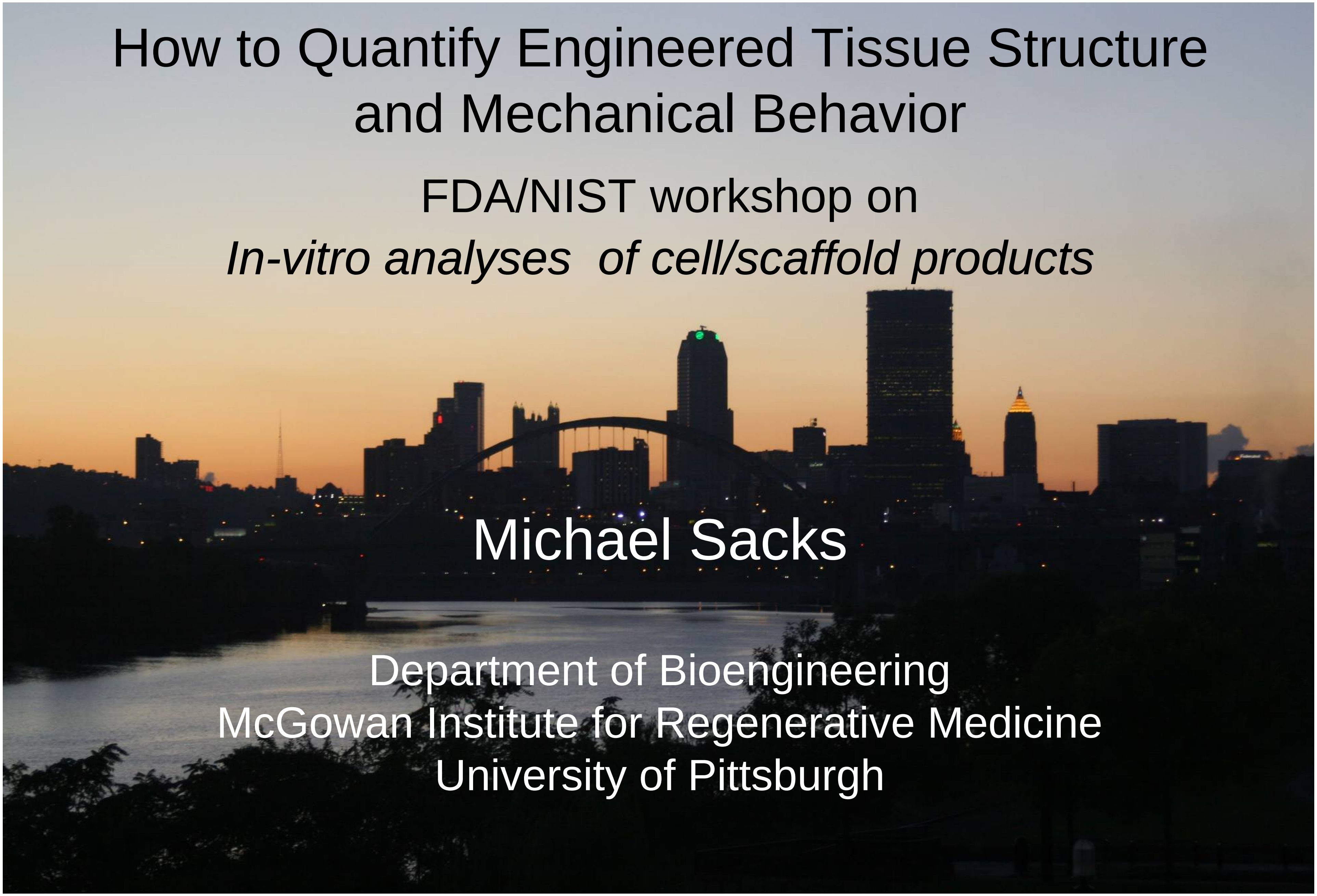


How to Quantify Engineered Tissue Structure and Mechanical Behavior

FDA/NIST workshop on

In-vitro analyses of cell/scaffold products

Michael Sacks

Department of Bioengineering
McGowan Institute for Regenerative Medicine
University of Pittsburgh

Tissue Engineering: Role of Biomechanics

- Many tissues and organs to be replaced have critical biomechanical functions
- “*Tissue Engineering*” was first coined by Y.C. Fung in 1987 for determining the biomechanical responses of cells and tissues in order to learn how to replace them

Contemporary issues in Biomechanics

Genomic structure
Genomic biomechanics
Genomic function
Molecular structure
Molecular biomechanics
Molecular function
Cell structure
Cell biomechanics
Cell behavior
Tissue structure
Tissue biomechanics
Tissue function

Organ structure
Organ biomechanics
Organ physiology
Human movement
Human performance

Biomechanics is the middle name between <u>structure</u> and <u>function</u>

Functional Tissue Engineering*

- What are thresholds of force, stress, and strain that the normal tissue must withstand during normal operation?
- What are their mechanical properties, during both normal and failure conditions?
- Which properties should be incorporated into TE designs?

*Butler et al., JBME, 2000

Functional Tissue Engineering

- When developing implants in culture, how to mechanical factors regulate cell behavior as compared to those experienced in-vivo?
- Do we have too exactly reproduce every feature of the native tissue to get acceptable levels of physiological function restoration?
- When evaluating TE repairs, how good is good enough?

Functional Tissue Engineering

1. In-vivo stress/strain histories need to be measured in normal tissues over the physiological range
2. Mechanical properties of the native tissues must be established for sub-failure and failure
3. A subset these mechanical properties must be selected and prioritized
4. Standards must be set when evaluating the repairs/replacements after surgery so as to determine “how good is good enough”

Primary considerations

In-vitro phase

- Enhancement of protein synthesis
- tissue formation and strength
- Strategic use of and mechanical/biochemical stimulation

Assessment of In-vivo function

- Invasive measures (explant)
- Non-invasive (primarily image based)

Major scaffold types

- Biologically derived
  - SIS, UBM
  - Decellurized tissues (e.g. aortic valve)
  - Collagen, fibrin, and GAG gels
  - Electrospun biopolymers (collagen)
- Synthetic
  - Wovens and fabrics
  - Gels and foams
  - Non-wovens made from PGA, PLLA
  - Electrospun biodegradable polymers

Mechanical Behavior

Driven by physiological functional requirements

a. Stress-strain response non-linearity, rapid transition of stiffness

b. Time-dependence
   i. viscoelasticity
   ii. poroelasticity

c. Anisotropy
   i. Appropriate knowledge of mechanical properties

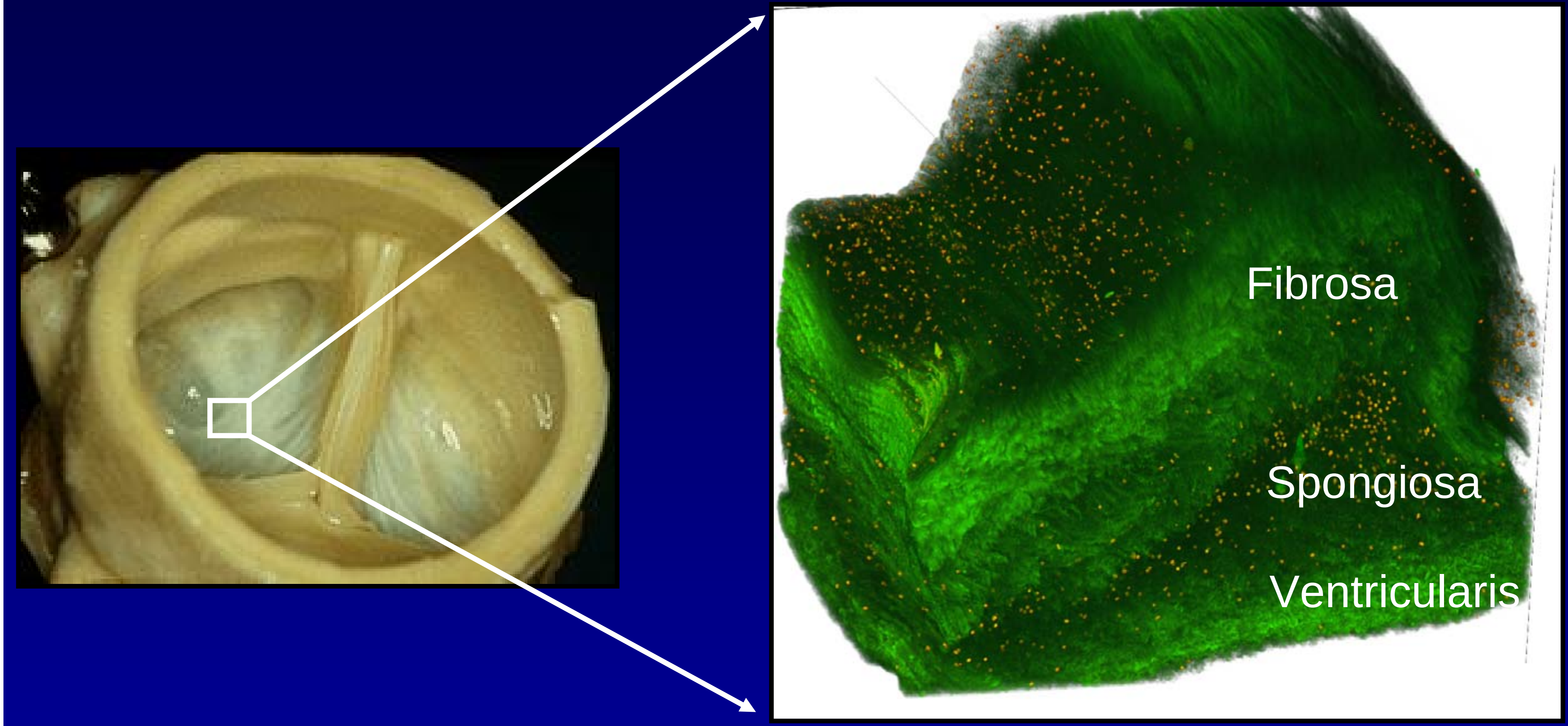
d. Dimensionality
   i. Uniaxial (tendon)
   ii. Planar biaxial (valve leaflet)
   iii. Full 3D (myocardium, cartilage) - No approach available

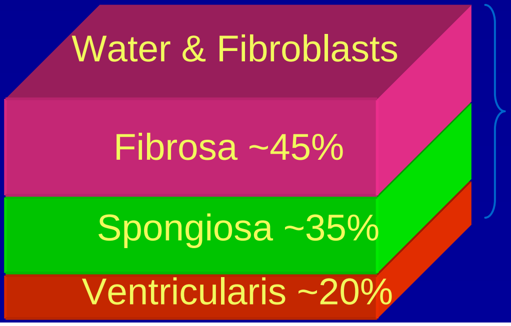
Mechanical Behavior

Driven by physiological functional requirements

1. Major modes
    a. Tension
    b. Compression
    c. Flexural
2. Meso/macro scale vs. local properties
    a. Local
        i. AFM, nano-indentation.
    b. Larger scales
        i. More relevant for physiological function
3. Need to link measures a various scales to make sense of cell and physiological behaviors

Leaflet tri-layered structure





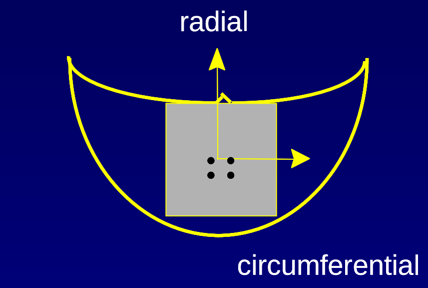
Water & Fibroblasts

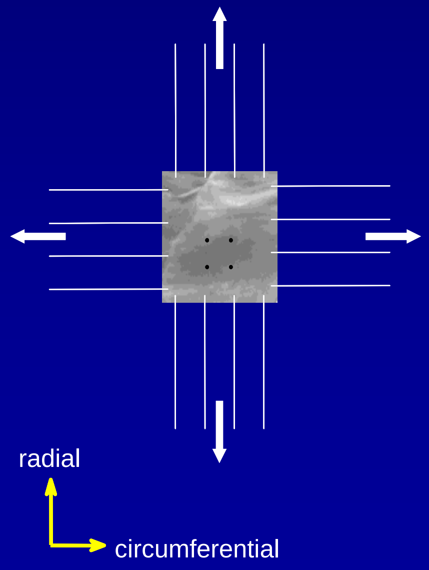
Collagen	Fibrosa ~45%
GAGs	Spongiosa ~35%
Collagen, Elastin	Ventricularis ~20%

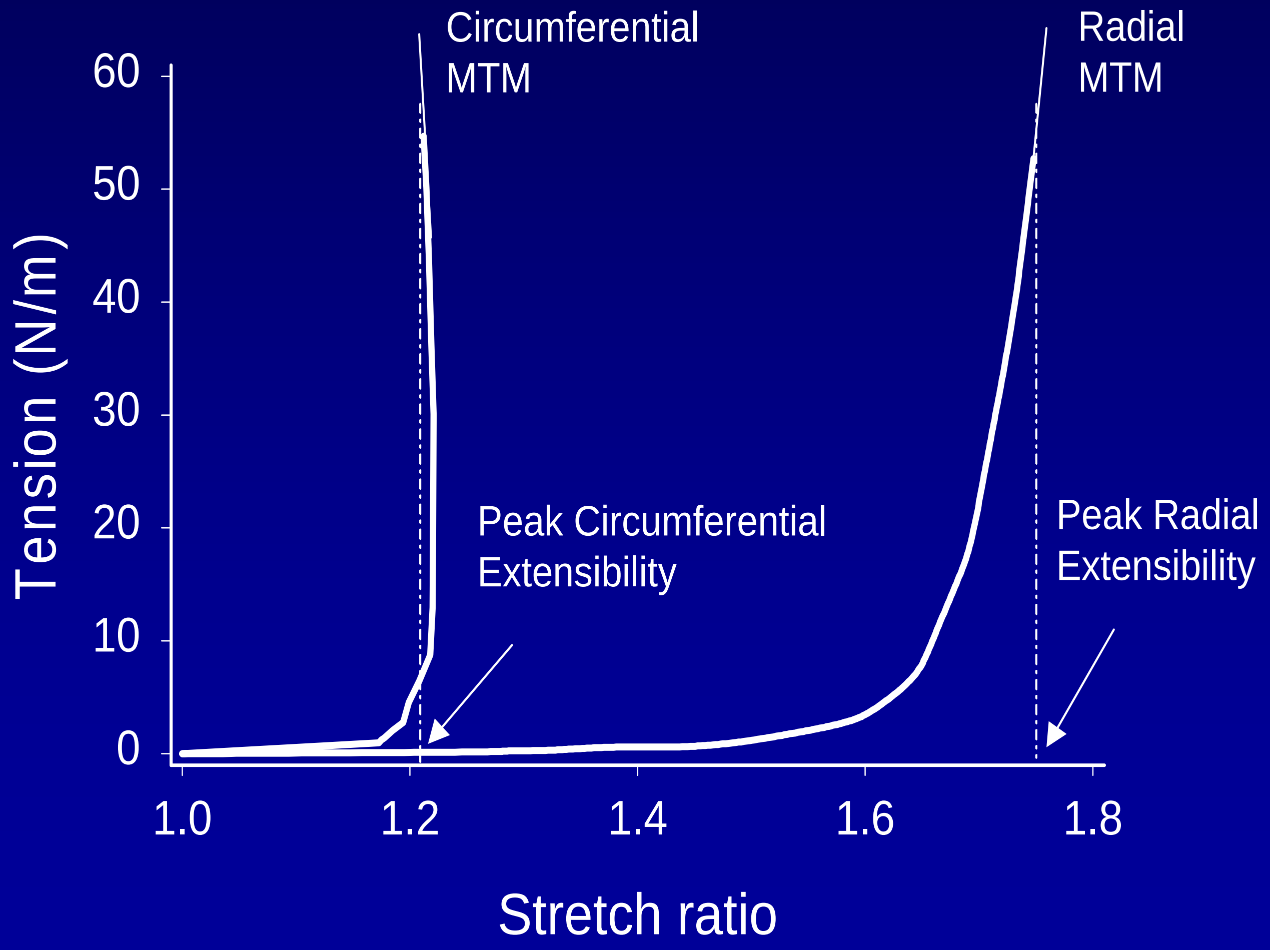
Total thickness ~300-700µm

Planar biaxial mechanical properties of the aortic valve leaflet

There is more to life than Young's modulus

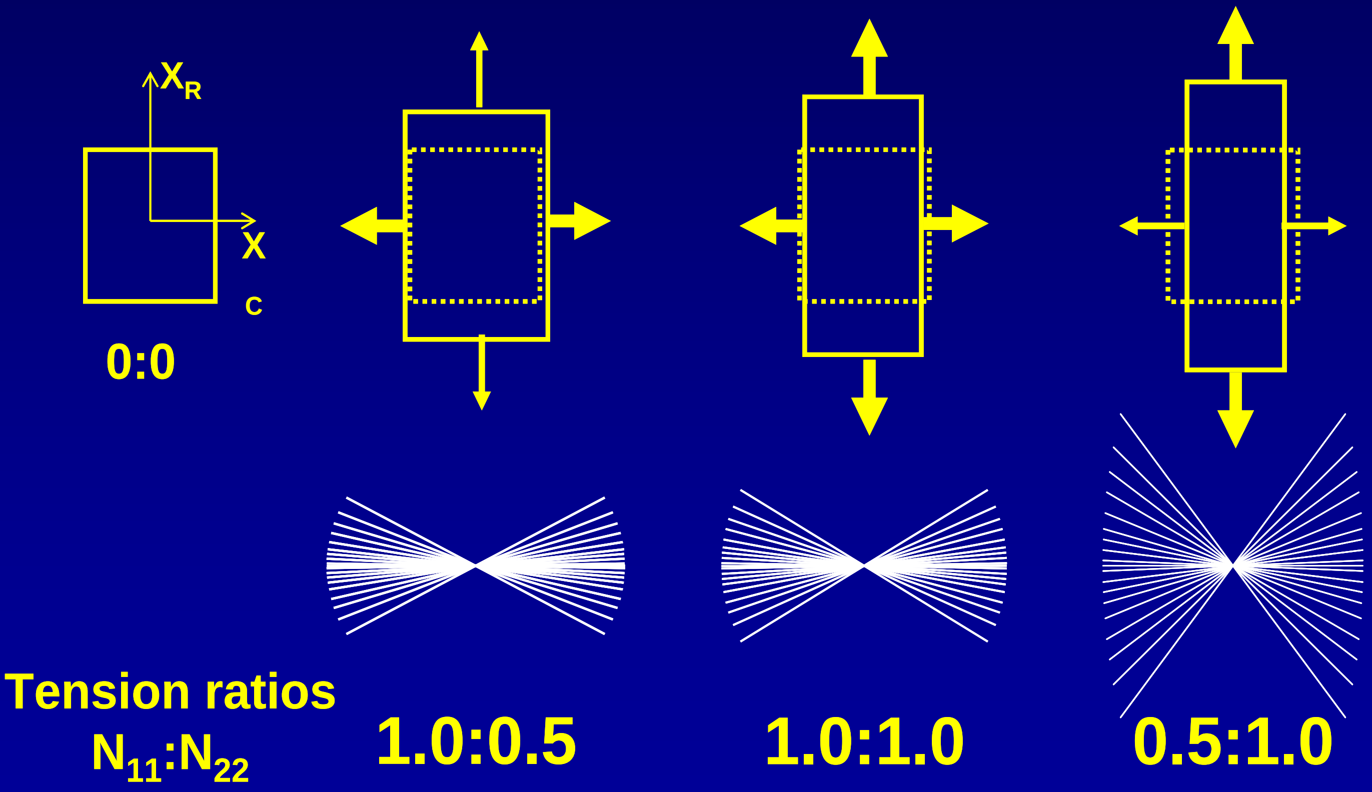






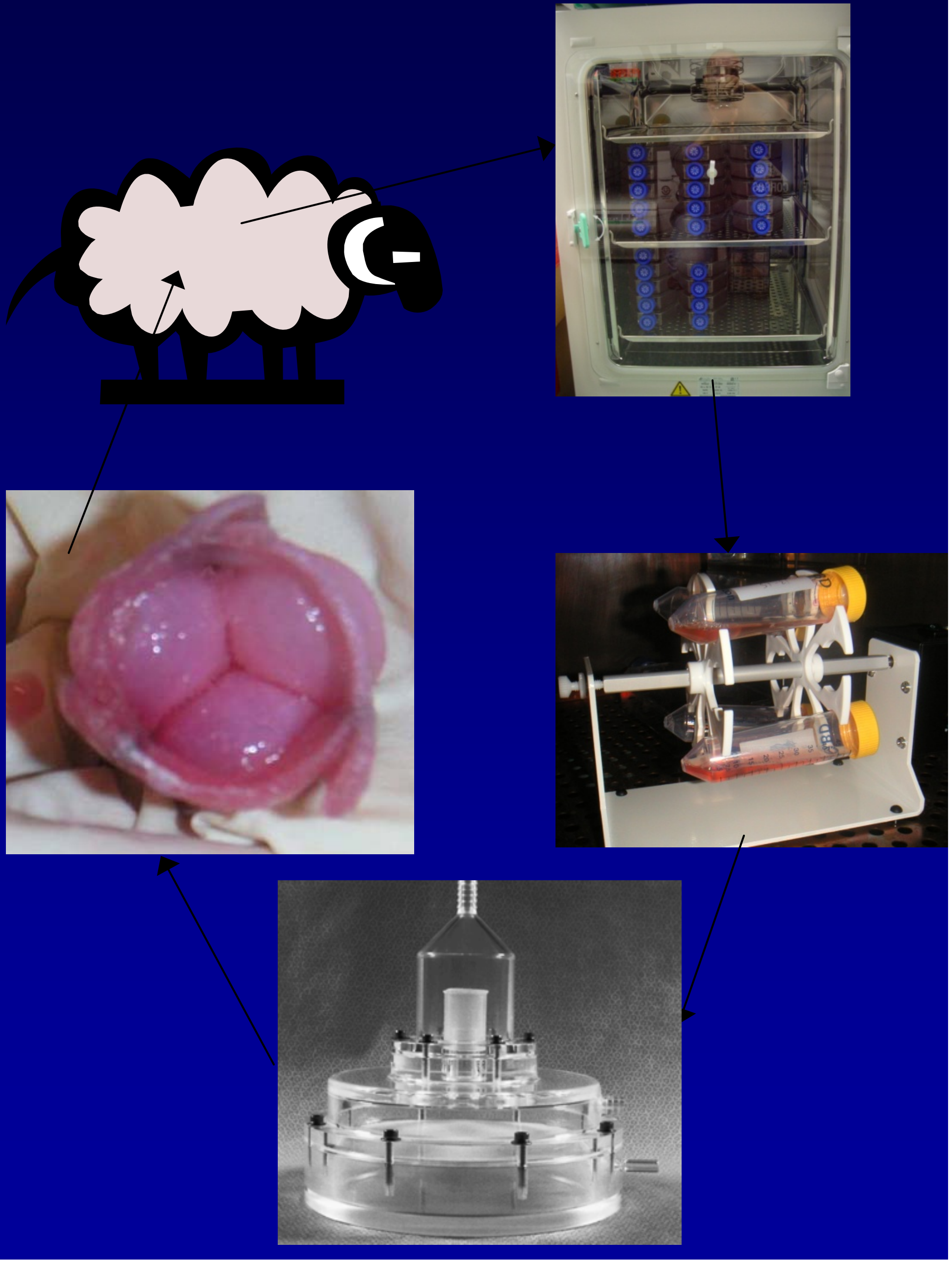
Structural basis for mechanical behavior

Anisotropy and due to fiber rotations, not stretch



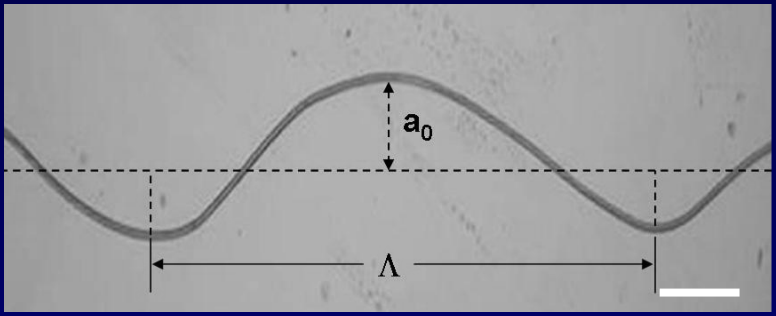
Tissue Engineered Heart Valves (TEHV)

- Living autologous cells + bioresorbable scaffolds
- Potentially have a capacity for growth, self-repair, & resistance to infection
- Presents opportunity to answer some fundamental bioengineering questions:
  - How do the scaffold and tissue interact to give rise to overall mechanical properties?
  - How do individual modes of mechanical loading affect tissue development?

