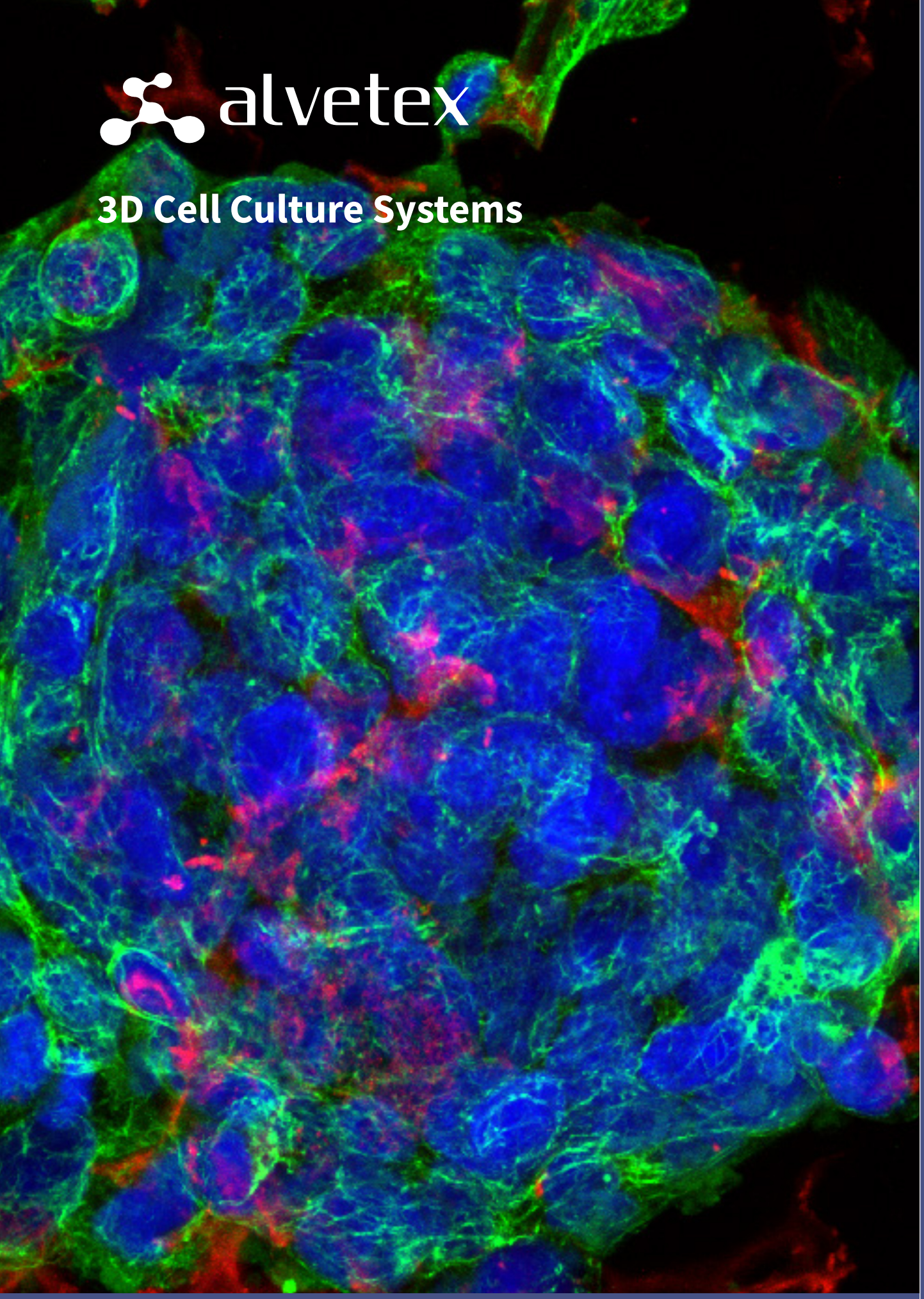
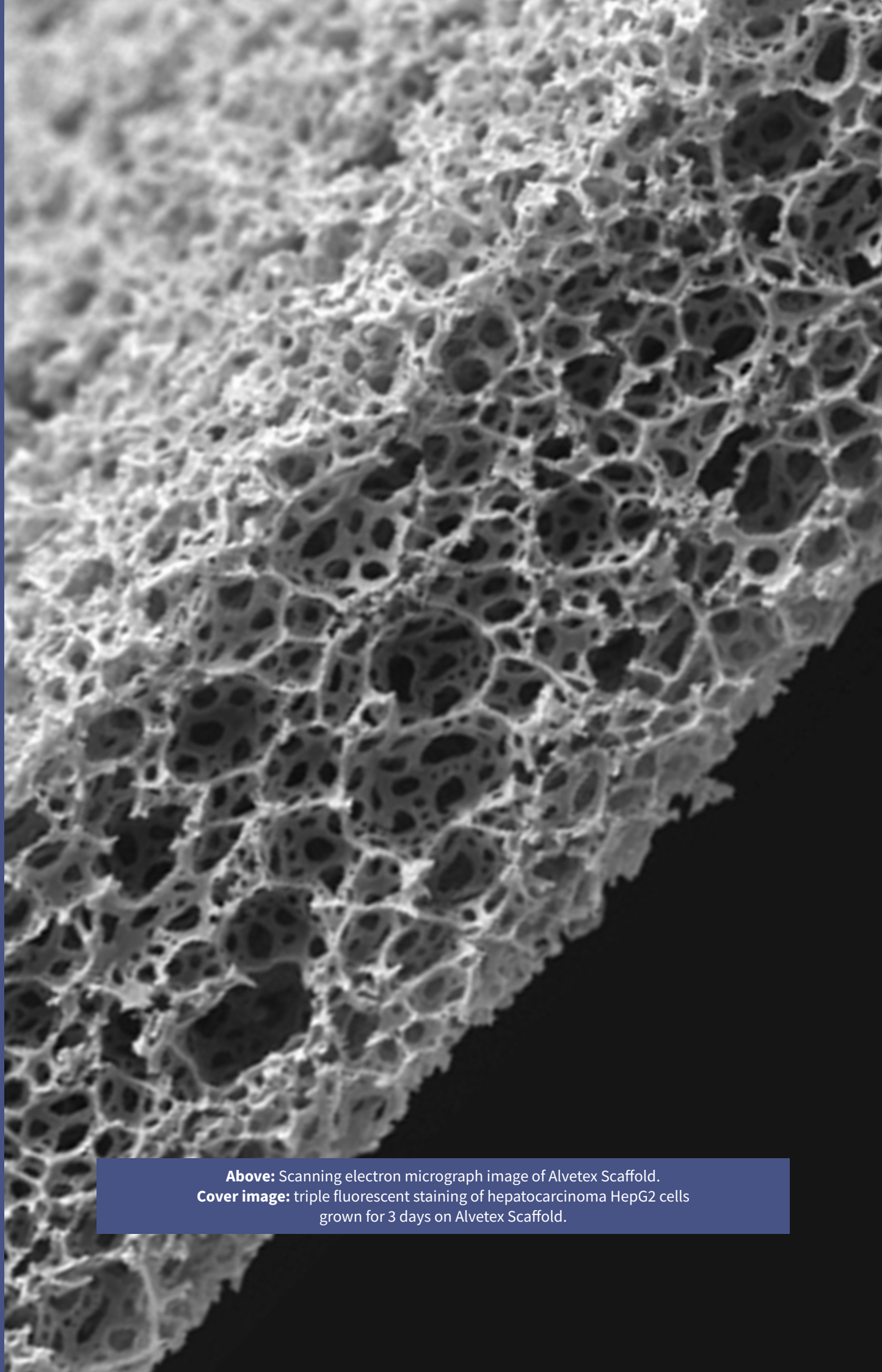


alvetex

3D Cell Culture Systems

REPROCELL

www.reprocell.com/alvetex



Above: Scanning electron micrograph image of Alvetex Scaffold.
Cover image: triple fluorescent staining of hepatocarcinoma HepG2 cells grown for 3 days on Alvetex Scaffold.

alvetex
A REPROCELL BRAND

Contents

Ordering information

https://www.reprocell.com/product-catalog/alvetex-3d-cell-culture-systems

REPROCELL

Culturing cells in three dimensions

The goal of three-dimensional (3D) cell culture is to eliminate the stress and artificial responses that cells experience as a result of cell adaptation to flat, 2D growth surfaces and to create suitable surroundings for optimal cell growth, differentiation and function.

Genuine 3D cell culture allows individual cells to maintain their normal 3D shape and structure with minimal exogenous support and interference. Cells are freely able to form complex interactions with adjacent cells and to receive and transmit signals, enabling a more natural environment to foster the creation of native architecture found in tissues.

Could the limitations of 2D cell culture be holding you back?

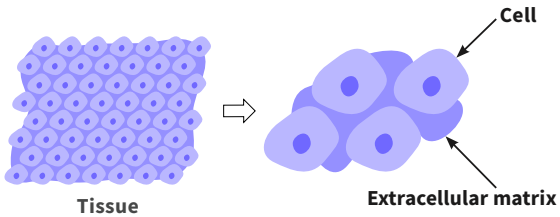
Finding experimental systems that model and provide useful information about *in vivo* biological processes is one of the most challenging tasks in scientific research. Cell culture enables the growth of cells outside the body in a controlled laboratory environment. Although convenient, culturing mammalian cells results in flat monolayer cultures. This is dramatically different to the 3D *in vivo* environment cells experience in the body.

In order to enable survival in 2D culture, cells are forced to make dramatic changes to their morphology. Gene expression mediated changes to the cytoskeleton result in a flattened cell morphology. These changes can impair cellular functions. Cells grown in the laboratory do not always grow and function in a realistic fashion. This has major implications for research and discovery.

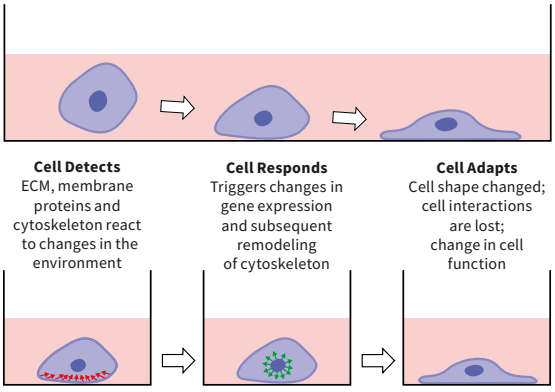
For example:

- Inaccuracy of predictive assays in the drug discovery process
- Modification of normal behavior of cells in response to external stimuli
- Generation of potentially inaccurate / misleading data
- Misunderstanding of complex biological phenomena
- Poor planning and direction of future research program

***In vivo* 3D environment:** typically cells maintain a 3D ellipsoidal structure and organization



***In vitro* 2D environment:** cells adopt flattened morphology in a monolayer

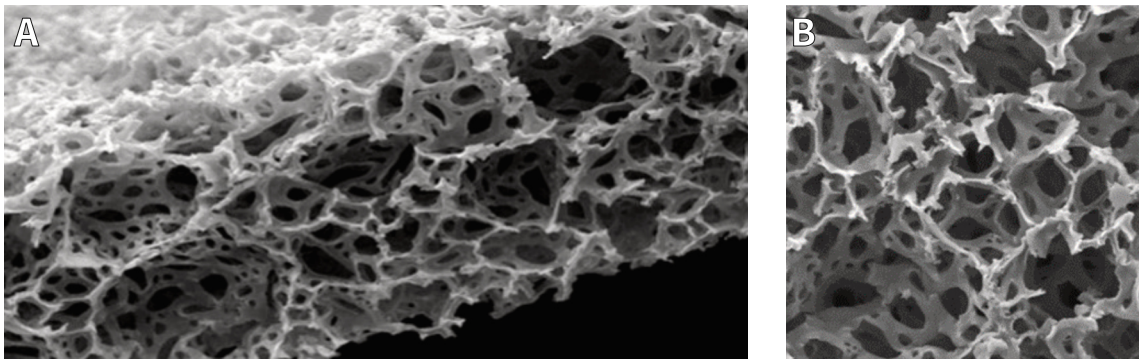


Alvetex is a unique micro-scale environment to support genuine 3D cell growth

The structure of Alvetex provides cultured cells with an environment and physical space in which to grow in three dimensions. The architecture of Alvetex, as viewed by a scanning electron microscope, illustrates voids that are interconnected by pores creating a material with > 90% porosity. Emulsion templating is used to control the size of the voids, optimizing the porosity of the material for 3D cell culture. The matrix structure has been designed to enable cells to reproduce an environment that is more consistent with the *in vivo* cellular environment.

Alvetex Scaffold

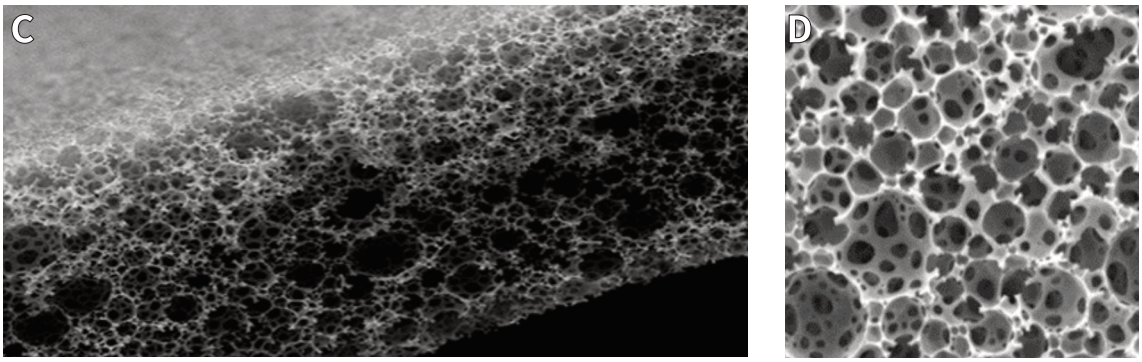
Our market leading product, Alvetex Scaffold is primarily designed for three dimensional culture of dissociated mammalian cells within the scaffold, forming three dimensional associations as they propagate and migrate.



Scanning electron microscope image of Alvetex Scaffold, to highlight its porous structure. **A**: a 200 µm thick Alvetex Scaffold disc. **B**: Close up of Alvetex Scaffold voids with dimensions of approximately 42 µm in diameter and interconnects of approximately 13 µm in diameter.

Alvetex Strata

Our second generation product, Alvetex Strata is primarily designed to support the growth of cells and intact tissues on the surface of the membrane.

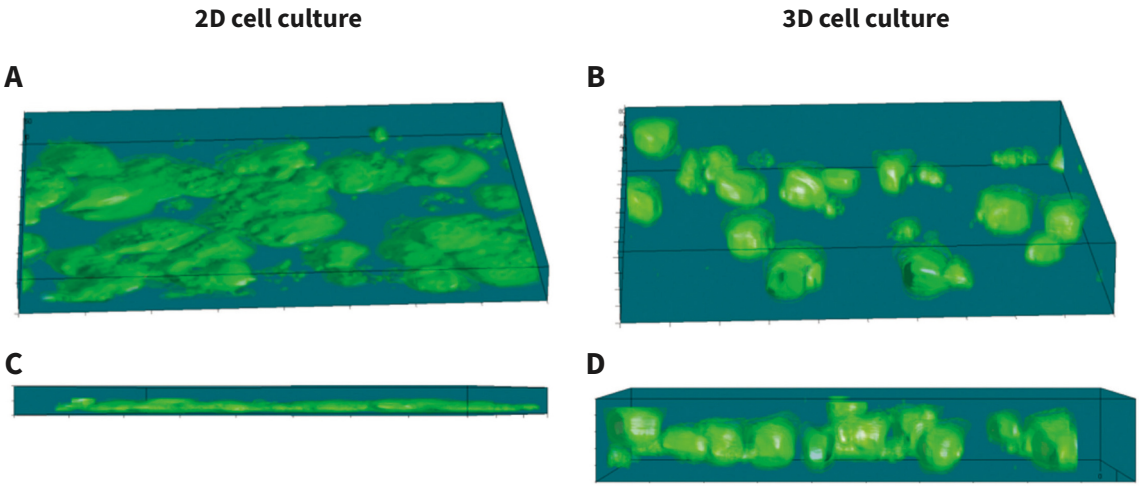


Scanning electron microscope image of Alvetex Strata. **C**: a 200 µm thick Alvetex Strata disc. **D**: Close up of Alvetex Strata voids with dimensions of approximately 15 µm in diameter and interconnects of approximately 5 µm in diameter.

Alvetex overcomes the limitations of culturing cells on flat plastic surfaces

The geometry and shape of a cultured cell is significantly affected by the physical environment in which it grows. Using Alvetex to culture cells, it is possible to maintain natural 3D cell morphology and to replicate the conditions for growth and development that occur within living tissues.

Unlike cells grown on conventional 2D substrates where cell morphology is much more varied in appearance, consisting of clumps and individual flattened cells, cells grown in Alvetex exhibit a morphology that is much more consistent with that found within the *in vivo* environment. The appearance of cells is more homogeneous with a high degree of 3D organization.



Cells grown on conventional 2D surfaces (**A** and **B**) adopt a typical flattened morphology covering a large surface area in horizontal x–y plane (**A**) and have a reduced height in the vertical z plane (**B**). In comparison, cells maintained in Alvetex Scaffold (**C** and **D**) retain a more cuboidal morphology and 3D cell structure, particularly in the z-plane.

