



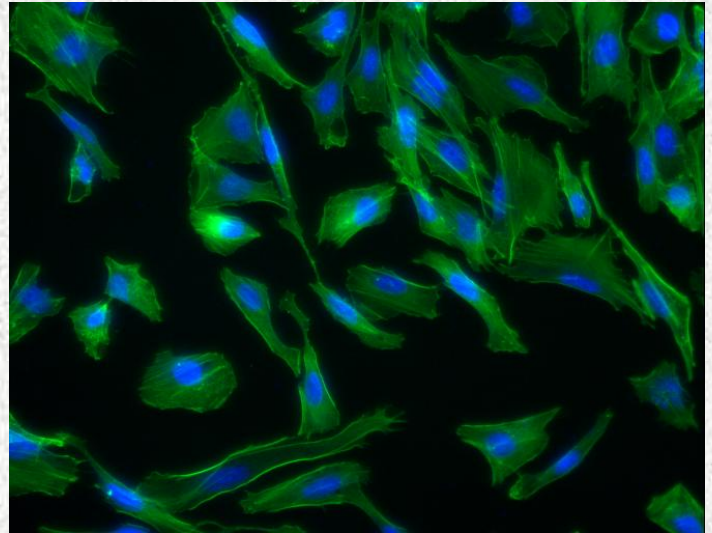
3-D Cell Culture for a 3-D World

Table of Contents

Introduction	3
Research Areas	4
Cancer Research	4
Stem Cell Research	4
Cell Migration Analysis.....	5
<i>In Vitro</i> Disease Models	8
Tissue Engineering Scaffolds	8
Schwann Cell Alignment	9
High Throughput Drug Discovery	11
Brain Cancer Drug Sensitivity	12
Successful Manufacturing of Artificial Trachea	17
Nanofiber Comparisons to Native Tissue.....	18
Products.....	19
Cell Seeding Protocol.....	20
References.....	21
Contact Us	22

Introduction

Historically, cell culture for life science research has been performed on flat, tissue culture polystyrene (TCPS) because it is cheap, optically clear, and many cells grow well on it. In reality, however, living organisms are made up of an extracellular matrix (ECM) that presents both aligned physical structure and mechanical support to the cells. TCPS lacks this three-dimensional (3-D) component and cells behave very differently on this flat, smooth substrate than they do in true biological settings.



Not surprisingly, **drugs developed using TCPS as an *in vitro* substrate experience a >99% failure rate in later testing.**

NFS vs basement membrane matrices (gels):

Compared with gels, Nanofiber Solutions synthetic scaffolds have the following benefits – they:

- *do not* require specialized transportation or storage
- supply a physical structure for cell adherence and growth
- allow for easy cell extraction for gene and protein analyses
- are sold in standard cell culture plate sizes and thus are adaptable for automation
- *do not* introduce a third-party animal contaminant in stem cell use.

NFS vs other 3D cell culture products:

Nanofiber Solutions scaffolds are engineered to mimic cellular-scale structures. Our standard plates include polycaprolactone (PCL) nanofibers averaging less than one micron in diameter. The fiber dimensions and specific physical properties are optimized to produce ideal synthetic *in vitro* models. *No other 3D cell culture product* matches our ability to physically mimic the *in vivo* environment and create a realistic scaffold for all types of adherent cells.

We also offer specialized plates using other polymers and fiber diameters tailored for specific cell types and extracellular matrix conditions (i.e., to mimic normal, pathological, and aged ECM environments).

Cancer Research

- Our nanofibers are optically transparent to allow for live-cell imaging and real time quantification of cell mobility using an inverted microscope.
- Nanofibers mimic the 3D topography found *in vivo* which produces a more realistic cellular response to therapeutics.
- More realistic cellular behavior means you can use fewer animals and decrease time-to-market for drug discovery and development.
- Our nanofibers can easily be coated with ECM proteins using existing protocols for standard lab ware. Cells can be easily removed for protein or gene analysis using trypsin, EDTA, etc.

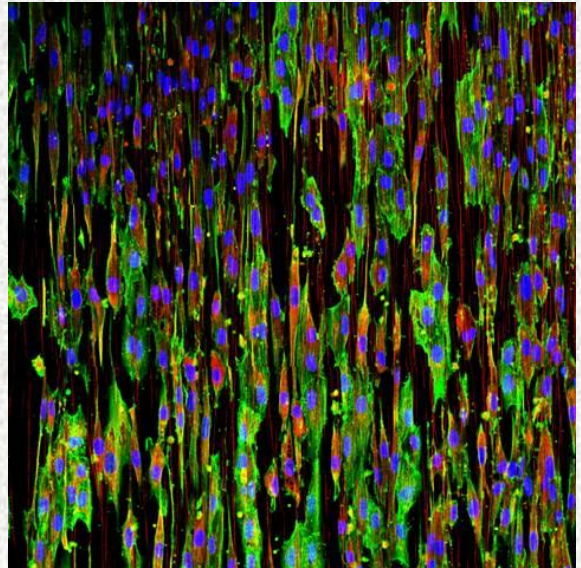


Figure 1. High content screening (HCS) of human kidney derived stem cells cultured on Nanofiber Solutions' aligned nanofibers.

Stem Cell Research

- Higher expansion rates of stem cells on nanofiber scaffolds versus traditional flat surfaces.
- Our nanofibers maintain stem cell pluripotency during expansion and help control differentiation into the desired cell type.
- Our nanofibers are synthetic with no animal derived by-products which facilitates higher reproducibility and clinical applications.
- Compatible with standard immunohistochemistry staining for validation of phenotypic markers.
- Our nanofiber scaffolds can be applied in large commercial bioreactors or in disposable bag bioreactors.



Figure 2. Human breast progenitor cells spontaneously form spheroids on Nanofiber Solutions' randomly oriented nanofibers.

Cell Migration Analysis

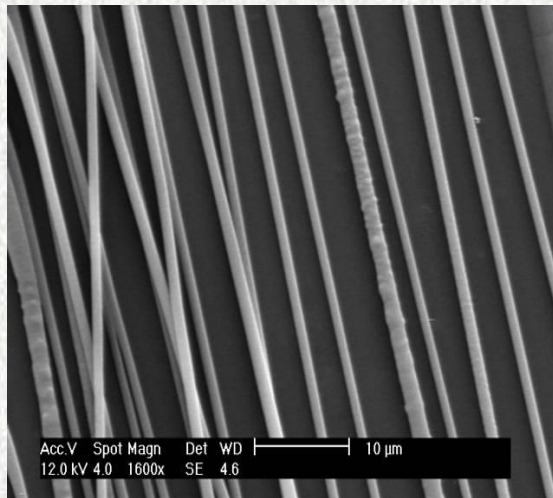
Malignant gliomas are the most common tumors originating within the central nervous system (CNS) and account for over 15,000 deaths annually in the US. Tumor cells exhibit a diffuse migration around the tumor core and disperse much further along anatomical fiber-like and tube-like structures of the brain, such as white matter tracts and blood vessels. This dispersion prevents complete surgical removal and contributes to tumor recurrence followed by a rapid, lethal outcome.