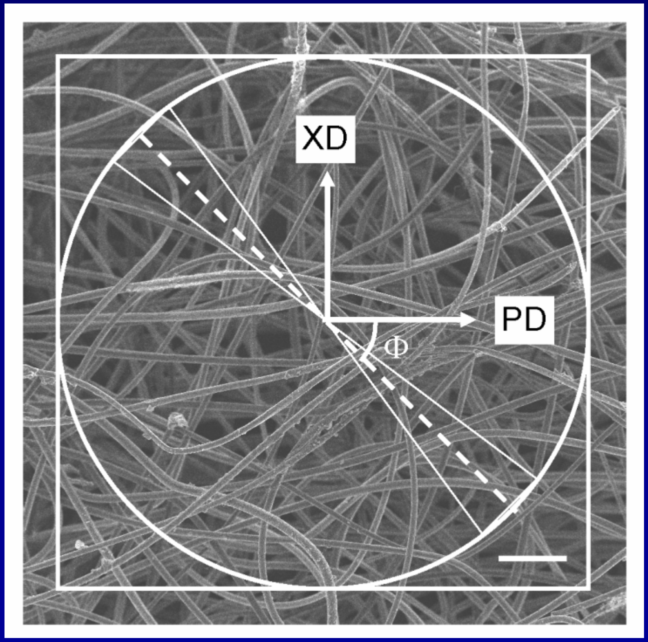


Hierarchal Structure of Nonwoven Scaffolds

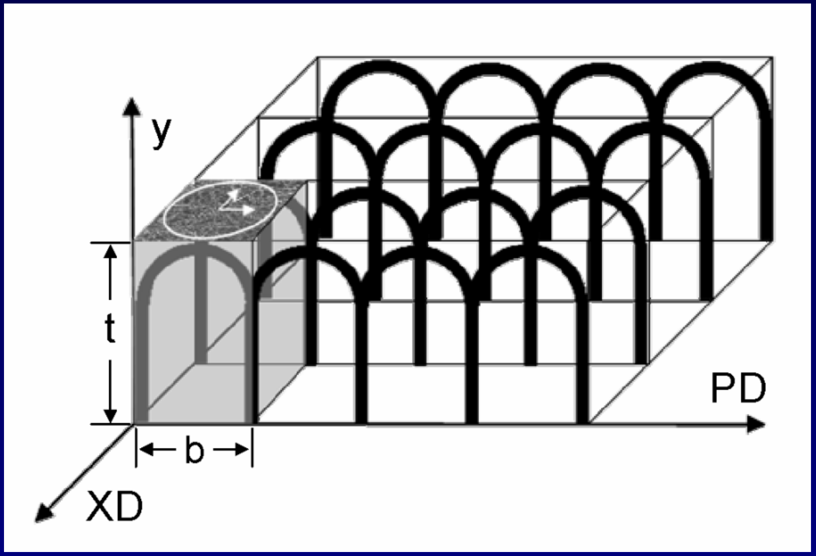
Primary: Fiber Crimp



Secondary: Fiber Orientation



Tertiary: Discretization by Needling



Freed L.E., et al, Bio/technology 1994;12:689-693.

Engelmayr, G.C. and Sacks, M.S., J Biomech Eng, 2006

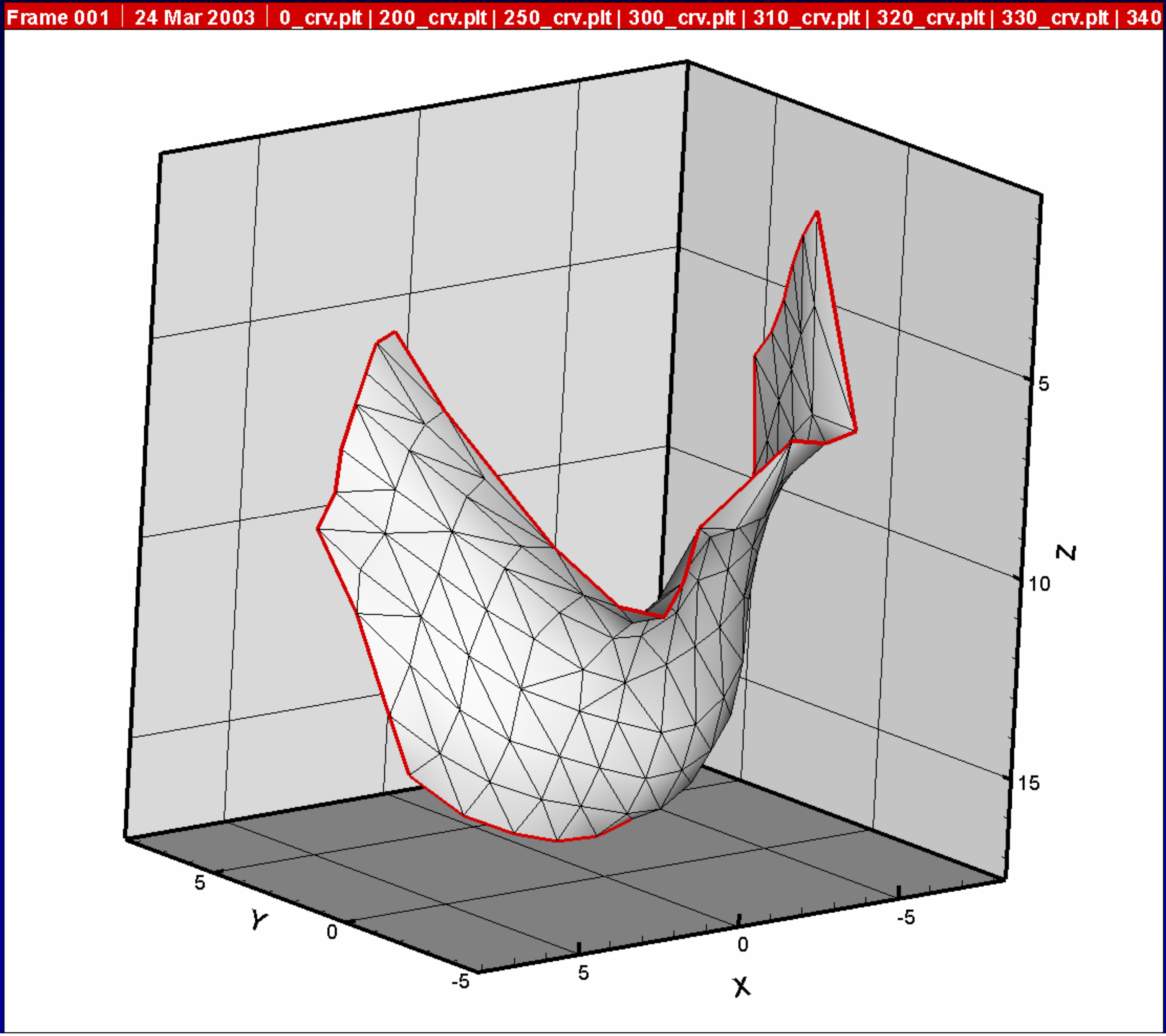
Rational for Cyclic Flexure Bioreactor

- Pulse duplicator / Flow loop bioreactor
  - Used to grow a TEHV for implantation
  - Anatomical geometry
  - Coupled mechanical stimuli

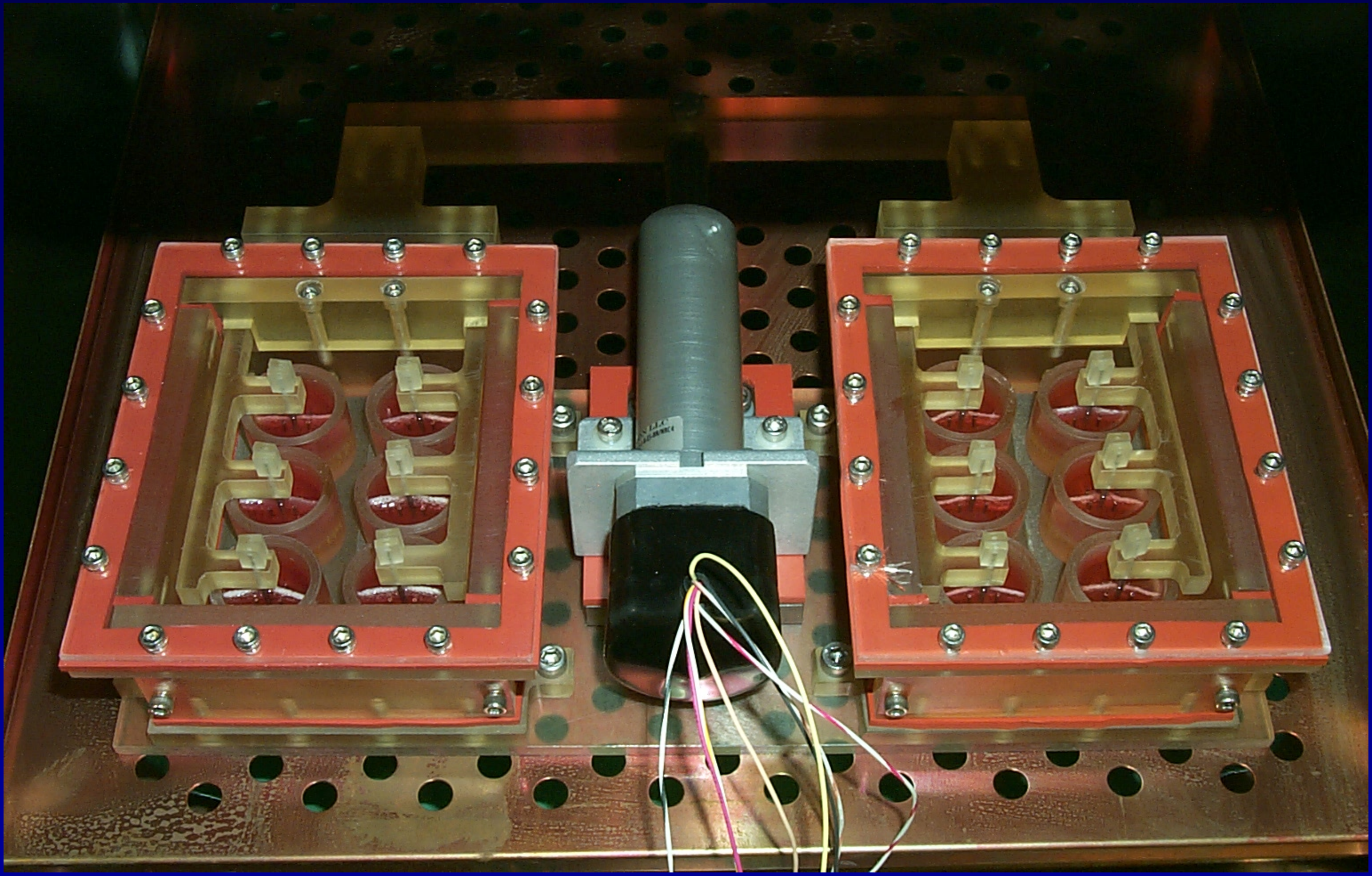
- Decompose complex mechanical environment into simple, independent modes of deformation:
  - Cyclic Flexure
  - Shear Stress
  - Tension
  - Pressure

- Why cyclic flexure?
  - Non-woven scaffolds are <u>not elastomeric</u>
  - Flexure is a mode of deformation innate to heart valves

Aortic valve leaflet dynamic motion



Cyclic Flexure Bioreactor

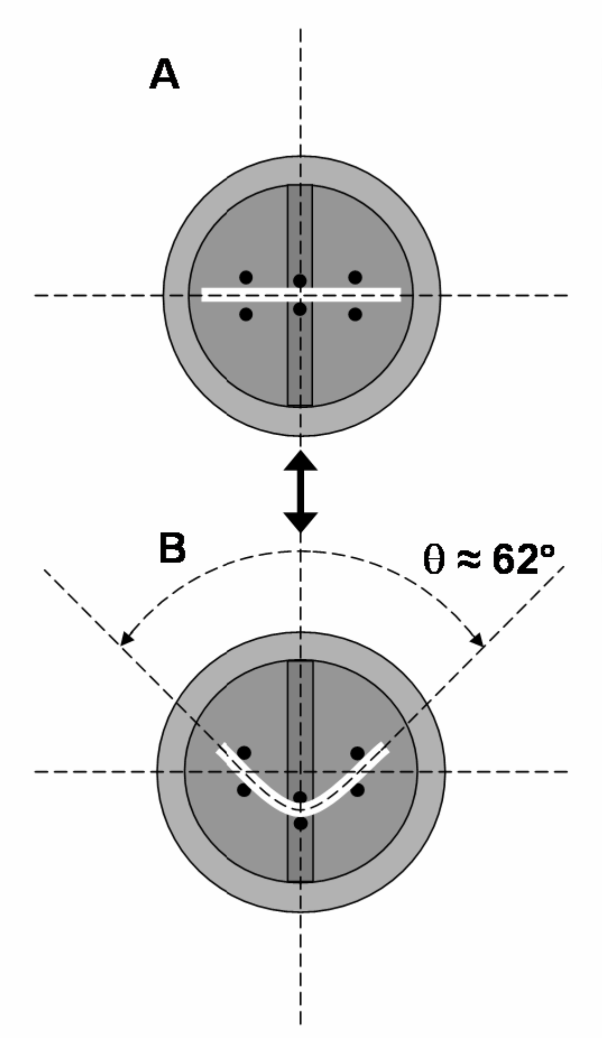


Engelmayr et al., Biomaterials, 2003, 24(14):2523-32

Engelmayr et al., Biomaterials, 2005, 26(2):175-87

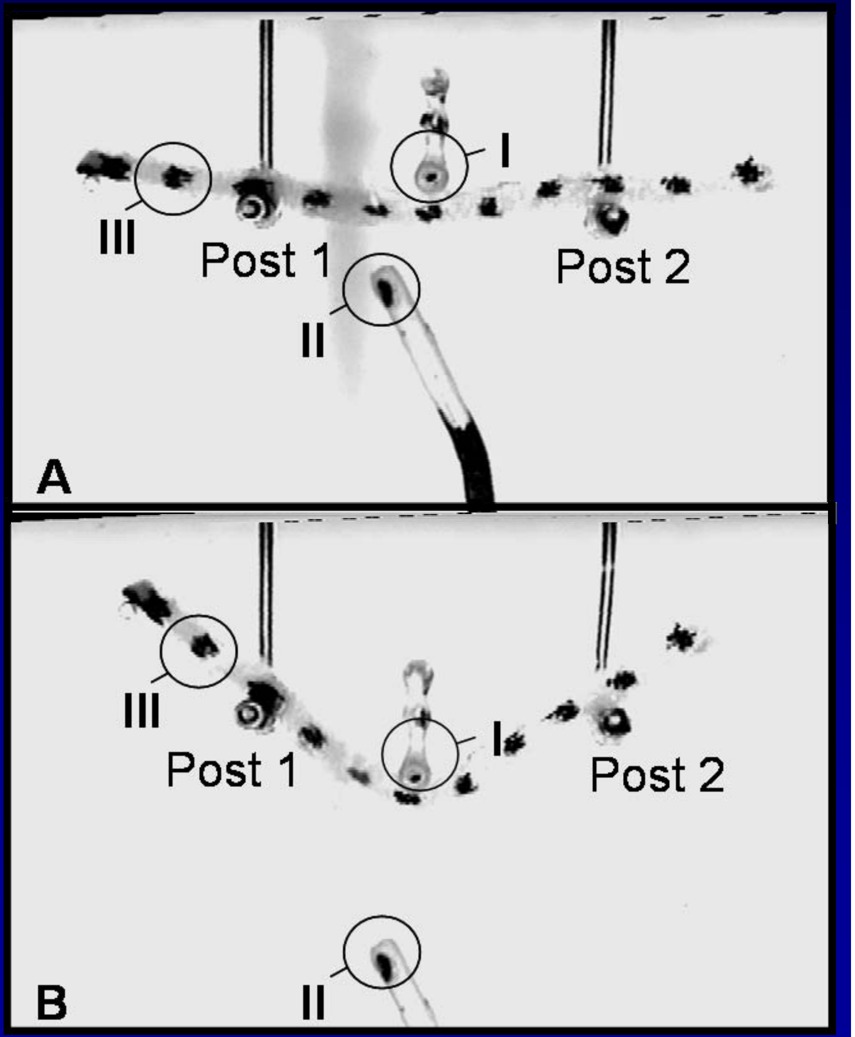
Physiologically Relevant Flexural Testing