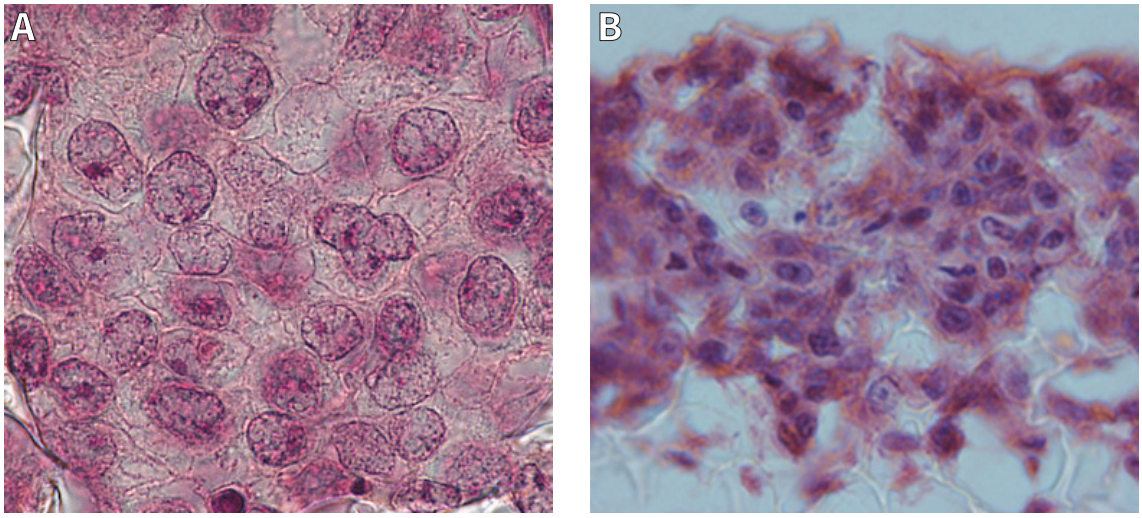
Data generated during a collaborative project between Reinnervate Ltd and LGC Standards – data now published in the following journal: Title: Rat primary hepatocytes show enhanced performance and sensitivity to acetaminophen during three dimensional culture on a novel polystyrene scaffold designed for routine use. Maaike Schutte, Bridget Fox, Marc Baradez, Alison Devonshire, Jesus Minguez, Maria Bokhari, Stefan Przyborski, Damian Marshall. *Assay and Drug Development Technologies*. DOI: 10.1089/adt.2011.0371. (Reinnervate Ltd. was acquired by REPROCELL Inc. in 2014, and merged with Biopta Ltd. to form REPROCELL Europe Ltd. in 2016.)

Alvetex enhances the biological relevance of your cell culture research

By accurately recreating the complex cellular organization and environment experienced by cells within their native tissues, Alvetex enables more accurate investigation into the study of cell behavior and function than ever before possible within conventional 2D model systems.

Cells maintain their natural 3D shape and structure within Alvetex, freely interacting with adjacent cells and laying down extra-cellular matrix which often leads to the formation of “mini slabs” of tissue-like structures. Using Alvetex, the cell biologist can create *in vitro* models which more accurately mimic the tissue environment, gaining a much deeper insight into the complexities of cell function and behavior.

Typical mammalian cells are around 10-25 µm in size and are rarely further than 0-50 µm from another cell or 100-200 µm from a source of nutrients via a blood capillary. Alvetex is made of the same polystyrene as used in traditional 2D cell culture plasticware. Alvetex has been designed to enable cells to reproduce natural shape and form to enable the cell biologist to maintain the integrity of the micro-scale *in vivo* cell environment within simple *in vitro* models.



Cells grown within Alvetex Scaffold maintain their natural shape and 3D organization. **A**: 3D cell culture of human pluripotent stem cells within Alvetex Scaffold. **B**: 3D cell culture of liver hepatocytes grown within Alvetex Scaffold.

Alvetex: Frequently Asked Questions

www.reprocell.com/alvetex/alvetex-faq

Changing cell culture environment impacts on cell behavior

	Traditional 2D Cell Culture	Alvetex 3D Cell Culture	Normal *in vivo* Environment
General dimensions and physical differences			
Maximum distance of cell from the source of nutrients	0	0-100 µm	0-200 µm
Resemblance to *in vivo* cellular environment	Low	High	N/A
Appearance of cell morphology	Flattened	3D shape	3D shape
Potential for 3 dimensional cell to cell interactions	Very low	High	High
Ability to form complex 3 dimensional cellular structures	Very low	High	High
Upon initial seeding of cells onto plasticware			
Degree of cellular stress placed upon cell structure	High	Low	N/A
Changes to protein and gene expression	High	Low	N/A
Cell surface area in contact with plasticware	At least 50%	0-50%	N/A
Post seeding phase			
Ongoing changes to cellular shape	High	Low	N/A
Need for remodeling of cytoskeletal architecture	High	Low	N/A
Deviation from normal *in vivo* morphology	High	Low	N/A
Cell surface area in contact with plasticware	At least 50%	0-50%	N/A
Opportunity for enhanced *in vitro* cell functionality	Low	High	N/A

Alvetex's unique scaffold dimensions are ideally suited to 3D cell culture

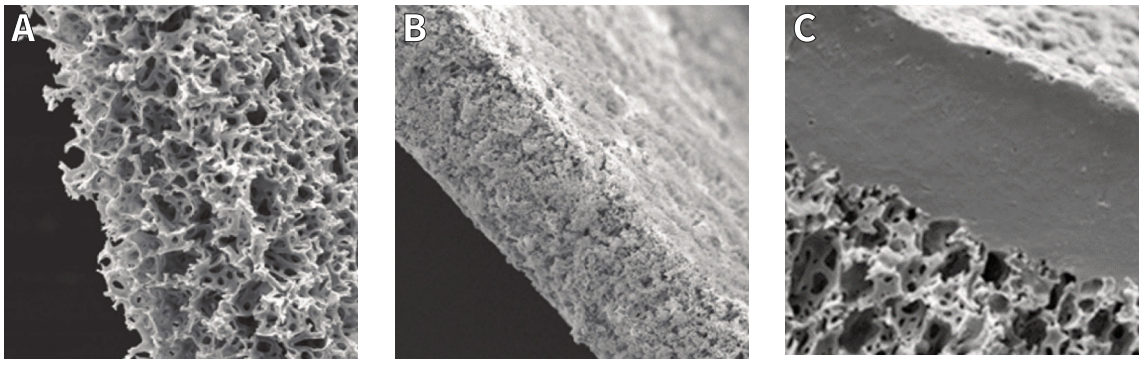
Alvetex Feature	Benefits of using Alvetex for 3D cell culture
Same polystyrene as existing cell culture plasticware	• Easily switch between 2D and 3D protocols • Inert – no effect on cell growth or function – no new experimental variables • Stable – does not degrade, no change throughout long-term studies • Can be pre-coated with ECM proteins
Consistent scaffold structure – extremely low batch to batch variability	• Reproducible, consistent results, low batch to batch variability • Genuine and homogeneous 3D cell growth
Entire scaffold is only 200 µm thick	• No cell is ever more than 100 µm away from nutrients and gasses – mimics *in vivo* conditions • Cells can feed and excrete via passive diffusion – mimics *in vivo* conditions
> 90 % Porosity	• Cells can easily penetrate scaffold and lay down ECM to more closely mimic *in vivo* conditions • Cells and media move freely through the matrix • Nutrients and waste exchanged by passive diffusion • Partial cell retrieval is possible
Alvetex Scaffold void dimensions approximately 42 µm	• Typically up to 75 cells may occupy a single void
Alvetex Strata void dimensions approximately 15 µm	• Highlight behavioral differences between non-invasive (e.g. epithelial) and invasive (eg. Fibroblast) cell types

Unlock the potential of your
in vitro cell culture with Alvetex.
Take your research
to a whole **new dimension.**

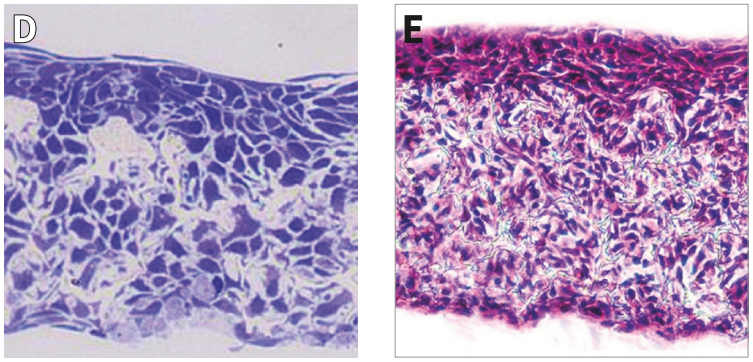
Alvetex 3D cell culture leads to the creation of “mini-slabs” of tissue

Cells grow and divide occupying the 3D space within the porous Alvetex scaffold. Cells form complex interactions with one another, behaving in a manner that far more closely mimics normal growth in tissues than is possible using traditional 2D techniques. Cells are free to migrate throughout the matrix, functioning as they would within their natural environment, laying down extracellular matrix and forming organized and complex *in vivo*-like structures.

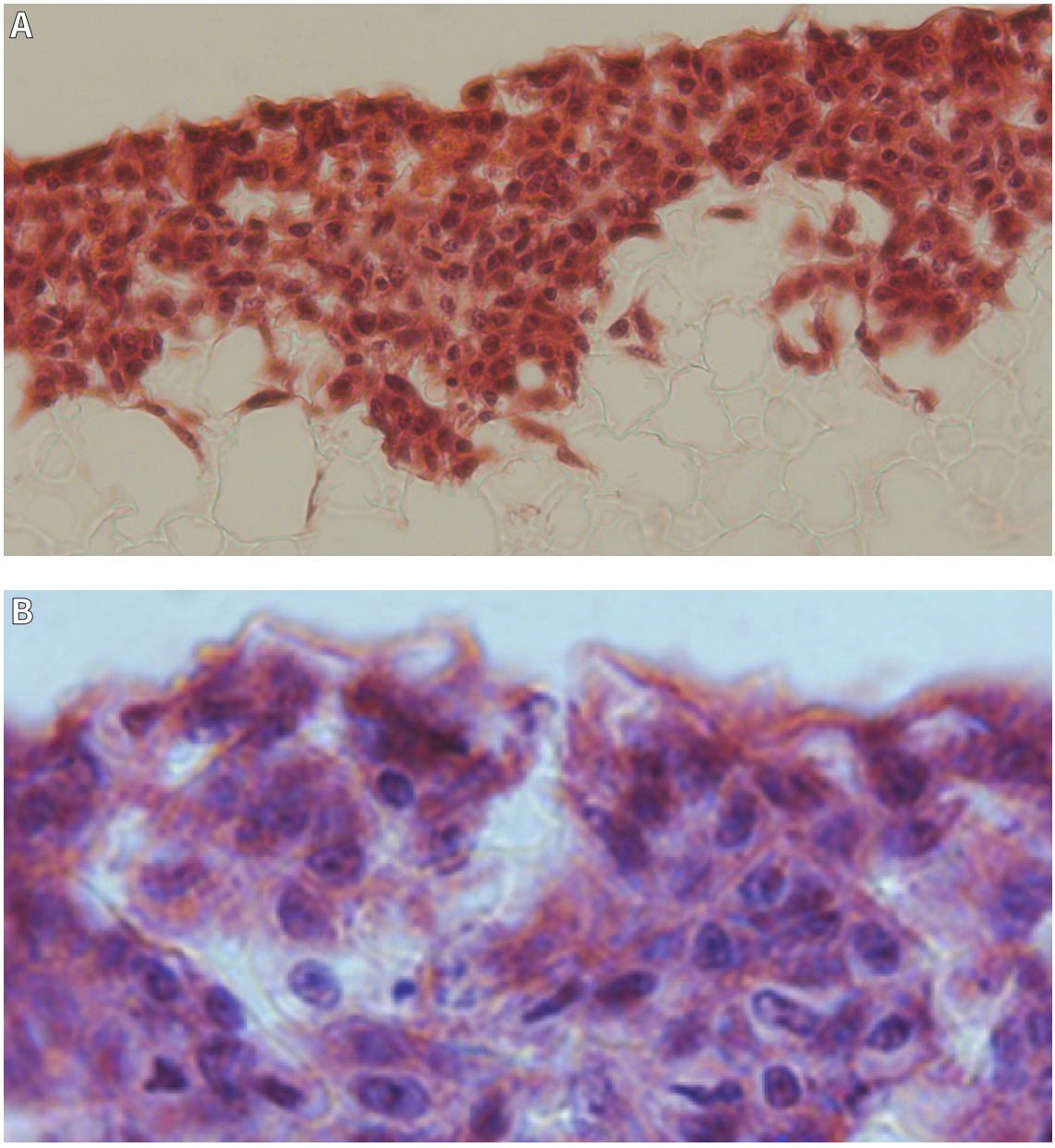
Alvetex derived cell cultures can be processed just like normal tissue samples and prepared for histology using standard procedures including fixation, embedding, thin sectioning and counter-staining.



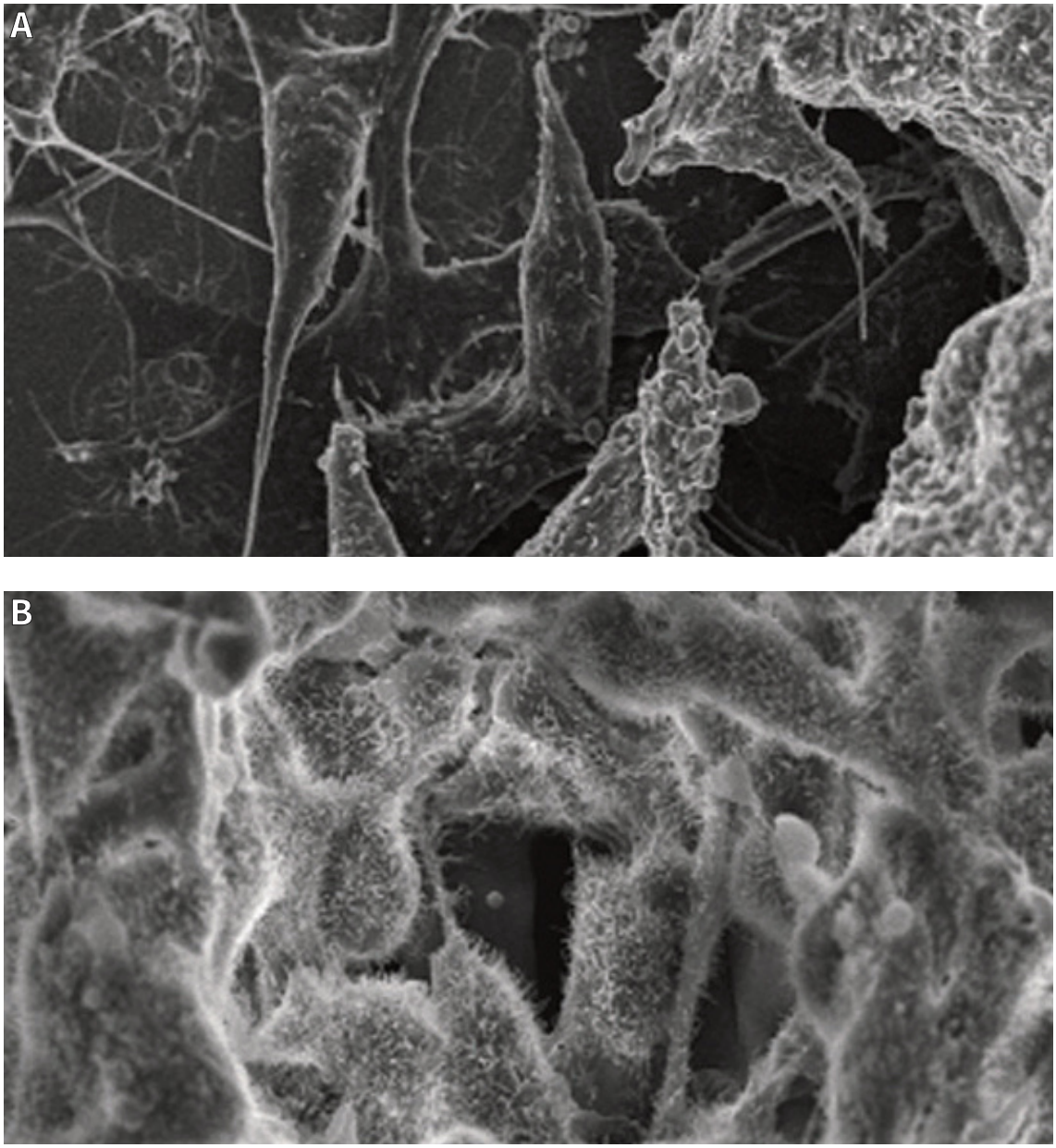
3D growth of cultured cells form 200 µm thick ‘slabs of tissue’. **A**: Naked Alvetex Scaffold before cell seeding viewed under scanning electron microscope to show the highly porous scaffold. **B**: Cells have grown throughout Alvetex Scaffold to the point where the scaffold is no longer visible. **C**: Hepatocytes form a thick multilayer on top of Alvetex Strata with maximal cell-cell contact as would be found within in *in vivo* tissue.



