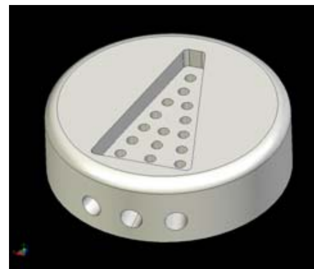
Tech Report 104:

Trapezoidal Trough Loader™

A Device for Fabricating 3-Dimensional Bioartificial Tissue Constructs



Authors: Ruwan Sumanasinghe, Ph.D., Albert J. Banes, Ph.D., Jie Qi, Ph.D.

Document: Trapezoidal Trough Loader Tech Report, Rev 1.3

08-18-2009

Culturing Cells in a Mechanically Active Environment™

Flexcell International Corporation • 437 Dimmocks Mill Road, Suite 28 • Hillsborough, NC 27278
800-728-3714 • (919) 732-1591 • FAX: (919) 732-5196 • www.flexcellint.com

COPYRIGHT © 2009 FLEXCELL® INTERNATIONAL CORPORATION

APPLICATION

The Trapezoidal Trough Loader™ is designed to create cell-seeded 3-dimensional (3D) bioartificial tissues (BATS) that simulate the shape of an Achilles tendon. The Trough Loader™ is used in conjunction with the Tissue Train® culture system to create trapezoidal shaped tissue constructs.

The Tissue Train® culture system utilizes a novel method to culture and mechanically load cells in a 3D matrix gel or cell-assembled matrix. The system consists of a flexible-bottomed Tissue Train® culture plate with mesh-like anchors at the culture well periphery for cell and matrix attachment. The anchor widths are designed to match the width of trapezoidal gels at each end (Fig. 1). A cell-populated matrix gel may be cast by placing the Tissue Train® culture plate on a gasketed baseplate and atop a planar-faced cylindrical post bearing a central trough centered beneath each well (Fig. 1-2). When a vacuum is applied, the flexible membrane is pulled downward into the trough deforming the membrane into the conformation of the trough and, thus providing a space for delivery of cells and gel (Fig. 3). Simultaneously, a matrix-bonded nylon mesh anchor stem is canted into each end of the trough as polar attachment points for the gel constructs (Fig. 3). The baseplate and culture plates are then transferred to a 37 °C, CO_2 incubator where the construct is held in position in the trough until gelation occurs. After gelation, the vacuum is released, and the cell-gel construct rises into the plane of the culture well. Growth media (3 ml) are added to each well, and the constructs cultured according to the experimental protocol.

Mechanical load may be applied to the cell-seeded constructs using a Flexcell® FX-5000™ Tension System (Fig. 4). Cell-gel constructs or cell-assembled matrices may be mechanically loaded uniaxially or equibiaxially using different shaped Loading Stations™ (Arctangle™ or cylindrical, respectively). A program defining the % elongation, frequency and duration of mechanical load can be regulated by the user in the FX-5000™ FlexSoft® software.

Figure 1. Tissue Train® culture plate for 3D culture of cell-matrix constructs. The top left well has a Trapezoidal Trough Loader™ beneath the flexible membrane. The 4 adjacent wells show the anchors for attachment of cells and gel for a uniaxial construct. The bottom left well shows an Arctangle™ loading post to deliver uniaxial load.

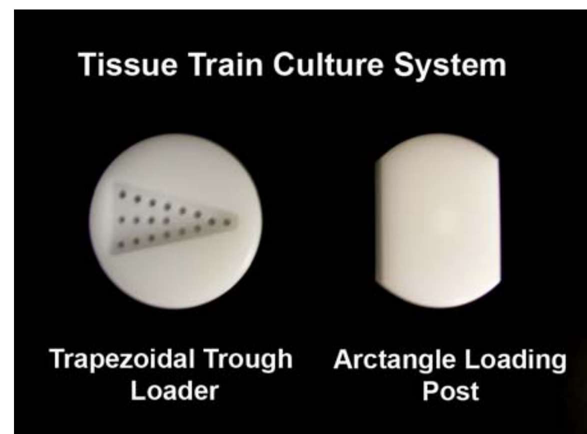


Figure 2. The Trapezoidal Trough Loader™ is used to fabricate 3D cell-gel matrices while the Arctangle™ loading post is used to apply uniaxial load to the 3D cell-seeded gel.