Cyclic Flexure Bioreactor Culture Well

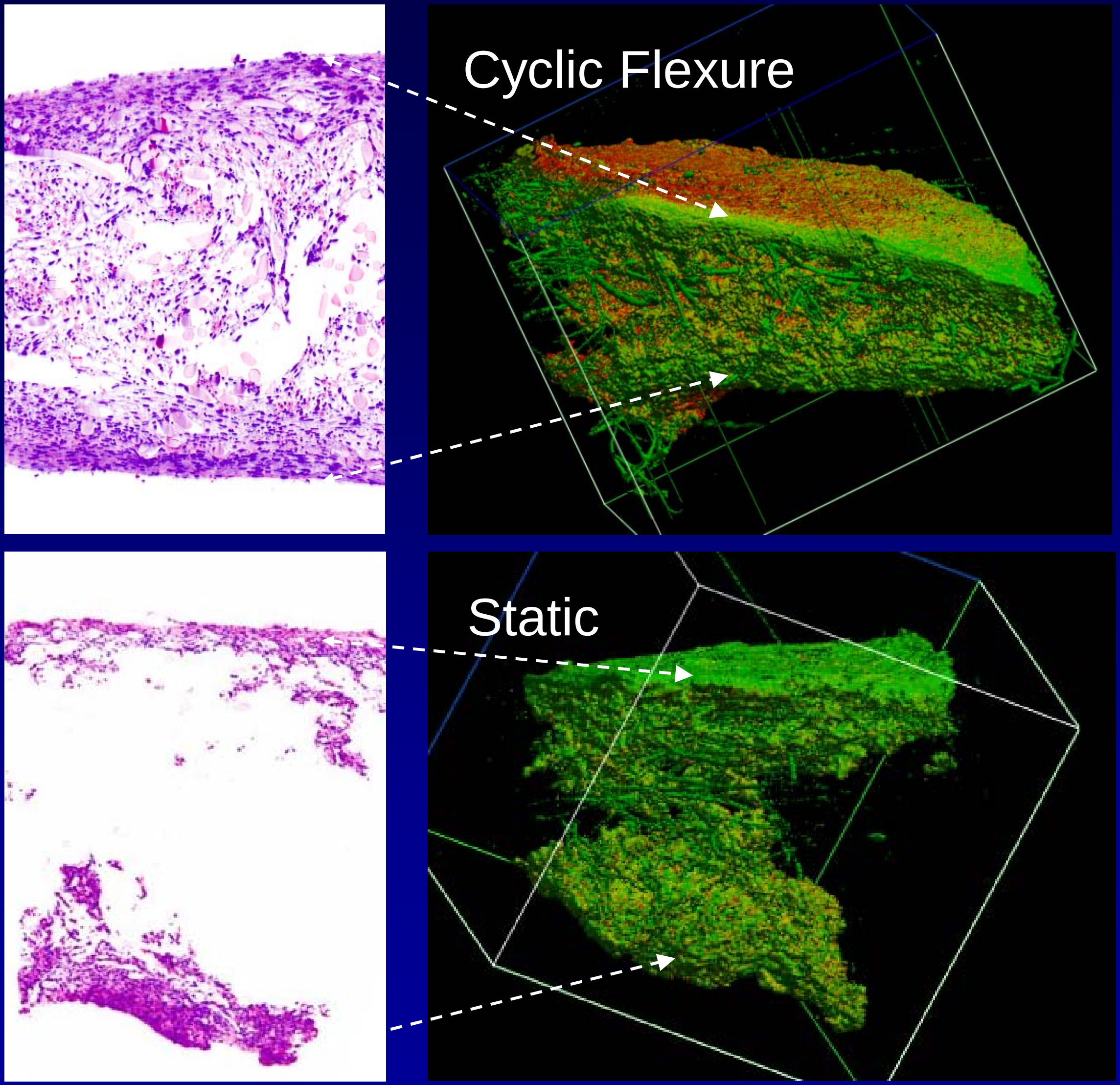


$$M = EI\Delta\kappa$$

Three-Point Bending Test

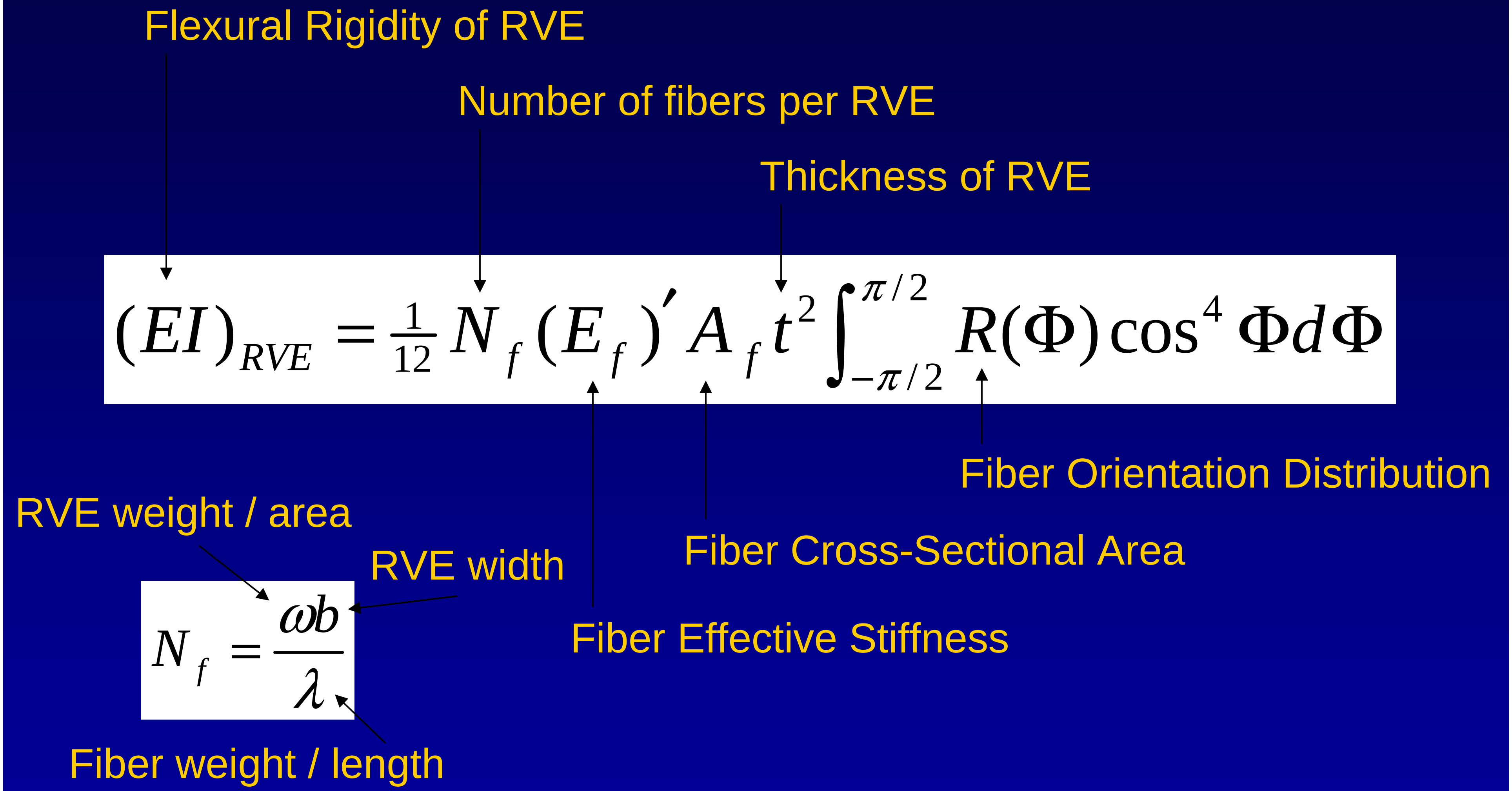


Effects of Cyclic Flexure on SMC-Seeded TEHV



RESView 3D Histology, Resolutions Sciences Corp., Corte Madera, CA

A Structural Model for Nonwoven Scaffolds



Flexural Rigidity of RVE

Number of fibers per RVE

Thickness of RVE

$$(EI)_{RVE} = \tfrac{1}{12} N_f (E_f)' A_f t^2 \int_{-\pi/2}^{\pi/2} R(\Phi) \cos^4 \Phi \, d\Phi$$

Fiber Orientation Distribution

RVE weight / area

RVE width

Fiber Cross-Sectional Area

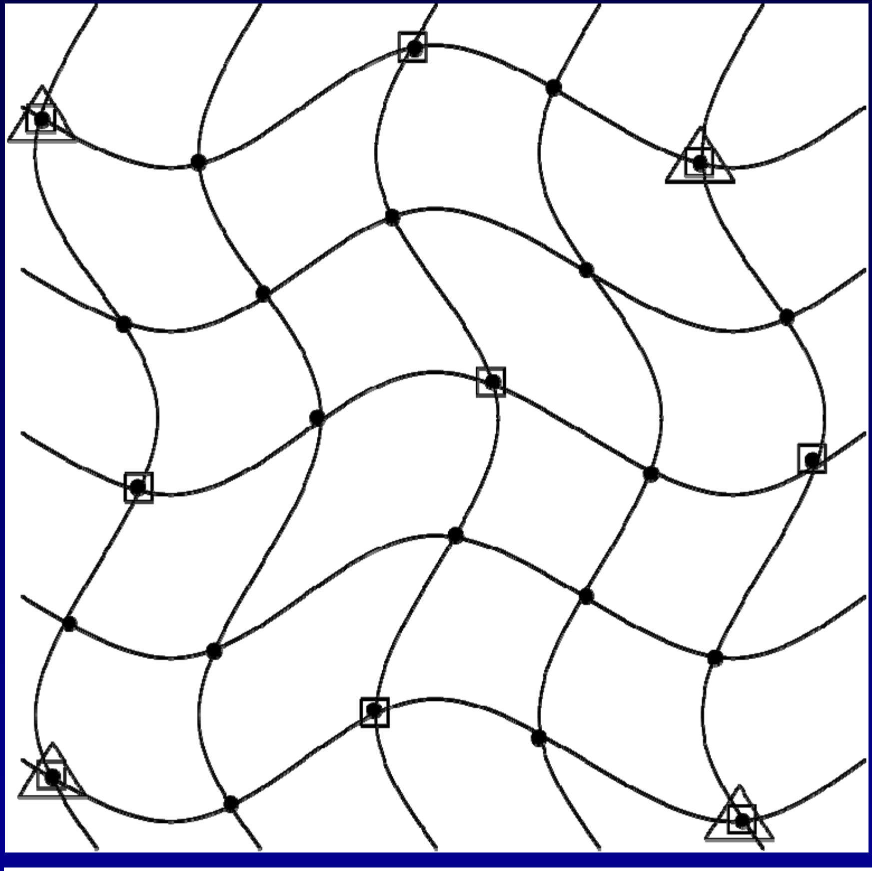
Fiber Effective Stiffness

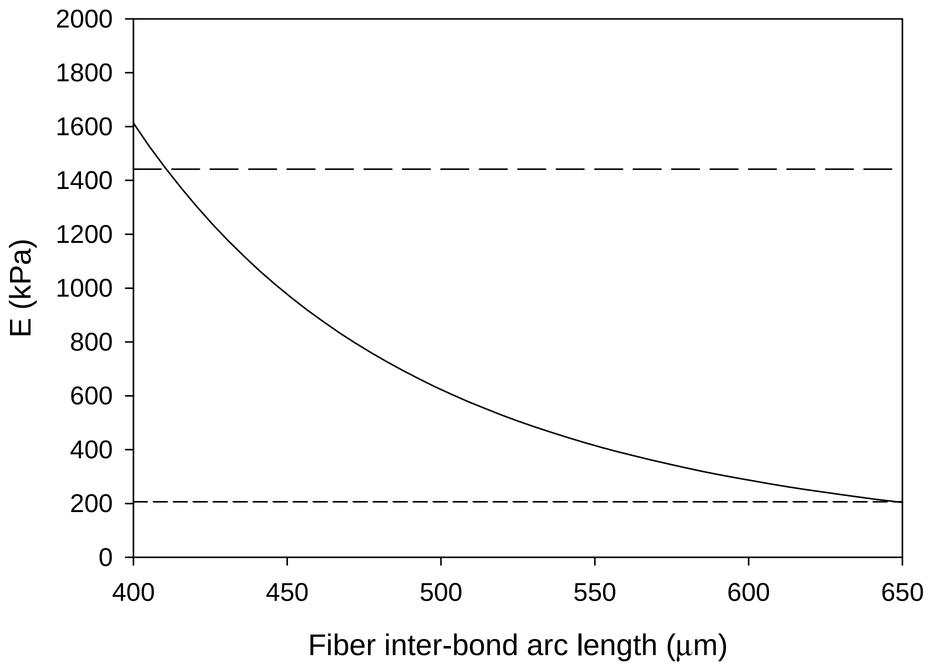
$$N_f = \frac{\omega b}{\lambda}$$

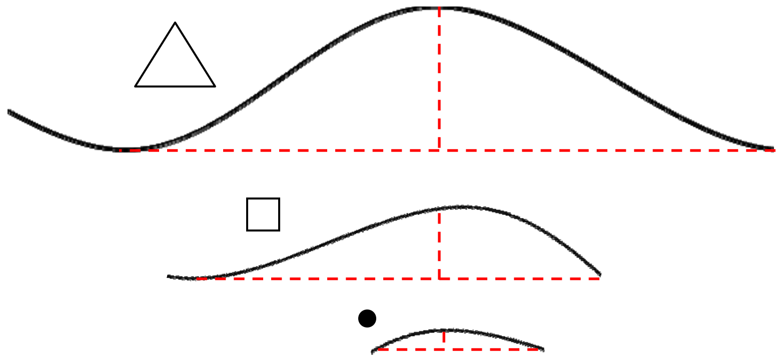
Fiber weight / length

Freeston, W.D., Jr., Platt, M.M., Textile Research Journal 1965;35(1):48-57

Nonlinear Reinforcement Effects of ECM







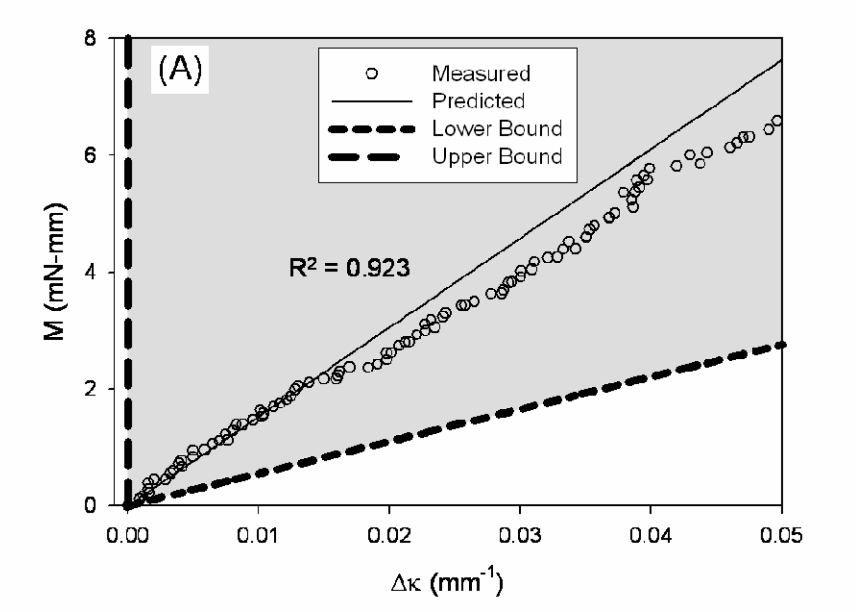
$E_{ECM} = 0$ $(E_f)' = 8896$ kPa E = 206 kPa

$E_{ECM} > 0$ $(E_f)' \sim 15430$ kPa E = 431 kPa

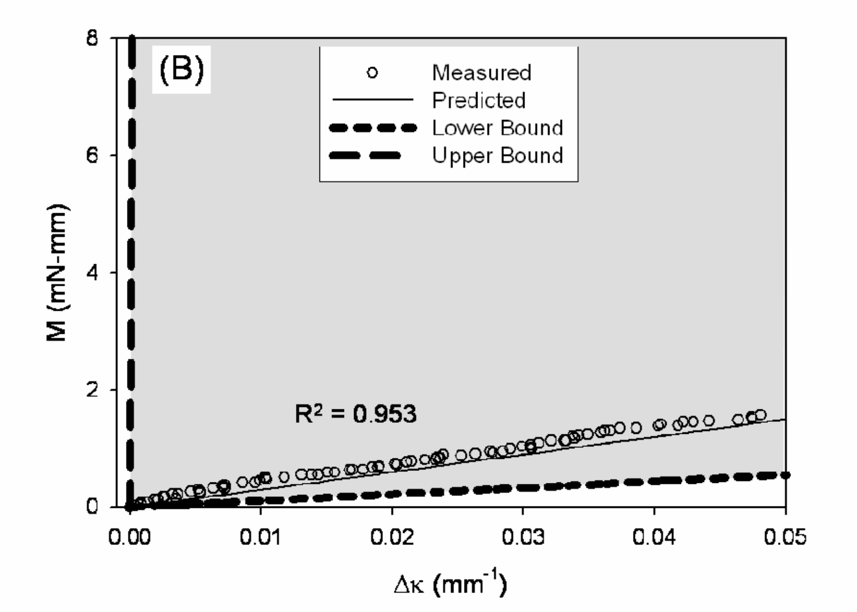
$E_{ECM} >> 0$ $(E_f)' \sim 55640$ kPa E = 1555 kPa

Structural Model Results

Preferred (PD) Fiber Direction

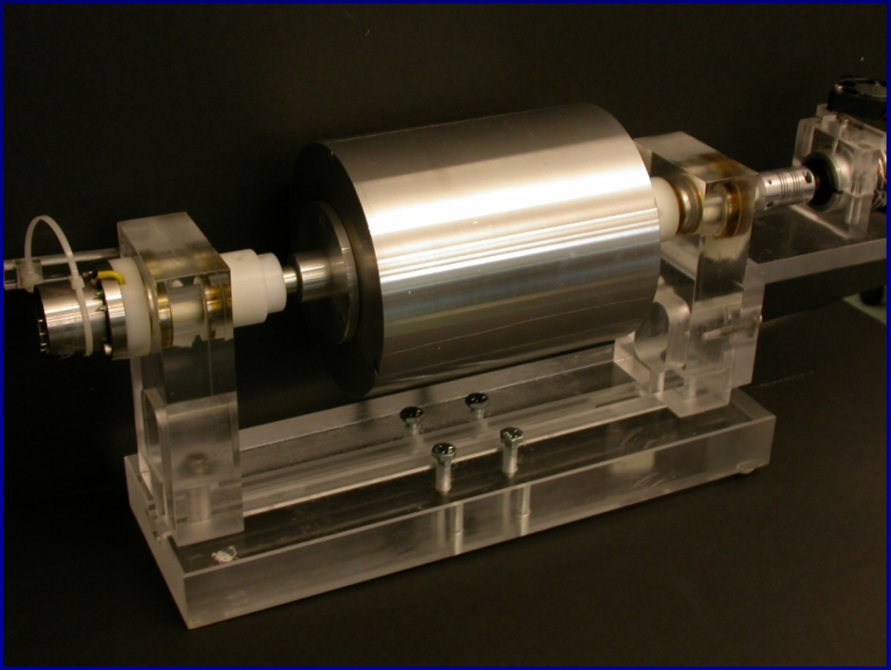


Cross-Preferred (XD) Fiber Direction

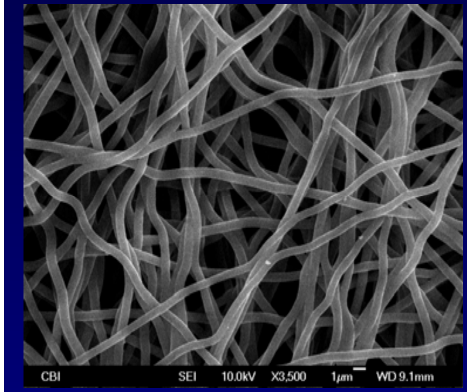


Electrospinning setup

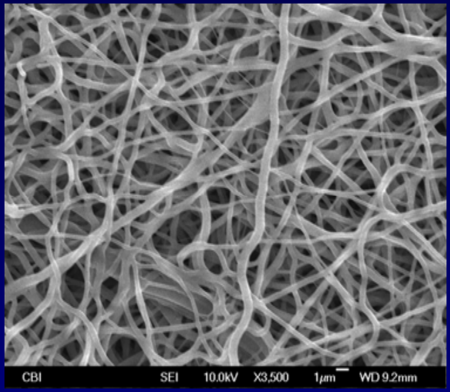
Mandrel:
50rpm to 2300rpm
or 0.3 m/s to 13.8 m/s