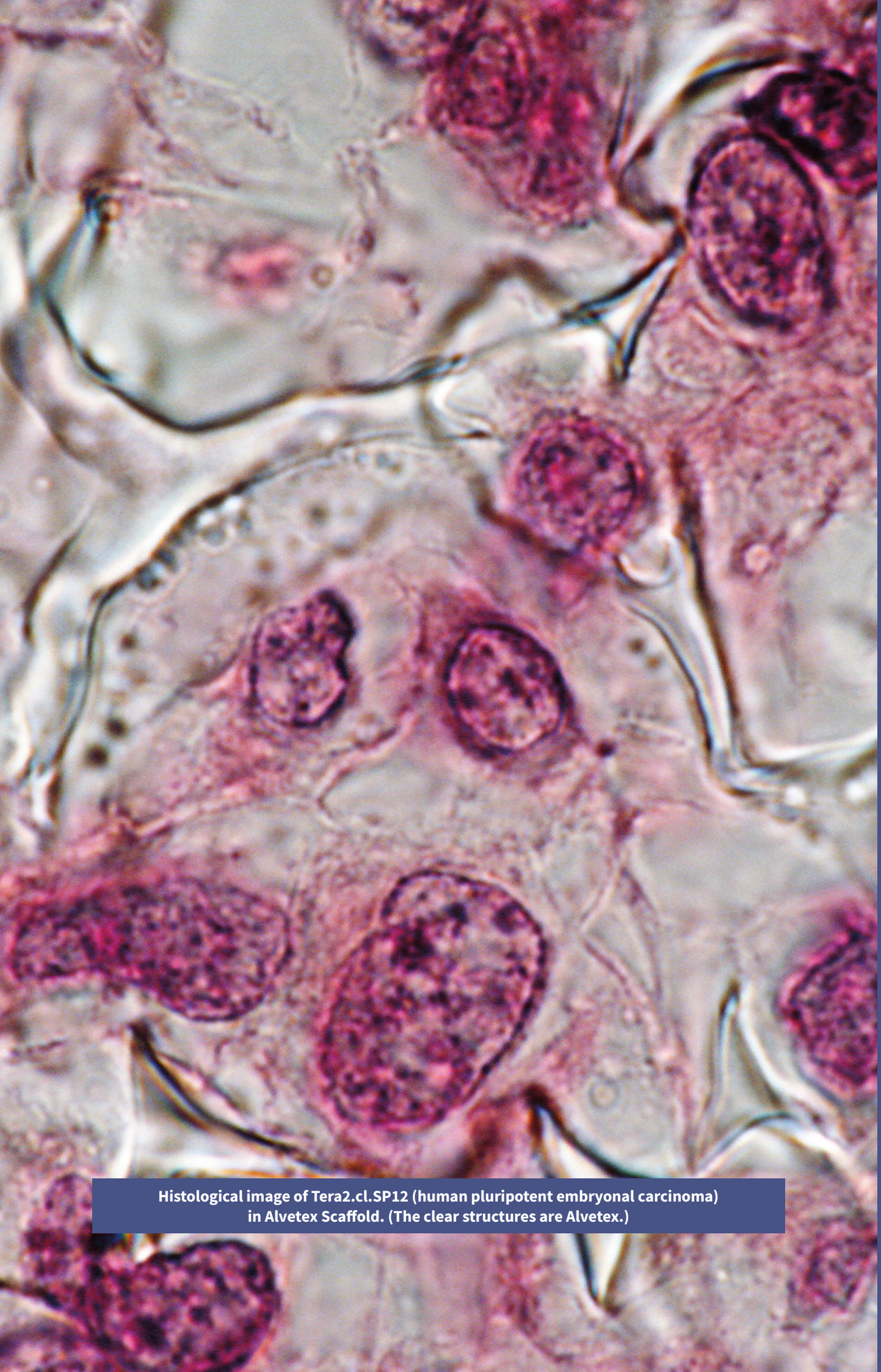
Tissue processing and staining highlight the complex organization of cells growing throughout Alvetex Scaffold. **D**: Resin sections of Alvetex Scaffold showing skin keratinocytes stained with Toluidine Blue. **E**: Paraffin sectioned skin keratinocytes counter stained with H&E viewed by bright field microscopy.



Structure of HepG2 cells cultured in 3D for 2 weeks on Alvetex Scaffold inserts. **A**: Low magnification. **B**: High magnification. In both images the clear structures are Alvetex.



Scanning electron microscopy image comparing the cell morphology and organization of HepG2 liver cells grown in Alvetex Scaffold Scaffold versus 2D culture. **A**: Structure of cells in 2D is very heterogeneous with poor organization. **B**: Cells in Alvetex Scaffold grow homogeneously and develop a 3D form characteristic of liver tissues in the body.



Histological image of Tera2.cl.SP12 (human pluripotent embryonal carcinoma) in Alvetex Scaffold. (The clear structures are Alvetex.)

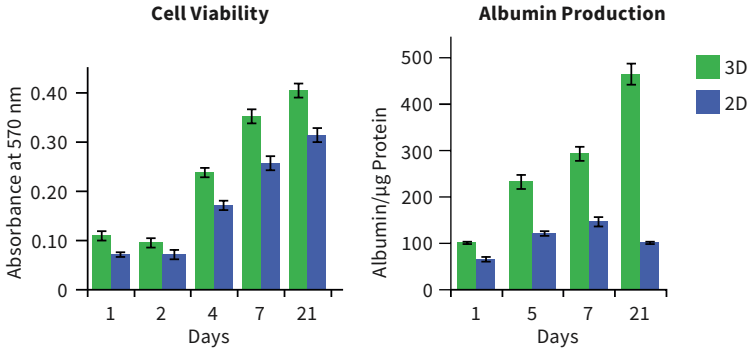
Maintaining *in vivo* cell structure in Alvetex results in improved function

By maintaining the shape and structure of cells and enabling a high level of cell-to-cell interaction, Alvetex enables a much deeper understanding of how cells function *in vivo*.

Enabling cells to maintain their natural morphology and 3D organization leads to improved cell function and responsiveness which is much more representative of the natural *in vivo* environment.

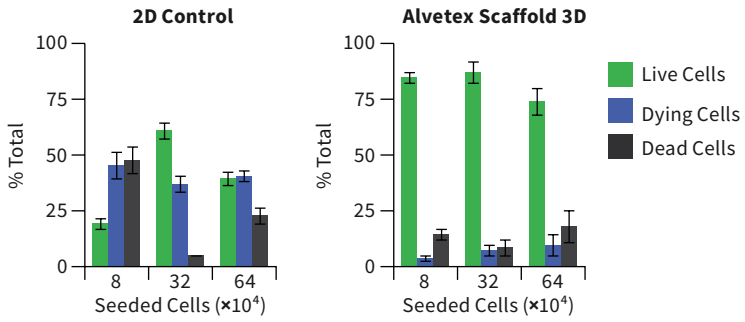
Alvetex delivers data of unmatched biological relevance. Factors such as cell viability and responsiveness have been demonstrated to be enhanced when growing cells in Alvetex in comparison to 2D monolayer cultures.

Example 1: Improved Cell Function and Responsiveness



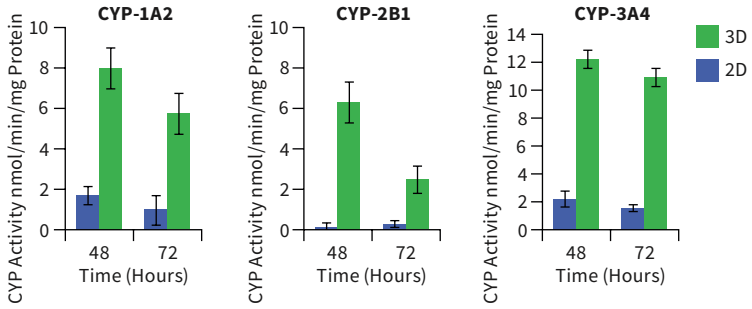
Assessment of HepG2 cells grown on 2D and 3D substrates. Cell viability was determined using a MTT assay and showed greater numbers of viable HepG2 cells in Alvetex Scaffold than on 2D substrates. Similarly, the secretion of albumin from 3D HepG2 cells was elevated compared to standard 2D cultures. In both cases, these data have been normalized to total protein per well to take into account differences in cell numbers. Overall, these results indicate the superior performance of HepG2 cells in 3D culture compared with their 2D counterparts.

Example 2: Increased Cell Viability



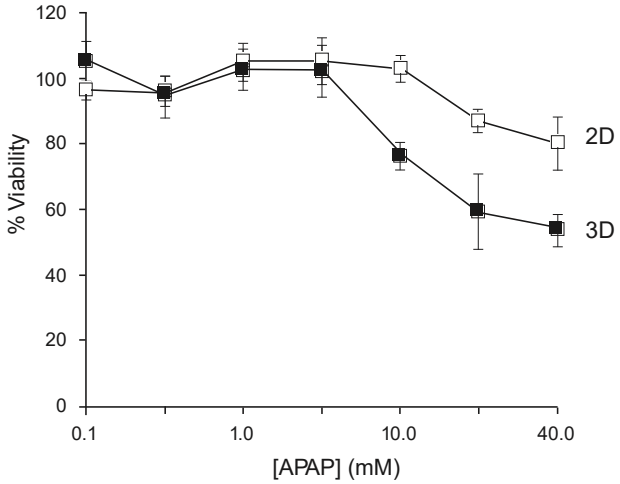
Cell viability of rat primary hepatocytes determined by quantification of live/dead cell staining of hepatocytes maintained for 24 hours on 2D plasticware or Alvetex Scaffold. Cells showed greater than 74% viability when grown on Alvetex Scaffold compared to 2D monolayer culture.*

Example 3: Increased Metabolic Responses



Metabolic responses to model toxicants were significantly enhanced using Alvetex Scaffold. Primary rat hepatocytes were cultured for 3 days in either 2D or 3D culture, Cytochrome p450 expression was induced in cells using a cocktail of model toxicants.*

Example 4: Increased Cell Sensitivity



Primary hepatocytes cultured on 2D plasticware versus Alvetex Scaffold were exposed to a range of acetaminophen (APAP) concentrations for a period of 20 hours and their viability was determined by a standard MTT assay. In general, these data demonstrate that rat primary hepatocytes cultured in 3D using Alvetex Scaffold show increased sensitivity to the model toxicant, acetaminophen.*

* Data generated during a collaborative project between Reinnervate Ltd and LGC Standards – data now published in the following journal: Title: Rat primary hepatocytes show enhanced performance and sensitivity to acetaminophen during three dimensional culture on a novel polystyrene scaffold designed for routine use. Maaike Schutte, Bridget Fox, Marc Baradez, Alison Devonshire, Jesus Minguez, Maria Bokhari, Stefan Przyborski, Damian Marshall. *Assay and Drug Development Technologies*. DOI: 10.1089/adt.2011.0371 (Reinnervate Ltd. was acquired by REPROCELL Inc. in 2014, and merged with Biopta Ltd. to form REPROCELL Europe Ltd. in 2016.)

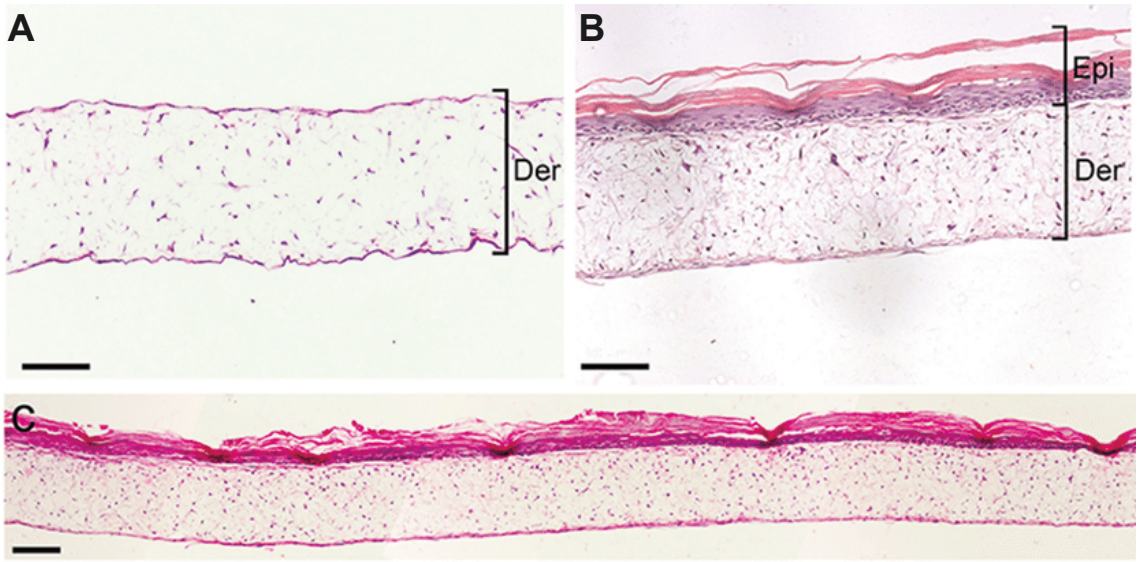
Alvetex protocols

www.reprocell.com/resources/protocols

Imaging reveals the integrity of *in vivo*-like structure and organization of cells grown in Alvetex

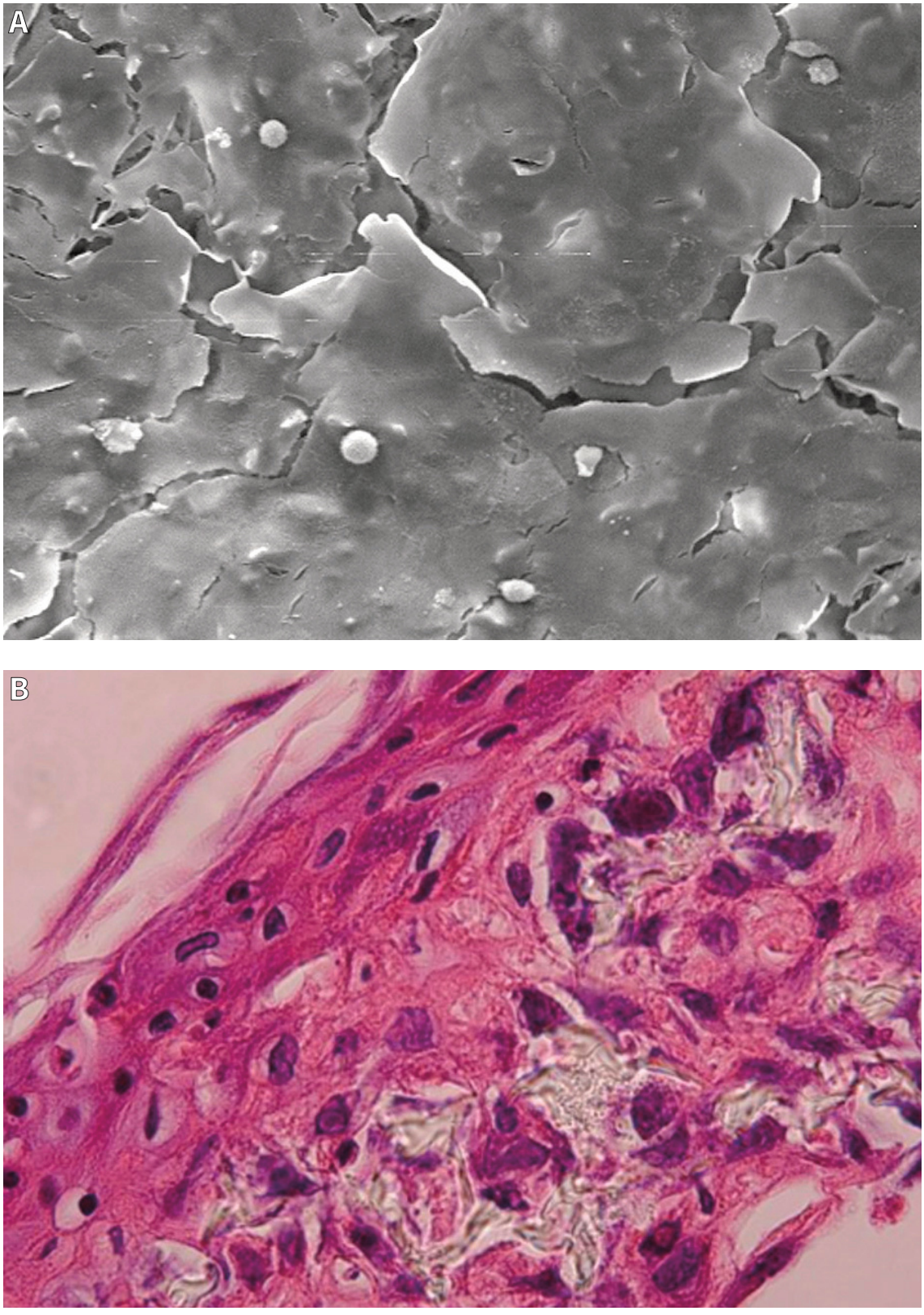
Changes in cellular morphology and function limit the value of cells grown in 2D cell culture. 3D culture systems enable cells to form more complex structures. Various models have been developed to create 3D skin constructs *in vitro*, including raft cultures. These methods are often technically challenging, involve multiple steps, show poor reproducibility and are difficult to practice routinely.

Alvetex provides an alternative method for 3D growth of keratinocytes, enabling reproduction of natural in vivo structures including the development of the stratum corneum, an essential component of the epidermal barrier. Skin constructs generated on Alvetex can then be used for drug and allergen penetration studies as well as assessment of barrier function.