In this context, there are no strategies formulated to act against migratory cells. Evidence suggests that current radiotherapy and anti-angiogenic approaches may actually cause an increase in invasion. The development of effective anti-invasive approaches has been largely hampered by the difficulty in modeling cell migration appropriately *in vitro*. A number of *in vitro* migration and invasion assays are in common use, but significant limitations restrict their potential to predict cell behavior *in vivo*. The traditional "wound healing" and the Boyden chamber (Transwell) assays, which measure motility on flat surfaces are quantitative but rely on cells arranged in flat monolayers on rigid substrates. This condition forces the cells to adopt a fibroblast-like morphology and motile behavior different from that *in vivo*. At the other end of the spectrum is the "organ assay." For gliomas this involves cells seeded on live brain slices supported by appropriate culture medium. This model challenges glioma cells with a true neural cytoarchitecture but is laborious, difficult to reproduce and includes poorly controlled variables such as the rapid degradation of myelin fibers and the death of neural cells within the slice.

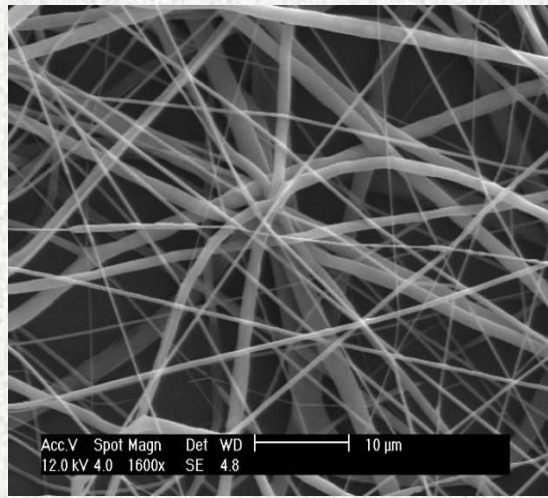
To design a more realistic assay for the study of glioma cell migration, Nanofiber Solutions has developed a product consisting of electrospun poly- ϵ -caprolactone (PCL) nanofibers precisely engineered to produce both aligned and random structures (Figure 3). Clear differences in migratory behavior between the two are observed as cells on randomly oriented substrates

Cell Migration Analysis (cont'd)

interact with many fibers and do not show preferential pseudopodia extension (Figure 4). Cells on aligned substrates interact with relatively few nanofibers and are highly elongated in the direction of alignment.

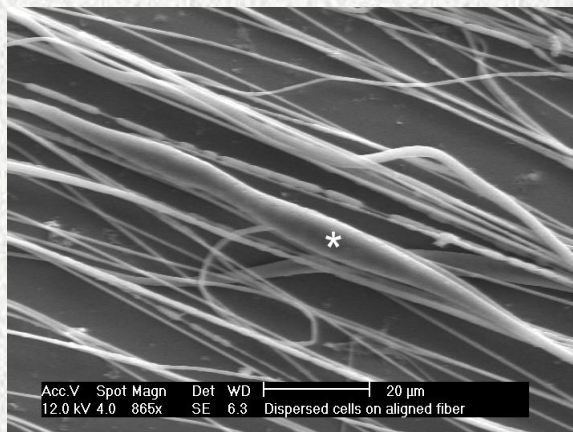


A

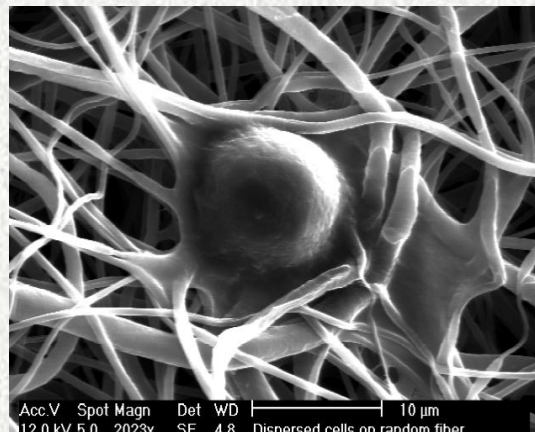


B

Figure 3. Scanning electron microscopy of randomly oriented PCL nanofibers (A; part#2401) and aligned PCL nanofibers (B; part#2402).



A



B

Figure 4. Scanning electron microscopy showing U251 glioma cells migrating on aligned (A) or randomly oriented (B) PCL fibers.

On aligned and random scaffolds, the migratory behavior of glioma cells displays clear, reproducible and quantifiable differences when challenged by aligned versus randomly oriented topographic cues (Figure 5) (Johnson et al). This mimics the behavior of these cells observed *in vivo*. Glioma neurospheres were also prepared and showed better adhesion to this substrate than to TCPS. Time-lapse confocal tracking of cells migrating from the spheres revealed that cells on

Cell Migration Analysis (cont'd)

random fibers did not detach from the original aggregate (Figure 6). Conversely, cells readily detached from neurospheres seeded on aligned fibers and showed decisive motion away from the neurosphere along the fiber axes (Figure 6). Cell dispersion along the fibers was on average ~6-fold higher than across the fibers. **These chemically and physically flexible assays underscore the relevance of Nanofiber Solutions products containing these structures as a valuable tool providing a basis for the *in vitro* development of improved pharmaceutical compounds.**

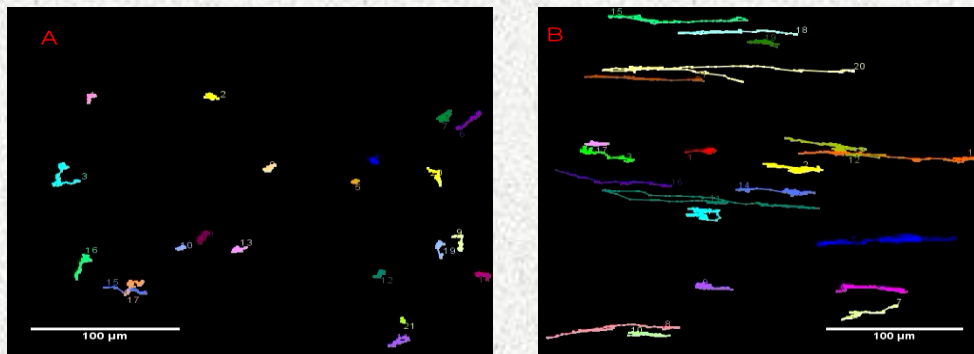


Figure 5. Motion cell-tracking of individual cells on random (A; part#2401) and aligned (B; part#2402) PCL nanofibers after a total tracking period of 36hrs. Scale bars: 100 μm