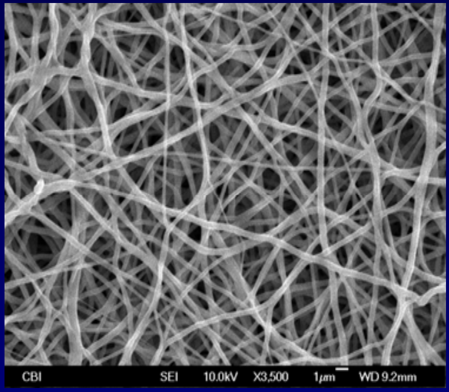
ES-PEUU microstructure



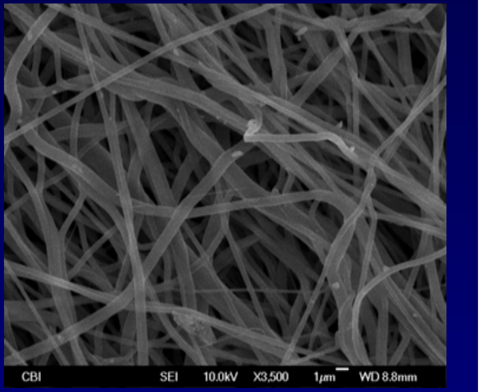
0.0 m/s



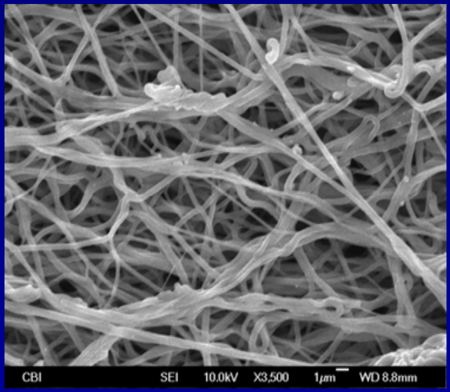
0.3 m/s



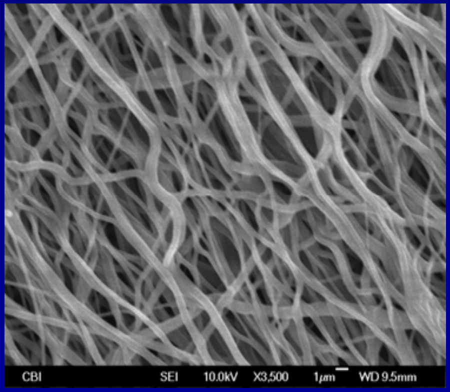
1.5 m/s



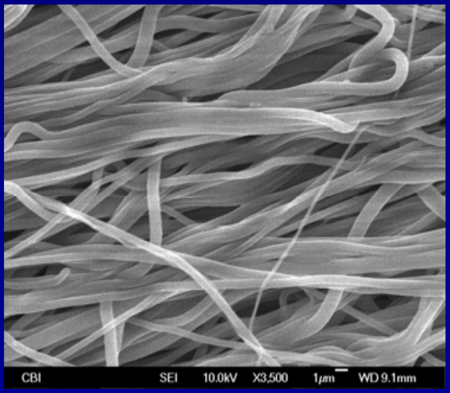
3.0 m/s



4.5 m/s



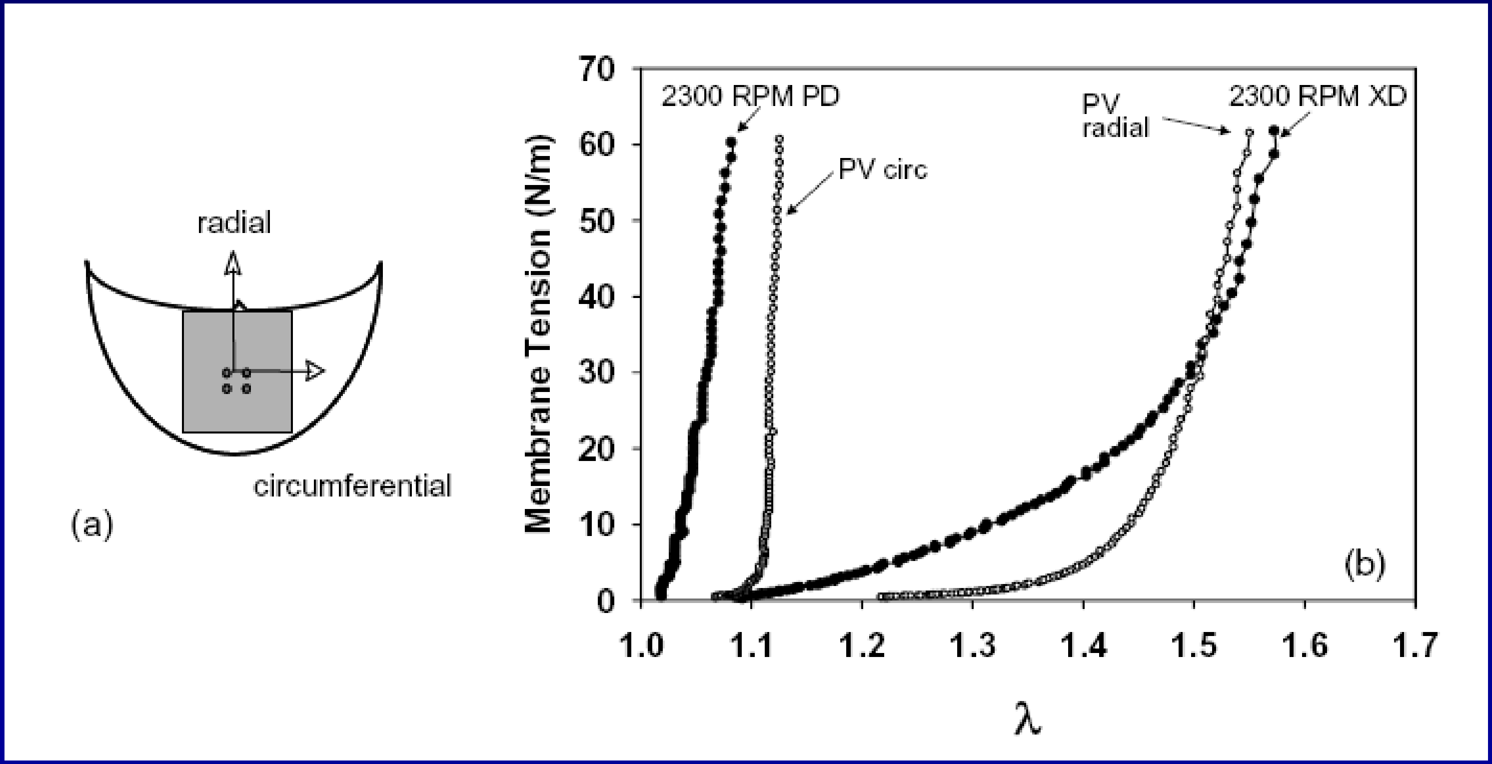
9.0 m/s



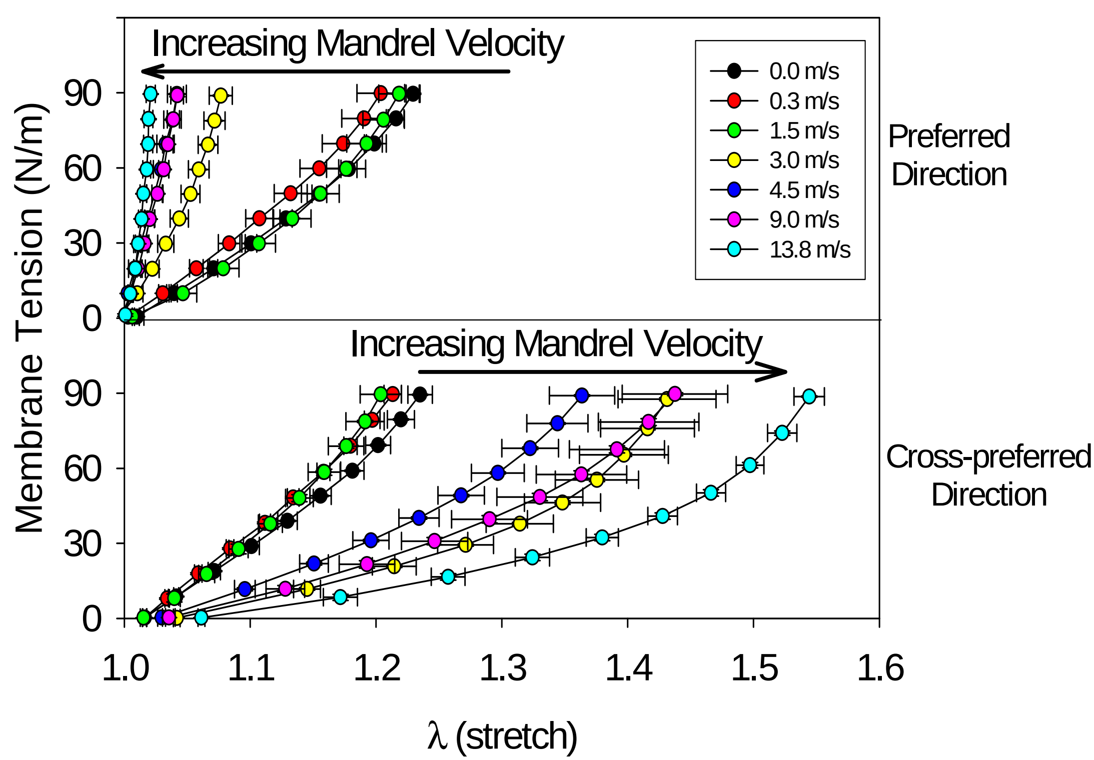
13.8 m/s

Why ES-PEUU scaffolds ?

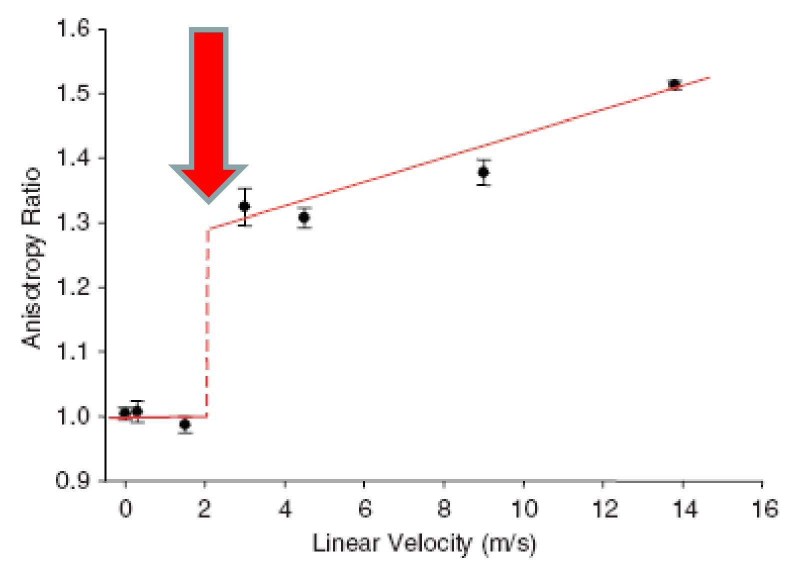
- Electrospun PEUU scaffolds exhibit
  - wide range of mechanical compliance and anisotropy
  - mechanical properties very similar to native tissue



Mechanical analysis



Equibiaxial stress-stretch results



Comparison to native pulmonary valve

Model formulation

Stress-stretch relations

$$P_{11} = \int_{-\pi/2}^{\pi/2} S_f[E_f(\theta)]\, R(\theta)\, (F_{11}\cos^2\theta + F_{12}\sin\theta\cos\theta)\,d\theta$$

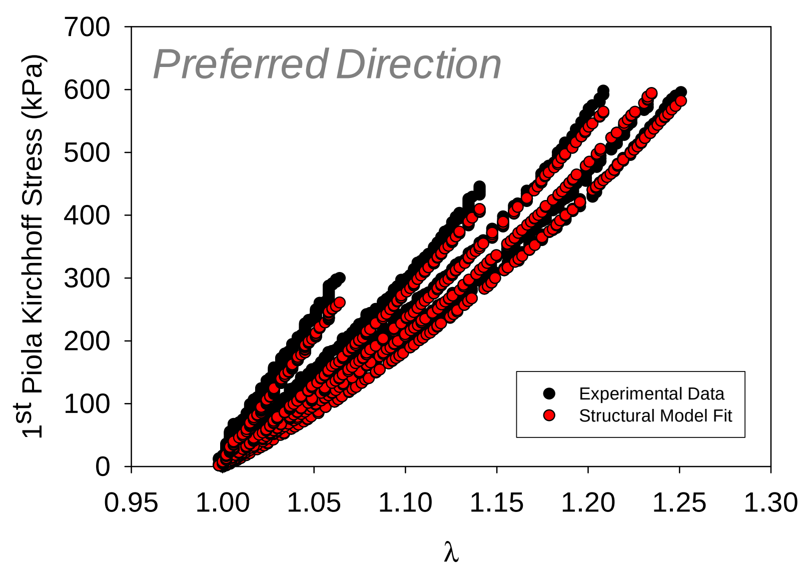
$$P_{22} = \int_{-\pi/2}^{\pi/2} S_f[E_f(\theta)]\, R(\theta)\, (F_{22}\sin^2\theta + F_{21}\sin\theta\cos\theta)\,d\theta$$

Effective fiber properties

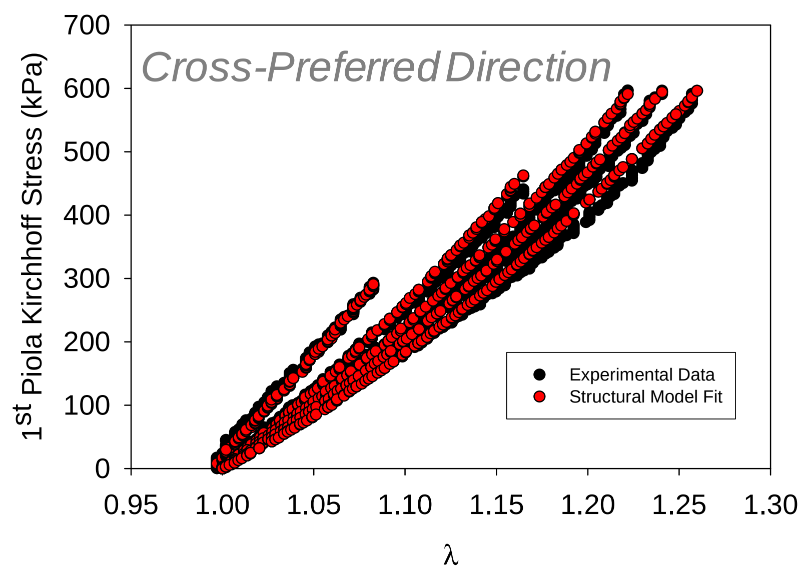
Effective fiber structure

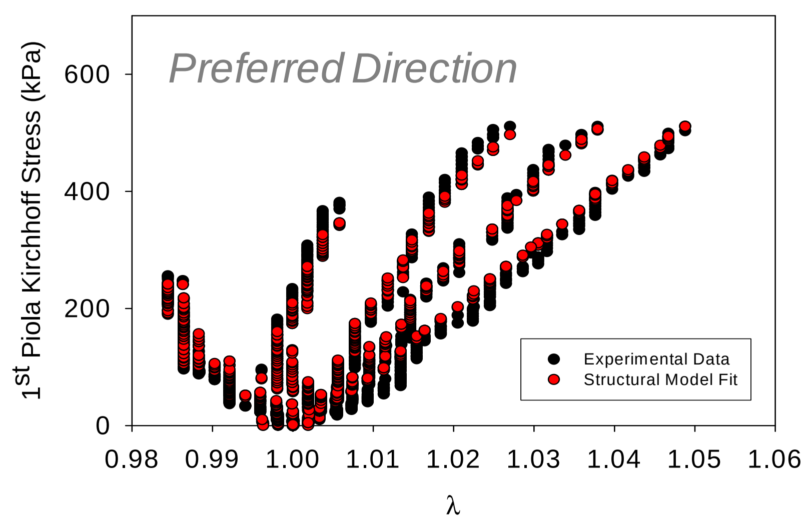
Kinematic terms based on experimental strains

Structural model fit

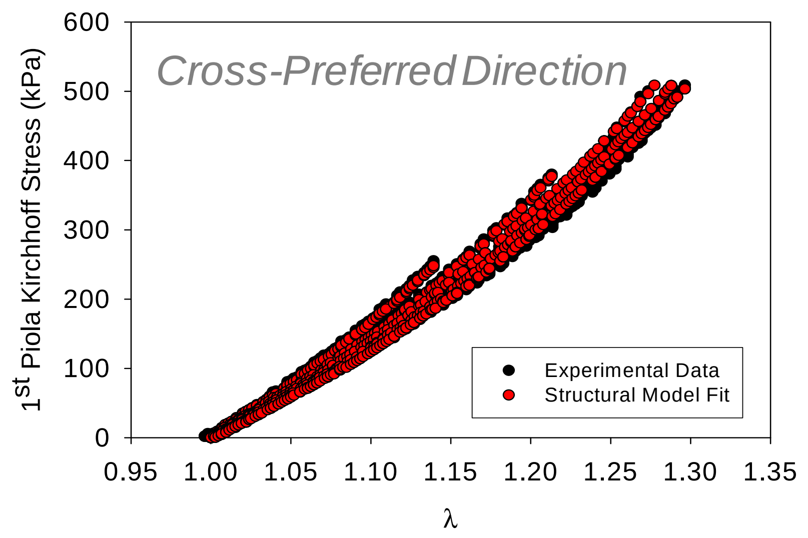


0.0 m/s
or
Random

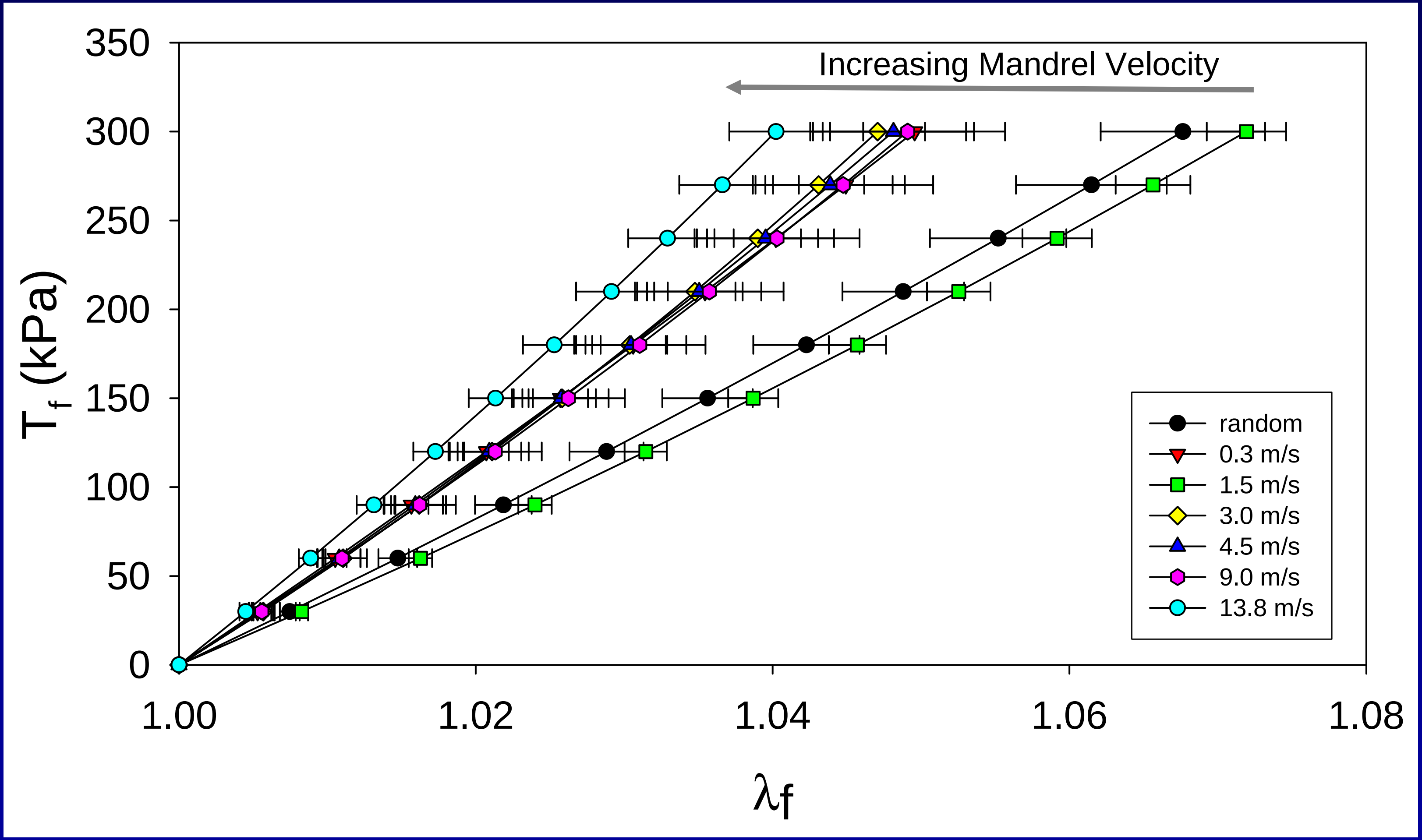




4.5 m/s
Or
750 rpm



Effective fiber stress-stretch



From this model we can

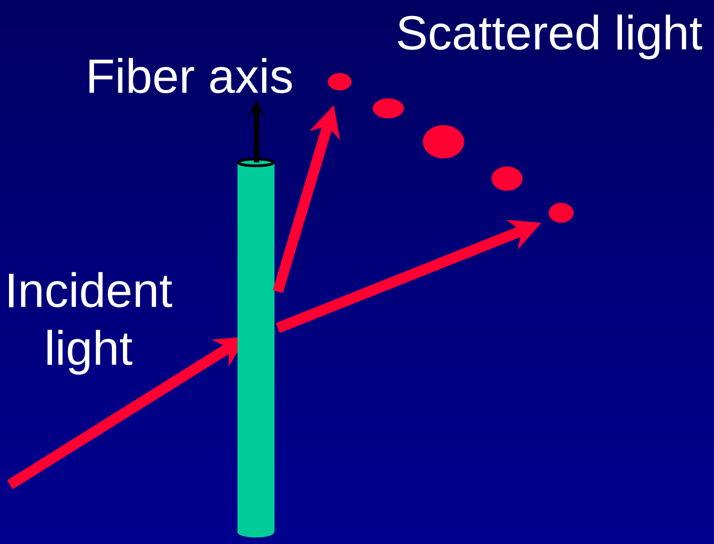
1. Obtain true fiber (polymer) moduli as opposed to effective fiber stress-strain response using exponential model used previously
2. Separate structural effects (e.g. orientation) from changes in fiber material properties
3. Allow derivation of true fiber (material) moduli independent of micro-structural features
4. Practical uses:
   - Guiding scaffold design for tissue or cell specific applications
   - optimizing in-vitro conditioning regimes to produce viable tissues for implantation.

Scaffold physical characterization-Structure

many methods are available

- Porosity and pore geometry
- Focus on fibrous architecture as this dictates both bulk properties and local cellular deformations
  - SALS for both native and non-wovens.
  - EM and CLSM fiber alignment image analysis
- Cellular deformations and it relation to local and global fiber architecture.
  - Native tissue as the functional endpoint
  - CLSM of cell micro-integrated scaffolds

Laser Light Scattering

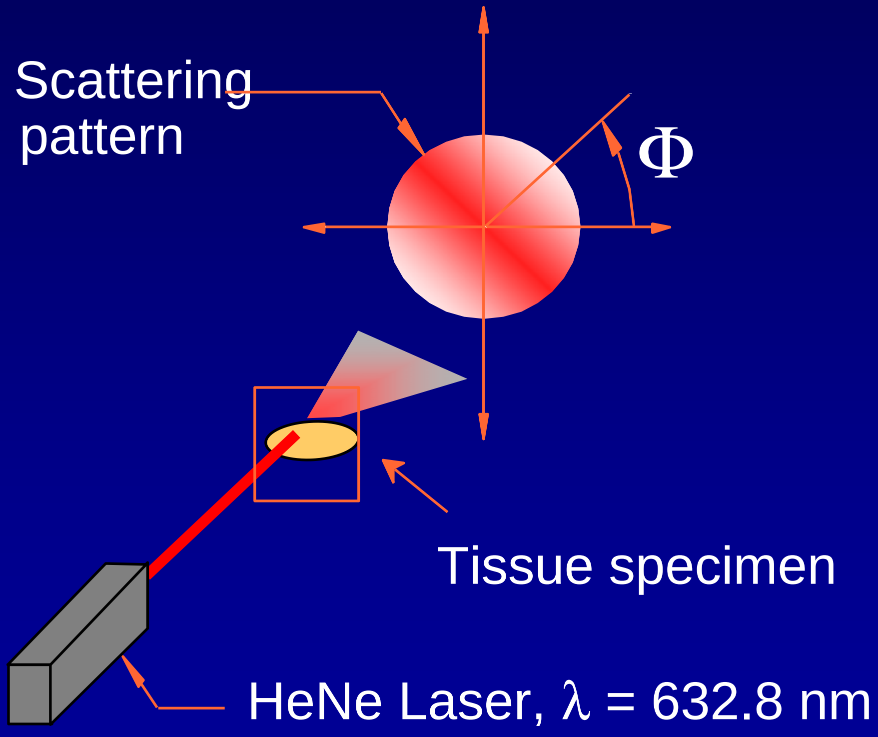


Scattered light

Fiber axis

Incident light

Light is scattered perpendicular to fiber axis



Scattering pattern

Φ

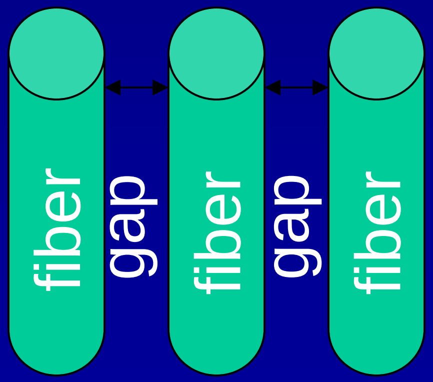
Tissue specimen

HeNe Laser, λ = 632.8 nm

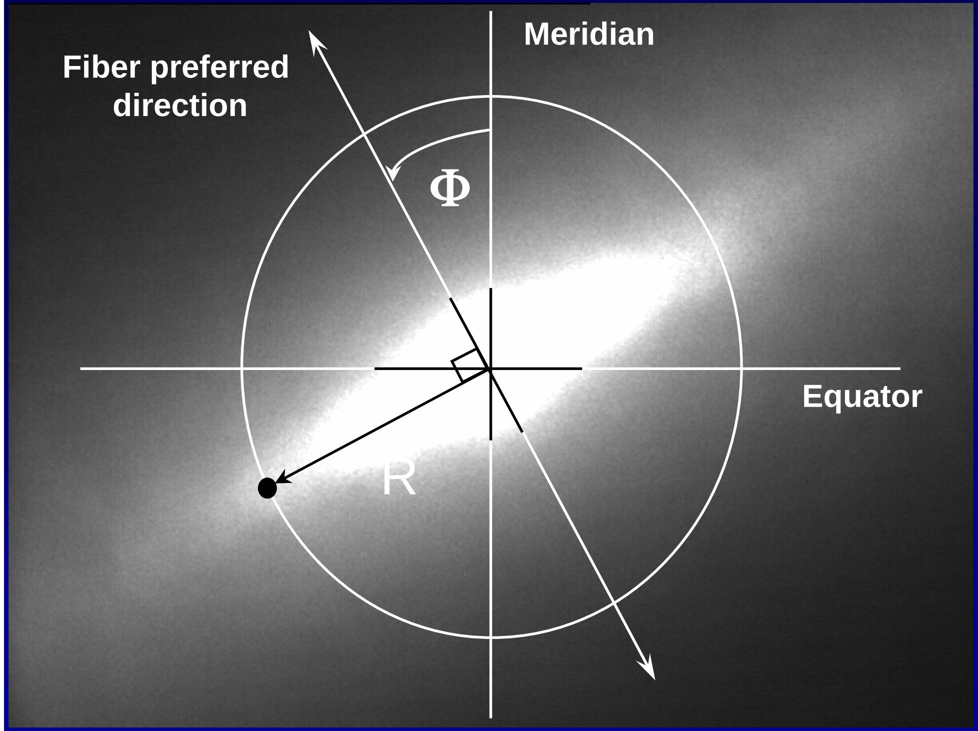
Laser Light Diffraction

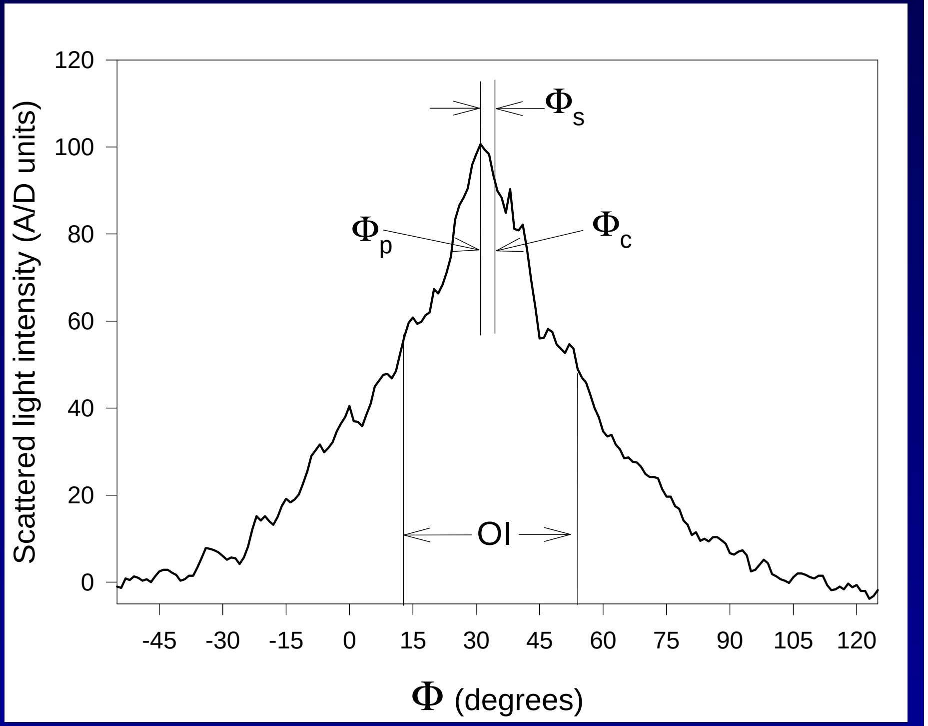
- In connective tissues, one “slit” is the fiber and the other the gap between fibers