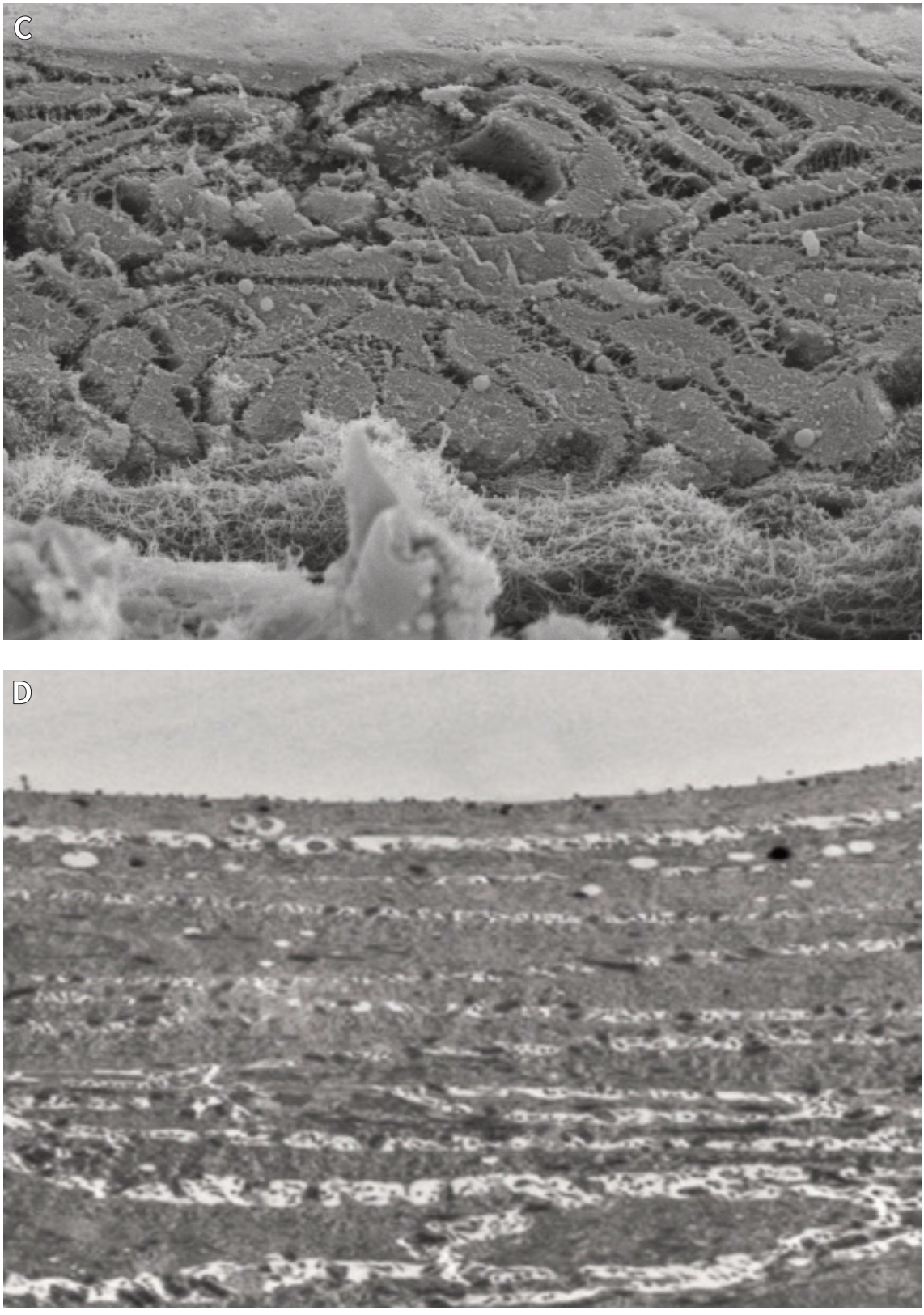


Histology images showing a full thickness skin construct grown by co-culturing human dermal fibroblasts inside Alvetex Scaffold and human keratinocytes on top, thus replicating both the dermal and epidermal compartments. Note that the presence of a stratum corneum is also evident. Validation of dermal and epidermal structure in full-thickness human skin equivalents. **A**: Representative photomicrographs of haematoxylin and eosin (H&E) stained Alvetex Scaffold seeded with human dermal fibroblasts after culture in Media A for 18 days. **B** & **C**: Representative photomicrographs showing H&E stained 35 day full-thickness human skin equivalents at 20× and 10× magnification respectively.*

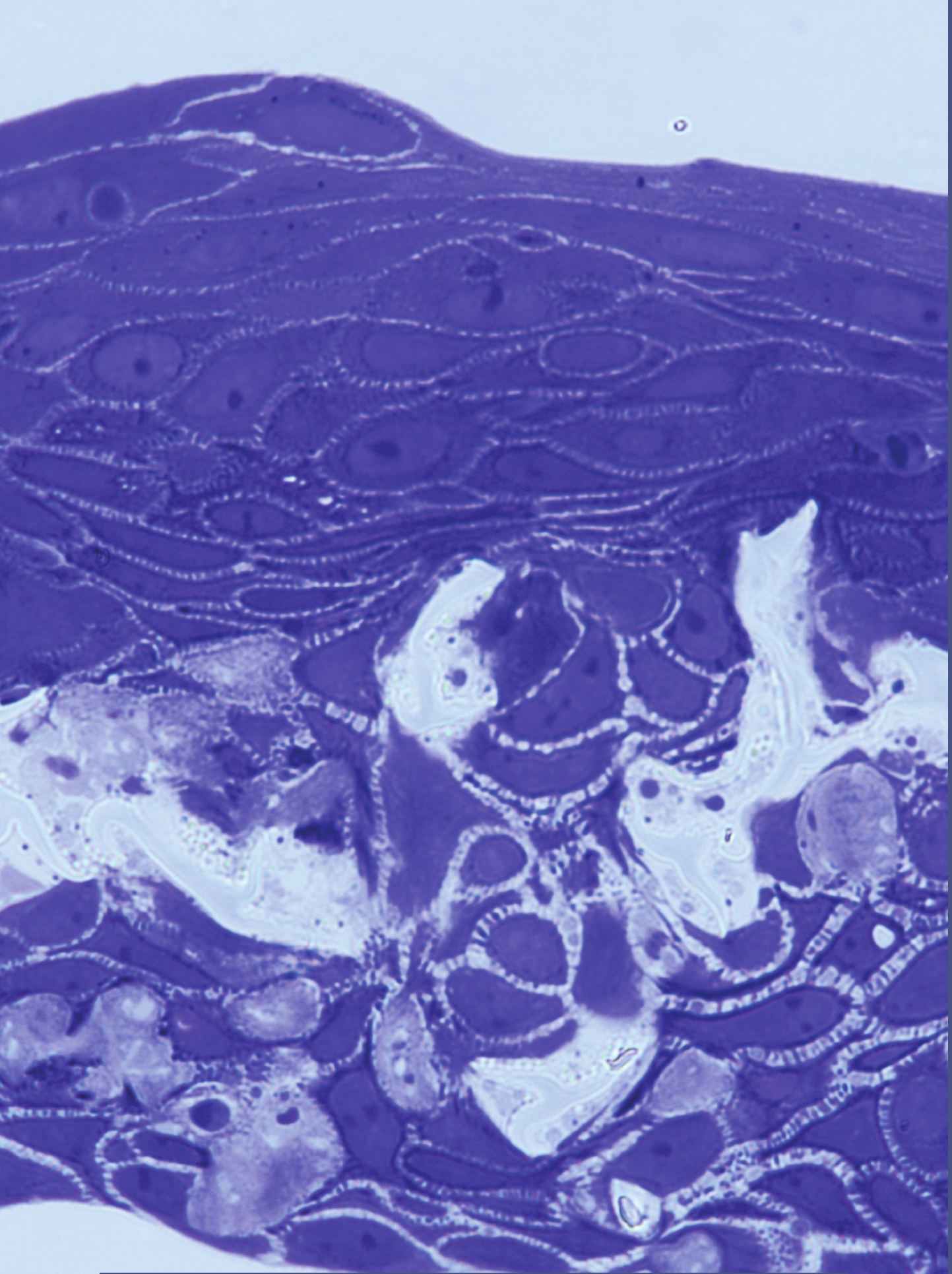
*Data generated during a collaborative project between Reinnervate Ltd and Newcastle University – data now published in the following paper: A Novel Fully Humanized 3D Skin Equivalent to Model Early Melanoma Invasion. Authors: D.S. Hill, N.D. Robinson, M. P. Caley, M. Chen, E.A. O'Toole, J.L. Armstrong, S. Przyborsky and P.E. Lovat. *Mol Cancer Ther*. 2015 November; 14(11): 2665–2673. doi:10.1158/1535-7163.MCT-15-0394. (Reinnervate Ltd. was acquired by REPROCELL Inc. in 2014, and merged with Biopta Ltd. to form REPROCELL Europe Ltd. in 2016.)



3D culture of skin keratinocytes on Alvetex Scaffold resulting in formation of 3D epidermis and maturation of the stratum corneum. **A**: Scanning electron microscope image illustrating the formation of the stratum corneum of a 21 day culture. **B**: Alvetex Scaffold sectioned and stained with H&E and viewed by light microscopy after 35 days.



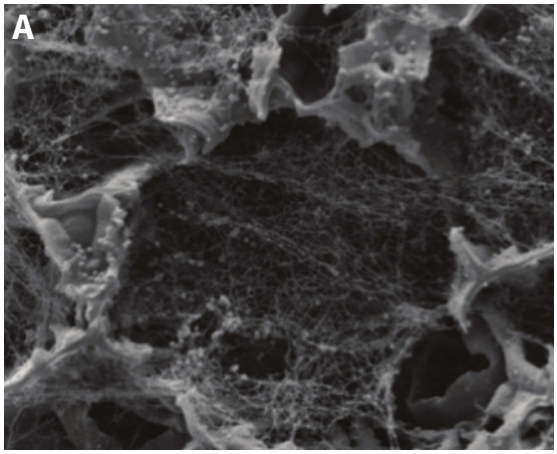
3D culture of skin keratinocytes on Alvetex Scaffold resulting in formation of 3D epidermis and maturation of the stratum corneum. **C**: Scanning electron microscope image illustrating the formation of the stratum corneum of a 21 day culture. **D**: Alvetex Scaffold sectioned and stained with H&E and uppermost portion of the stratified epidermis viewed by transmission electron microscopy (TEM).

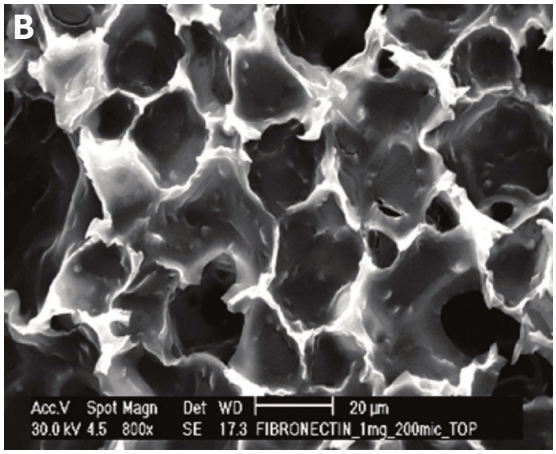


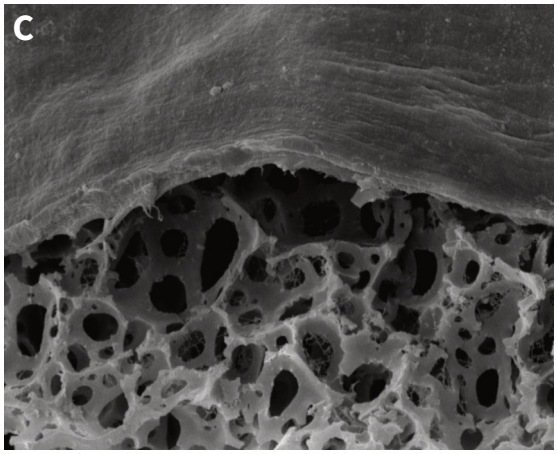
Histological image of HaCaT (human keratinocyte) cells grown on Alvetex Scaffold, forming layers on top of the scaffold. (The clear structures are Alvetex.)

Alvetex can easily be coated with ECM proteins

Alvetex can be coated with extracellular matrix (ECM) proteins and other reagents commonly used to treat cell culture substrates, notably: Collagen I; Collagen IV; Fibronectin; Laminin; Poly-D-lysine; Poly-L-lysine; Poly-D-lysine and Laminin; Poly-L-orthinine and Laminin; Matrigel™; PuraMatrix™.







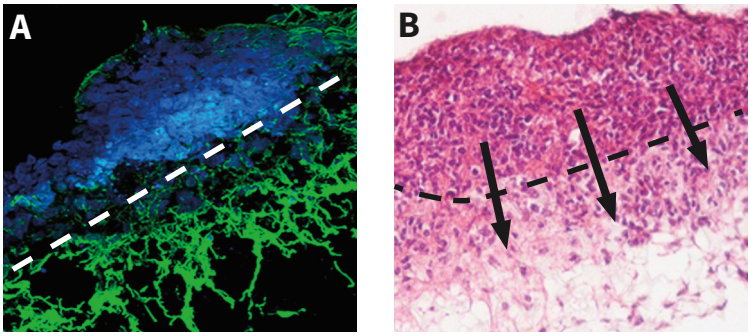
A: Scaffold pre-loaded with Collagen IV.
B: Coating Alvetex Scaffold with fibronectin.
C: Coating Alvetex Strata with Collagen I (2 mg/mL shown). The ECM proteins form a web of fibers spanning voids into which cells can grow and migrate in 3D. Depending on the ECM concentration used, this coating can either encourage cell invasion into the scaffold or create a barrier between two co-cultured cell populations.

"**Alvetex** should enable the routine and reproducible creation of 3D cell cultures in the laboratory and extend the concept of 3D culture beyond the simple, reconstituted extracellular matrices to complex cellular structures."

H. Steven Wiley, Lead Biologist at the Environmental Molecular Sciences Laboratory (EMSL) at Pacific Northwest National Laboratory (Richland, WA, USA) and award judge of *The Scientist* Magazine Top Ten Life Science Innovations 2010.

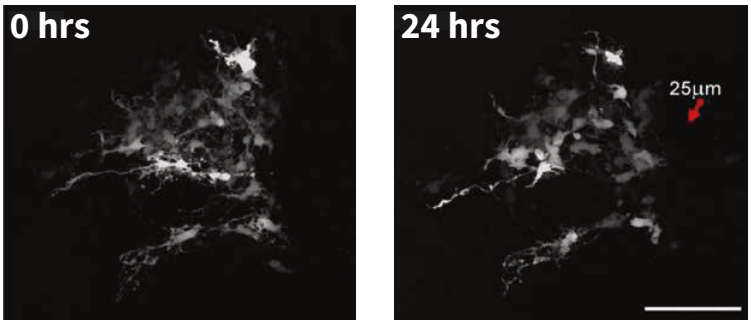
Cells can be explanted directly into Alvetex

Cells can move into Alvetex directly from pieces of primary tissue or cell aggregates, migrating freely into the scaffold and spreading throughout its structure. Depending on cell type and characteristics, cells may proliferate as well as migrate. By enabling cells to be explanted in this way, Alvetex creates the opportunity for many different applications including tumor cell biology, separation of alternative cell types and establishing and maintaining 3D cultures *de novo* directly from primary sources, etc.



Examples of cells from tissue pieces placed on top of Alvetex Scaffold migrating into the structure of the scaffold. **A**: A neural aggregate gener-ated on a low-adherence plate before transfer to Alvetex Scaffold shows extensive neurite elongation within the thickness of the scaffold. **B**: Cells from an embryonal carcinoma aggregate readily invade Alvetex Scaffold.

Freshly-obtained intact tissues can also be maintained directly on Alvetex Strata for improved adherence and stability during imaging.



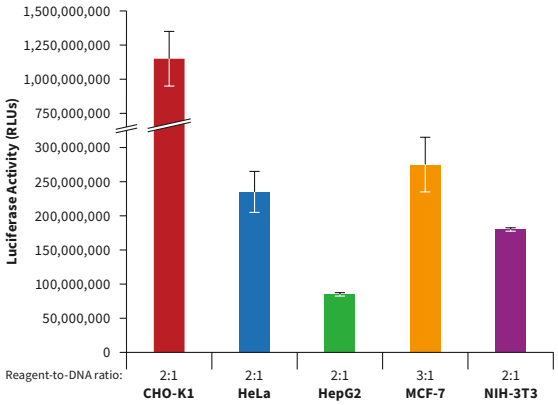
Time lapse imaging of spinal cord tissue slice maintained on Alvetex Strata demonstrates minimal tissue drift over a period of 24 hours.

(Images courtesy of Kieran McDermott, University of Cork.)

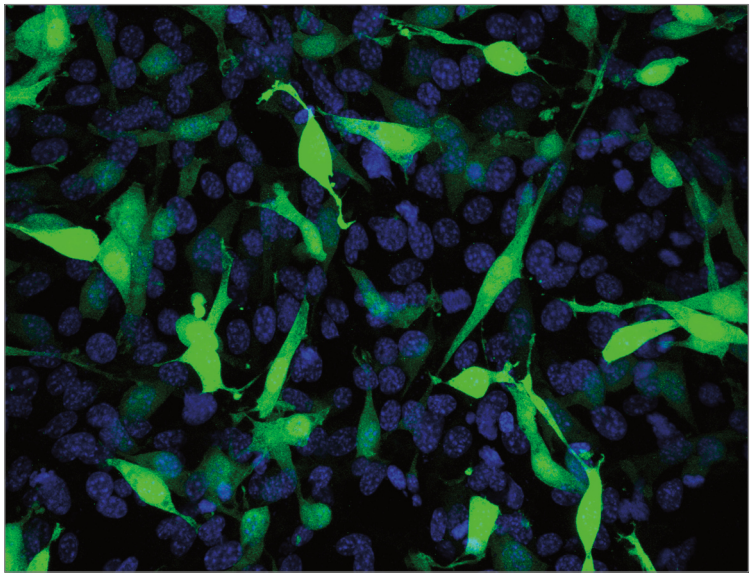
Transfection of various cell types using Alvetex 3D cell culture

In collaboration with Mirus Bio, methods have been developed that enable the transfection of cells grown in Alvetex 3D culture.

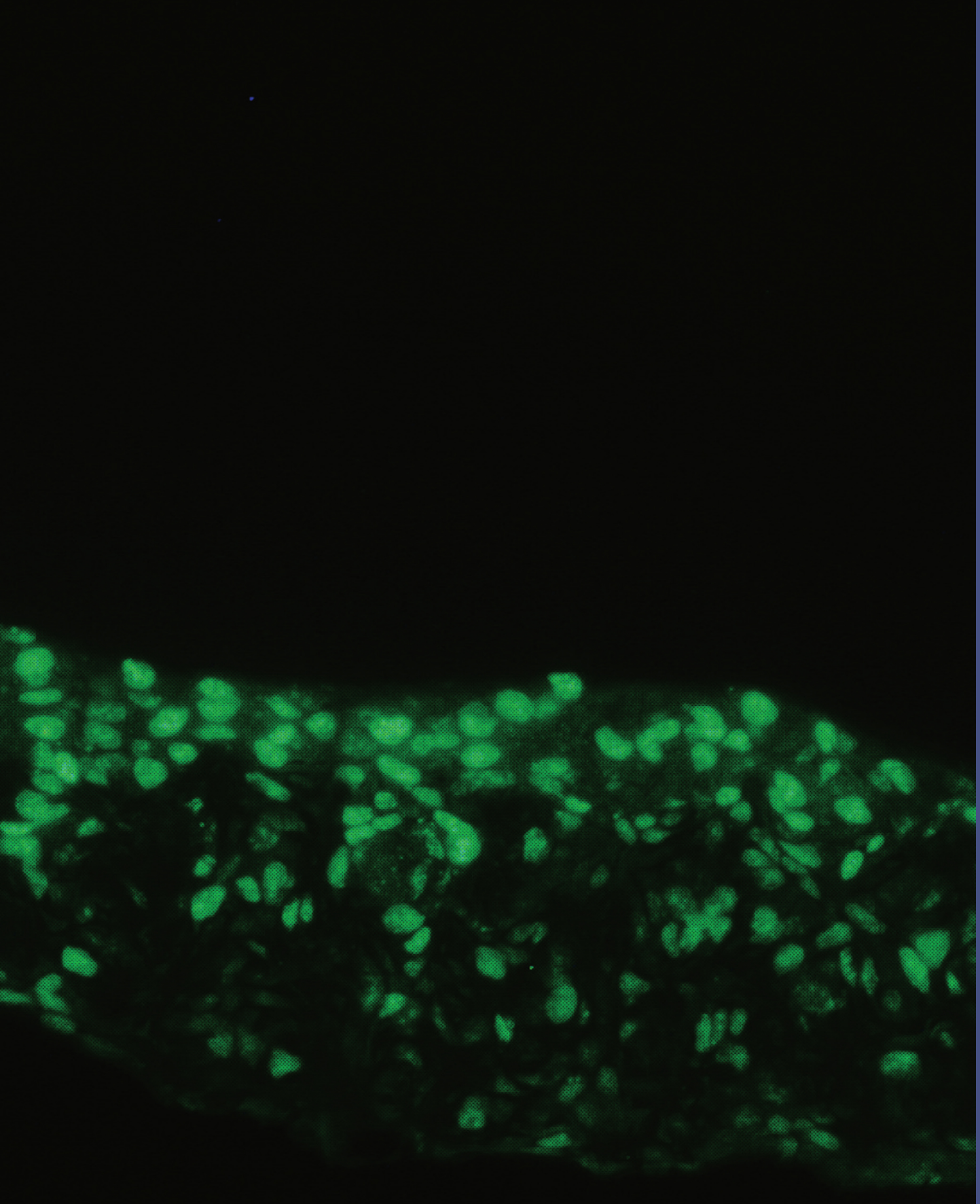
Common cell types (CHO-K1, HeLa, HepG2, MCF-7 and NIH-3T3) were seeded at optimized cell densities in 12 well Alvetex Scaffold 3D plates and adapted to 3D culture conditions for 48 hours. After adaptation, cells were transfected with a novel Mirus Bio formulation combined with a plasmid encoding firefly luciferase at the reagent-to-DNA ratios indicated beneath the bars. Luciferase activity was measured 24 hours post-transfection using a conventional assay. High expression was detected in all cell types demonstrating the efficiency of the Mirus Bio TransIT® 3D Transfection Reagent (MIR 5804) when used with Alvetex Scaffold 3D culture plates.