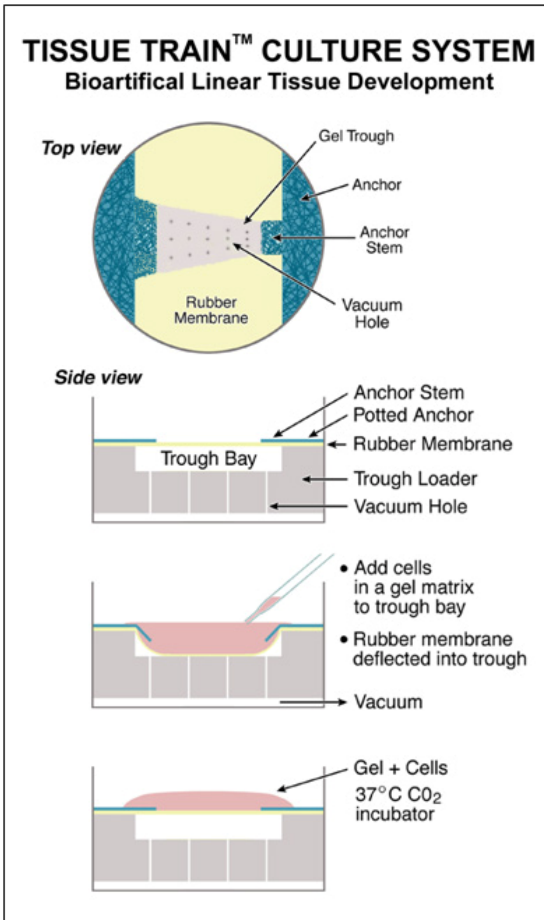


Figure 3. A cell-gel construct in a Tissue Train® well attached to the anchor stem (top view). When vacuum is applied, the rubber membrane is deformed downward conforming to the trough and creating a space for the gel solution (side view).

Figure 4. The Flexcell® FX-5000™ Tension System is used to apply regulated strain to the 3D cell-seeded gel constructs.

BACKGROUND

Formation of tissues *in vitro* that are structurally and functionally viable requires several basic conditions, such as 1) cells 2) matrix 3) media and growth factors and 4) mechanical stimulation. These conditions are linked to each other and act in conjunction to form a structurally robust tissue that can withstand biomechanical forces. As a tissue is developed, its cells create an extracellular matrix of which the composition is based on the environmental stimuli to which the cells are exposed. Several signal pathways may be involved in generating the composition of the extracellular matrix. Some of these pathways are regulated by mechanical deformation of cell matrix and transmitted into the cell via membrane bound proteins such as integrins, focal adhesion complexes (mechanosensory complex), cell adhesion molecules and ion channels. Cells can also respond to ligands, such as cytokines, hormones or growth factors that are released as a result of matrix deformation (Banes et al., 1995, 2002). In order to maintain the integrity and strength of musculoskeletal tissues, the cells may require maintaining a certain level of intrinsic strain. In the absence of this intrinsic strain, the tissue will lose its strength leading to failures or fractures. Culturing cells in a mechanically active environment increases cell metabolism and alters cell shape and other properties. Therefore, it is vital to create and maintain a mechanically active environment (i.e., tension, shear stress or compression) for the cells during the formation of tissues *in vitro*.

To generate a tissue *in vitro* that is more or less equal to the native tissues, it is utmost important to create an environment that would mimic the *in vivo* conditions. This includes the type of environment (2D or 3D)

and size and shape of the tissue matrix. A three dimensional matrix would assist in resembling the *in vivo* organization of cells while size and shape of the tissue matrix would directly affect the type, magnitude, direction and distribution of physiological forces within the tissue matrix. The composition of tissue may also depend on the types of forces that the tissue undergoes. Depending on the anatomical location some tissues may experience both tension and compression forces in different areas within the tissue leading to multiple compositions. For an example, the midsubstance (where tensile forces exist) of an Achilles tendon is comprised of dense fibrous connective tissue while the area where tendon presses against calcaneus (where compression forces exist) is comprised of fibrocartilaginous tissue. The shape of the tissue also plays a major role in the location of its failure. Most failures in Achilles tendons occur at the calcaneal junction where it joins the bone and have the least thickness. Therefore, it is clear that the native shape of the tissue needs to be simulated *in vitro* to facilitate studying the failure mechanism as well as the healing mechanism of tissues. The Trapezoidal Trough Loader™ was developed to address these needs in tissue engineering. When used in conjunction with the existing Tissue Train® Culture System, the investigator will be able to fabricate a trapezoidal shaped, 3D, cell-seeded tissue construct that connects to nylon anchors at both ends. The investigator can also apply regulated strain to the growing tissue using a regimen with set values for strain frequency, elongation and duration based on the physiological condition that needs to be simulated. The strain regimen can be controlled by a Flexcell® FX-5000™ Tension System (Fig. 4) that can apply static or cyclic equibiaxial or uniaxial strain.

MATERIALS AND METHODS

The following is a method by which to culture cells in a 3D matrix and to apply strain to cells in a 3D gel material.

A. Preparation of Cells in 3D Trapezoidal Gels in a Tissue Train® Culture Plate.

1. Prepare cells according to the laboratories established protocol for primary cultures or continuous cell lines in the medium of choice.