- Since the gap must follow the fiber geometry, it turns out that this distinction is not necessary

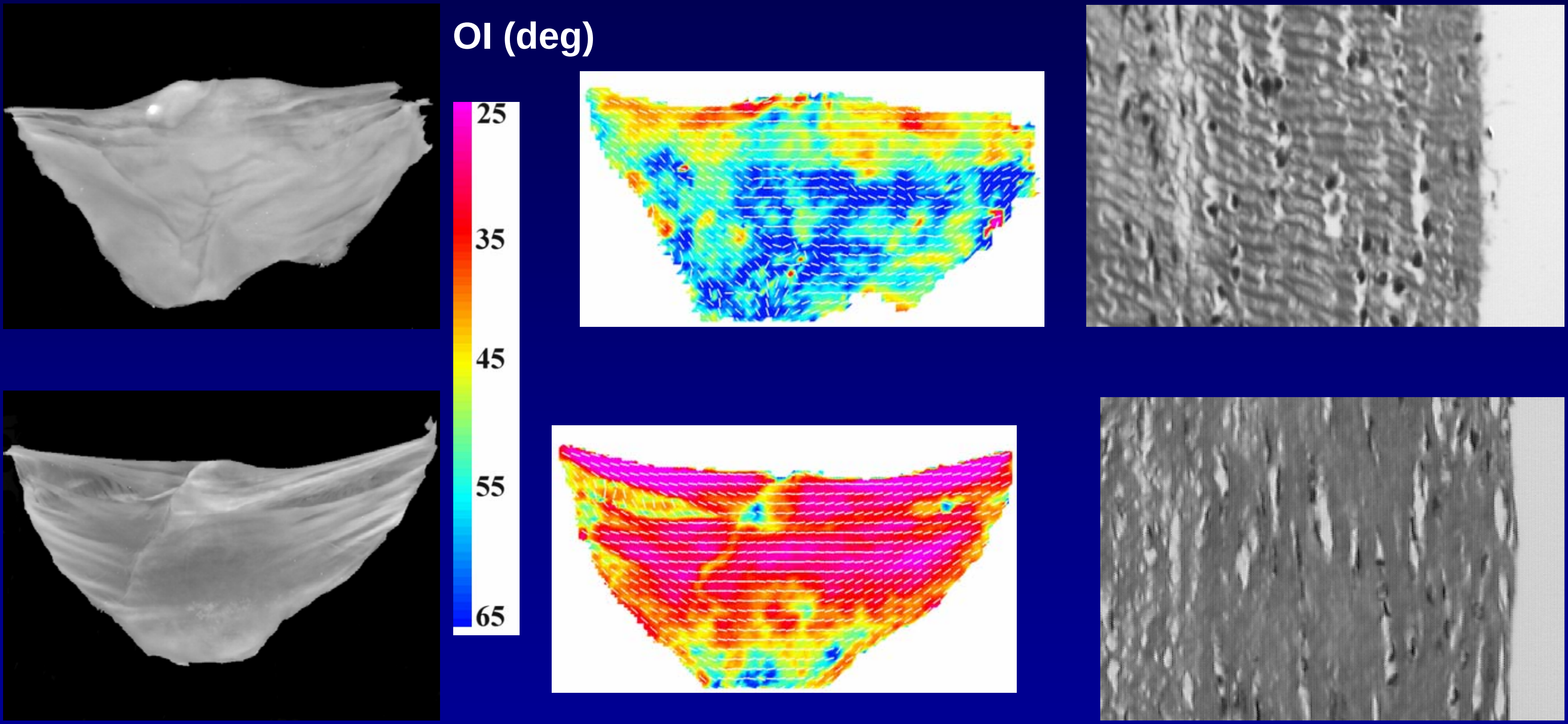


Angular Fiber Distribution from SALS



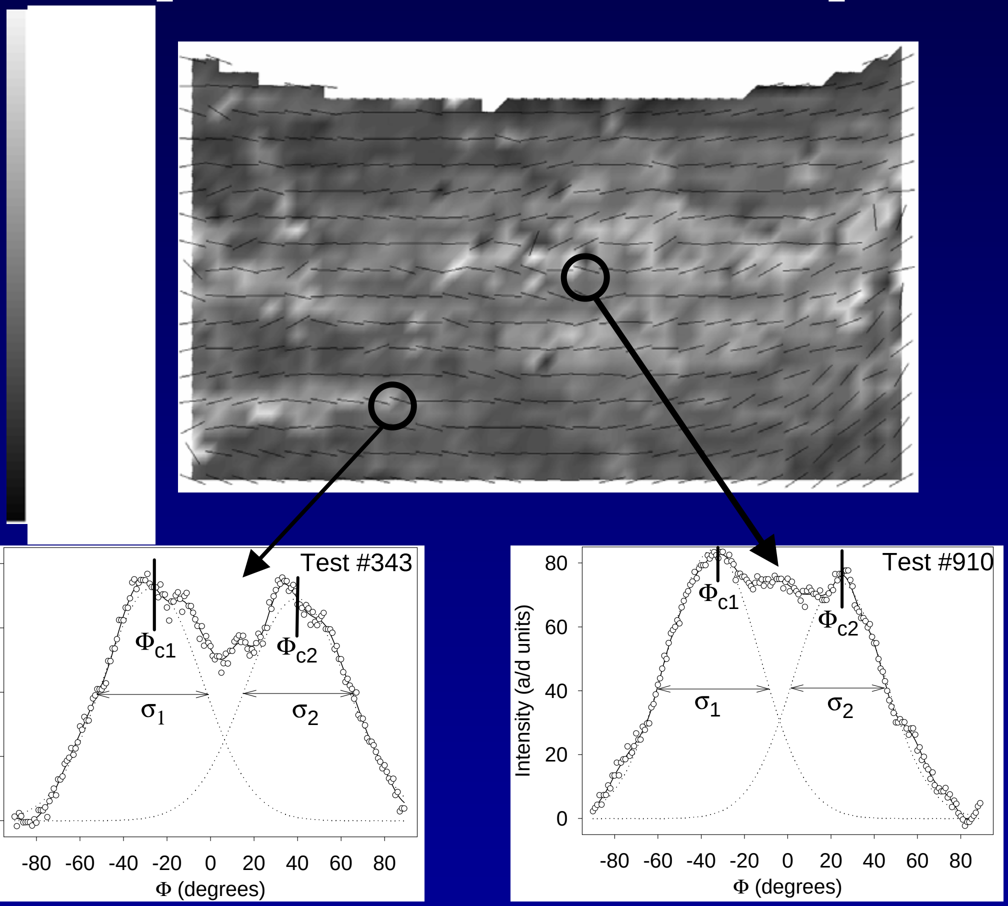


Native valvular tissue



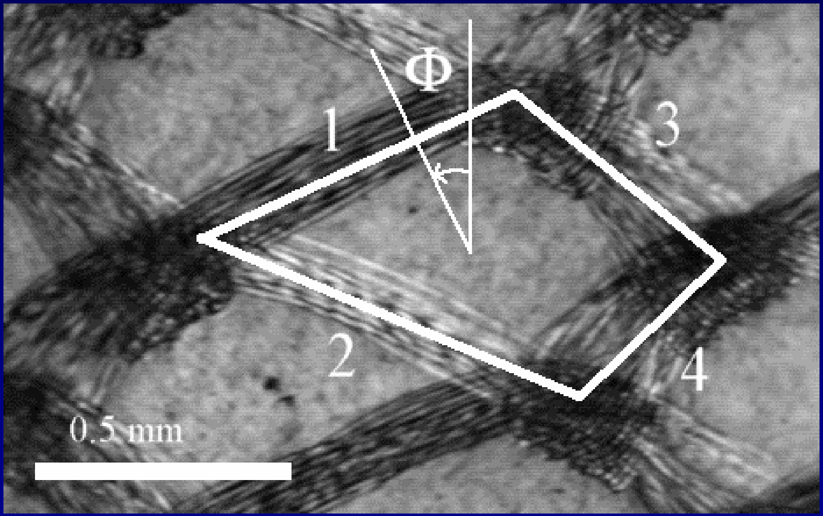
SALS was used to compare the changes in fiber distributions between pressure-fixed aortic valve cusps (bottom row) and non-pressure fixed (top row). The changes in crimp due to the two preparations are on the right. The SALS data (center) shows a much higher alignment in the pressure-fixed cusp.

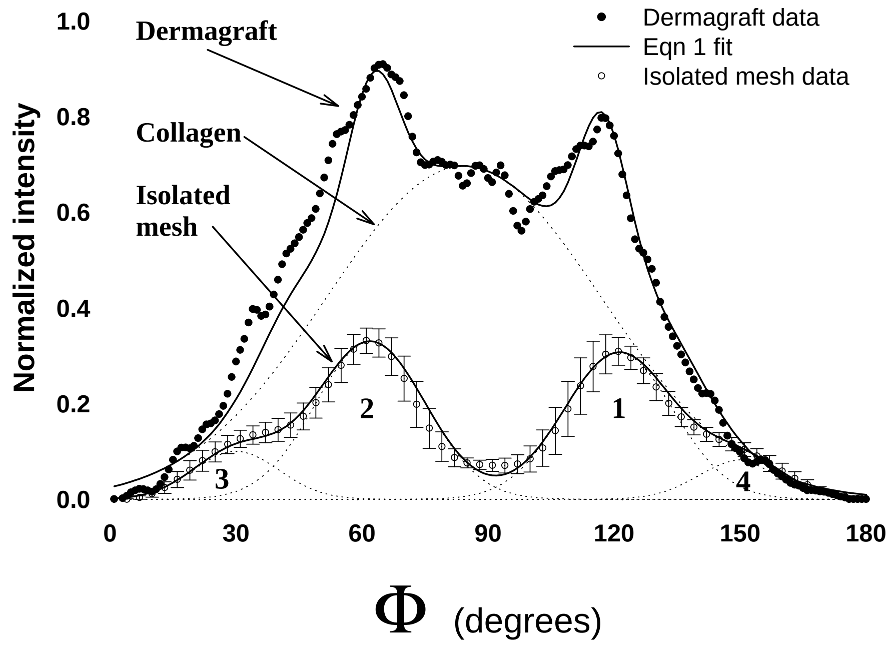
SIS Multiple Fiber Populations



SALS can also indicate the presence of multiple fiber populations, which can then be deconstructed using mathematical techniques to investigate the results of multiple fiber populations on mechanical performance.

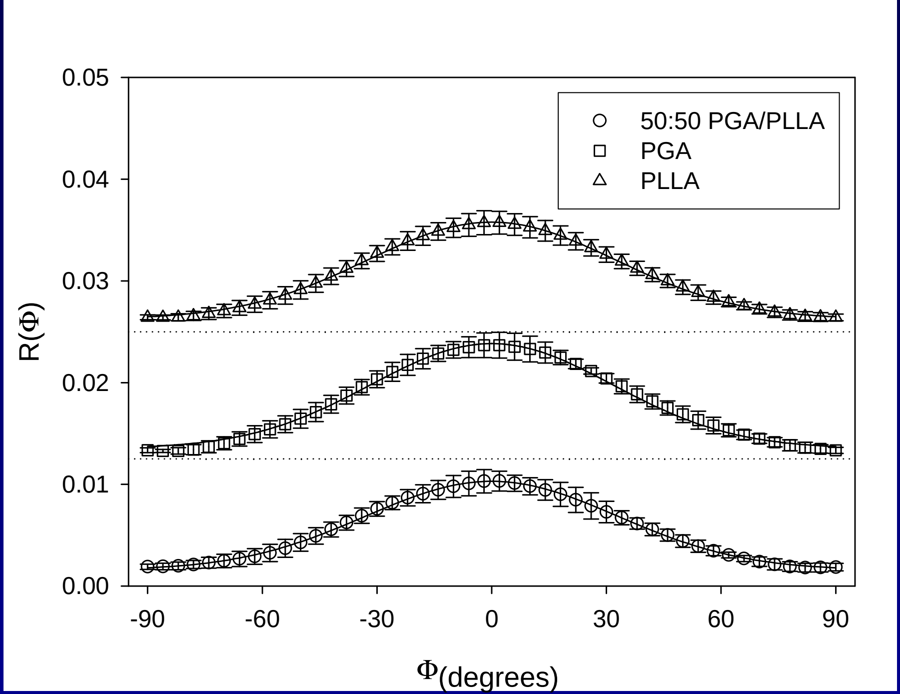
Engineered Biomaterials





SALS can be used to evaluate the structural properties of composite biomaterials such as the Dermagraft (Advanced Tissue Sciences). This material is composed of a biodegradable mesh embedded in a collagen matrix. Both the collagen and mesh components of the fiber distribution are observed in the SALS signal (right).

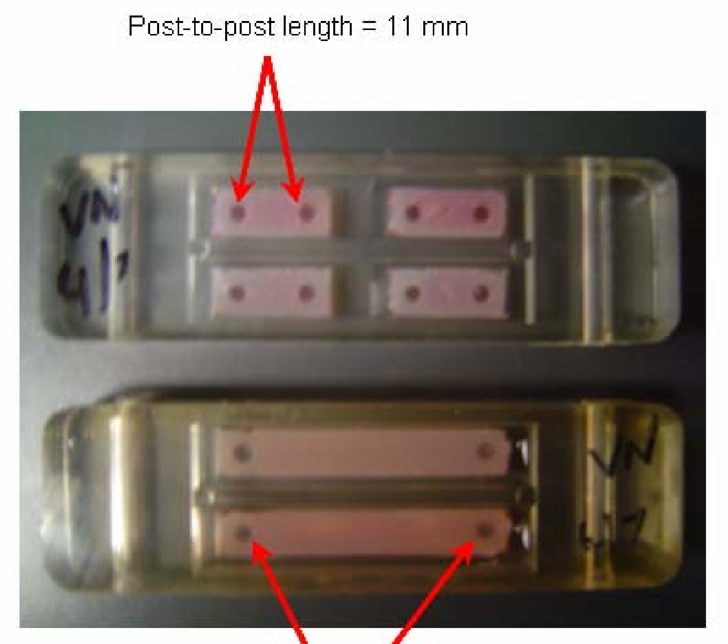
R(Φ): Normalized Fiber orientation distribution of non-wovens

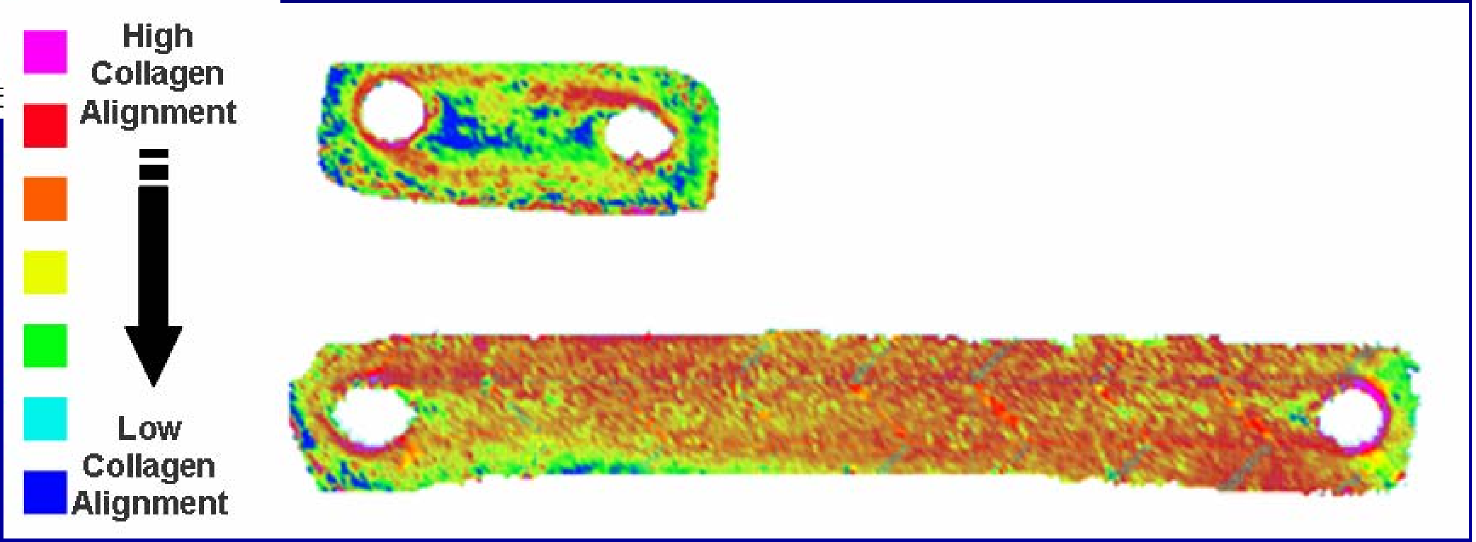


$$R(\Phi)=\frac{\frac{\sigma}{y_0}+\frac{1}{\sigma\sqrt{2\pi}}\exp\left(-\frac{\Phi^2}{2\sigma^2}\right)}{\int_{-\pi/2}^{\pi/2}\left[\frac{\sigma}{y_0}+\frac{1}{\sigma\sqrt{2\pi}}\exp\left(-\frac{\Phi^2}{2\sigma^2}\right)\right]d\Phi}$$

Material	Thickness (μm)	Bulk Density (mg/cm^3)	Mean Fiber Orientation Distribution, *R*(Φ) Normalized Gaussian Model (μ= 0)		
			σ(degrees)	y_o	R^2
PGA	948 ±28	69.0	31.66	25000	0.9951
PLLA	1153 ±37	61.9	33.79	20000	0.9986
50:50	889 ±6	61.75	32.97	15000	0.9987