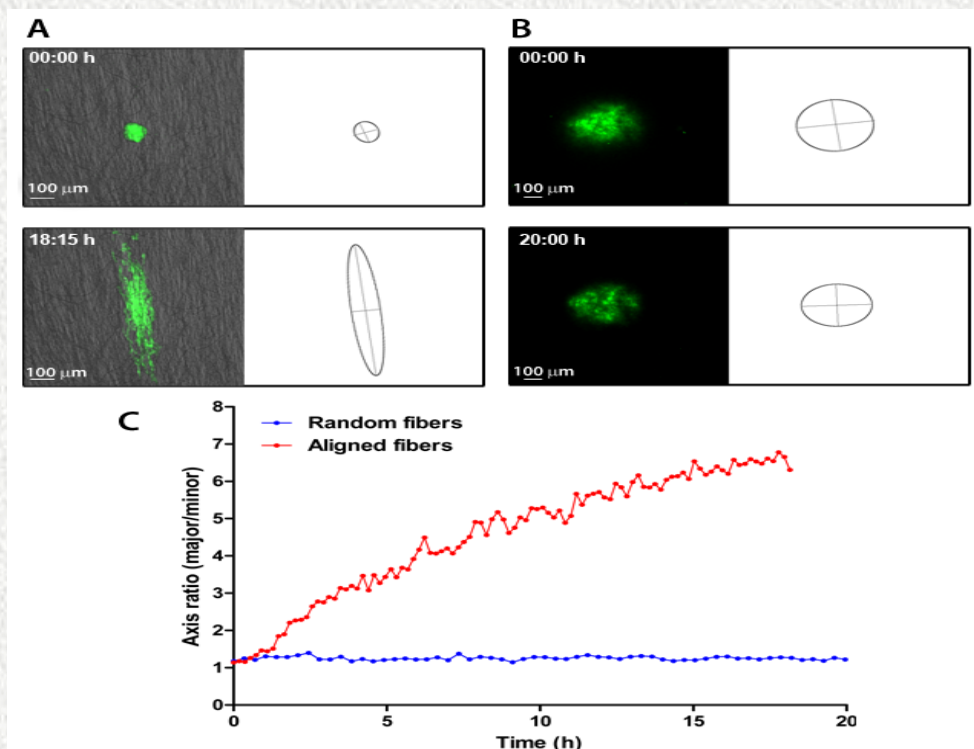


Figure 6. Representative frames showing cell dispersion from neurospheres seeded on aligned (A; part #2402) and random (B; part #2401) PCL fibers. The corresponding bounding ellipses were estimated by principal component analysis. The change in the ratio of the elliptic axes over time revealed a 6-fold increase in along-axis versus across-axis migration on aligned fibers (C).

In Vitro Disease Models

- Cell based assays incorporating cardiomyocytes (Fig. 7) or hepatocytes on nanofiber scaffolds for more realistic toxicity testing.
- Organ derived coatings may be applied to the nanofiber scaffolds to recapitulate specific microenvironments.
- Neuronal models on the aligned nanofibers may allow advanced *in vitro* models of Alzheimer's and Parkinson's.
- Our aligned nanofibers facilitate the orientation of myoblasts for *in vitro* muscle

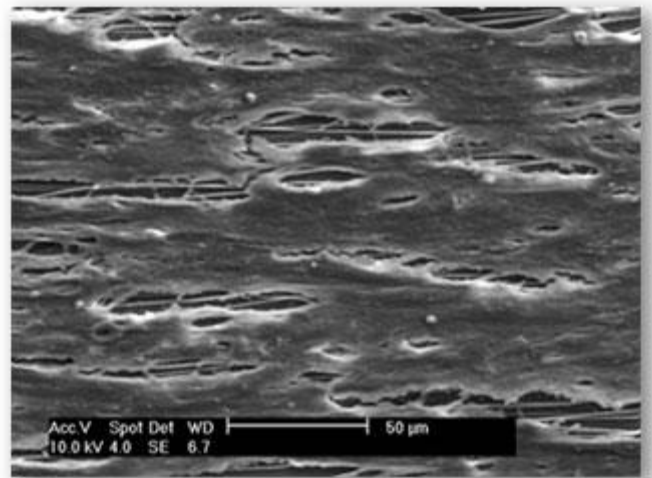


Figure 7. Alignment of human mesenchymal stem cells on Nanofiber Solutions' aligned PCL for cardiotoxicity testing.

Tissue Engineering Scaffolds



Figure 8. Blood vessel made from nanofibers, pre-seeded with HUVEC's and implanted into the femoral artery of a porcine model.

- Nanofiber scaffolds can be any shape or size.
- Made from nearly any synthetic or natural polymer.
- Can be implanted *in vivo* or simply used *in vitro*.
- Mechanical properties of the nanofibers can be tailored.
- Nanofiber scaffolds may be degradable or non-degradable depending on your needs.

Schwann Cell Alignment

Schwann cells are known for their roles in supporting nerve regeneration and have been widely investigated for applications in neurological repair. Studies have demonstrated positive results and potential for Schwann cell transplantation as a therapy for spinal cord injury, both in aiding regrowth and myelination of damaged CNS axons. Schwann cells can guide regeneration by forming a 'tunnel' that leads toward the target neurons. The stump of the damaged axon is able to sprout, and those sprouts that do grow through the Schwann-cell 'tunnel' do so at the rate of approximately 1 mm/day in good conditions. Regenerating axons will not reach their targets unless Schwann cells are there to support and guide their motion.

