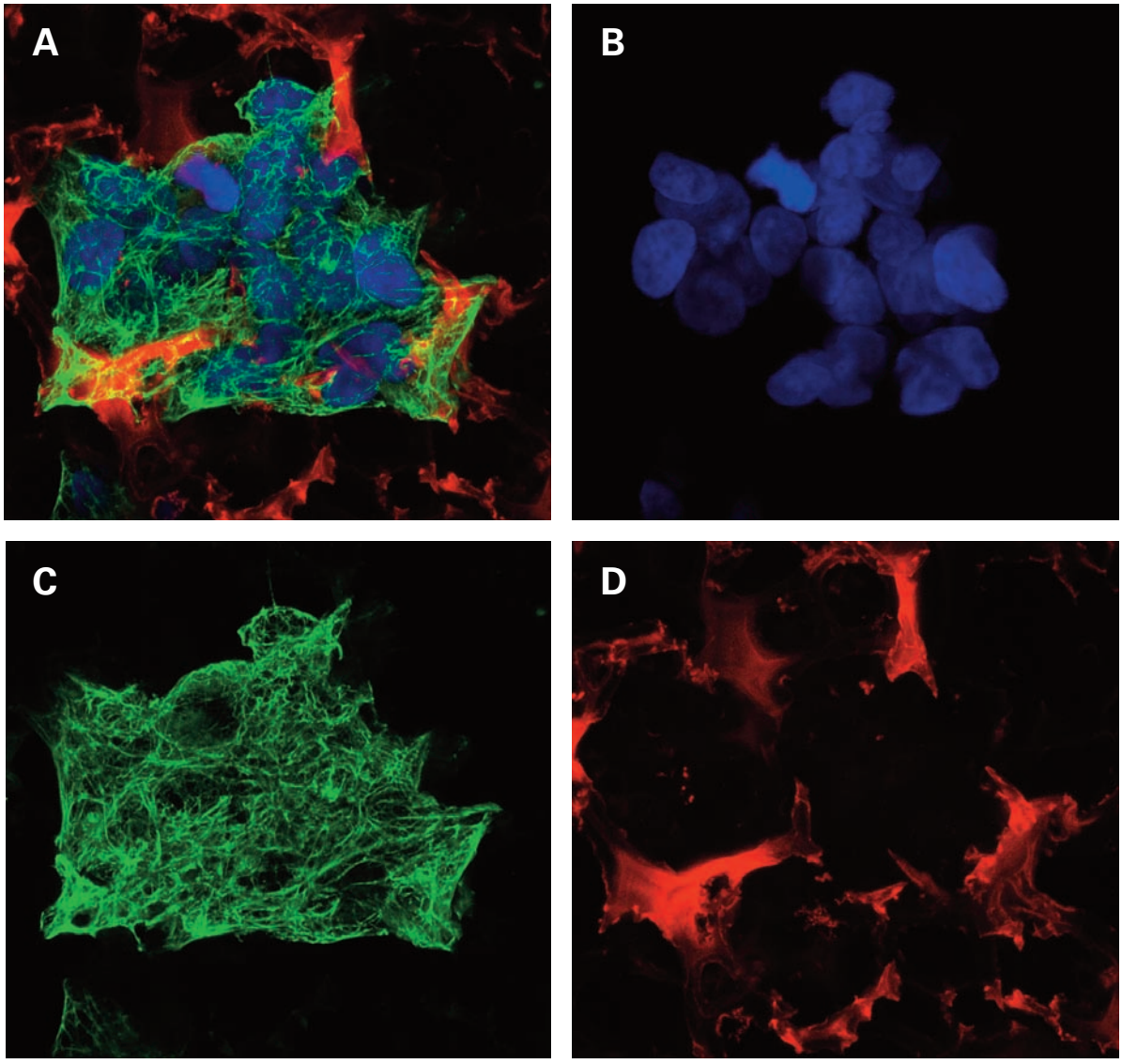


Figure 10*. HepG2 cells grown for 3 days in Alvetex®Scaffold 12-well plate format. Cells were stained with Hoechst 33342 (blue), cytokeratin 8 (green) and Nile Red (red). Pictures were taken on a Zeiss LSM 510 confocal microscope. Note the background signal from Alvetex®Scaffold in the blue and green channels is very low. For full experimental details refer to 'Confocal protocol' located at* ***www.reinnervate.com/alvetex/protocols****.*

Histology:

Histology is seen as the gold standard of cell visualisation in tissues, and therefore is very suitable for 3D cell culture. Histology is essentially the art of observing a thin section of fixed material under either a light microscope or electron microscope. The ability to specifically identify cellular components can be enhanced by the addition of histological stains. Common stains include: Haematoxylin and Eosin, which are generally used together to visualise gross cell architecture; Toluidine Blue stain, which is also a general cell stain, staining most proteins; and Masson's trichrome which is a three-colour staining protocol producing red keratin and muscle fibres, blue or green collagen and bone, light red or pink cytoplasm, and dark brown to black cell nuclei.