SALS
Tendon tissue engineering*

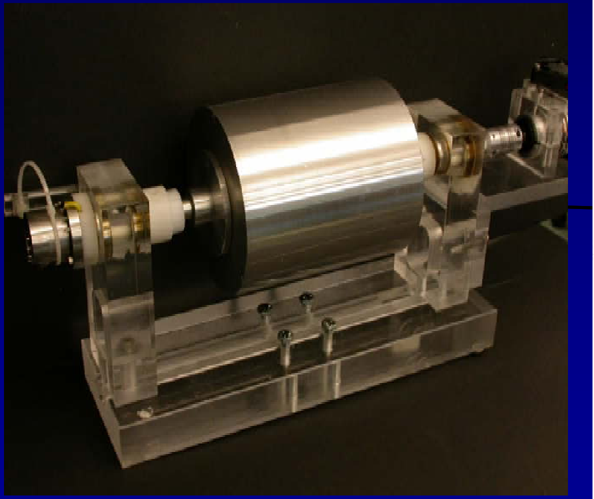


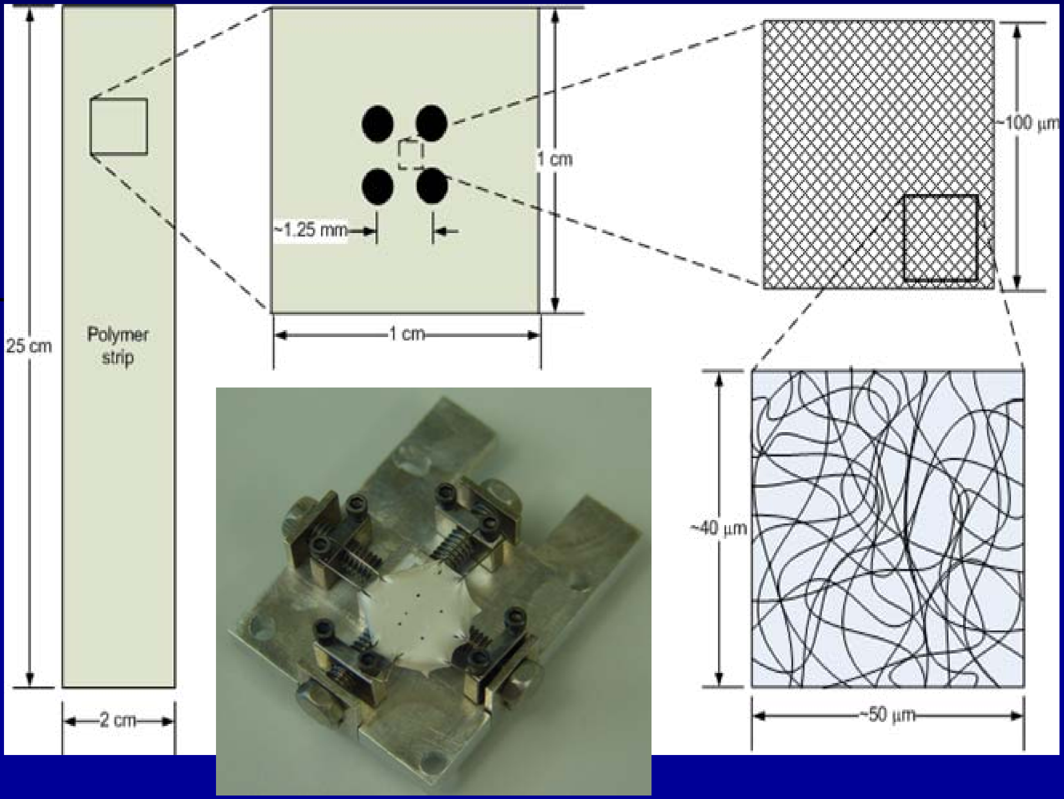


*Nirmalanandhan VS, Rao M, Sacks MS, Haridas B, Butler DL., JB 2007

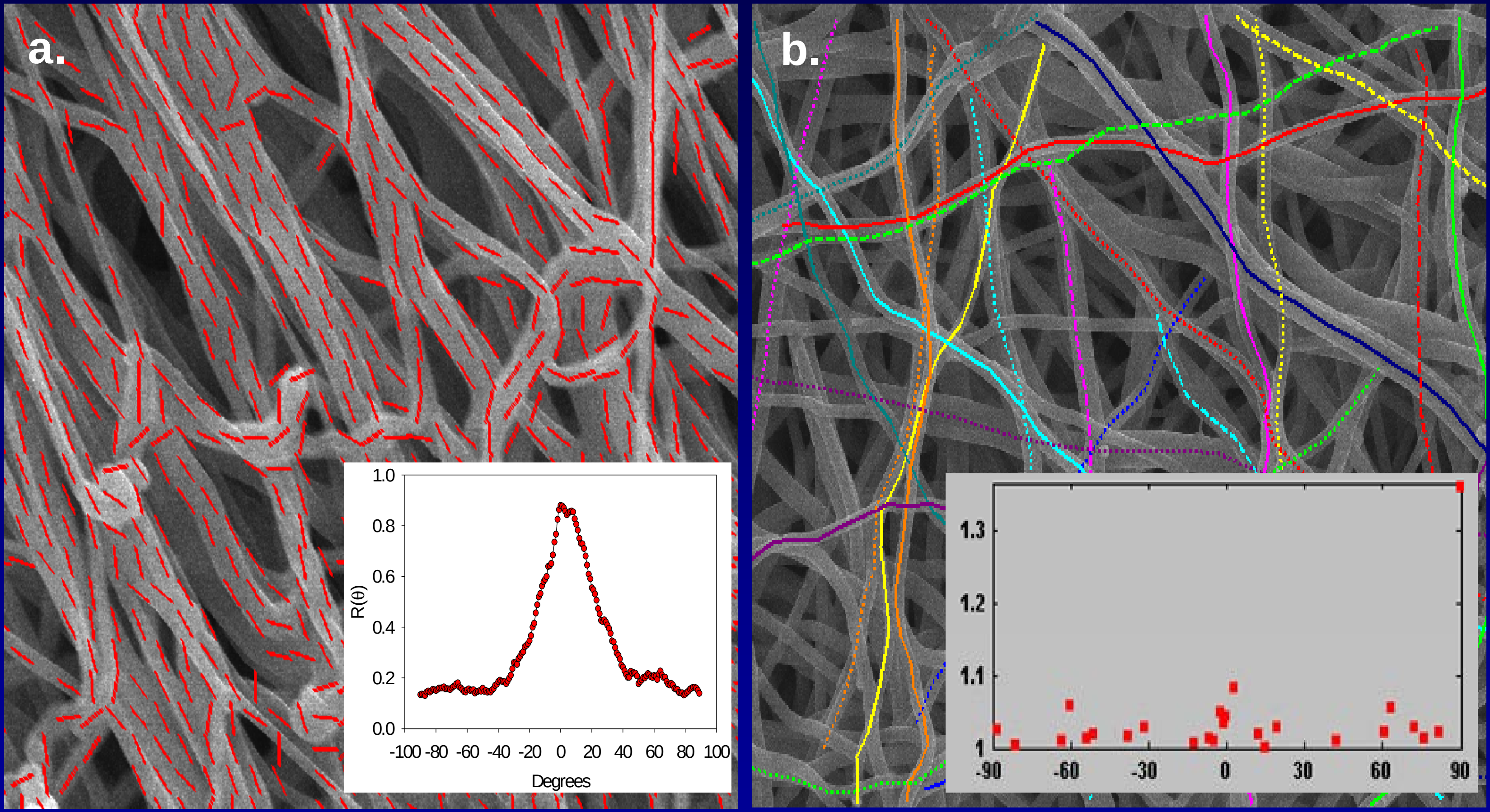
Global deformations vs. local fiber response

- Illustration of multi-scale characteristics encountered when relating global deformations to local fiber responses





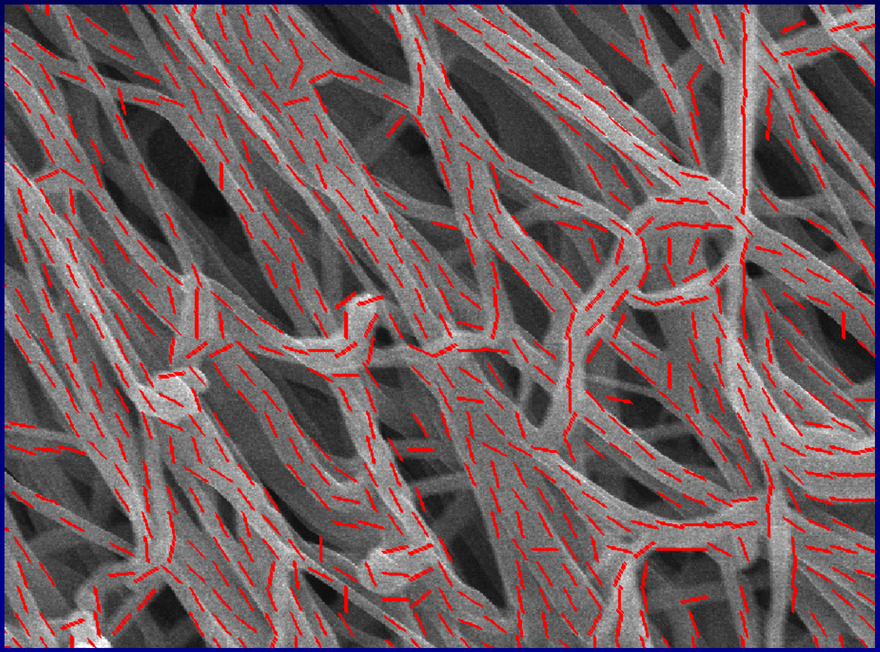
Fiber orientation and tortuosity tracking

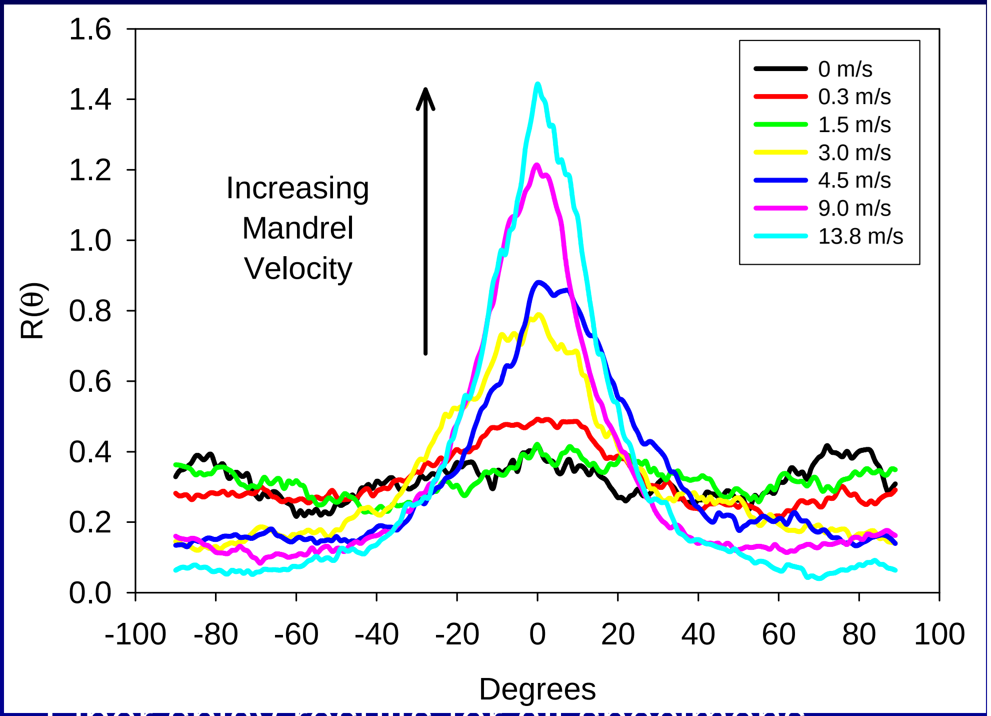


Custom image analysis to quantify (a) orientation and (b) tortuosity

Fiber architecture analysis*

Tracking fiber splay

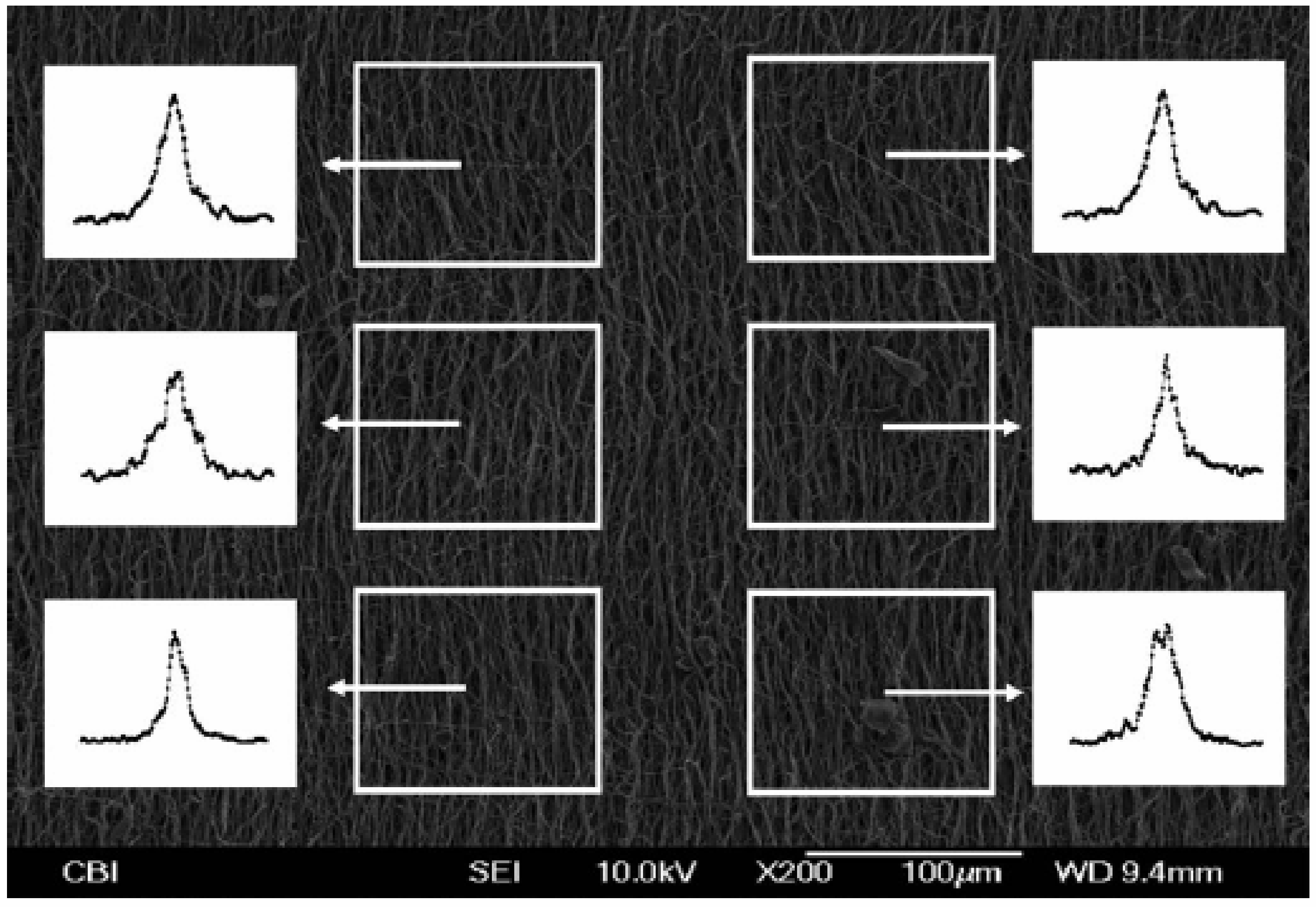




Fiber splay results for all specimens

*Courtney et al., Biomaterials, 2006

Structural uniformity



Functional Tissue Engineering

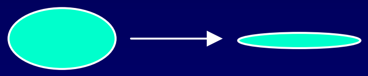
Effects of changes in tissue formation with time

a. Effects of tissue formation.
    i. Physical stimulation to enhance tissue generation.
    ii. Methods to assess effects of tissue formation.
b. Scaffold degradation
    i. Mass changes
    ii. Surface vs. bulk erosion
    iii. Stress-transfer considerations.

Mechanical training paradigm

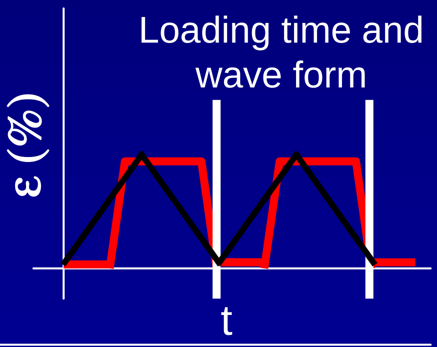
INPUTS

1.



Controlled cell deformation

2.



Loading time and wave form

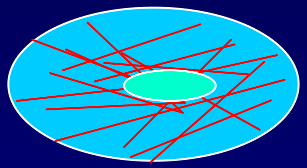
3. # of cycles

4. Addition of:
growth factors
ascorbic acid

Mechano-dependent, phenotypic/biosynthetic response

OUTPUTS

1.



Phenotype changes

Biosynthetic levels

2.

Robust ECM formation

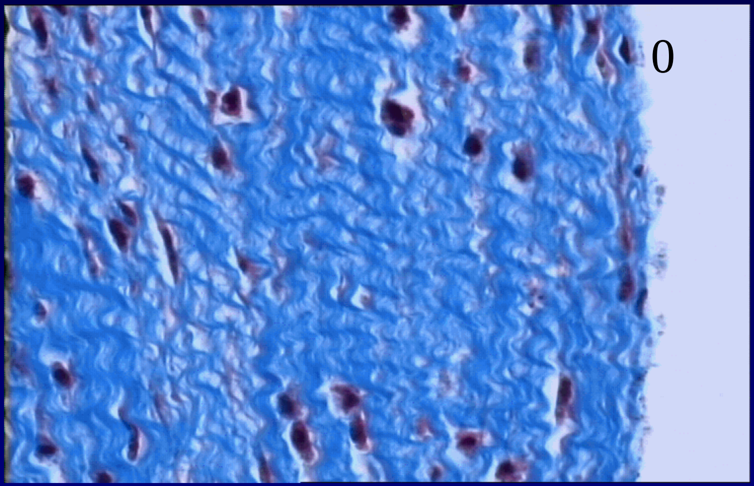
Scaffold degradation

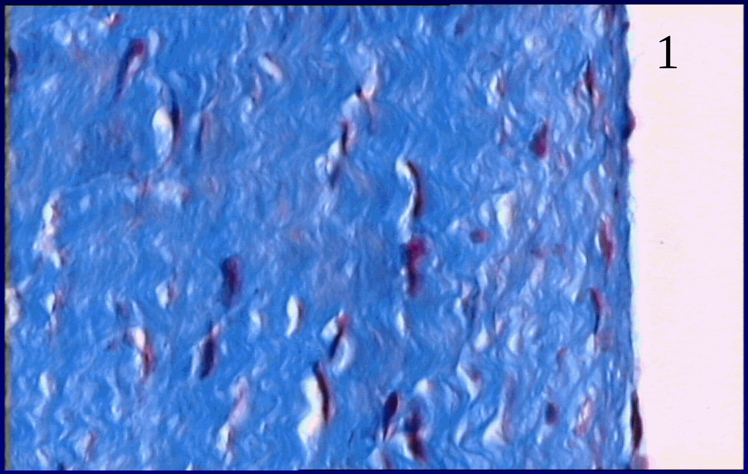
Related studies

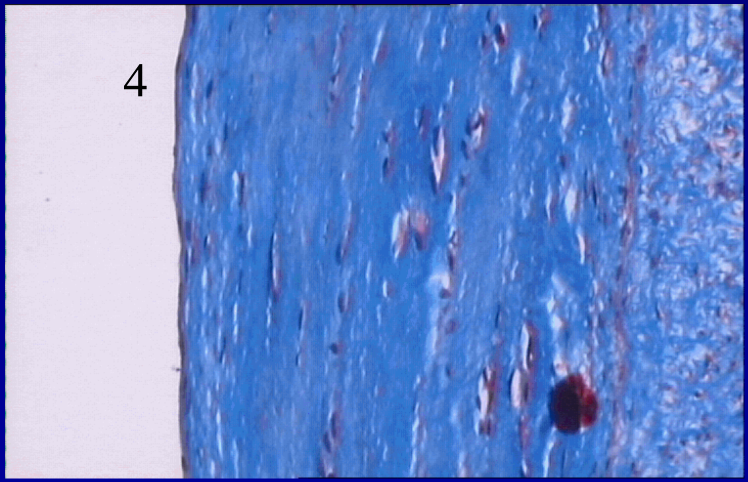
- Relating the microenvironment experienced by a cell in response to global tissue deformation is a reoccurring question
  - Cellular deformation influences biosynthetic activity
- Mow et al. – Chondrocyte deformation and local tissue strain in articular cartilage
- In recent studies, Huang et al. investigated the response of aortic valve interstitial cells (AVIC's) with increasing transvalular pressure[†]
- Cell nuclear aspect ratio was used to measure cell deformation

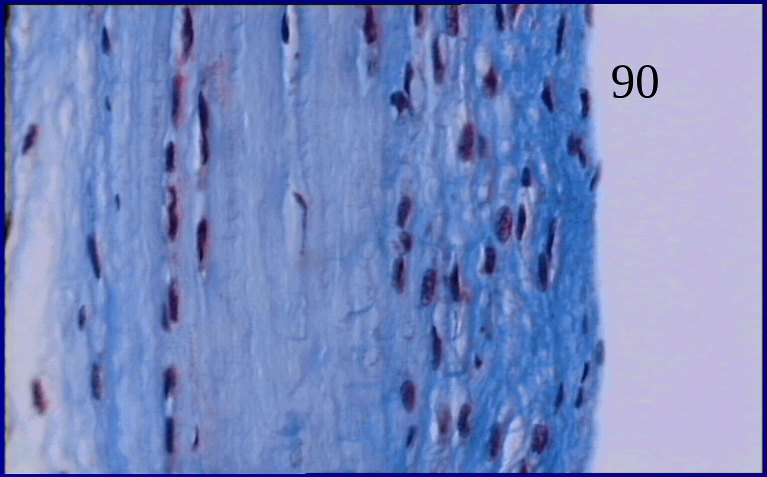
[†] Huang, et al. Effects pf transvalvular pressure on the aortic valve interstitial cell nuclear aspect ratio. JBME. In-press