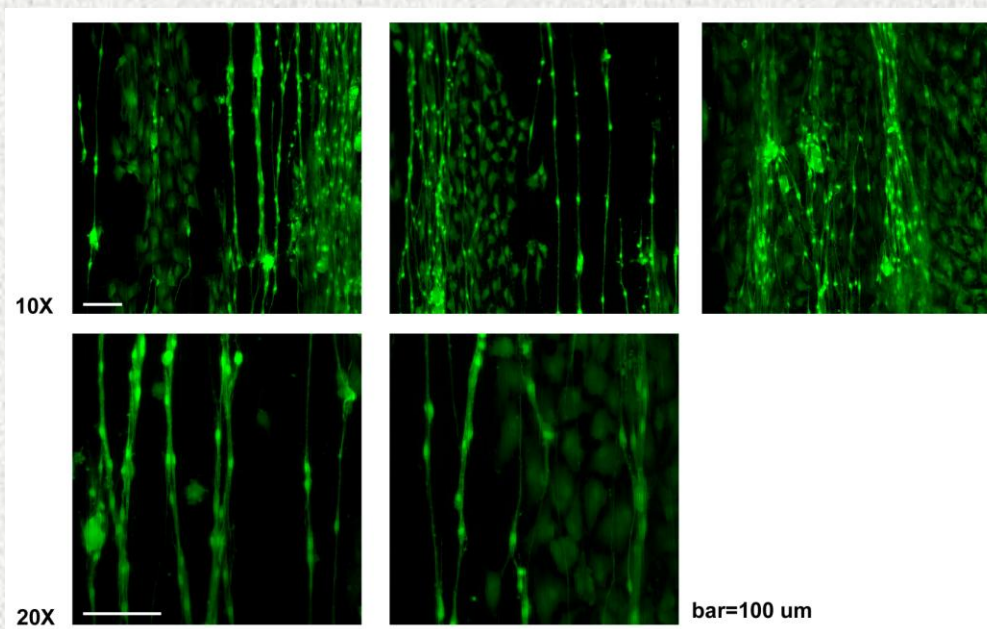


Figure 9. Schwann cells after one week of culture on aligned Nanofiber Solutions products.

Nanofiber Solutions aligned nanofiber products provide a consistent means of guiding Schwann cells toward assuming directional configurations in a convenient multi-well plate format. Figure 9 shows Schwann cells after one week of culture. This culture contains mixed spinal ganglion cells and Schwann cells on aligned Nanofiber Solutions part#9602. The live cells were stained with calcein-AM (green).

Schwann Cell Alignment (cont'd)

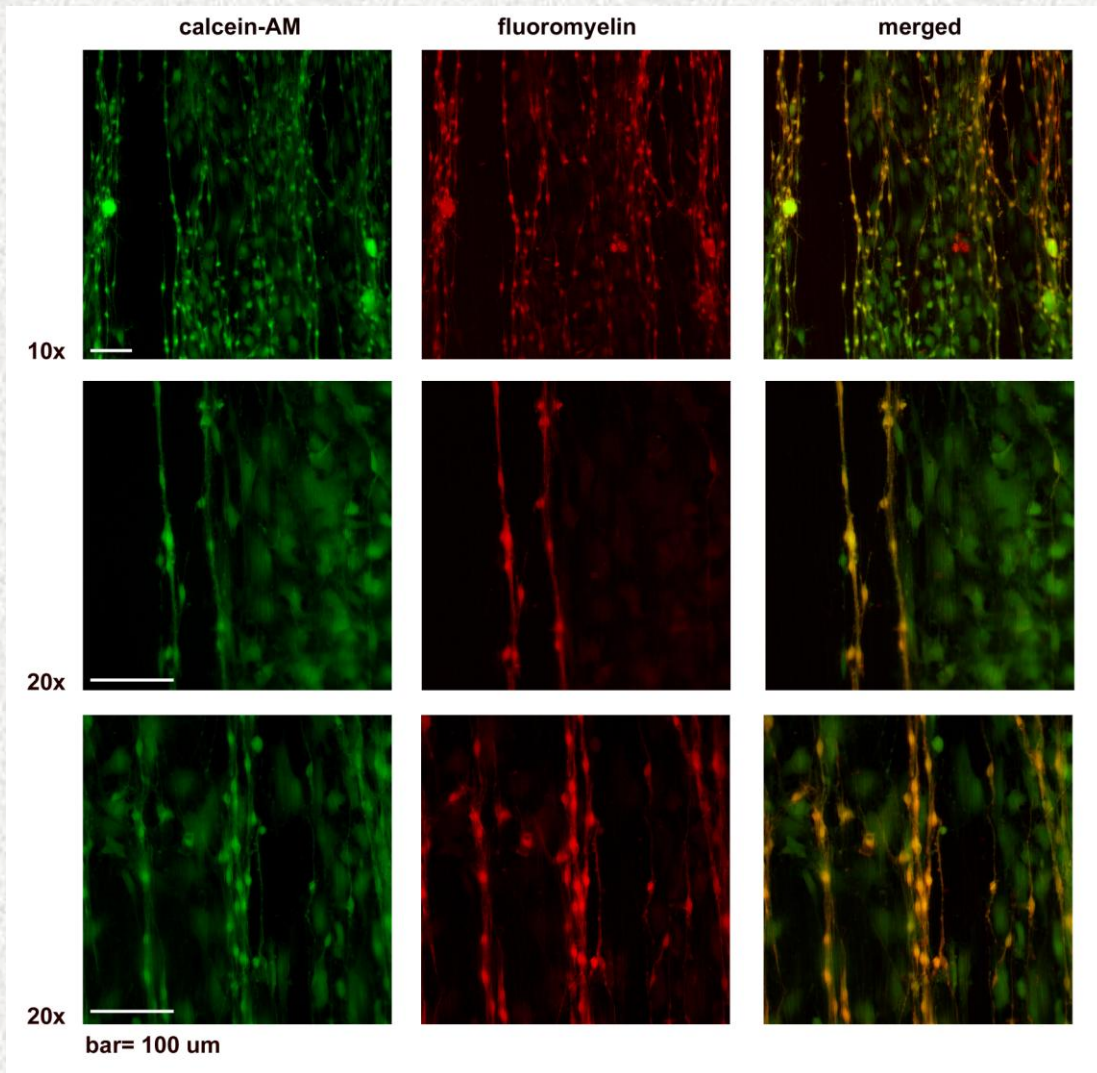


Figure 10. Schwann cells after two weeks of culture on aligned Nanofiber Solutions products.

Figure 10 shows the same Schwann cells following two weeks in culture. The conditions are the same as Figure 9, but the cells were stained with calcein (green) and fluoromyelin (red) to detect myelination; the myelin staining is largely restricted to the Schwann cells. The Schwann cells are highly elongated along the axis of the fibers. Other round cells observed in the same culture take up less calcein and are likely fibroblasts.

High Throughput Drug Discovery

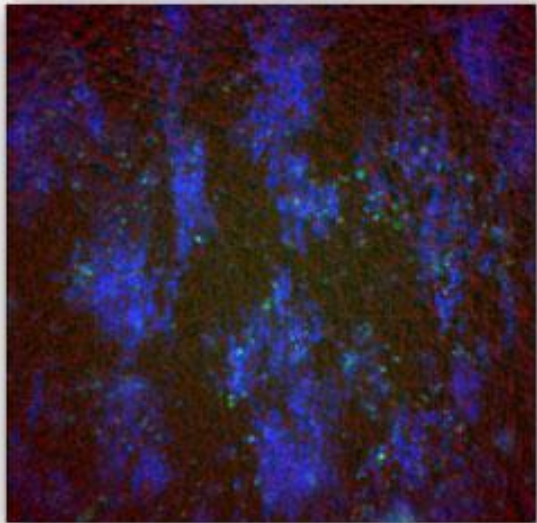


Figure 11. HeLa cells (blue) imaged in real time using an automated plate reader while being cultured in one of our 384 well plates (part#38401) shown

- Improved estimates of *in vivo* drug efficacy and high content screening (HCS).
- Better screening for potential toxicity using a synthetic, reproducible substrate.
- Shortened time to market and reduced clinical failures via a far more realistic substrate.
- Cellular responses to our 3-D substrates significantly decrease the need for animal testing.
- Our nanofiber plates easily integrate into standard automated plate handling and imaging equipment.