3D transfection of multiple cell types.



Fibroblasts grown in 3D using Alvetex Scaffold were successfully transfected with a GFP construct and imaged using confocal microscopy. In brief, cells were transfected with the new Mirus Bio Transfection Reagent for 3D transfection at a reagent-to-DNA ratio of 3:1 using a GFP-expressing plasmid. Cells were seeded at 48 hours prior to transfection, and the cultures were fixed 24 hours post-transfection. Cells were imaged using a confocal microscope (Zeiss LSM510). The data shows a 40 µm integrated stack of multiple images as viewed from above the intact 3D culture. The position of all the cell nuclei are visualized with Hoechst 33342 (blue) and the positively transfected cells express GFP (green).



Skin keratinocytes grown in Alvetex Scaffold with antibody staining for Ki67 showing dividing cells. Ki67 positive cells imaged in green using FITC labelling.

Using Alvetex to create co-culturing experiments

Advancing from single cell mono-cultures and co-cultures in conventional 2D models, Alvetex Scaffold provides the next step towards replicating the *in vivo* environment by providing the architecture necessary for 3D cell culture *in vitro*. Alvetex's extremely high porosity allows cells to penetrate, grow and proliferate throughout the material for highly effective and reproducible 3D cell culture. Cells are freely able to form complex interactions with adjacent cells and receive and transmit signals, enabling a more natural environment to foster the native architecture found in tissues.

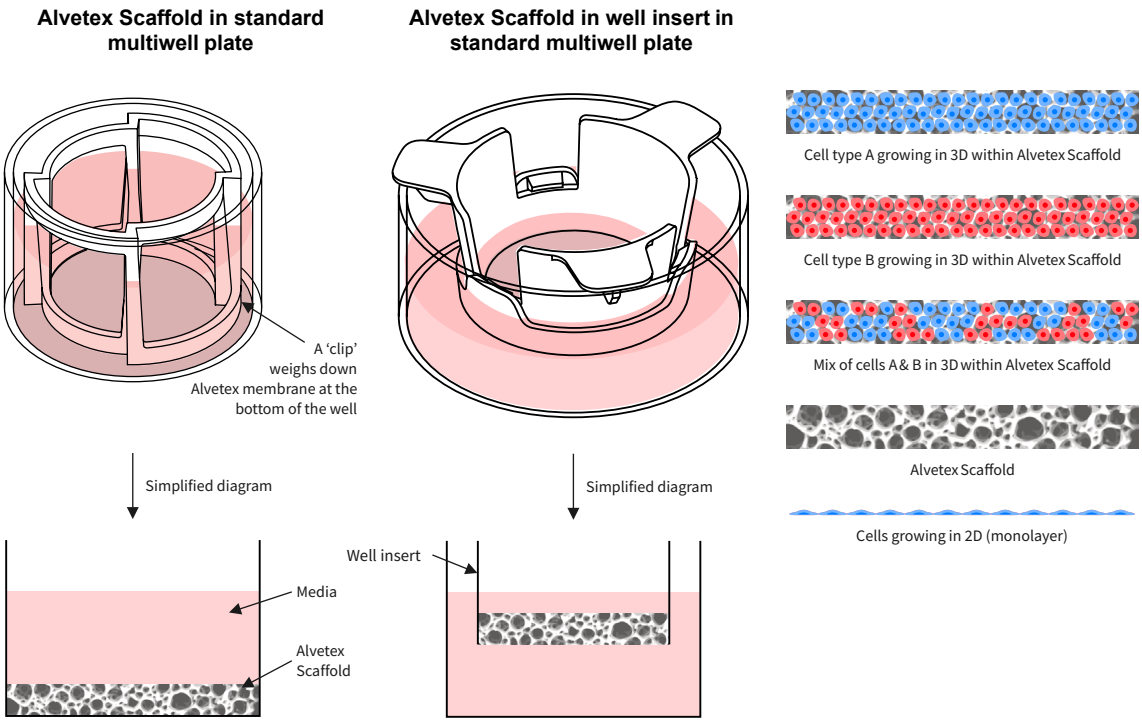
As well as being able to study single cultures in 3D, Alvetex Scaffold provides a support that enables the co-culture of more than one cell type. The structure of tissues is often comprised of discrete layers of distinct cell types. Growing different cell types in 3D, inside and on the surface of Alvetex Scaffold, enables users to re-create such tissue structures *in vitro*. A variety of cell co-culture scenarios can be set up to study different cell-cell interactions, according to the requirements of the cells and the dynamics under investigation.

Key Benefits and Applications

- Study interactions between distinct cell types in 3D culture
- Recreate in vivo tissue morphology
- Recreate specific niche environments for disease modelling or drug testing
- Customize co-culture setup to suit the cell types involved

Several alternative approaches for co-culture design can be utilised. Alvetex Scaffold is a versatile technology that enables users to create co-culture models in many different ways.

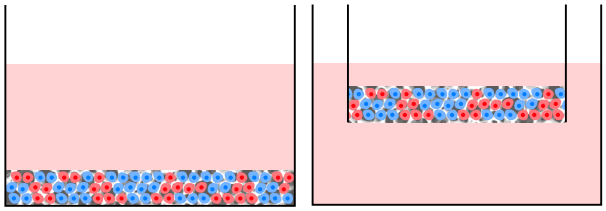
Key to image parts:



Assembly option 1

3D co-culture in multiwell plate or well insert

Description: Different cell types cultured together within the same scaffold
Application: Emulate the structure of a tissue comprised of more than one cell type

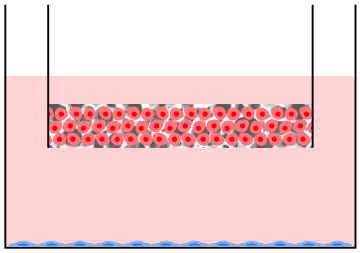


Assembly option 2

3D / 2D co-culture in multiwell plate and well insert combined

Description: Different cell types cultured together within the same scaffold within a well insert

Application: Approach used to study the secretion of factors and signaling molecules

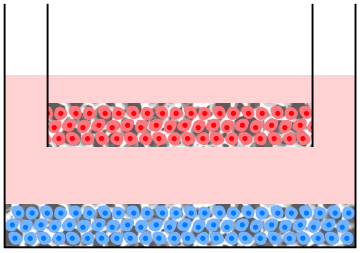


Assembly option 3

3D co-culture in multiwell plate and well insert combined

Description: Two independent 3D cultures. Contact is via medium – communication via paracrine factors

Application: Approach used to study the secretion of factors and signaling molecules

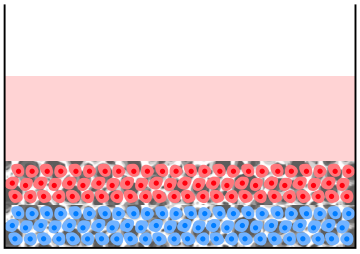


Assembly option 4

3D / 3D co-culture in multiwell plate

Description: Two 3D cultures in direct contact with one another

Application:
- Study the direct interaction of cells in contact with one another
- To establish layers of alternate cell types in 3D to mimic tissue structures
- To investigate invasion and migration of different cell types amongst each other

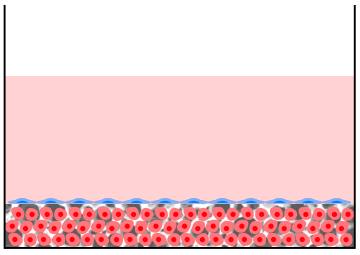


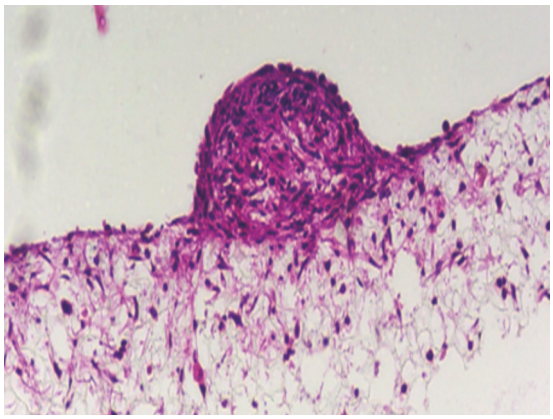
Assembly option 5

2D / 3D co-culture in multiwell plate

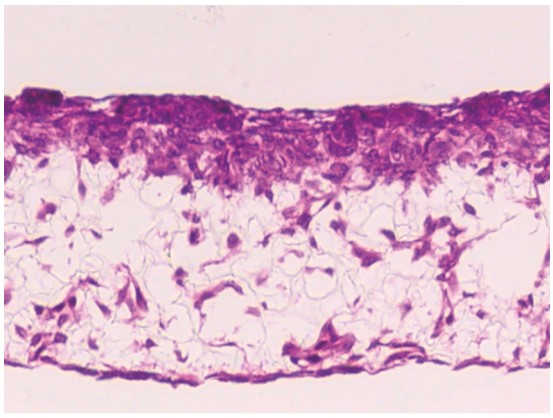
Description: One 2D (monolayer) and one 3D culture layered in direct contact with one another

Application:
- Study the direct interaction of cells in contact with one another
- To establish layers of alternate cell types in 3D to mimic tissue structures
- To investigate invasion and migration of different cell types amongst each other

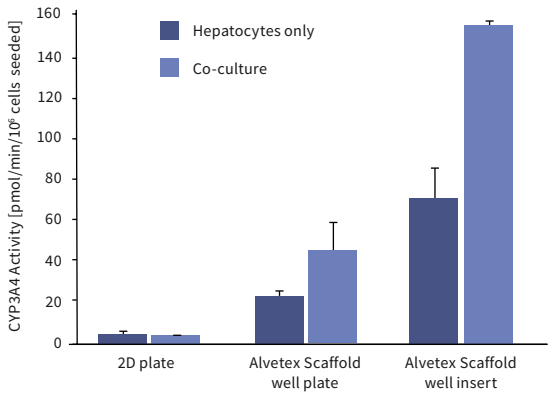




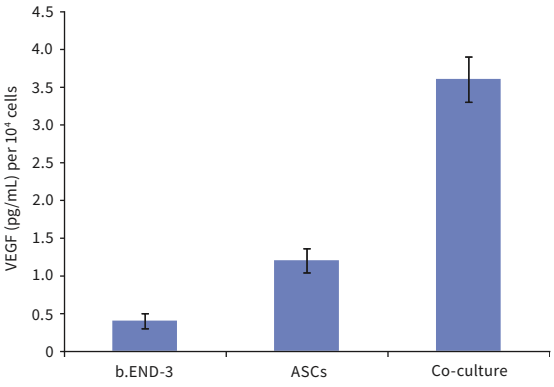
Co-culture of glial and neural cells to model brain tissues. Brightfield micrograph showing the structure of a human stem cell-derived neurosphere co-cultured for 7 days with U118-MG glial cells on Alvetex Scaffold presented in the 12-well insert in 12-well plate format.



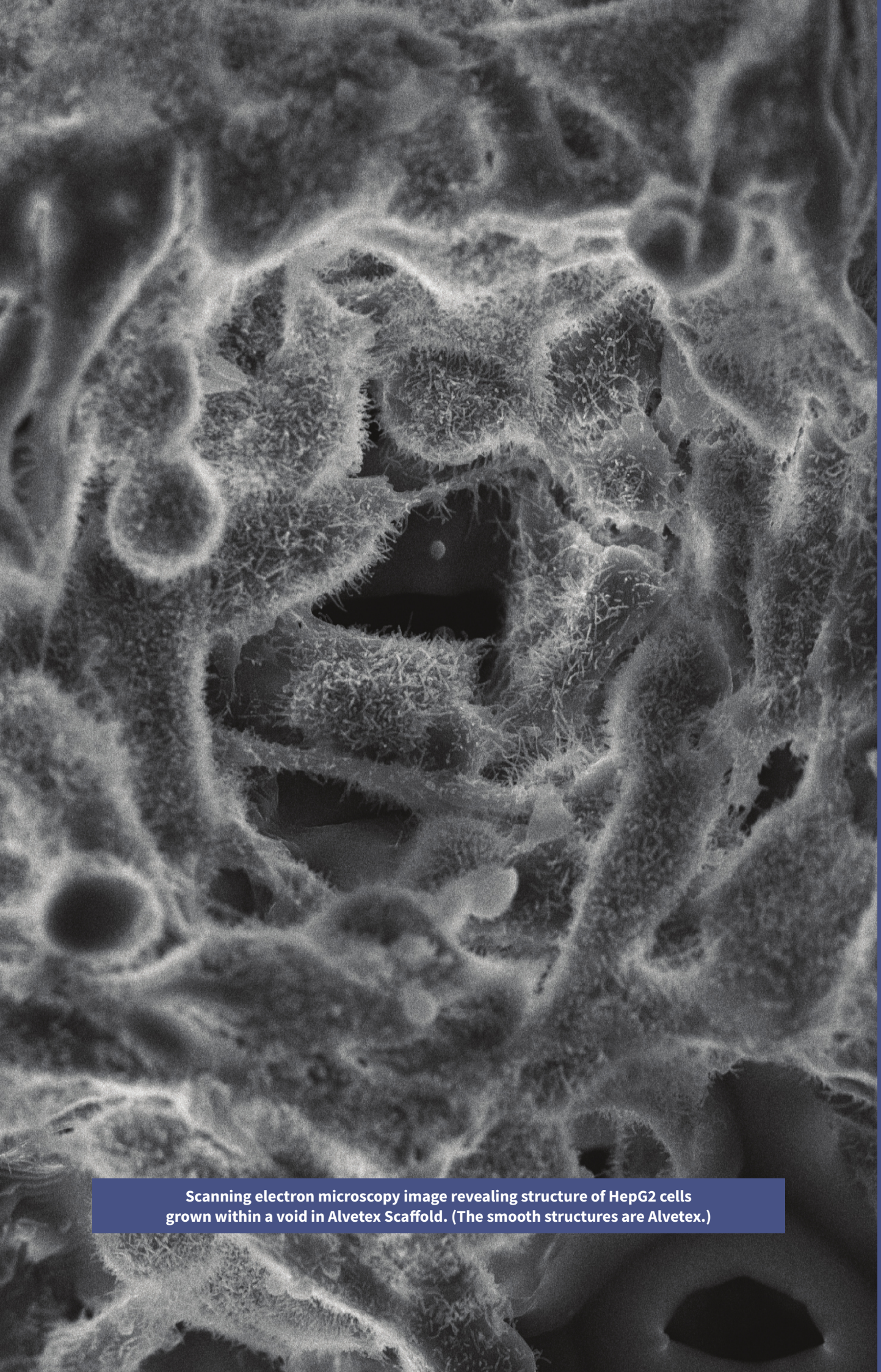
Cell invasion into Alvetex Scaffold. Brightfield micrograph showing the structure of SW480 colon adenocarcinoma cells co-cultured for 7 days with established 3D cultures of 3T3 fibroblasts on Alvetex Scaffold.



Enhanced cell function with hepatocyte and endothelial cell co-culture. The activity of CYP3A4 in upcyte® hepatocytes cultured on a 2D plate and on Alvetex Scaffold presented in both a 12-well plate and 6-well insert formats. Hepatocytes were grown as mono-cultures and also co-cultured with upcyte® micro-vascular endothelial cells for 10 days. For further details please visit www.medicyte.com.



Co-culture of adipose tissue-derived stem cells with endothelial cells influences their differentiation. Adipose tissue-derived stem cells (ASCs) and endothelial cells (b.END-3) were cultured independently and as co-cultures in Alvetex Scaffold 12 well plate format for 3 days. Data from 3 sample replicates. VEGF levels normalized to the number of cells at the time of seeding, expressed as pg/mL per 10 cells. For further details please refer to Neofytou, E.A., *et al.* Adipose tissue-derived stem cells display a proangiogenic phenotype on 3D scaffolds. *J Biomed Mater Res A.* 2011; 98(3): 383-93.

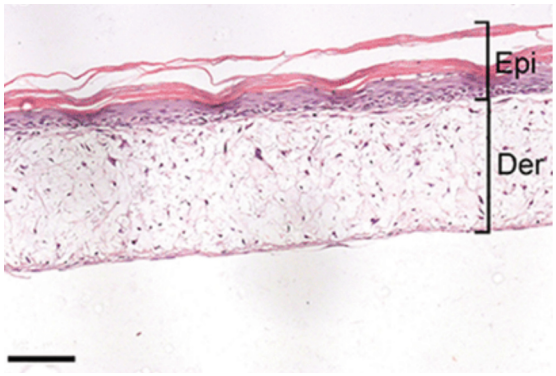


Scanning electron microscopy image revealing structure of HepG2 cells grown within a void in Alvetex Scaffold. (The smooth structures are Alvetex.)