VIC deformations within HV tissues

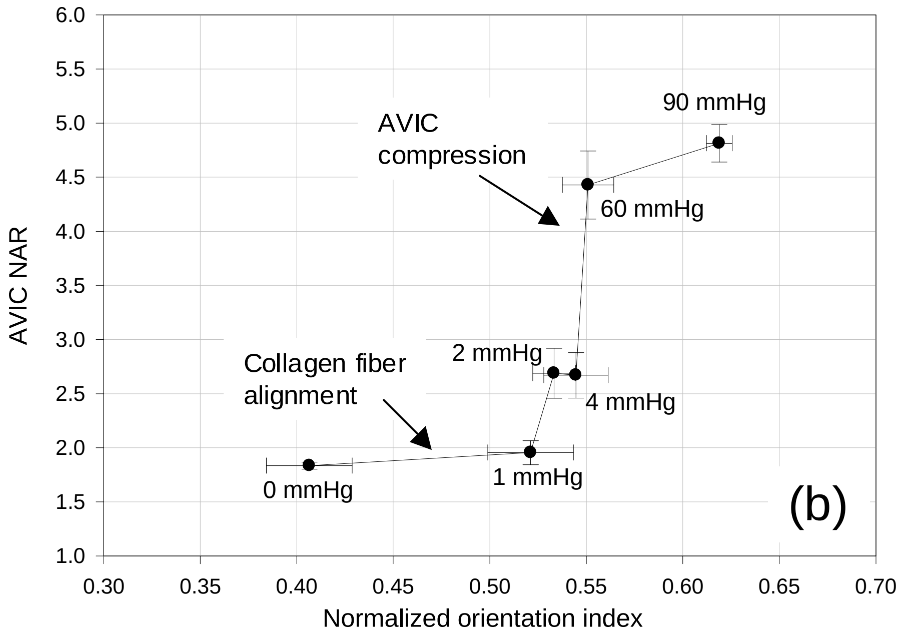


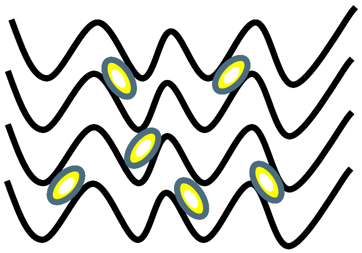




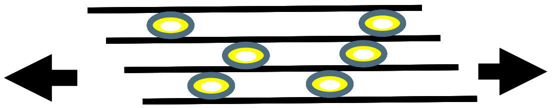


Collagen alignment-VIC aspect ratio

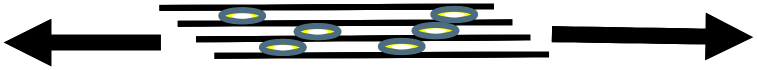




TVP 0 mmHg

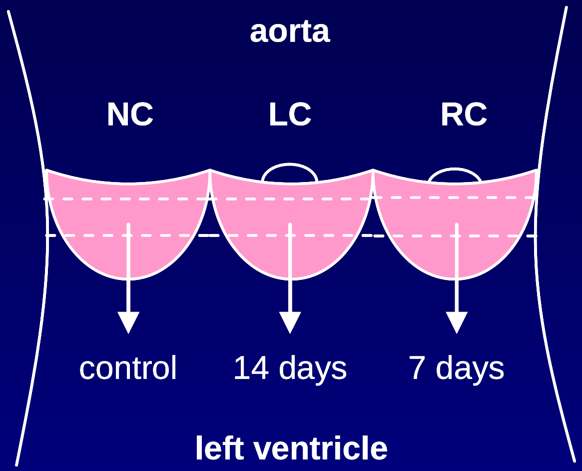


TVP ~4 mmHg



TVP ~60 mmHg
Compaction effects dominate

Mechanical stimulation of heart valve tissues[1]

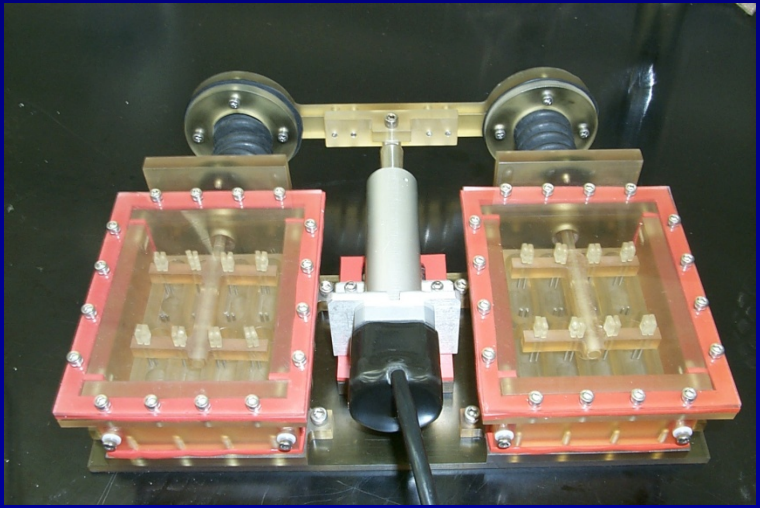


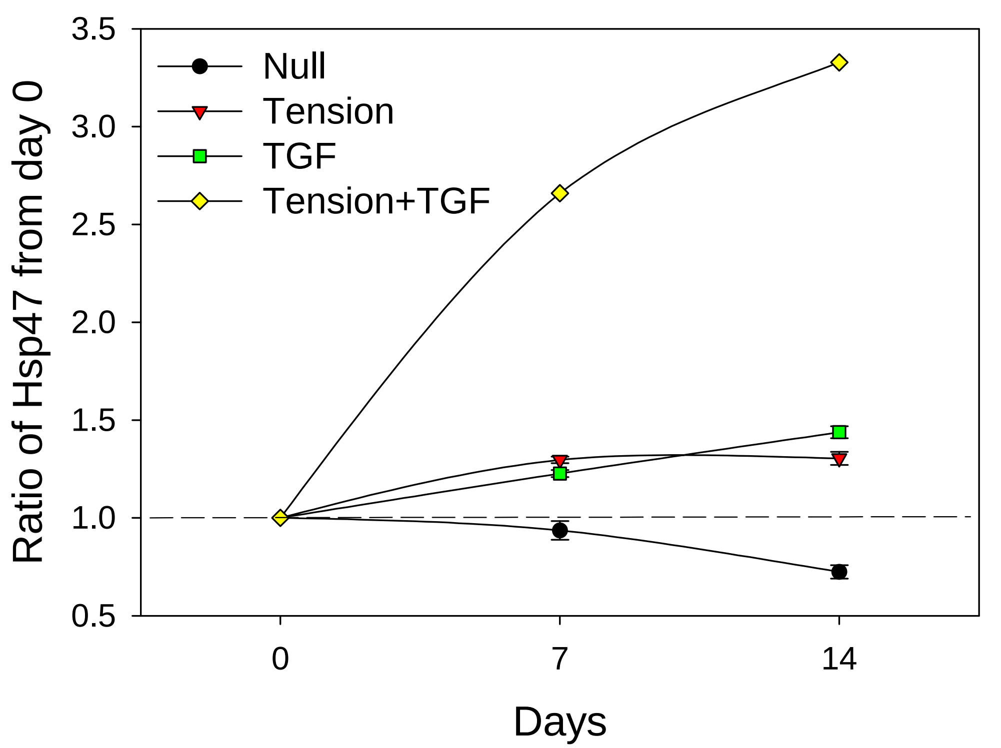
aorta

NC **LC** **RC**

control 14 days 7 days

left ventricle





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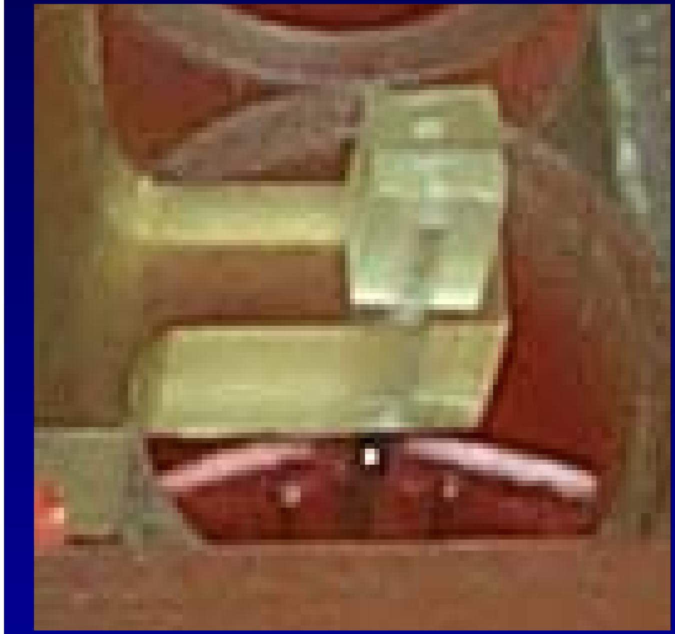
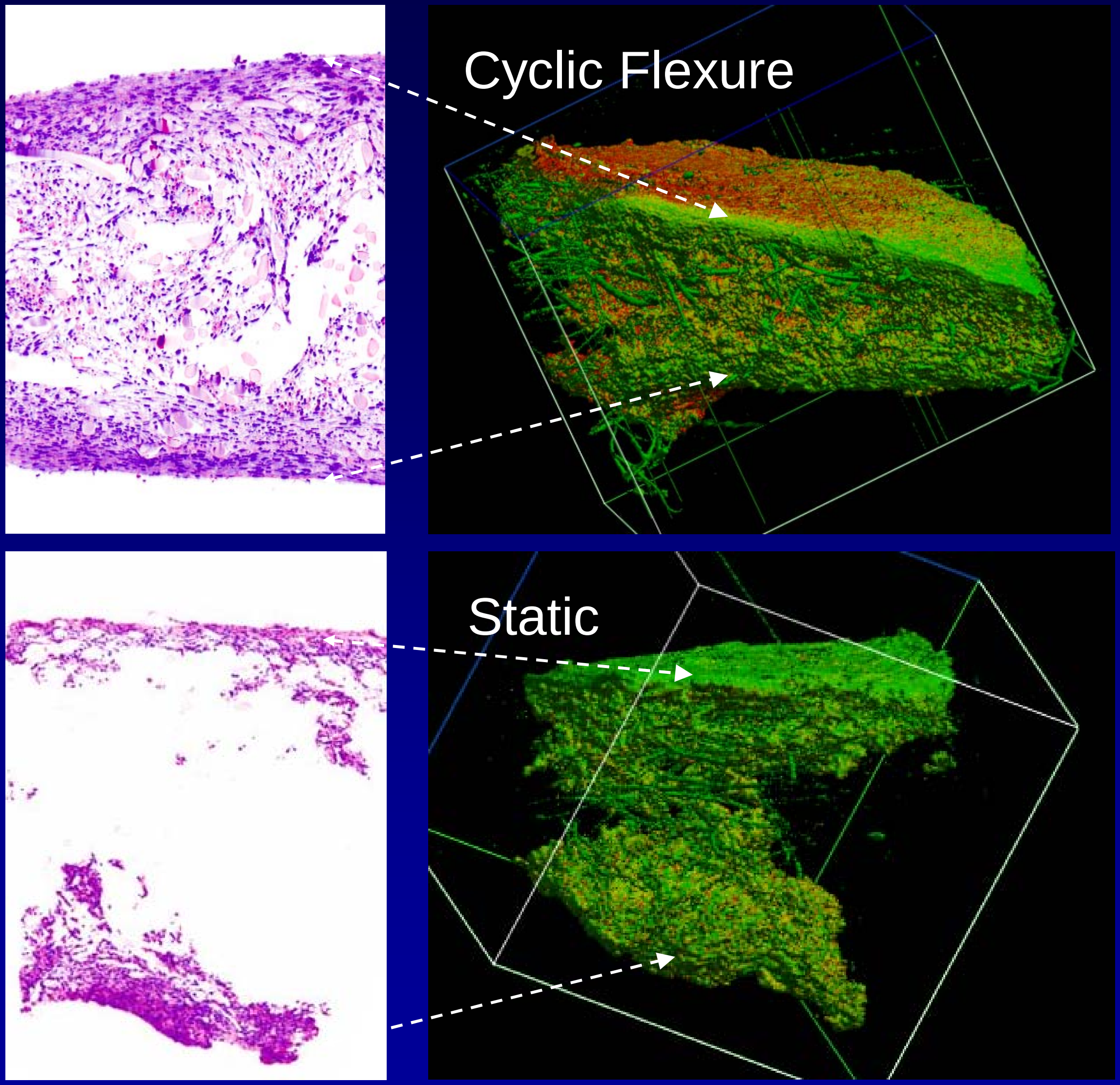
[1] Merryman et al. Cardiovascular Pathology, in-press

Effects of Cyclic Flexure on SMC-Seeded TEHV



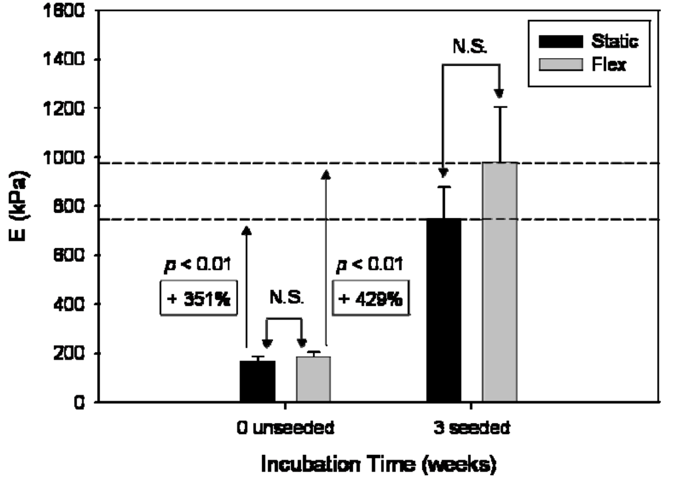


RESView 3D Histology, Resolutions Sciences Corp., Corte Madera, CA

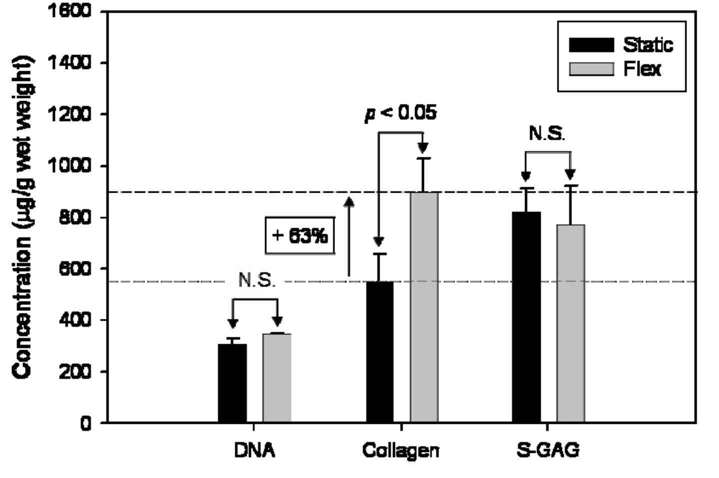
Key Results from Cyclic Flexure Studies

Mechanical Stimulation

Trend of Increased effective stiffness with cyclic flexure compared with static

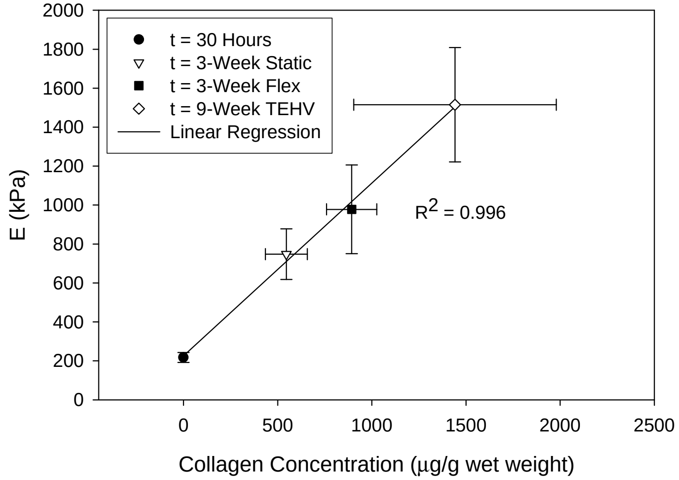


63% increase in collagen concentration with cyclic flexure

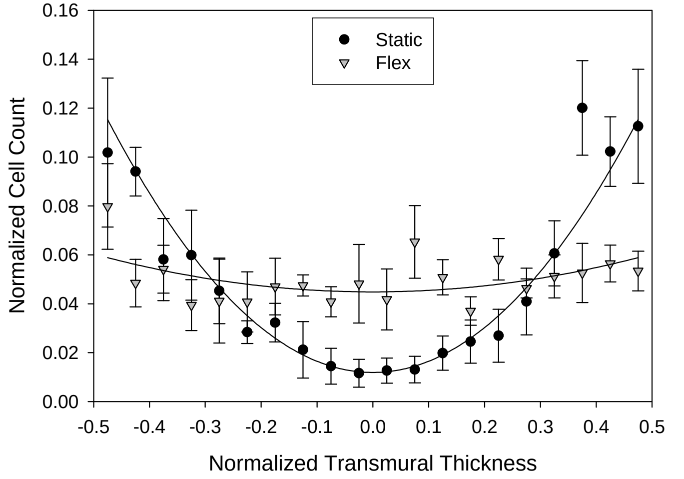


Structural Mechanics

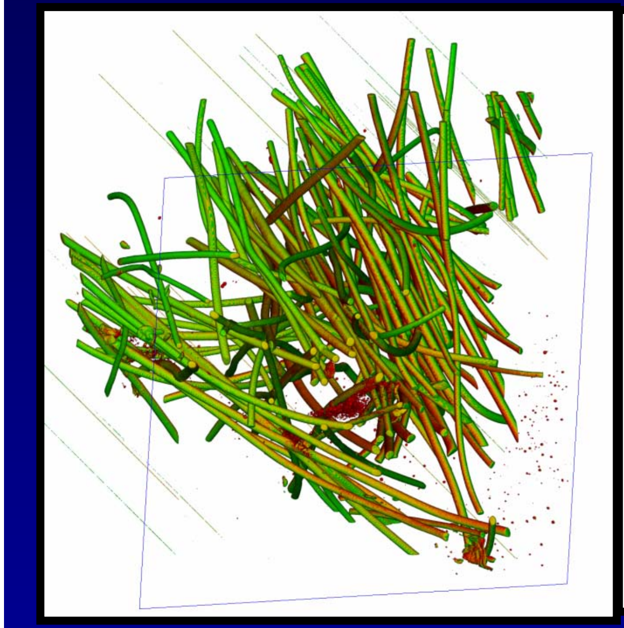
Effective stiffness is highly dependent on collagen concentration



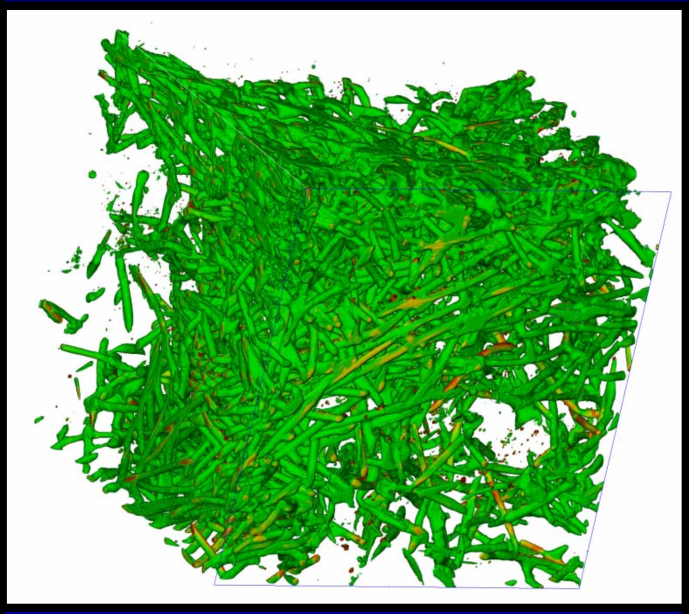
Cyclic flexure can homogenize the transmural cell and ECM distribution

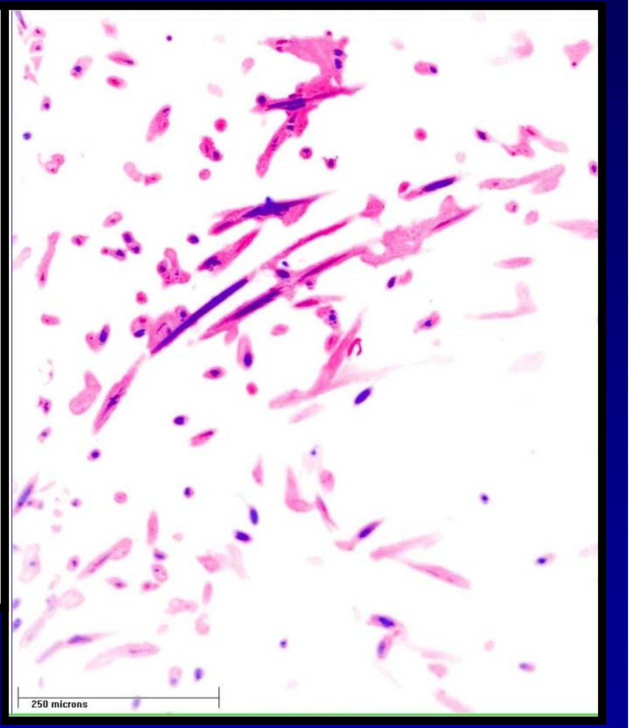


Engineered Heart Valve Tissue



PGA scaffold





TEHV scaffold after 18 day dynamic incubation

Meso-Scale Model for Nonwoven-ECM

$$(EI)_{RVE} = \int_0^w \int_{-t/2}^{t/2} E(y)y^2 dydx$$

ECM-Coupling Parameter

ECM Effective Stiffness

Scaffold Effective Stiffness

$$E(y) = R \cdot E_{ECM}(y) + E_s$$

[Collagen]

$$E_{ECM}(y) = C(y) \cdot B_c \cdot \bar{E}_c$$

Normalized Transmural Collagen Concentration

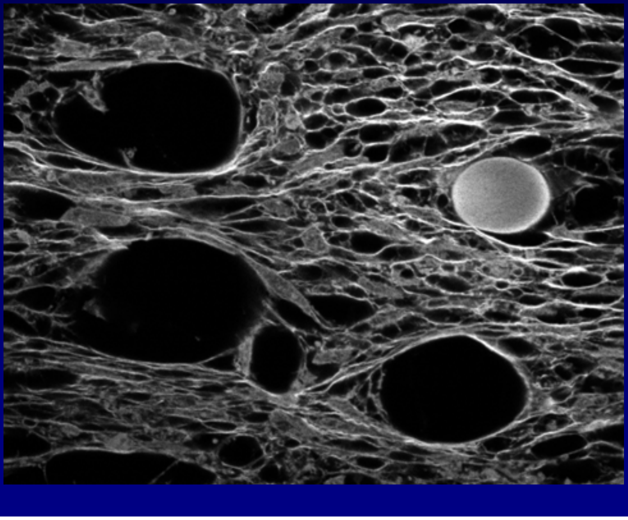
Collagen Specific Stiffness (i.e., stiffness/quantity)

$$(EI)_{RVE} = \int_0^w \int_{-t/2}^{t/2} \left[R\left(C(y) \cdot B_c \cdot \bar{E}_c\right) + E_s \right] y^2 dydx$$