Brain Cancer Drug Sensitivity

Anti-cancer drugs developed using TCPS approach 100% failure when transitioning into clinical settings. Motile brain cancer/glioma cells are known to be more resistant than non-motile cells to apoptotic stimuli. Current evidence suggests that conventional therapies may in fact trigger glioma cell dispersion. Anti-migratory approaches against gliomas have targeted cell adhesion molecules or tumor-associated proteases, following anti-metastatic strategies utilized in other solid tumors. However, these approaches have been largely ineffective in the clinical setting, partly due to the ability of brain tumor cells to shift between different mechanisms of cell adhesion as well as proteolytic and non-proteolytic modes of migration. This underscores the need for better cell culture tools that can identify anti-migratory compounds capable of targeting the master regulators of tumor cell locomotion.

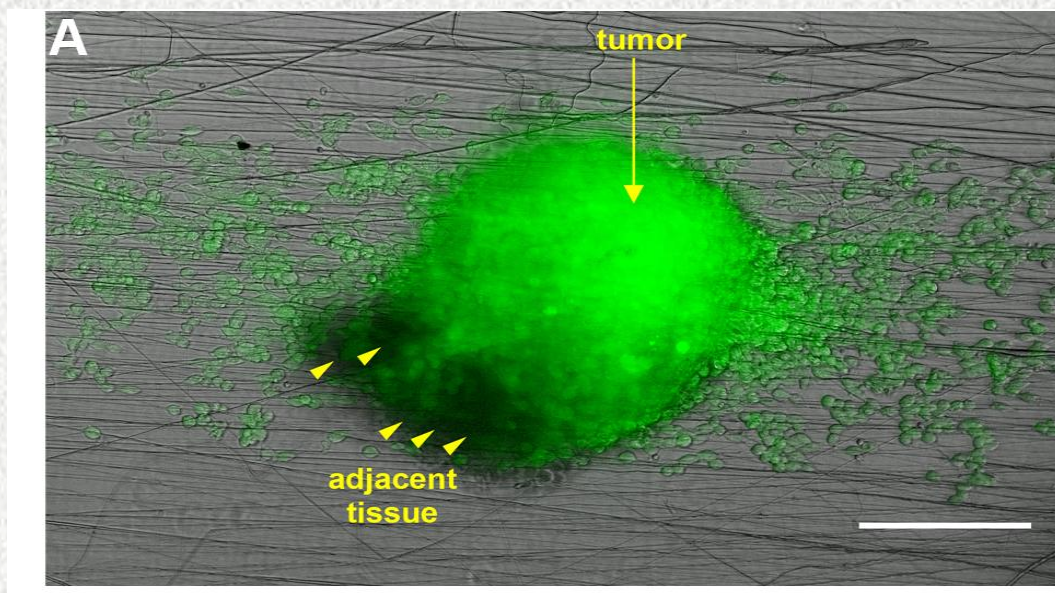


Figure 12. Cell dispersion from a human brain cancer tumor explant.

Nanofiber Solutions product #9602 has been used to promote cell dispersion out of human-derived primary tumors (Figure 12). Cancer cell dispersion in the brain occurs along preferential patterns, in many cases following the orientation of thin, elongated anatomical structures such as

Brain Cancer Drug Sensitivity (cont'd)

white matter fibers (Figure 13). Nanofiber Solutions products mimic these structures to promote identical behavior *in vitro*. Standard assays devised to study glioma cell motility do not incorporate such topographical cues known to guide cell adhesion and traction *in vivo*, often focusing instead on cell motility on rigid (i.e., TCPS or glass) surfaces.

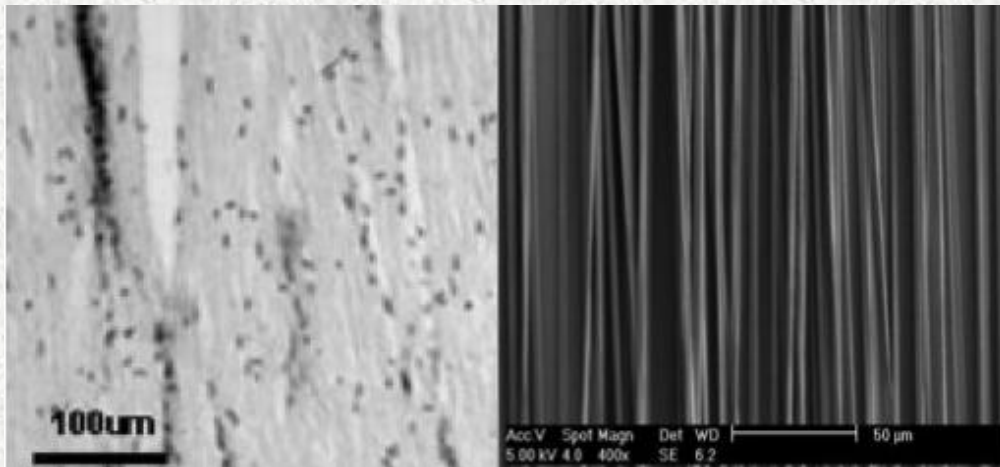


Figure 13. Aligned white matter tracts in the brain (left) compared to aligned Nanofiber Solutions nanofiber (right).

Nanofiber Solutions provides a range of 3D multiwell plates for drug discovery. Both primary tumors and tumor-derived cell lines can be studied on these 3D culture environments. Fiber density, alignment, and stiffness can be controlled in these scaffolds, thus providing the cells with a topographically-complex substrate. Glioma cells growing on aligned nanofibers reproduced the morphologies observed for these cells migrating through neural tissue.

Here, Dr. Mariano Viapiano's lab (Agudelo-Garcia et al) shows that migration of glioma cells on nanofiber scaffolds reproduces not only the morphology, but also characteristic molecular features of 3D migration to result in a pattern of gene expression dependent on fiber alignment.