Alvetex is compatible with many downstream applications

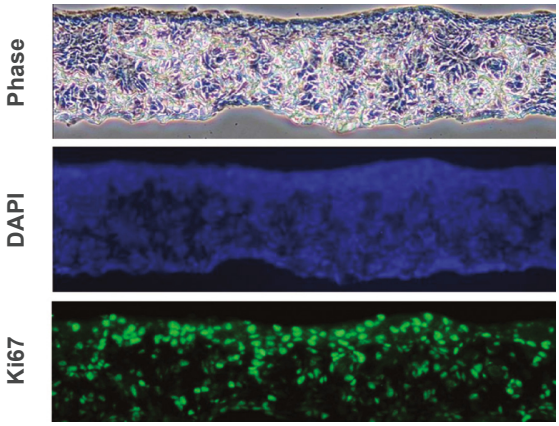
- Tissue processing, fixation, embedding and sectioning
- Brightfield microscopy and photographic imaging
- Cryostat sectioning
- Fluorescence microscopy, confocal, laser capture
- Flow cytometry and cytospinning
- Biochemical assays
- Histological staining, in situ hybridization
- Electron microscopy – both SEM and TEM
- Immunocytochemistry
- Isolation of viable cells
- Extraction of nucleic acid and total protein
- And more . . .

Sectioning and Counterstaining



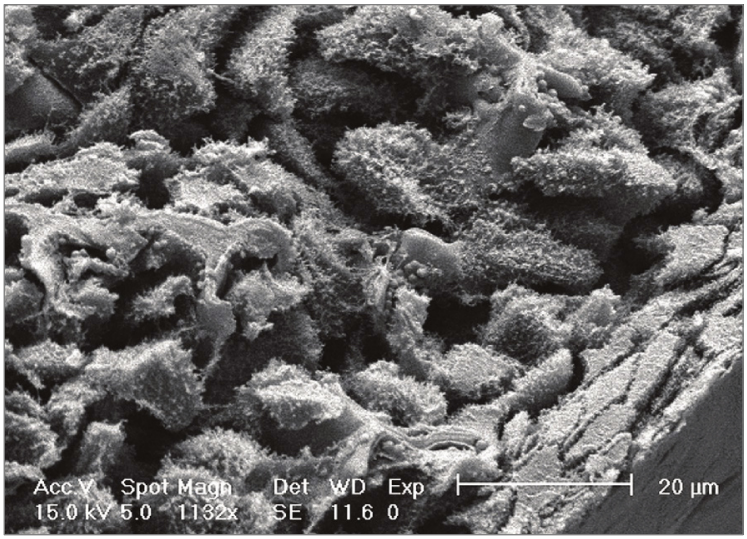
Unlike other 3D cell culture supports, Alvetex can easily be processed like a standard tissue sample. Frozen and paraffin embedded samples can be sectioned and stained to reveal the native cellular structures inside Alvetex. In this example, cells have been fixed in 4 % paraformaldehyde, embedded in paraffin wax, sectioned (7 µm) before staining with H&E and cover-slipped.

Immunocytochemistry

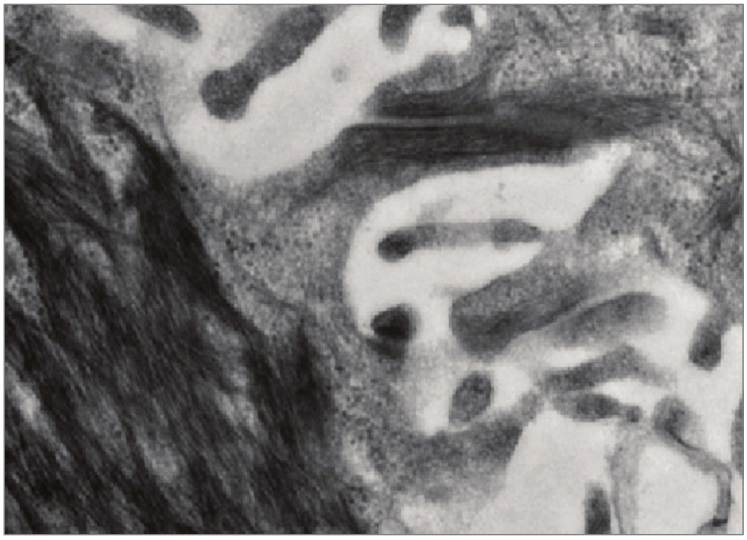


Immunocytochemistry used to visualize the expression of specific protein markers. In this example, cell cultures have been fixed in 4 % paraformaldehyde, embedded in paraffin wax and sectioned (10 µm). Antigen retrieval followed by immunocytochemical analysis with the proliferation marker Ki67 (green) and the nuclear stain DAPI (blue) was perform-ed following standard immunocytochemical methods.

Scanning Electron Microscopy



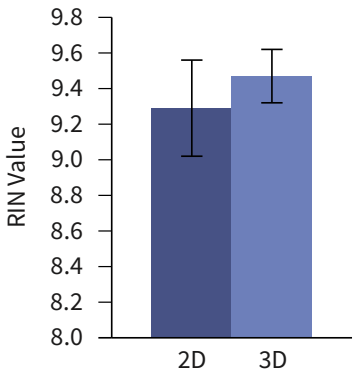
Visualization of the structure of 3D cultures in Alvetex Scaffold is made possible using scanning electron microscopy (SEM). Samples are prepared in the same manner as would normally be used for tissues. In this example, skin, cells have penetrated throughout the scaffold and some have stratified on the surface (lower right corner).



The ultrastructure of cells grown in Alvetex Strata can be analyzed by standard trans-mission electron microscopy (TEM). At high magnification, cellular structures such as these specialized bile canalicular cell protrusions are readily visualized.

Gene Expression Analysis

Quality and yield of RNA isolated from primary rat hepatocytes

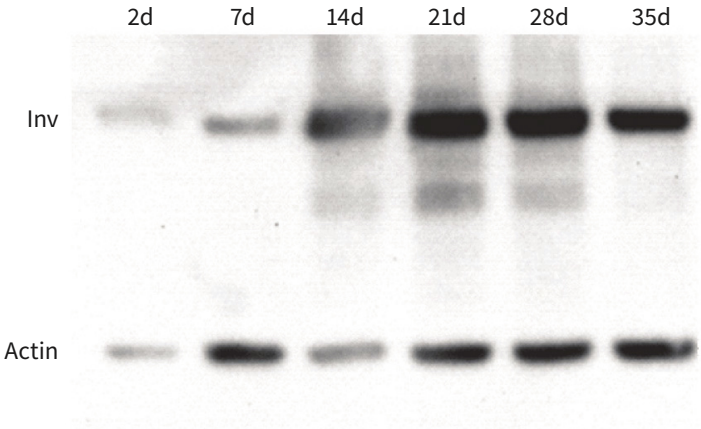


	Sample	ng/µL	RIN
2D	DMSO	82	9.3
3D	DMSO	91	9.5

Isolation of nucleic acid from rat primary hepatocytes grown in Alvetex and conventional 2D cultures. RNA quality was determined by the RIN (RNA Integrity Number) and showed that the quantity and quality of RNA isolated from cells grown on Alvetex Scaffold was the same if not better than that isolated from standard 2D cultures. Data generated in collaboration with LGC (unpublished).

Protein Expression Analysis

Protein isolation and western blotting

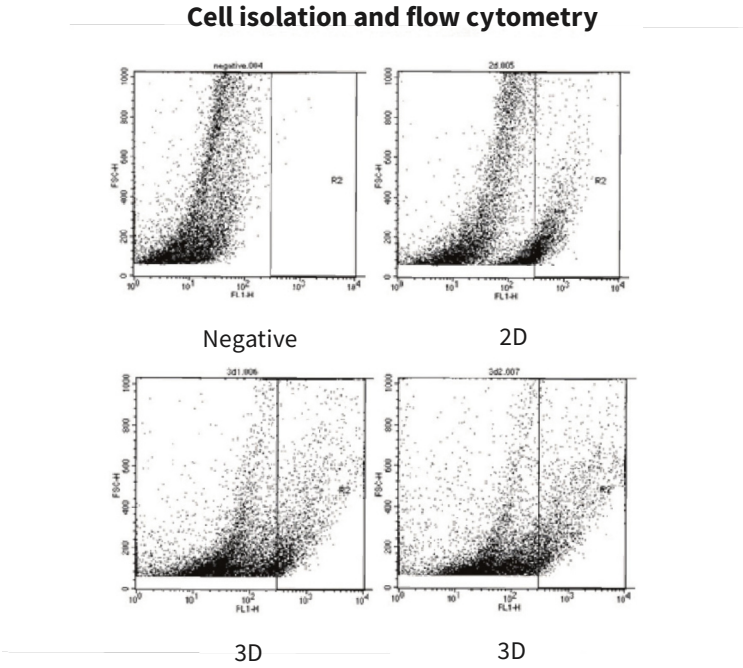


With standard lysis protocols, total protein can be efficiently isolated from cells growing inside Alvetex. This allows for more biologically relevant protein expression analysis experiments to be carried out. Here we showed the increase over time of involucrin (Inv) expression in maturing keratinocytes by western blot analysis.

Alvetex protocols

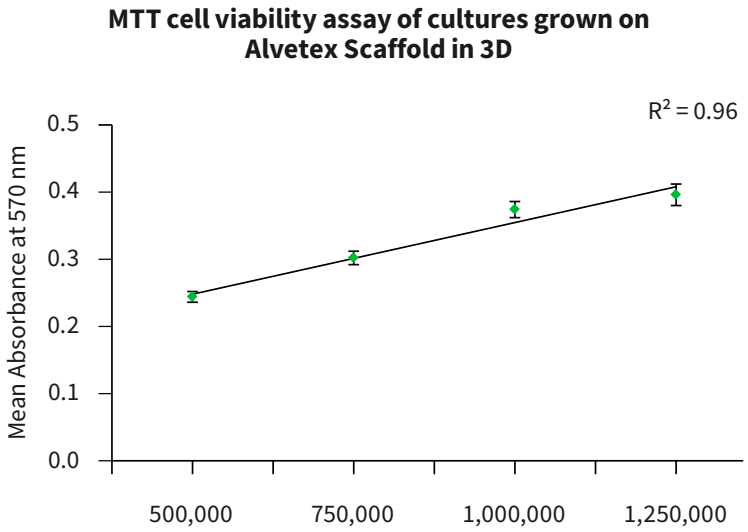
www.reprocell.com/resources/protocols

Isolation of cells from Alvetex

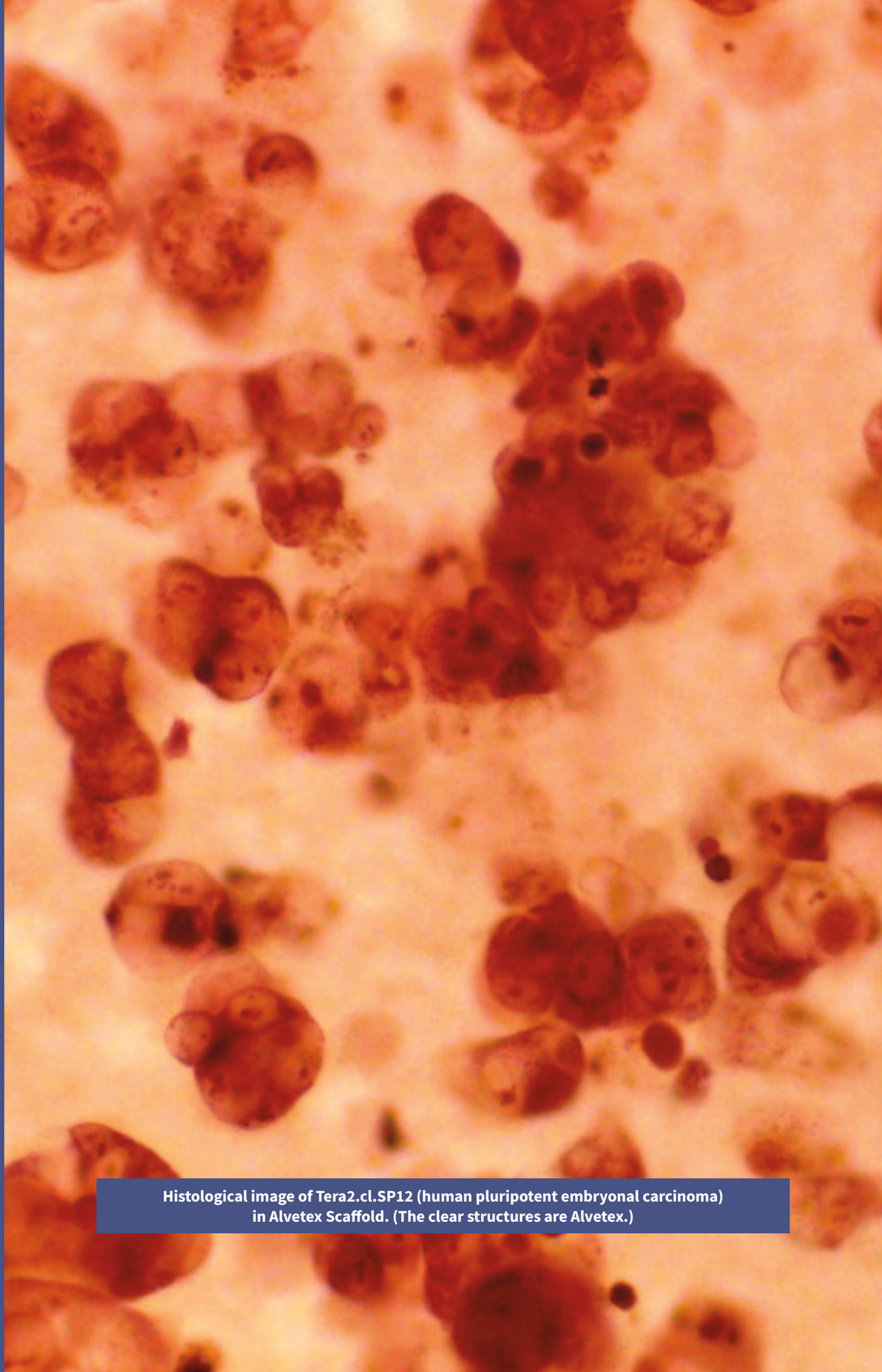


It is possible to isolate some viable cells from Alvetex for downstream experiments such as flow cytometry, cytospinning and for subculture. Here, mesenchymal stem cells induced to form adipocytes were isolated from Alvetex Scaffold and 2D cultures. Cells were subsequently stained with Nile Red to detect the presence of lipid and analyzed by flow cytometry.

Biochemical assays



Cells growing in 3D inside Alvetex can be studied using typical biochemical assays such as cell viability assays, apoptosis assays, cell proliferation assays etc. Here we show the measurement of cell viability using a standard MTT assay.



Histological image of Tera2.cl.SP12 (human pluripotent embryonal carcinoma) in Alvetex Scaffold. (The clear structures are Alvetex.)

Alvetex defines the gold standard for 3D cell culture

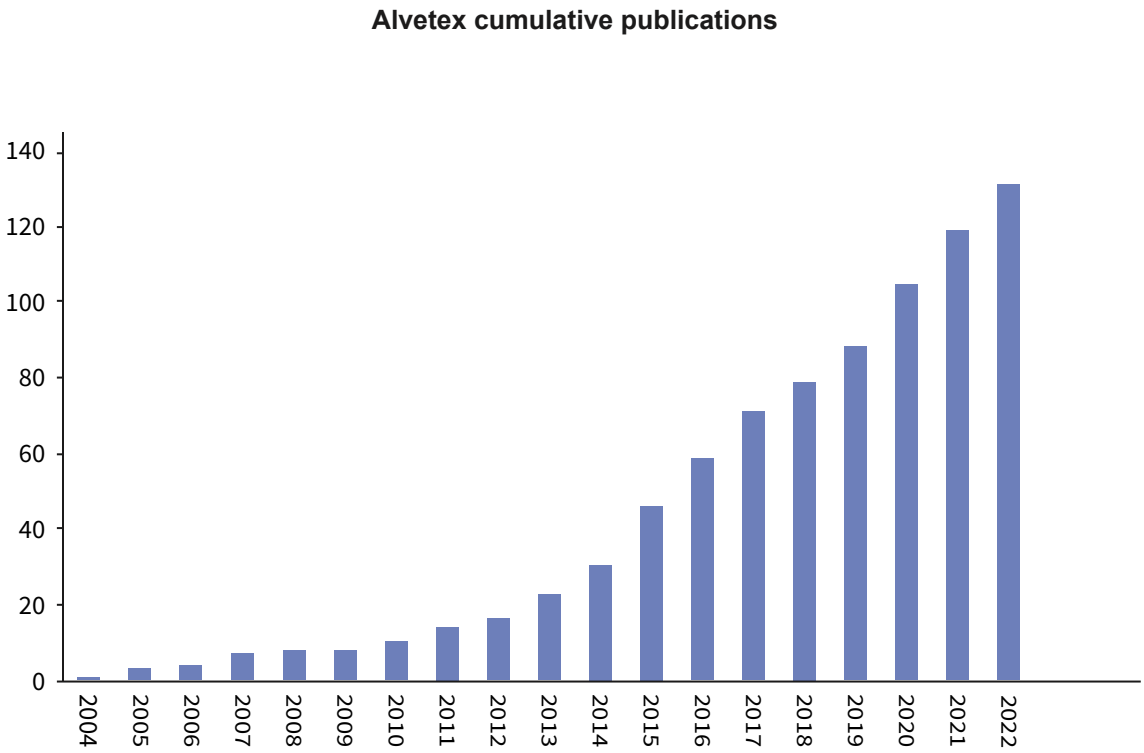
- Creating suitable surroundings for 3D cell growth, differentiation and function ☑
- Allowing cells to adopt a natural 3D shape and structure ☑
- Encouraging cells to form complex interactions with adjacent cells ☑
- Reducing stress and aberrant responses as a result of the growth substrate ☑
- Enabling a more natural environment to mimic native tissue structures ☑
- Consistent 3D cell culture growth within the matrix ☑
- High batch-to-batch reproducibility ☑
- Assay compatibility ☑
- Consumable, off-the-shelf product ☑
- Developed for routine use ☑

"**Alvetex** is an example of innovation to move us closer to better models for mimicking *in vivo* behavior of cells with the control of *in vitro* conditions."