Unlike other 3D cell culture supports, Alvetex®Scaffold can easily be processed like a standard tissue sample, allowing established histology protocols to be followed with excellent results as seen in Figure 11.

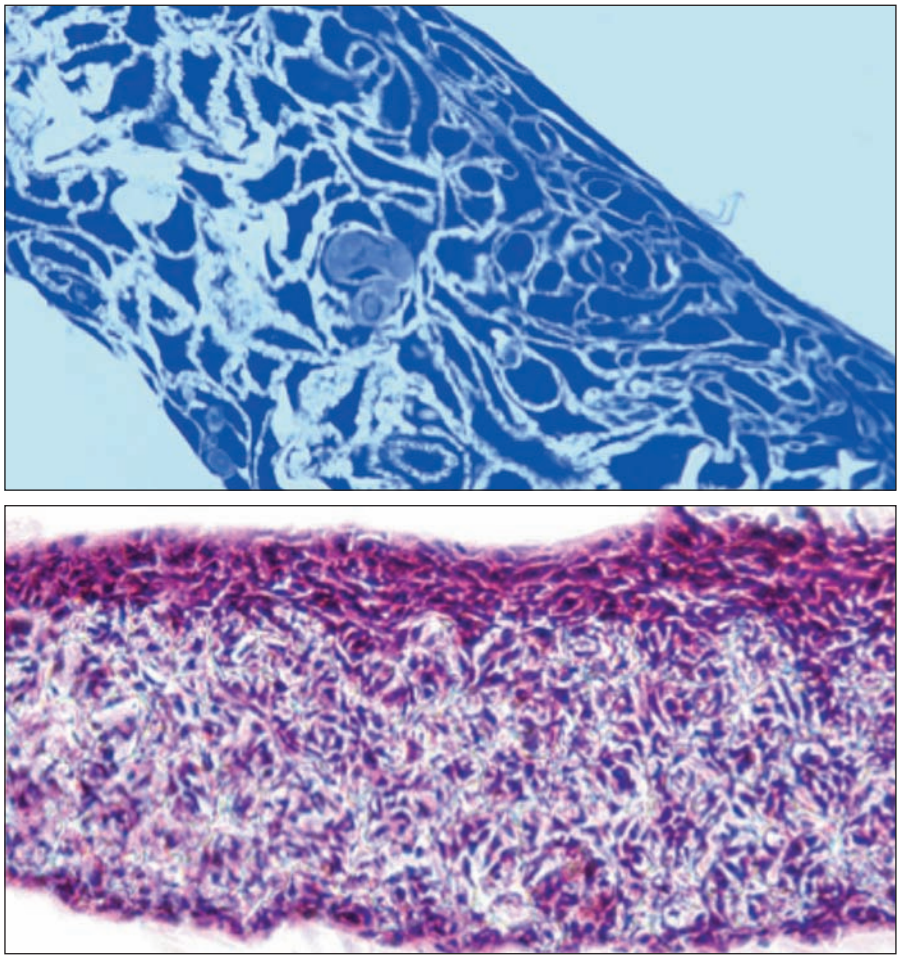


Figure 11: *Cells grown in Alvetex®Scaffold can be fixed and processed for histological analysis using standard methods. Both staining examples are carried out on human keratinocytes (HaCaT) grown in Alvetex®Scaffold for 7 days. The sample on the top shows a culture that was fixed and processed for resin embedding (L R White resin). Resin sections (1 µm) were stained with Toluidene Blue for structural analysis by light microscopy. The sample on the bottom shows a culture that was fixed and processed for paraffin embedding. Sections (10 µm) were stained with Haematoxylin and Eosin for morphological analysis by light microscopy. For full experimental details refer to the 'Histology Series of protocols' located at* ***www.reinnervate.com/alvetex/protocols.***

Electron Microscopy:

Scanning electron microscopy (SEM) is becoming a popular method of visualisation of cultures grown in 3D. SEM is a form of electron microscopy where images are obtained by scanning samples using a high-energy beam of electrons. As the samples are scanned the electrons interact with the sample surface, and these interactions are detected and processed, leading to high-resolution images depicting the sample topography and composition.