Brain Cancer Drug Sensitivity (cont'd)

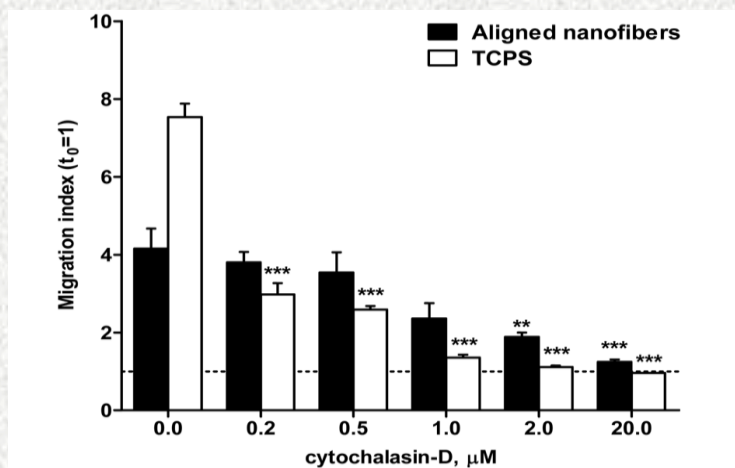


Figure 14. Simple 2D cell dispersion on TCPS is significantly reduced even at the lowest concentration of cytochalasin D (0.2 μM). Dispersion on Nanofiber Solutions part#0602 required 10 to 100 times higher concentrations to be significantly inhibited.

Cell migration on nanofibers is less sensitive to stress fiber disruption than it is on TCPS. Quantification of radial dispersion (Figure 14) of U251 cells shows that 2D cell dispersion on TCPS was significantly reduced even at the lowest concentration of cytochalasin D tested (0.2 μM). Dispersion on Nanofiber Solutions aligned nanofiber part#0602 required 10 to 100 times higher concentrations to be inhibited.

Active cell migration on Nanofiber Solutions products correlates with activation of the transcription factor STAT3, a central regulator of tumor progression and metastasis in solid cancers. Inhibition of STAT3 specifically reduced glioma cell migration on nanofibers (Figure 15), but not on TCPS, suggesting that Nanofiber Solutions' technology can be used for screening of anti-migratory compounds. Dissociated U251 cells (5×10^5 cells/ml) were plated on aligned (A; part#2402) or randomly oriented (R; part#2401) nanofibers and collected 6 or 24 hours after attachment. Cells recovered from aligned nanofibers showed a substantial increase in Y^{705} -phosphorylated STAT3 ($p\text{-STAT3}$) compared to cells recovered from randomly-oriented nanofibers. When U251 cells were plated on conventional TCPS they showed high levels of expression of total and active STAT3 at all times tested, revealing the unsuitability of TCPS for mechanistic studies of cell migration.

Brain Cancer Drug Sensitivity (cont'd)

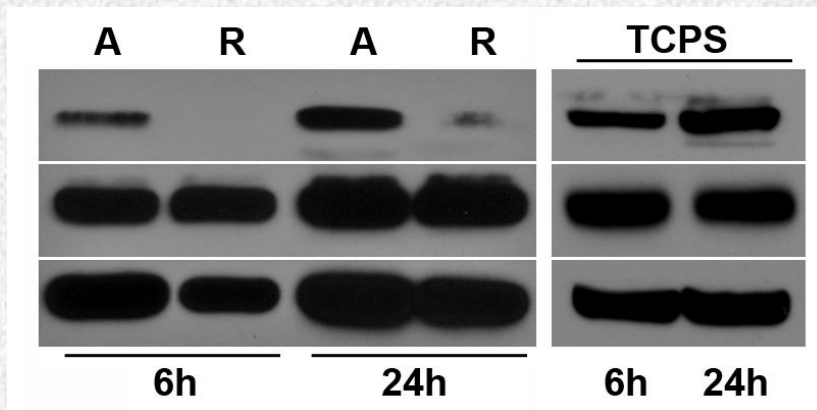


Figure 15. U251 cells plated on TCPS show high levels of expression of total and active STAT3 at all times tested. On Nanofiber Solutions products, however, inhibition of STAT3 reduced glioma cell migration.

In addition, STAT3 inhibition reduced MLC2 phosphorylation of cells cultured on aligned nanofibers, but not on TCPS (Figure 16). U251 glioma cells were cultured on aligned nanofibers or TCPS for 24 hours in the presence of 1 μ M static, collected, and processed for Western blot analysis. Results showed that a low concentration of the STAT3 inhibitor partially reduced STAT3 phosphorylation in cells of myosin II, MLC2. In contrast, neither STAT3 nor MLC2 phosphorylation was affected by the same treatment when cells were cultured on TCPS.