Neil Kelleher (Award Judge of *The Scientist Magazine* Top Ten Life Science Innovations 2010)
Northwestern University (Chicago, IL, USA).

A growing body of peer-reviewed scientific literature

Alvetex 3D cell culture has been successfully used my many scientists and groups around the world, as well as in pharma and biotech companies. Where research has been reported in peer-reviewed scientific journals, we have been keeping a record – see the Alvetex publications page at www.reprocell.com/resources/publications. The graph below shows the cumulative number of publications in which Alvetex systems have been used with success.



www.reprocell.com/resources/publications

Alvetex enables you to experience all the benefits of **3D cell culture** simply, consistently and reproducibly.

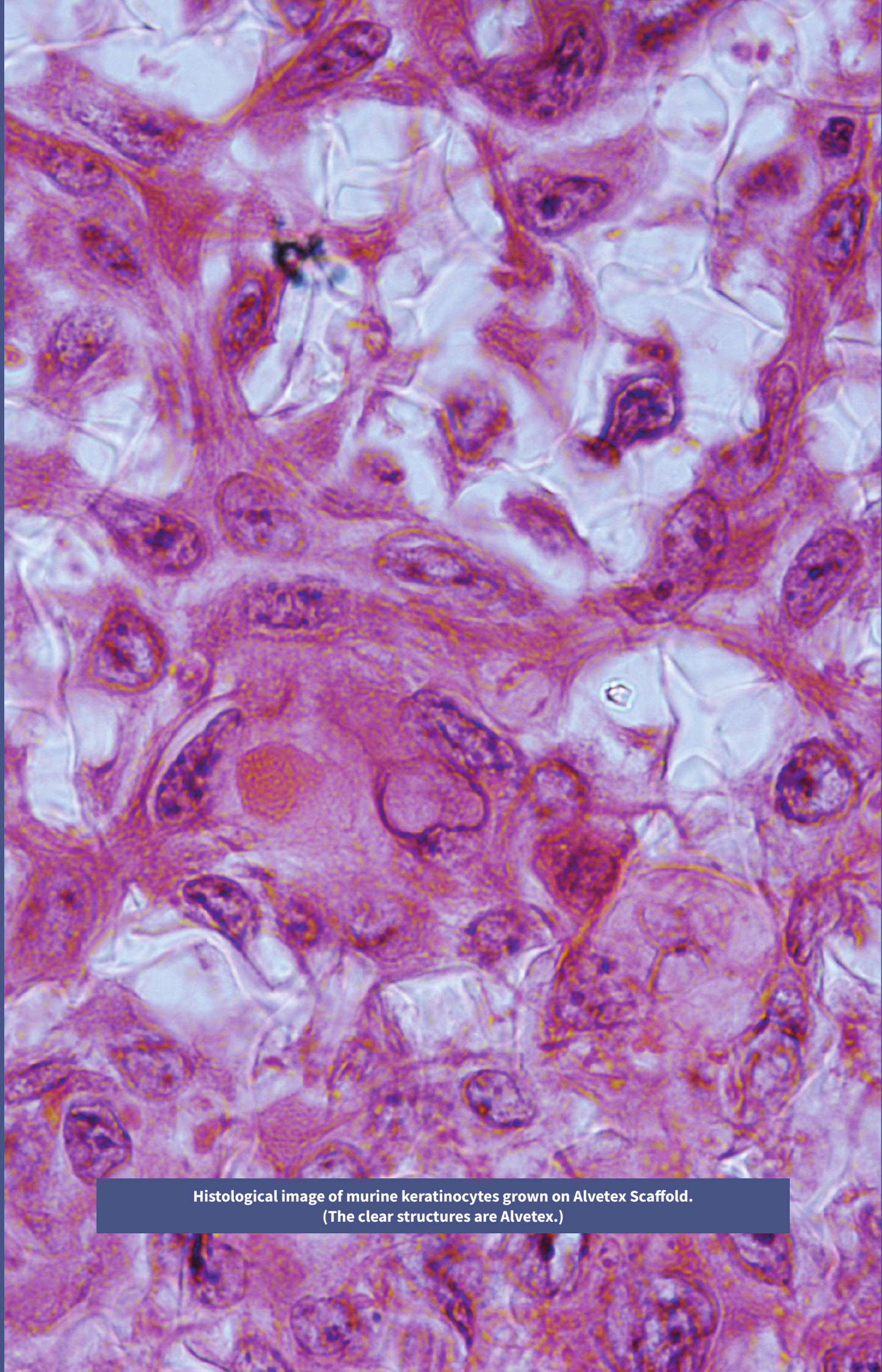
Alvetex protocols

www.reprocell.com/resources/protocols

Example cell types that have been successfully cultured on Alvetex

Following is an inexhaustive list of examples of cell types that have been successfully grown in Alvetex 3D cell culture. Many of these have been grown by REPROCELL scientists (see the Alvetex protocols page at www.reprocell.com/resources/protocols), while others have been reported in scientific journals (see the Alvetex publications page at www.reprocell.com/resources/publications-alvetex).

- 3T3 cells
- HaCaT cells
- HepG2 cells
- TERA2.cl.SP12 cells
- CHO-K1 cells
- LN229 cells
- Full thickness skin equivalent
- SW480 cells
- SW620 cells
- PC3 cells
- BT474 cells
- MG63 cells
- Primary rat MSCs
- MCF-7 cells
- Caco-2 cells, and the co-culture of Caco-2 with CCD-18co cells
- CCD-18co cells
- H1299 cells
- U118-MG glioblastoma cells
- Bone marrow stromal cells
- Primary hepatocytes
- Upcyte® hepatocytes
- Neurospheres
- Adipose tissue-derived stem cells
- Human pluripotent stem cell-derived neurons
- cylindroma primary cells
- MET4 squamous carcinoma cells
- L929 mouse fibroblasts
- Neural crest cells
- Human nucleus pulposus cells
- Chondrocytes
- Primary chick embryonic tissue
- Brain tissue slices
- Equine oviduct cells
- Spermatogonial stem cells
- Human disc cells
- Melanocytes
- A375/A2058 melanoma cells
- 3T3-L1 cells
- Prostate cancer cells and prostate stem cells
- H9 human embryonic stem cells
- CRL-11372 osteoblasts
- HEK293 human embryonic kidney cells
- JJ012 chondrosarcoma cells
- NG108 stem cells
- MBT-2 cells
- MB49 mouse bladder cancer cells
- Rat primary urothelial cells
- Primary retinal pigment epithelium
- Urothelial cells
- Myofibroblasts
- Astrocytes
- Primary and secondary ESC-derived hepatic cells
- Primary keratinocytes
- Pancreatic duct and stromal cells
- GSC glioblastoma stem cells
- b.END-3 endothelial cells
- HCT116 cells

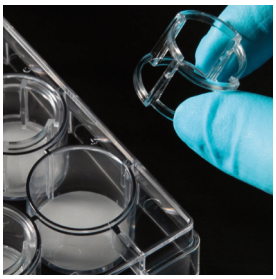
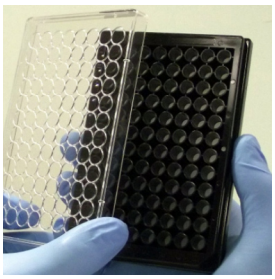


**Histological image of murine keratinocytes grown on Alvetex Scaffold.
(The clear structures are Alvetex.)**

For more information on Alvetex plasticware formats, see
https://www.reprocell.com/alvetex/alvetex-plasticware-formats

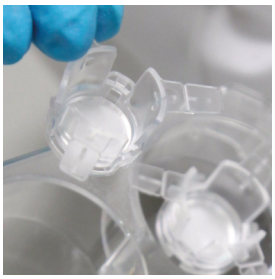
Alvetex plasticware: a range of formats for all your applications

Alvetex well plate formats

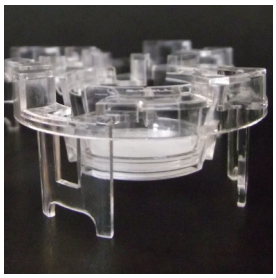
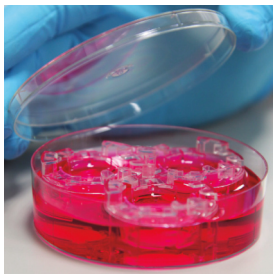
- Ideal for 3D culture in the top half of the scaffold, or layered above
- Cells are fed from the top of the scaffold only
- Useful for when restricted cell penetration is required
- An option for use with expensive cells, reducing cell number (e.g. cell transfection)
- Allows high throughput applications in 3D

Alvetex well insert formats

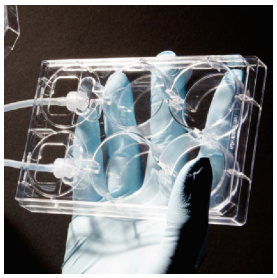
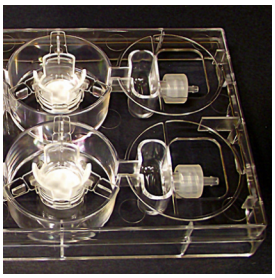
- Enables cells to be fed from above and below simultaneously
- Ideal for longer term 3D culture
- Growth of cells at the air/liquid interface
- Readily transfer 3D cultures to a fresh plate
- Enables co-culture studies (2D+3D, or 3D+3D)

Alvetex well insert holder and deep Petri dish

- Reduces frequency of media changing as up to 95 mL of medium
- Can be used to support a single 3D culture
- Ideal for maintaining long term 3D culture experiments of up to several weeks
- Facilitates the use of a magnetic stirrer to increase media circulation if required

Alvetex perfusion plates

- Inter-changeable Luer locks can be adapted to a range of tubing diameters
- Tissue fragments, 3D cultures in Alvetex well inserts and 2D cells can be maintained in the perfusion plate (for 2D cells, the plastic-ware may require coating with the appropriate cell culture reagent to promote cell adhesion)
- Unidirectional flow between separate wells allows the study of the interaction between cell populations grown in separate wells through the release of paracrine factors

Alvetex plasticware formats

www.reprocell.com/alvetex/alvetex-plasticware-formats

The Alvetex product range

Alvetex is supplied as 200 µm thick discs that each provide a highly porous polystyrene scaffold in which cells can grow and interact in 3D. Compatible with conventional tissue culture plasticware, Alvetex is available in several multi-well plate and well insert sizes.

Alvetex Scaffold, our market leading product, is primarily designed for three dimensional culture of dissociated mammalian cells within the scaffold, forming three dimensional associations as they propagate and migrate.

Alvetex Strata, our second generation product, is primarily designed to support the growth of cells and intact tissues on the surface of the membrane. Mini-slabs of intact tissue or embryoid bodies can be maintained on the upper surface of Alvetex Strata, being fed from culture medium below or around.

Each product unit has been terminally sterilized by gamma irradiation and remains sterile until its blister pack is opened. Alvetex requires an ethanol wash prior to use to render it hydrophilic. Alvetex does not degrade during normal use.

	Alvetex Scaffold	Alvetex Strata
Void size	33-55 µm	> 20 µm
Interconnect size	10-20 µm	> 5 µm

Buy Alvetex anywhere in the world

https://www.reprocell.com/product-catalog/alvetex-3d-cell-culture-systems

Prodouct name	Product Code	Presentation
Alvetex Scaffold multiwell plate formats		
Alvetex™ Scaffold 12 Well Plate (with lid)	AVP002-2	2 × 12 well plates
	AVP002-10	10 × 12 well plates
	AVP002-80	80 × 12 well plates
Alvetex™ Scaffold 24 Well Plate (with lid)	AVP006-2	2 × 24 well plates
	AVP006-10	10 × 24 well plates
	AVP006-80	80 × 24 well plates
Alvetex™ Scaffold 96 Well Plate (with lid)	AVP009-2	2 × 96 well plates
	AVP009-10	10 × 96 well plates
	AVP009-80	80 × 96 well plates
Alvetex™ Scaffold 384 Well Plate (with lid)	AVP0010	Made to order – contact REPROCELL for details
Alvetex Scaffold / Alvetex Strata well insert formats		
Alvetex™ Scaffold 6 Well Insert	AVP004-12	12 inserts
	AVP004-48	48 inserts
	AVP004-96	96 inserts
Alvetex™ Strata 6 Well Insert	STP004-12	12 inserts
	STP004-48	48 inserts
	STP004-96	96 inserts
Alvetex™ Scaffold 12 Well Insert	AVP005-12	12 inserts
	AVP005-48	48 inserts
	AVP005-96	96 inserts
Alvetex™ Strata 12 Well Insert	STP005-12	12 inserts
	STP005-48	48 inserts
	STP005-96	96 inserts
Alvetex™ Scaffold 24 Well Insert	AVP012-12	12 inserts
	AVP012-48	48 inserts
	AVP012-96	96 inserts
Alvetex Tools		
Alvetex™ Well Insert Holder and Deep Petri Dish (with lid)	AVP015-2	2 units
	AVP015-10	10 units
Alvetex™ Perfusion Plate (with lid and Luer locks)	AVP011-2	2 units
	AVP011-10	10 units
Alvetex Kits		
Alvetex™ Scaffold Plate Starter Kit	AVP-KIT-1	1 × 12 well plate 1 × 24 well plate 1 × 96 well plate
Alvetex™ Scaffold Well Insert Starter Kit	AVP-KIT-2	6 × 6 well inserts 6 × 12 well inserts 1 × Alvetex Well Insert Holder in a deep Petri dish
Alvetex™ Strata Well Insert Starter Kit	STP-KIT-2	6 × 6 well inserts 6 × 12 well inserts 1 × Alvetex Well Insert Holder in a deep Petri dish
Alvetex Perfusion Plate and Alvetex Scaffold 6 Well Inserts Kit (small)	AVP-KIT-3	2 × Alvetex Perfusion Plates with Luer locks 12 × Alvetex Scaffold 6 well inserts
Alvetex Perfusion Plate and Alvetex Scaffold 12 Well Inserts Kit (small)	AVP-KIT-4	2 × Alvetex Perfusion Plates with Luer locks 12 × Alvetex Scaffold 12 well inserts
Alvetex Perfusion Plate and Alvetex Scaffold 6 Well Inserts Kit (large)	AVP-KIT-5	5 × Alvetex Perfusion Plates with Luer locks 48 × Alvetex Scaffold 6 well inserts
Alvetex Perfusion Plate and Alvetex Scaffold 12 Well Inserts Kit (large)	AVP-KIT-6	5 × Alvetex Perfusion Plates with Luer locks 48 × Alvetex Scaffold 12 well inserts

Choosing the right Alvetex format based on assay type

Types of assay	Alvetex Scaffold							Alvetex Strata	
	6 well insert	12 well insert	24 well insert	12 well plate	24 well plate	96 well plate	384 well plate	6 well insert	12 well insert
Viability/Proliferation/ Metabolic Activity Assays	+++	+++	+++	+++	+++	+++	+++	+++	+++
Toxicity Assays	+++	+++	+++	+++	+++	+++	+++	+++	+++
Gene Expression assays (qPCR/microarray)	+++	+++	+++	+++	+++	+++	+++	+++	+++
Protein Expression assays (e.g. western blot)	+++	+++	+++	+++	+++	+++	+++	+++	+++
Air-liquid Interface assays	+++	+++	+++	n/a	n/a	n/a	n/a	+++	+++
Cell Signaling assays	+++	+++	+++	+++	+++	+++	+++	+++	+++
Permeability assays	+++	+++	+++	n/a	n/a	n/a	n/a	+++	+++
Transfection assays	+++	+++	+++	+	+	+	+	+++	+++
Co-culture assays	+++	+++	+++	++	++	++	++	+++[C]	+++[C]
Invasion assays	+++	+++	+++	+	+	+	+	++[C]	++[C]
Migration assays	+++	+++	+++	+	+	+	+	++[C]	++[C]
Histology	+++	+++	+++	++	++	++	++	+++	+++
Immunostaining (IHC/IF)	+++	+++	+++	++	++	++	++	+++	+++
Confocal microscopy	+++	+++	+++	++	++	++	++	++	++
Live cell imaging[A]	+++	+++	+++	++	++	++	++	++	++
Ex vivo tissue maintenance	+++	+++	+++	++	++	++	++	+++	+++
Live cell retrieval[B]	++	++	++	++	++	++	++	++	++

Suggested guidelines for the use of Alvetex formats for cell applications and assays.