2. Release cells from their substrates with 0.05% trypsin, trypsin-EDTA, 0.05% bacterial collagenase or other means. Add serum containing media to the cells to neutralize the trypsin or collagenase.

3. Count cells and determine the number of cells needed. For a trapezoidal gel, approximately 700,000 cells in 700 µl will be required for each well of a 6-well Tissue Train® culture plate.

4. Wash cells 2 times with medium to remove trypsin or collagenase.

5. Combine cells with a matrix protein collagen gel solution at a cell concentration of 1000 cells/ µl of gel solution.

NOTE: *Before mixing with cells, the matrix protein gel solution should be neutralized to a pH 7.0 using 1M sodium hydroxide.*

Cells may be reconstituted in one volume of media containing 10% fetal calf serum, 70% matrix protein gel fluid and 20% 5X MEM. The objective is to achieve an overall gel MEM concentration of 1X. The suggested formula for the cell/matrix protein gel fluid combination is as follows:

- 70% by volume matrix protein gel fluid

- 20% by volume of 5X MEM to yield an overall 1X concentration by total volume
- 10% fetal calf serum
- Cells to provide an overall 1000 cells/ µl concentration by total volume.

6. The Tissue Train® culture plate with trapezoidal anchor configuration can be used for creating the gel constructs. Place a Trapezoidal Trough Loader™ in a Loading Station™ beneath the flexible membrane of a Tissue Train® culture plate so that the anchor stems are aligned with the long axis of the Trough Loader™. Apply a thin layer of lubricant to the top surface of Trough Loader™ prior to placement of the Tissue Train® culture plate. The lubricant will facilitate uniform and unrestricted conformation of the membrane in the trough.

7. Place the Tissue Train® culture plate on a BioFlex® baseplate with gaskets and connected to a Flexcell® Tension System or other regulated vacuum source.

NOTE: *FX-2000™ users should connect the baseplate directly to a regulated vacuum source to achieve the suggested vacuum level of -84 kPa. The FX-2000™ will deliver a maximum of only -40 kPa.*

Vacuum should be applied to the baseplate in a steady "hold" mode so that the flexible membrane is deformed and held in the space in the Trough Loader™. To supply the proper vacuum level with the FX-5000™ system, it is recommended that a maximum of 20% elongation be used with the Tissue Train® Loading Station™ (24 mm) platform setting. This is the equivalent of -90 kPa. Be sure that you allow enough vacuum tubing for your baseplate to reach from your incubator to your tissue culture hood.

NOTE: *When using the Flexcell® Tension System use the minimal amount of tubing required, as longer tubing decreases cyclic strain performance.*

8. Pipette the cell and matrix protein gel solution into the "trough" in each Tissue Train® well. First pipette a small drop of gel at each end of the trough, under the anchor stems. Then press the anchor stems into the trough and pipette over top of them. Finally, fill the middle of the trough with gel, moving the pipette back and forth to create a uniform strip of gel in the well.

9. Place the baseplate with culture plates in a 37 °C incubator and allow the solution to gel. After gel setting, release the vacuum and add 3 ml of serum-containing media to each well. The gels should appear as a trapezoidal band of gel attached at each anchor end in the Tissue Train® well (Fig. 5). Remove culture plates from the BioFlex® baseplate, if needed.

10. Culture trapezoidal constructs according to the laboratories established protocol. Cultures may be observed or assayed for various functions. The user can monitor cell shape, organization, migration, division, gene expression, protein expression /secretion, mediator secretion DNA and protein synthesis in the gels. Cultures may also be mechanically loaded (see part B below) at any time after gelation occurs in the matrix or after cells have self-assembled their own matrix. However, the elongation, frequency and duration of the applied strain to the cell must be determined by the investigator for his/her specific use.

Typically, spherical-shaped cells can be observed in the gel using an inverted phase contrast microscope directly after plating. By day one, the cells will begin to attach to and spread into the matrix. Cells will then form attachments to each other and

intercommunicate. By days 3-5, cells will reorganize and contract the matrix into a band (Fig. 5).

Figure 5. Trapezoidal shaped 3D linear tissue constructs in a Tissue Train® culture plate.

B. Application of Regulated Strain to Cells in a 3D Gel using the Tissue Train® Culture System

1. Cell-matrix constructs can be mechanically loaded in the 3D matrix by using a Flexcell® FX-5000™ Tension System to apply a regimen of controlled elongation (strain), frequency or duration with added rest periods.

2. The regimen parameters must be tested beforehand to determine if the cell-constructs can withstand the applied strain regimen. Usually, the cell matrix constructs can be stretched at 1-3% elongation for several minutes to several hours per day without matrix failure.