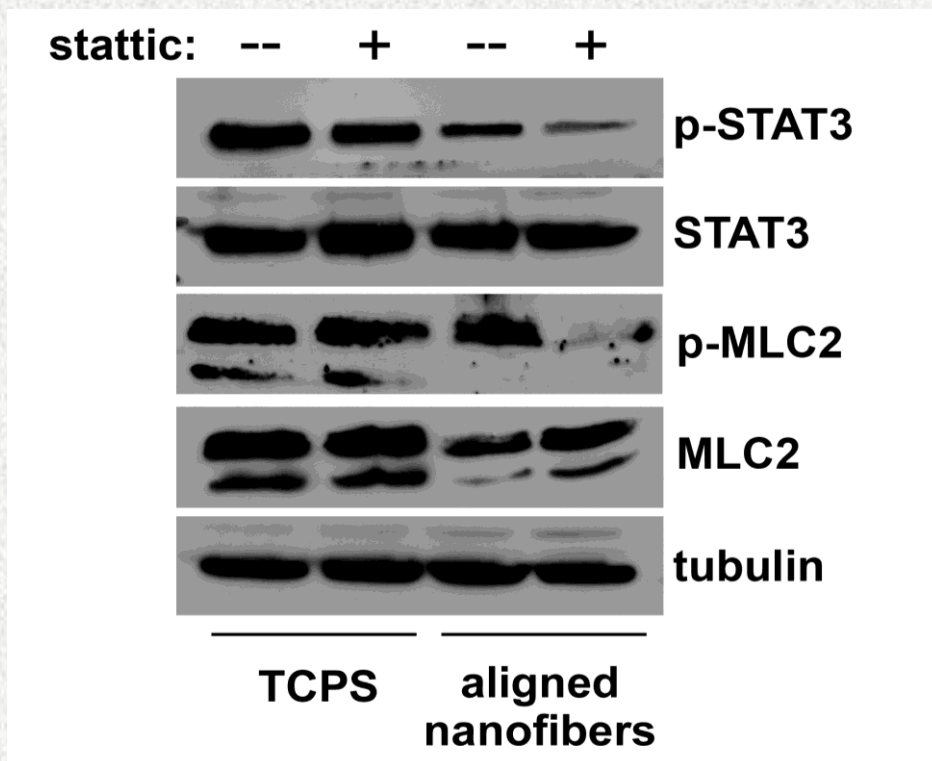
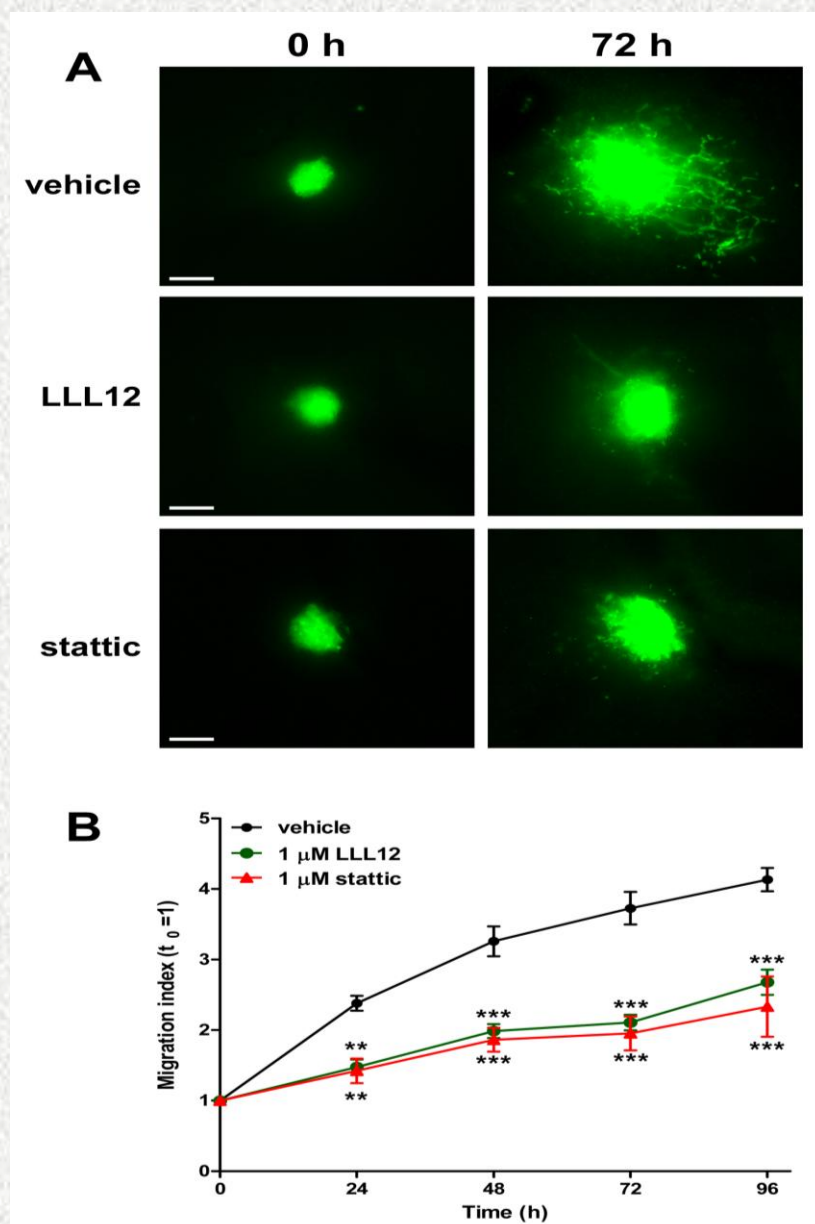


Figure 16. STAT3 inhibition reduces MLC2 phosphorylation of cells cultured on aligned Nanofiber Solutions products, but not on TCPS.

Brain Cancer Drug Sensitivity (cont'd)

Nanofiber Solutions products also more closely reproduce cell behavior on cultured brain slices (the current gold standard for cell migration) than TCPS. G9 glioma cells were treated with 1 μ M static of 1 μ M LLL12 overnight and deposited on brain slices. Dispersion of the cells on the tissue slice was followed by fluorescence microscopy for 96 hours (Figure 17). Cell migration followed a pattern of dispersion with typical trails of cells dispersing out of tumor spheres, which was abolished by the pharmacological treatments.



Quantitative results indicated that cell dispersion had been significantly reduced by treatment with low concentrations of the STAT3 inhibitors, in agreement with the results observed using nanofiber

Figure 17. As previously observed on nanofiber scaffolds, cell dispersion on brain slices is significantly reduced by treatment with low concentrations of STAT3 inhibitors. Migration on TCPS does not exhibit this behavior.

Tissue Engineered Nanofiber Solutions Scaffolds + Stem Cells = Trachea Replacements



Figure 18. Artificial Nanofiber Solutions trachea successfully implanted into Mr. Chris Lyles.

A Baltimore man became only the second patient to receive a completely synthetic trachea, fabricated by Nanofiber Solutions, to replace once ravaged by cancer.

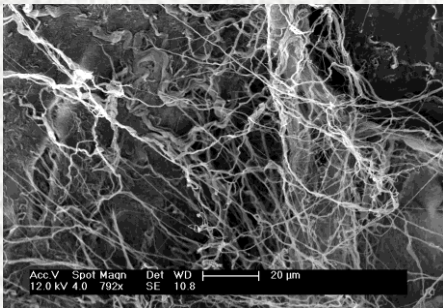
Swedish surgeons, Dr. Paolo Macchiarini, director of the Advanced Center for Translational Regenerative Medicine at the Karolinska Institute (Stockholm, Sweden), performed the transplant after removing Christopher Lyles' diseased trachea. Macchiarini then utilized a Nanofiber Solutions scaffold made from polyethylene terephthalate nanofibers that was modeled from CT scans. This scaffold was slowly saturated in bioreactor in a solution of stem cells derived from Mr. Lyles' own bone marrow. The stem cells, which can become the proper blend of cells needed to replace tracheal tissue when introduced back into the patient, populated the nanofiber scaffold. Because the cells growing the new trachea were Mr. Lyles' own, there was no risk of implant rejections as with cadaver organs.

Read more: <http://healthland.time.com/2012/01/13/cancer-patient-receives-a-man-made-windpipe/#ixzz1joKsysoN>

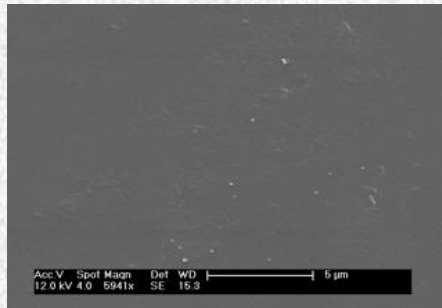
How Nanofiber Compares to Native Tissue

The tremendous potential of nanofiber substrates for cell culture can be further demonstrated by comparing SEM images of native human tissue and Nanofiber Solutions products. As can be seen in the pictures below, Nanofiber Solutions products mimic the natural environment found within a living organism to a much greater degree than does the widely-used TCPS.