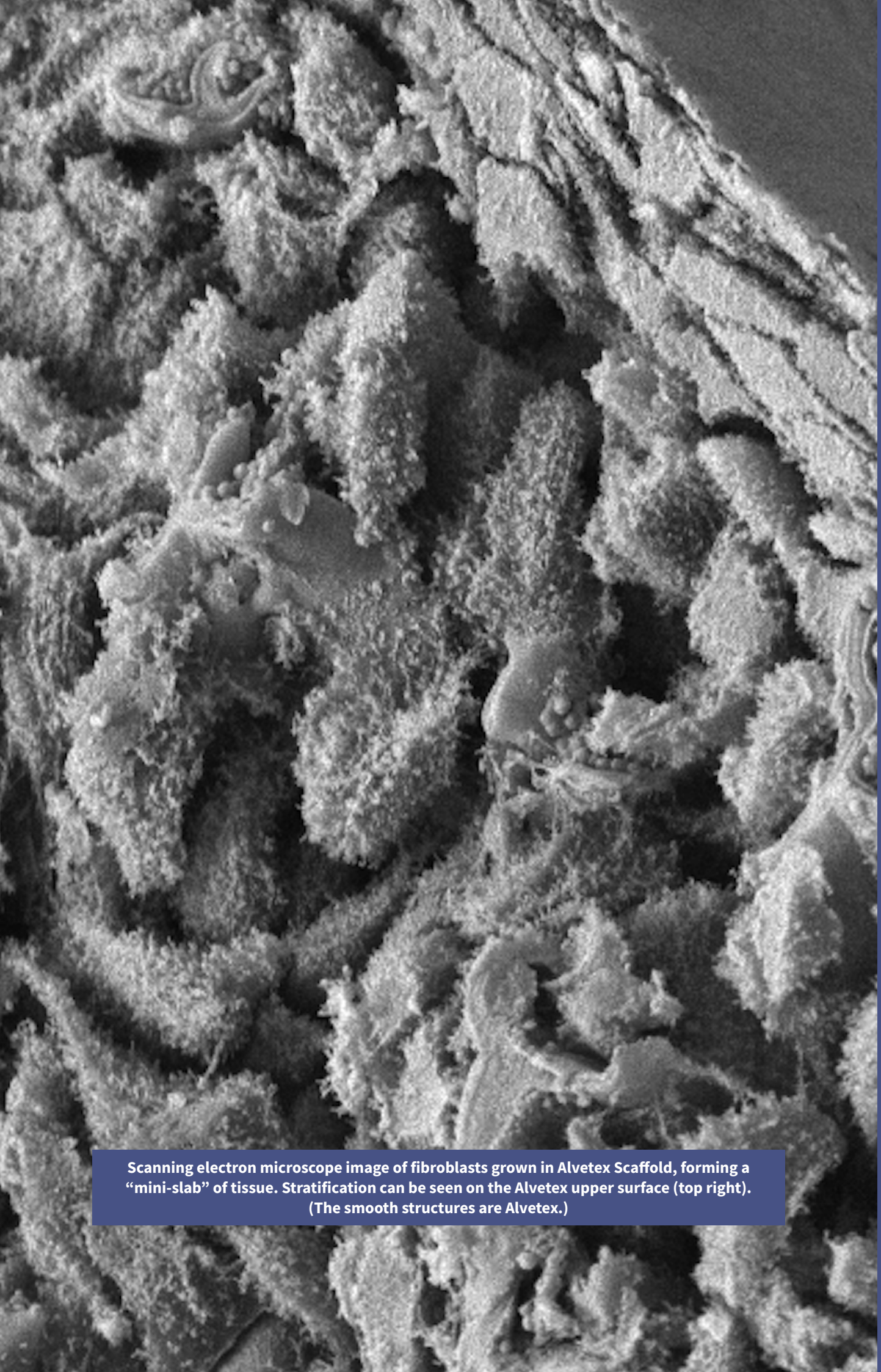
+++	=	most suitable
++	=	suitable
+	=	least suitable
n/a	=	not applicable

Ranking is based on Alvetex disc format suitability, the likely cell yields and therefore signal generation, and whether exogenously added chemicals and/or cells can be contained to only one side of the membrane.

A. The growth of cells cannot be followed by traditional light microscopy as in 2D, but as with *ex vivo* tissues, 3D structures have to be evaluated using histology or confocal microscopy. Alternatively cell proliferation can be monitored using a viability assay such as the MTT.

B. The exact number of cells retrieved from Alvetex varies with the invasiveness of the cell line cultured, e.g. epithelial vs. fibroblastic. Although the three-dimensional structure of Alvetex precludes all 100% of the cells from being routinely retrieved, calls can be retrieved in adequate numbers for quantitative down-stream processes, e.g. flow cytometry.

C. When designing co-culture, invasion or migration set-ups for Alvetex Strata, please keep in mind that some cell lines (e.g. epithelial) have a tendency to multi-layer on top of the substrate rather then invade into it.



Scanning electron microscope image of fibroblasts grown in Alvetex Scaffold, forming a "mini-slab" of tissue. Stratification can be seen on the Alvetex upper surface (top right). (The smooth structures are Alvetex.)

REPROCELL's Alvetex 3D Cell Services

REPROCELL's *Centre for Predictive Drug Discovery* (Glasgow, UK) has developed a range of 3D assay services using bioengineered tissue equivalents. All our services are designed to meet the contract research needs of academic and industrial customers.

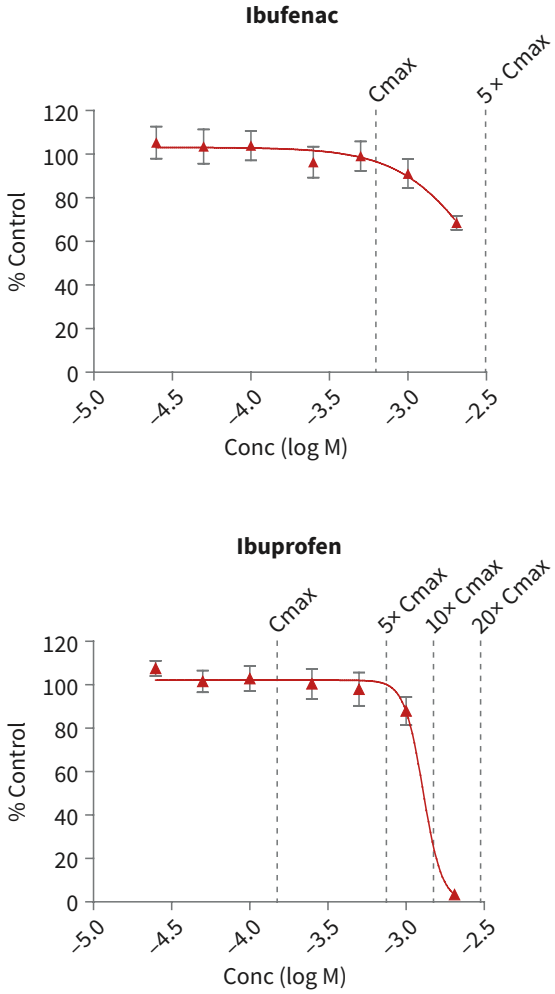
3D Hepatotoxicity Assay

Utilizing HepG2 cells grown in Alvetex Scaffold in 96 well plate format.

- Increase confidence and reduce drug development costs with a good *in vitro* prediction of clinical drug induced liver injury (DILI).
- Save money and time with a large number of test wells and a small compound volume.
- Assess chronic drug toxicity with cells stable for at least 28 days, allowing multiple days dosing.
- Innovation award-winning 3D substrate supported by multiple peer-reviewed publications.

Compound	EC_{90} 3D Alvetex (µM)	5× Clinical Cmax (µM)*	Clinical +ve or –ve DILI	Correctly Predicted
Ibuprofen	948.4	749.0	Negative	YES
Ibufenac	837.5	3125.0	Positive	YES
Entacapone	51.3	30.0	Negative	YES
Tolcapone	24.6	109.5	Positive	YES
Rosiglitazone	>75	5.2	Negative	YES
Troglitazone	72.4	32.0	Positive	NO
Acetaminophen	8871.6	650.0	Positive	NO
Gemfibrozyl	496.6	700.0	Positive	YES

Table of EC_{90} (10% cell death) values for HepG2 cells pre-cultured in Alvetex Scaffold 96-well plates for 7 days and subsequently exposed to DILI +ve or –ve compounds for a further 7 days. Note that 3/3 DILI –ve and 3/5 DILI +ve compounds are correctly predicted (cell viability assessed by Promega CellTiter-96 Aqueous Cell Proliferation Assay, single EC_{90} value calculated from an average of n = 4 replicate wells +/- standard error, * clinical Cmax values from Bale *et al*. 2014, *Exp Biol Med* 239(9):1180-1191). HepG2 offers an inexpensive and robust high screening system widely used in industry. It is however recognized that it has some limitations (Gerets *et al.* 2012, *Cell Biol Toxicol* 28:69-87).

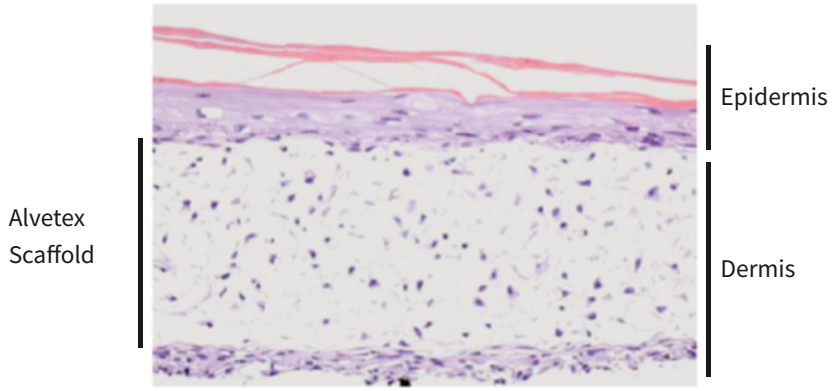


Example concentration-response curves. Ibuprofen and Ibufenac shown.

Full-Thickness Skin Model

REPROCELL and Alcyomics® have collaborated to develop the full-thickness skin equivalent model Skimune® 3D using healthy primary human skin cells and autologous immune cells.

This assay is ideal for testing adverse immunological reactions to large proteins such as monoclonal antibodies, biosimilars and biobetters.

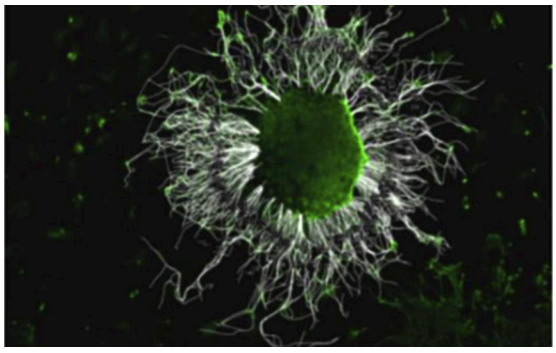


Skimune® 3D: skin equivalent model

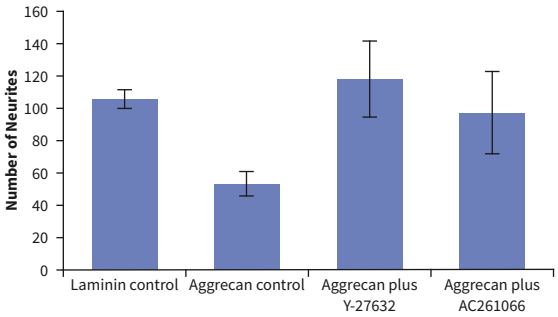
Neurite Outgrowth Model

REPROCELL has developed an Alvetex 3D assay for the inhibition and recovery of neurite outgrowth, in partnership with Durham University UK.

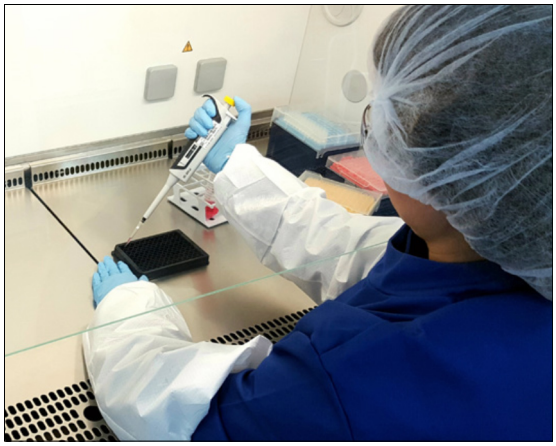
- Utilizing REPROCELL's iPSC-derived StemRNA™ Neuro cells, available as healthy wild-type and Alzheimer Disease patient-derived (presinilin 2 mutation) to assess pathological changes *in vitro*.
- Explore Alvetex 3D assay versatility with physiological and pathological ECM coatings (laminin, CSPGs), known pathway inhibitors and therapeutic lead compounds.



Neurite outgrowth from a neurosphere.



Recovery from neurite inhibitionTERA2.SP12 neurospheres transferred onto Alvetex Scaffold coated with four different ECM formulations.

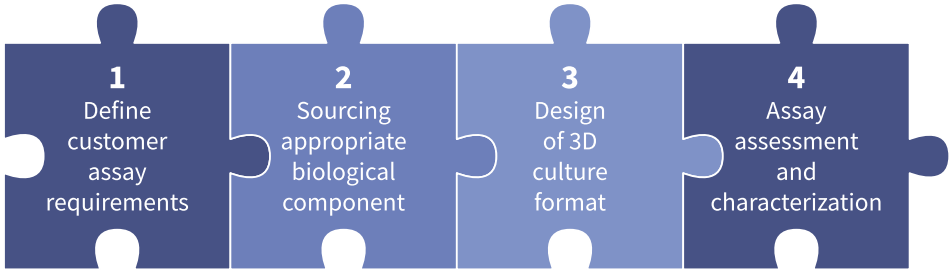


Inside the laboratories of REPROCELL Europe's Centre for Predictive Drug Discovery, Glasgow UK.

Custom Assay Development Services

Innovative 3D models have demonstrated evident advantages over conventional 2D culture, providing more physiologically relevant and predictive data. REPROCELL's scientists have a unique background and experience in 3D assay development with a variety of cell types and cell culture formats, and are on hand to create custom 3D assays for specific customer needs.

Our team of expert scientists are available to discuss how our assays can answer your contract research needs and to design a protocol that will fulfil your study requirements.



Alvetex assay services

www.reprocell.com/alvetex/alvetex-assay-services

Alvetex – past, present and future

Alvetex 3D cell culture technology was pioneered in the laboratory of Professor Stefan Przyborski at Durham University, UK.

Professor Stefan Przyborski

Growth of mammalian cells in 3D has been practiced for many decades using various materials and approaches. The Alvetex membrane was originally pioneered in the laboratory of Professor Stefan Przyborski at Durham University (UK).

Stefan has more than 35 years of research experience within the fields of cell and tissue biology with specialization in stem cell science, cell differentiation and the development of advanced technologies that enable the construction of human tissues *in vitro*.

Prof. Przyborski's interdisciplinary research at the boundaries of physical science and biology led to the development of Alvetex in the early 2000's. He founded the company Reinnervate Ltd in 2002 with the purpose of commercializing and further developing Alvetex products and services.

Alvetex was named among the winners of *The Scientist* magazine's "Top 10 Life Science Innovations of 2010" and was voted one of the "Top 100 Innovative Products of 2011" at the *R&D 100* Awards. Several other awards have since followed. Alvetex is arguably the market leading physical scaffold product that supports robust and routine 3D cell culture.

In 2013, Alvetex was chosen by a research group at Massachusetts General Hospital (USA) for 3D culturing of murine osteocytes on the International Space Station (ISS) for the study of bone loss during space flight. These microgravity experiments took place onboard the ISS in 2015.

Recognizing the future of 3D cell culture applications and tissue engineered in vitro models for drug discovery and regenerative medicine, REPROCELL Inc. (Japan) acquired Reinnervate in 2014 and merged it with Biopta Ltd. (Glasgow, UK) to form REPROCELL Europe Ltd. in 2016.

Reinnervate (formerly), the REPROCELL Group, Professor Przyborski's academic lab at Durham University, and many scientists around the world have used Alvetex with success in their research. This research has generated significant amounts of data which has been published extensively in peer-reviewed journals (see https://www.reprocell.com/resources/publications-alvetex). This body of scientific literature continues to grow year on year, and it exemplifies the use of Alvetex in multiple applications with many cell and tissue types.

Stefan Przyborski has remained active in the organization and currently serves as Chief Scientific Officer of the REPROCELL Europe Ltd, in addition to his academic role as Professor of Cell Technology in the department of Biosciences at Durham University (UK). He runs an active research laboratory at Durham University consisting of post-doctoral and postgraduate researchers. His group is well funded and regularly publishes their work in peer reviewed journals.

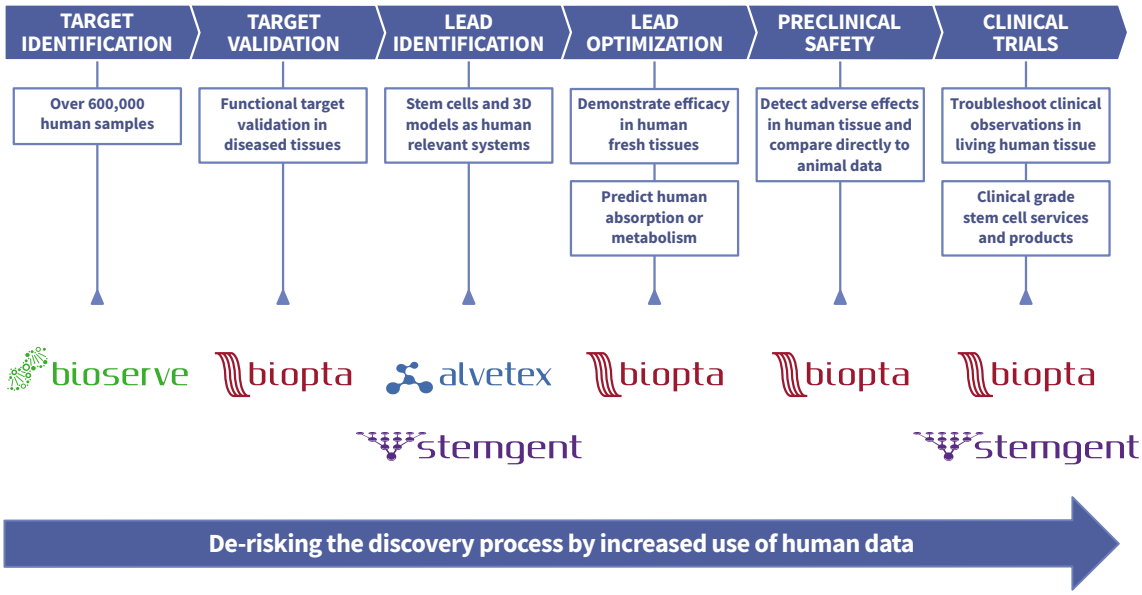
In both his Durham University lab and in REPROCELL Europe, Prof. Przyborski continues to lead research and development of new applications for 3D cell culture technologies and the generation of novel human tissue models that can be used in academia and industry for basic research, drug screening and safety assessment.

Alvetex 3D cell culture technology continues to be developed, produced and distributed by REPROCELL worldwide. And with our own expertise and experience, REPROCELL also now offers worldwide 3D cell culture and bioengineered human tissue model services built around the Alvetex platform.

Discover Alvetex online

www.reprocell.com/alvetex

REPROCELL provides products and services across the entire drug discovery process



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REPROCELL BRANDS

bioserve

- Extensive biorepository of human tissue samples
- Network of clinical sites for prospective sample collection
- Molecular services

stemgent

- RNA reprogramming systems and services
- Reagents for pluripotent cell culture and differentiation
- Extensive portfolio of small molecules

alvetex

- 3D cell culture technology creating *in vivo*-like cell environment
- Protocols for stem cell, oncology and other tissue research applications

biopta

- Experts in human tissue research services for drug development
- Predictive safety, efficacy and ADME assays in human and animal tissues

REPROCELL

Improving human health
through biomedical innovation and discovery

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