Alvetex®Scaffold can easily be processed like a standard tissue sample, allowing established methods to be followed with excellent results (Figure 12).

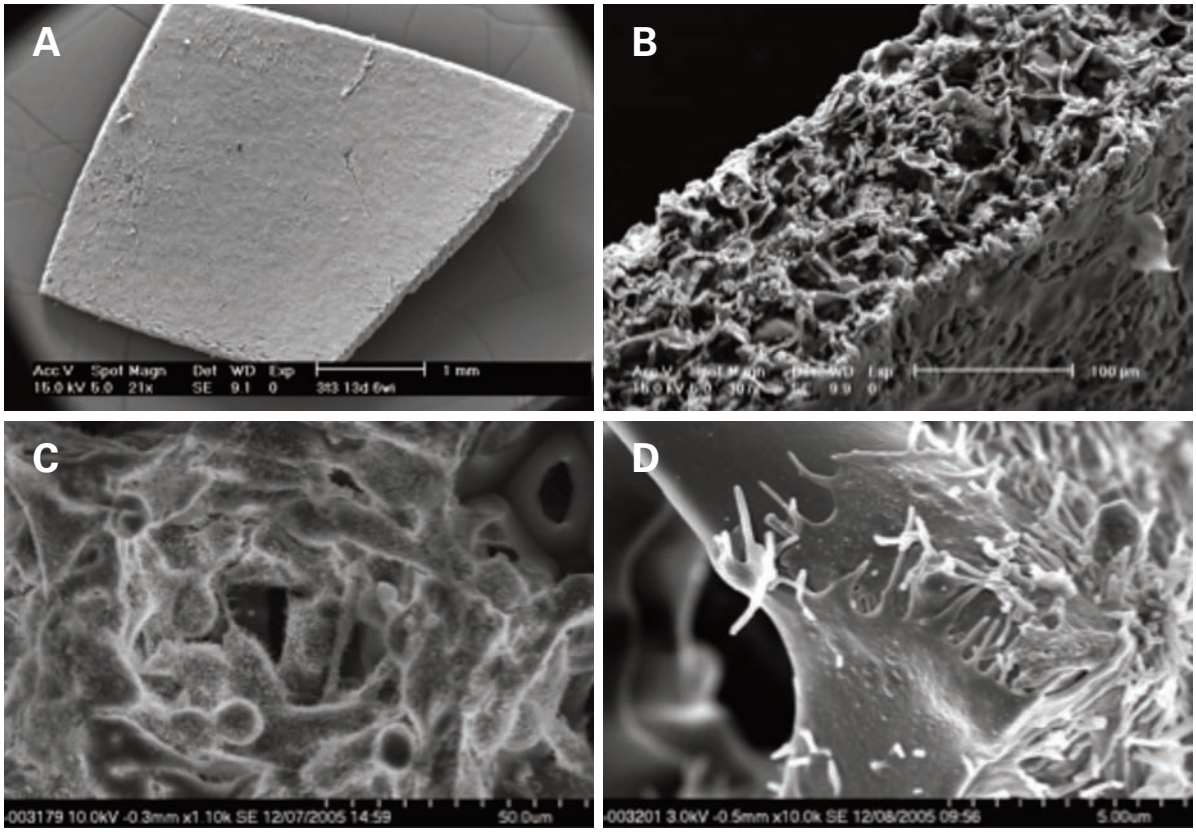


Figure 12. *Detailed structure of 3D cultures can be visualised using scanning electron microscopy. Inspection of pieces of Alvetex®Scaffold at low magnification shows homogeneous coverage by cultured cells* ***(A)****. Higher magnification imaging in this transverse section reveals cells growing throughout the scaffold* ***(B)****. Increasingly higher magnification micrographs reveal how cells interact with each-other and the Alvetex®Scaffold* ***(C & D)****. See scale bar inserts. For full experimental details refer to 'SEM protocol' located at* ***www.reinnervate.com/alvetex/protocols****.*

Transmission electron microscopy (TEM) can also be performed on cell cultures grown in Alvetex®Scaffold (Figure 13). TEM allows the ultrastructure of cells to be visualised due to the extreme high magnification achieved. Samples are processed in a similar way to SEM, however, instead of being sputter coated in gold the samples are embedded in resin and sectioned into ultrathin sections (1 µm). Samples are loaded into the TEM and a beam of electrons is transmitted through the sections. The electron beam interacts with the sample architecture and it is these interactions which are detected, processed and images are obtained.