Engelmayr and Sacks, Biomech Model Mechanobiol, 2006, in preparation

C(y): Normalized Transmural Collagen Concentration Distribution

Fluorescence Microscopy of Picro-Sirius Red Stained Sections



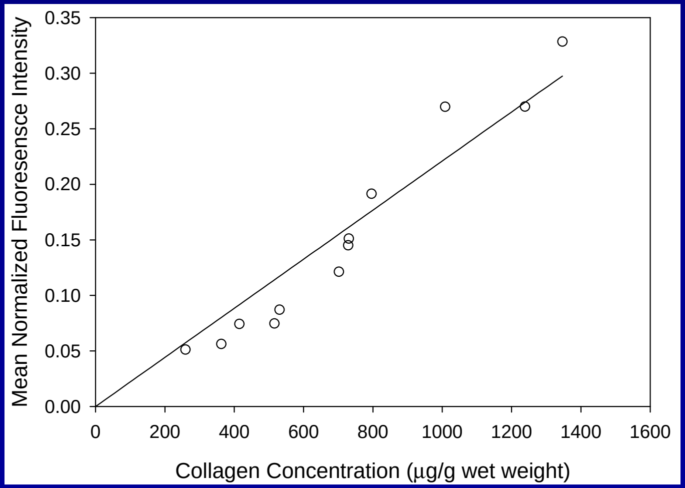
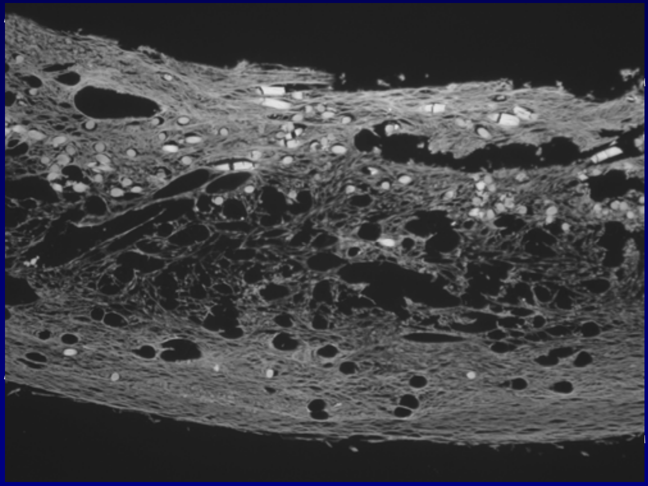
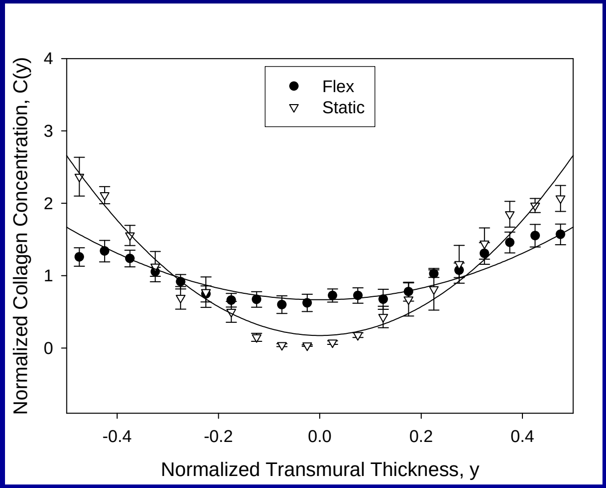


Image Analysis for Normalized Fluorescence Intensity Distribution



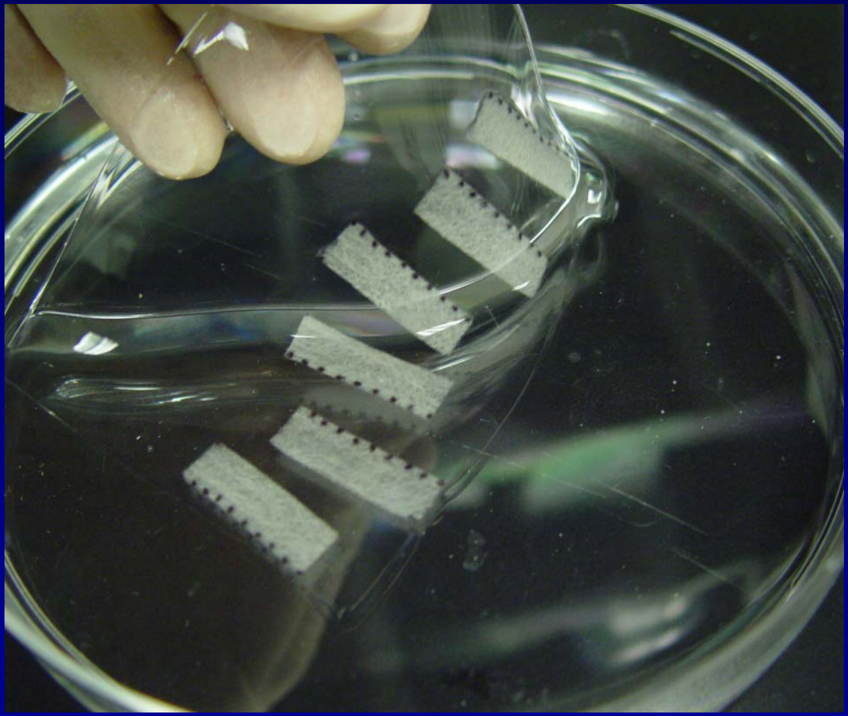


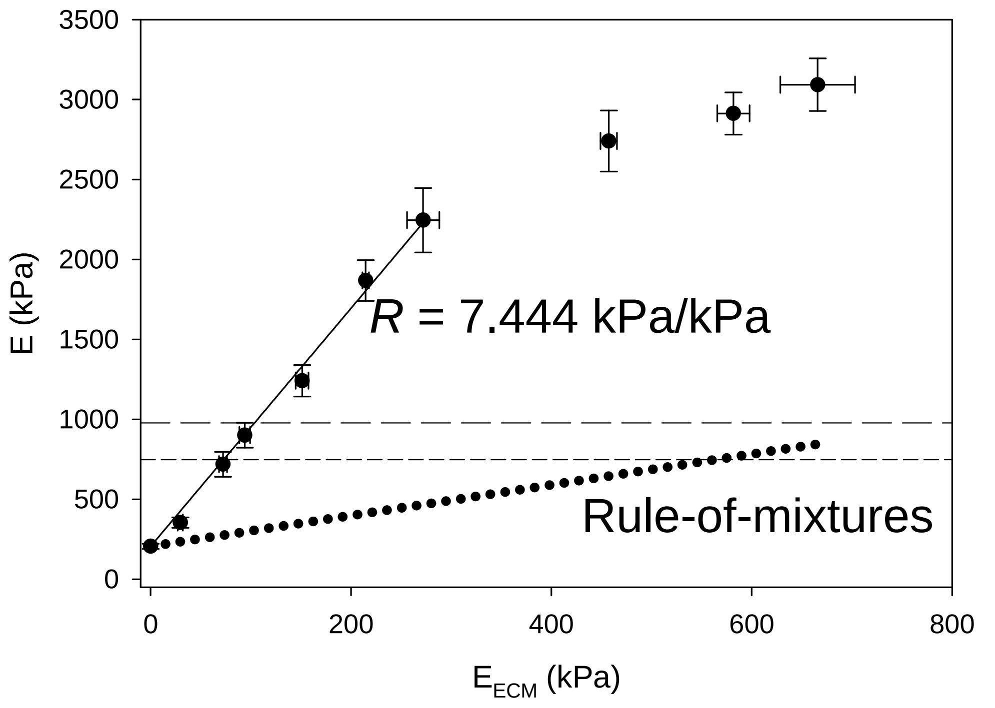
Dolber, P.C. and Spach, M.S., J Histochem Cytochem, 1993, 41(3):465-9.

R: Nonwoven-ECM Coupling Parameter

Physical Models of ECM and TEHV:
Polyacrylamide (Pam) gel and
Pam gel-infiltrated nonwoven scaffold

R is determined from linear region of E versus E_{ECM} plot



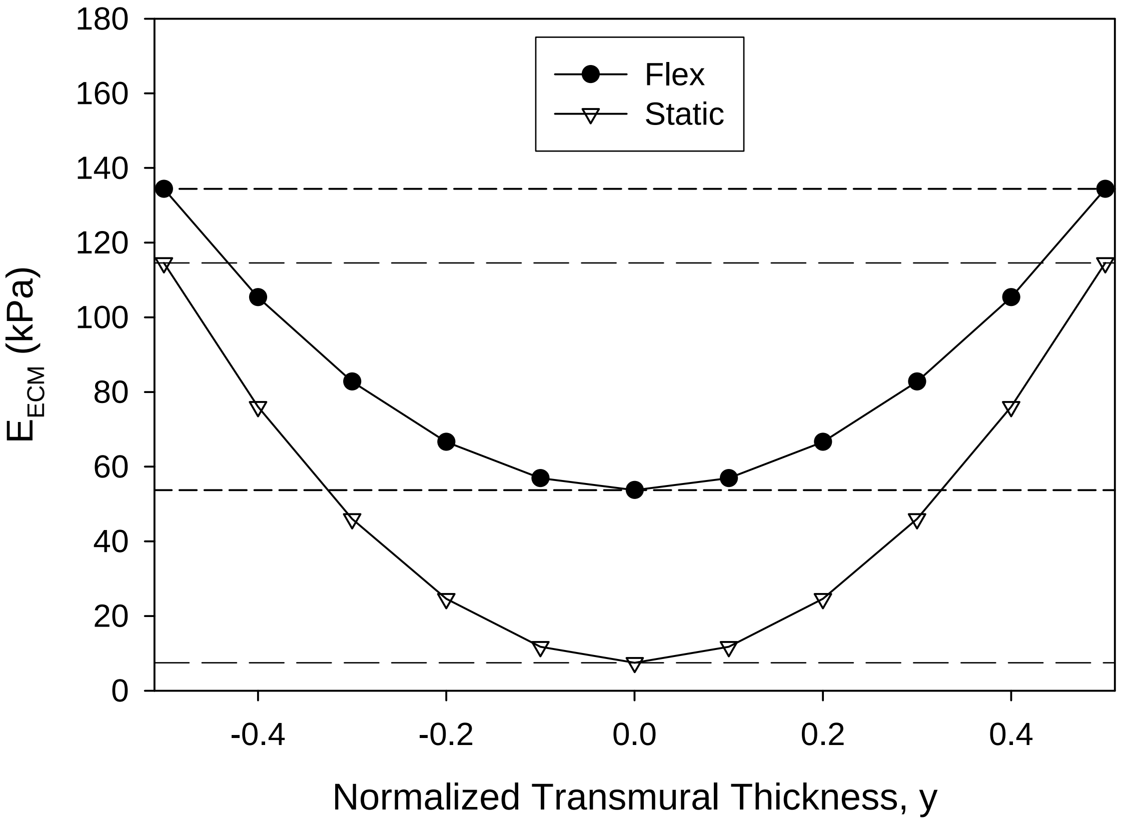


$$E = R \cdot E_{ECM} + \quad {}_{s}$$

$$\cancel{E = E_{ECM} v_{ECM} + E_s}$$

E_{ECM}(y): Transmural ECM Effective Stiffness

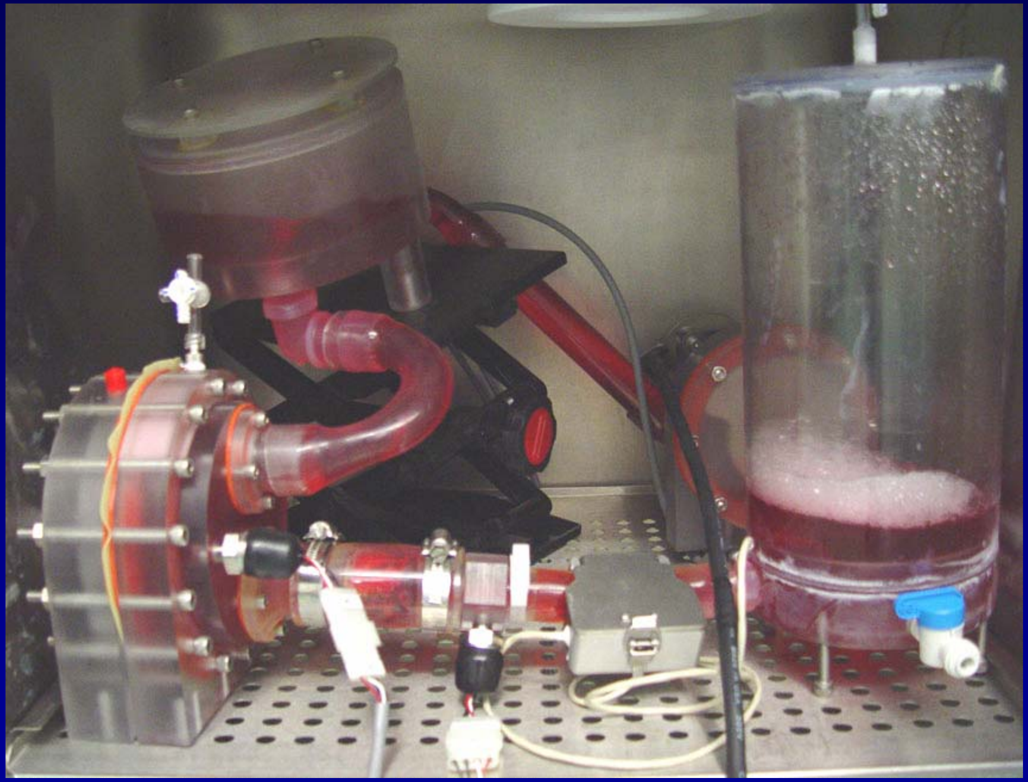
Experimental Group	E (kPa)	B_c (µg/g wet weight)	$C(y) = ay^2 + c$		$\bar{E}_c$ (kPa/(µg/g wet weight)
			a	c	
Static	748 ± 130	893 ± 133	9.946	0.173	0.0789
Flex	978 ± 228	546 ± 111	4.009	0.667	0.0904



Next steps: Scale up

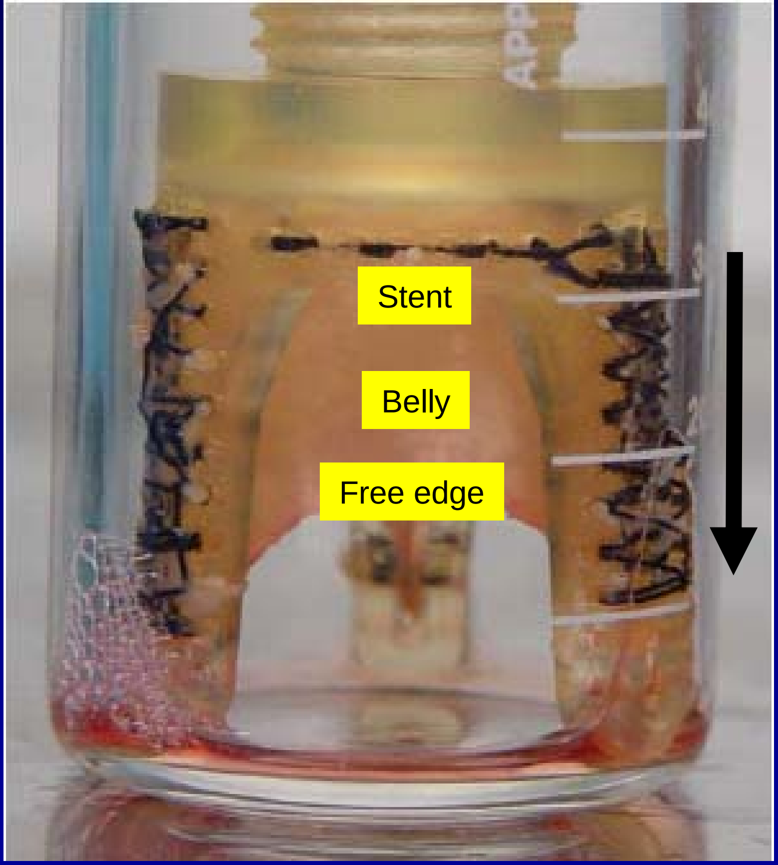
- Local cellular deformations need to be controlled at the macro-level in an intact valve
- Need to balance need for controlled biomechanical stimulation with other valve design requirements

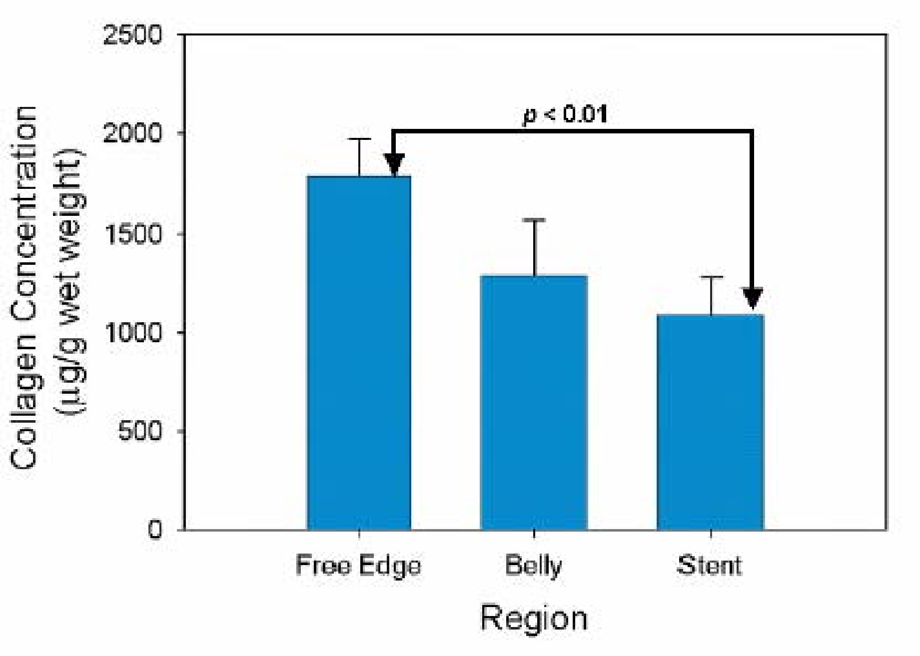
Physiological flow loop bioreactor[1]



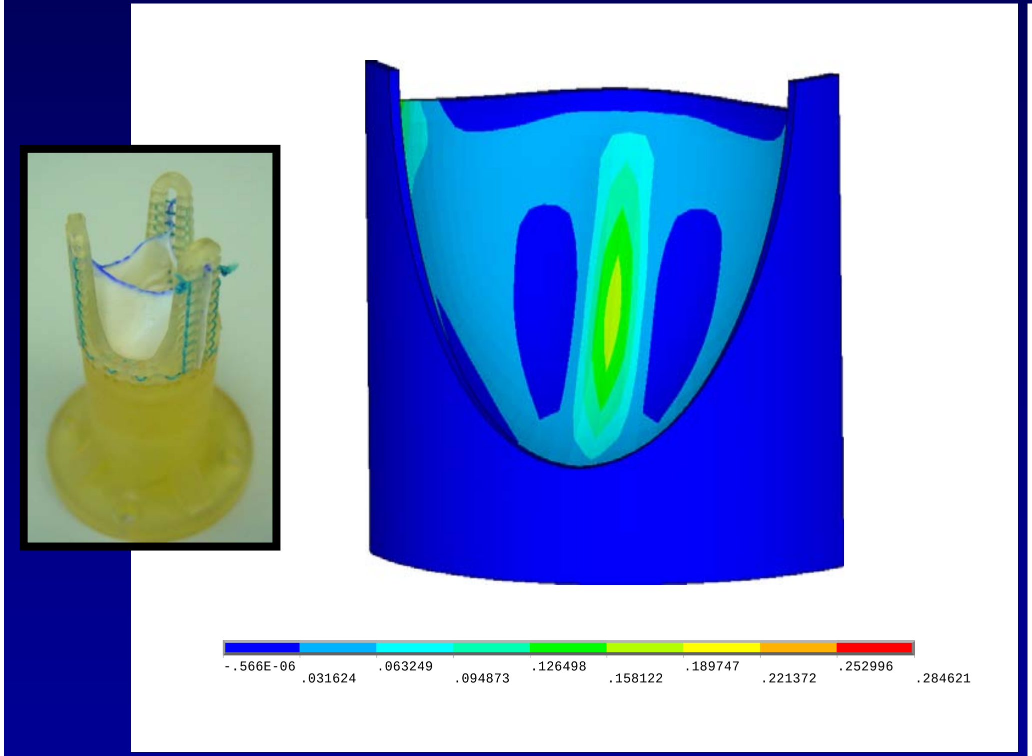
[1] Hildebrand et al. Annals of Biomedical Engineering 2004

Physiological flow loop bioreactor

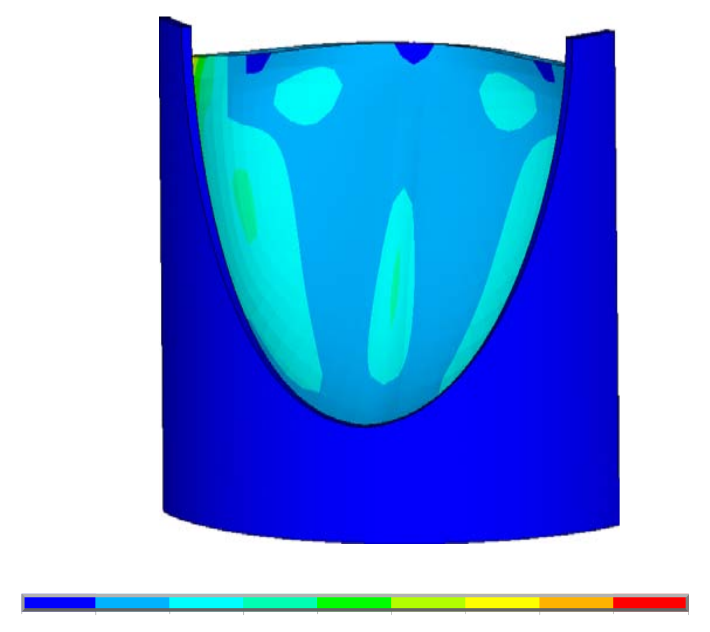




FE simulations of leaflet principal strain (quasi-static loading)



Isotropic fiber distribution



2300 rpm fiber distribution

Ongoing issues and future trends

- Lots of techniques and approaches – what is the correct approach?
  - Driven by functional understanding and application.
- There is more to life than Young’s modulus – what do you measure?
- Biomechanical studies usually require large specimens and large number of specimens due to variability – cost/benefit.

Ongoing issues and future trends

a. Need for non-destructive simultaneous cell/tissue imaging during in-vitro incubation and in-vivo development.
    i. Optical methods
    ii. US
    iii. MRI
b. Need for standardization of approaches – ASTM?
c. Need for low cost, high throughput, physiologically meaningful tests.
    i. Role of commercial sector.

Acknowledgments!

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NHLBI R01s: HL-68816 and HL17649

NIBIB T32 “Biomechanics in Regenerative Medicine”