3. When using the Tissue Train® plates, regimens can be programmed with strains ranging from 0-20% and frequencies from 0.01-5.0 Hz. When downloading regimens, choose the Tissue Train Loading Station (24mm) platform setting.

NOTE: *Higher frequencies will limit the system's ability to maintain higher strain ranges.*

4. When using the Tissue Train® with the FX-2000™ system, use the provided vacuum vs. strain data for Tissue Train® plates.

NOTE: *The FX-2000™ will not allow vacuum levels above -40 kPa and, therefore, can apply a maximum of 6% strain to the Tissue Train® construct.*

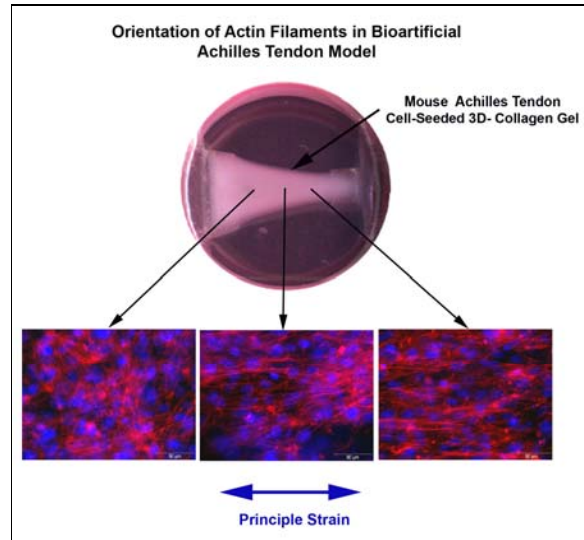


Figure 6. Orientation of actin filaments at multiple locations in a mouse Achilles tendon cell-seeded bioartificial tendon model (strained at 1% and 1 Hz). Actin filaments (red), cell nuclei (blue).

REFERENCES FOR TISSUE TRAIN®

Sumanasinghe, R.D., Qi, J., Wang, J., Tsuzaki, M., Wall, M., Pfeiler, W., Loboa, E., Hart, D.A., Banes, A.J., Cell Orientation and local strain distribution in an in vitro Achilles tendon tissue engineering model. Podium presentation. International symposium on ligaments and tendons-VIII, Annual conf., March 1, 2008, Stanford, CA.

Garvin, J., Qi, J., Maloney, M., and Banes, A.J., A Novel System for Engineering Bioartifical Tendons and Application of Mechanical Load. Tissue Engineering Journal, Oct. 2003, Vol. 9, No. 5, 967-979.

Triantafillopoulos, I.K., Karas, S.G., Bowman, Jr. K.F., Yang, X., Evans, G.A. and Banes, A.J. Anabolic Steroid and Load Enhance Matrix Remodeling and Biomechanical Strength of Bio-artificial Tendons. Abstract 49th Annual Meeting, ORS, Feb 2-5, 2003, New Orleans, LA.

Banes, A.J., Qi, J., Maloney, M., Almekinders, L., and Bynum, D. Bioartificial Tendons: A Model 3D System for Testing Tenocyte Responses to Drugs, Cytokines and Mechanical Load ex vivo. American Academy of Orthopaedic Surgeons, Accepted 12, 2002.

Garvin, J., Baldwin, N., and Banes, A.J., Novel Culture System for the Development of a Bioartifical Tendon. Abstract 48th Annual Meeting, ORS, Feb 10-13, 2002, Dallas, TX.

Guilak, F., Jones, W.R., Ting-Beall, H.P., and Lee, G.M. The Deformation Behavior and Mechanical Properties of Chondrocytes in Articular Cartilage. Spine 1999 Dec. 1, 24(23); 2475-2483.

Banes, A.J., Tsuzaki, M., Yamamoto, J., Fisher, T., Brigman, B., and Miller, L. Mechanoreception at the cellular level; The detection, interpretation, and the diversity of responses to mechanical signals. Biochem. & Cell Biology Vol.73; 349-365, 1995.

Banes, A.J. Mechanical strain and the Mammalian cell; Physical Forces and the Mammalian Cell. Academic Press, ed., John Frangos, pg 81-123, 1993.

FLEXCELL® PATENTS

Flexcell® International Corp.'s Tissue Train® Culture System is protected by one or more of the following United States or International Patents, with other patents pending:

US Patent	4,789.601
US Patent	4,822,741
US Patent	4,839,280
US Patent	5,122,470
US Patent	5,518,909
US Patent	5,593,891
US Patent	6,037,141
US Patent	6,048,723
US Patent	6,218,178
US Patent	6,472,202
US Patent	6,586,235
Japanese Patent	25-28174
German Patent	3855631.6
United Kingdom Patent	0,365,536
Canadian Patent	2,204,862