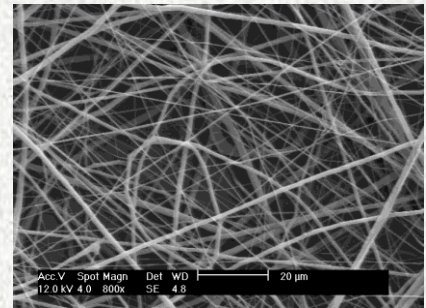
Nanofiber Solutions Products and Lung Tissue



Native Tissue

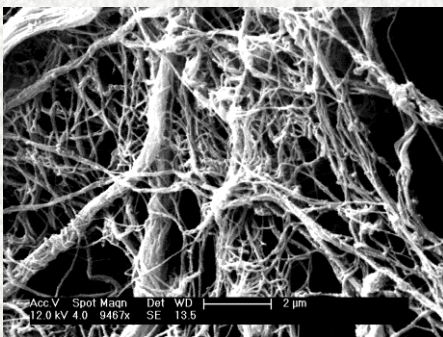


Tissue Culture Polystyrene

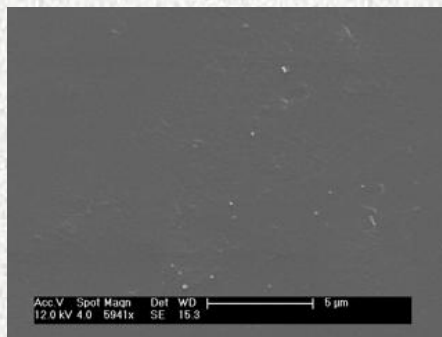


Nanofiber Solutions Product

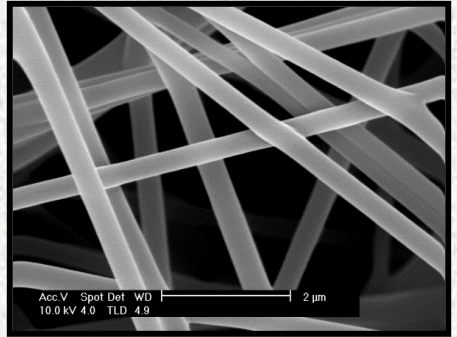
Nanofiber Solutions Products and Blood Vessel Tissue



Native Tissue



Tissue Culture Polystyrene

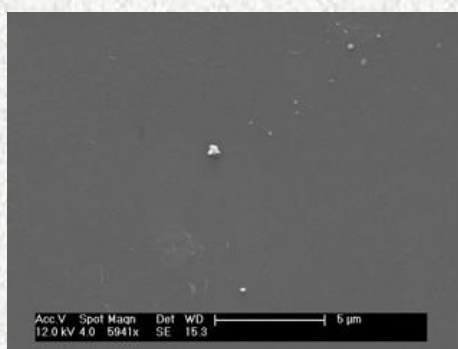


Nanofiber Solutions Product

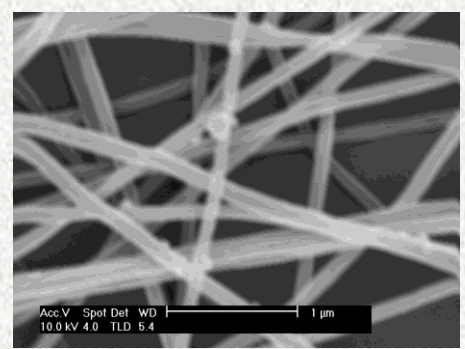
Nanofiber Solutions Products and Mesenchymal Tracheal Tissue



Native Tissue



Tissue Culture Polystyrene



Nanofiber Solutions Product

Product Part Numbers

<i>Plate Type</i>	<i>6 Well</i>	<i>24 Well</i>	<i>96 Well</i>	<i>384 Well</i>
Aligned PCL Nanofibers	0602	2402	9602	38402
Randomly Oriented PCL Nanofibers	0601	2401	9601	38401

<i>Insert Type</i>	<i>6 Well (6 pack)</i>	<i>12 Well (12 pack)</i>	<i>24 Well (12 pack)</i>
Aligned PCL Nanofibers	060602	121202	122402
Randomly Oriented PCL Nanofibers	060601	121201	122401

<i>Other Products</i>	<i>60mm Dish</i>	<i>100mm Dish</i>	<i>8 Chambered Slide</i>	<i>6 Well IMEMS</i>
Aligned PCL Nanofibers	6002	10002	0802	IMEM02
Randomly Oriented PCL Nanofibers	6001	10001	0801	IMEM01

Custom scaffolds – tubes, shaped sheet structures, etc. – available upon request.

Protocols

Cell seeding and use of Nanofiber Solutions multi-well plates*

Our culture plates are sterilized after packaging and may be used as direct replacements for tissue culture polystyrene in any traditional cell culture application. Nanofiber physically mimics the extracellular matrix found *in vivo* to provide a more realistic substrate to either culture or expand cells or to observe aspects of cell motility. Our nanofibers are fully synthetic and are plasma treated to acquire whatever cell media components you normally employ. Before adding your cells we suggest rinsing once followed by a pre-incubation in the media + biological components of interest for at least 30 minutes and up to 24 hours at 37°C, aspirating off the media and finally adding your cells and media. If desired, specific protein coatings or gels (i.e., collagen or Matrigel) may be added to the as-received nanofibers using the same protocols as traditional culture dishes. Initial cell densities of 10^4 - 10^5 cells/cm² (the cell density can be more or less based on your cell type and experimental needs) are suggested. While this number of cells may be higher than your normal usage, due to the higher surface area of our nanofibers we have found them necessary. During longer-term experiments, exchange the media at normal rates.

Following proliferation/migration, cells+scaffold may be fixed for post-processing and/or stained for immunocytochemistry utilizing normal protocols. Bear in mind that these nanofibers are composed of biodegradable polycaprolactone which dissolves in organic solvents such as acetone or toluene. Fixing in 4% paraformaldehyde for 10-20 minutes, followed by a PBS rinse and permeabilization with cooled methanol for 5-10 minutes at -20 °C has worked well. Adherent cells can be trypsinized or lysed for gene or protein expression, microRNA analysis, etc. If you have difficulty removing your cells from the nanofibers, try using a higher trypsin concentration. Many Nanofiber Solutions products are transparent to light to allow cells to be directly imaged using phase contrast or fluorescence microscopy by staining the cells with normal protocols.

*Suggested procedure, please adjust according to your experimental needs. For more protocols, please visit our website www.nanofibersolutions.com.

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