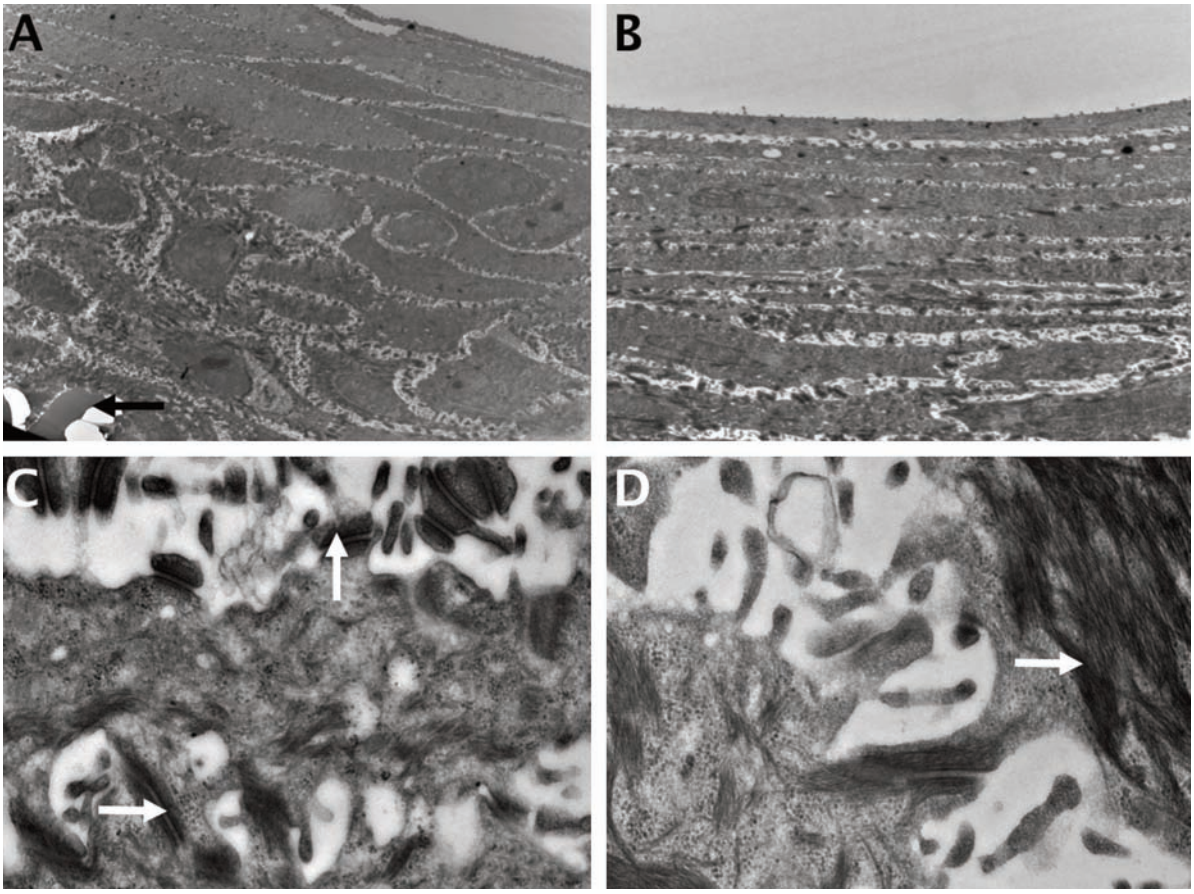


Figure 13. (A) *TEM image of keratinocytes cultured at air-liquid interface without collagen or fibroblasts for 14 days. The scaffold is indicated by a black arrow.* ***(B)*** *TEM image showing cells progressively flattening towards the upper surface of the culture after 14 days at air-liquid interface.* ***(C)*** *High magnification image showing desmosomes, indicated with white arrows.* ***(D)*** *High magnification image showing bundling of keratin filaments underneath cell membrane, indicated with white arrows. (Scale bar 500 µm) For full experimental details refer to 'TEM protocol' located at* ***www.reinnervate.com/alvetex/protocols***.

alvetex scaffold

Conclusions:

As outlined throughout the document, there are many imaging techniques which can be implemented to visualise cultures and cells grown within Alvetex®Scaffold in. For following culture progress, dyes stain cells contrasting them against the scaffold background allowing visualisation via light microscopy. For more in depth culture analysis, a range of more complex techniques can be implemented similar to those performed on tissue samples with excellent results.

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