3D Biotek, LLC

Stem Cell Expansion using Precision 3D Micro-fabrication Scaffolds and Perfusion Bioreactor for Stem Cell Therapy

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3D Biotek LLC

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Outline

- **Introduction**
- **Limitation of 2D cell culture**
- **Development of Novel 3D Scaffolds**

  (3D **Cell Culture: The Ideal Scaffold)**
- **Design of 3D Stem Cell Bioreactor**
- **Stem cell expansion using 3D Bioreactor**

  (challenges and solutions of cell detachment from scaffolds)
- **Summary**

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Introduction

- **3D Biotek LLC:** An Innovative privately held Medical Device Company founded in April 2007. Specialized in research and development of novel 3D Cell Culture Devices

- **Proprietary Platform Technology (US Patent approved 2013)**
  - Precision 3D Micro-Fabrication
  - Advanced Bio-manufacturing Coating Process
- **Current Products**
  - 3D Cell Culture Products for tissue engineering, drug discovery & stem cell research application
  - 3D Perfusion Bioreactor System for R&D

- **Products to be launched soon**
  - 3D Perfusion Bioreactor for production use
    - For Adipose-Derived or Bone Marrow Stem Cells Expansion
    - For therapy (regenerative Medicine) or Bio-banking

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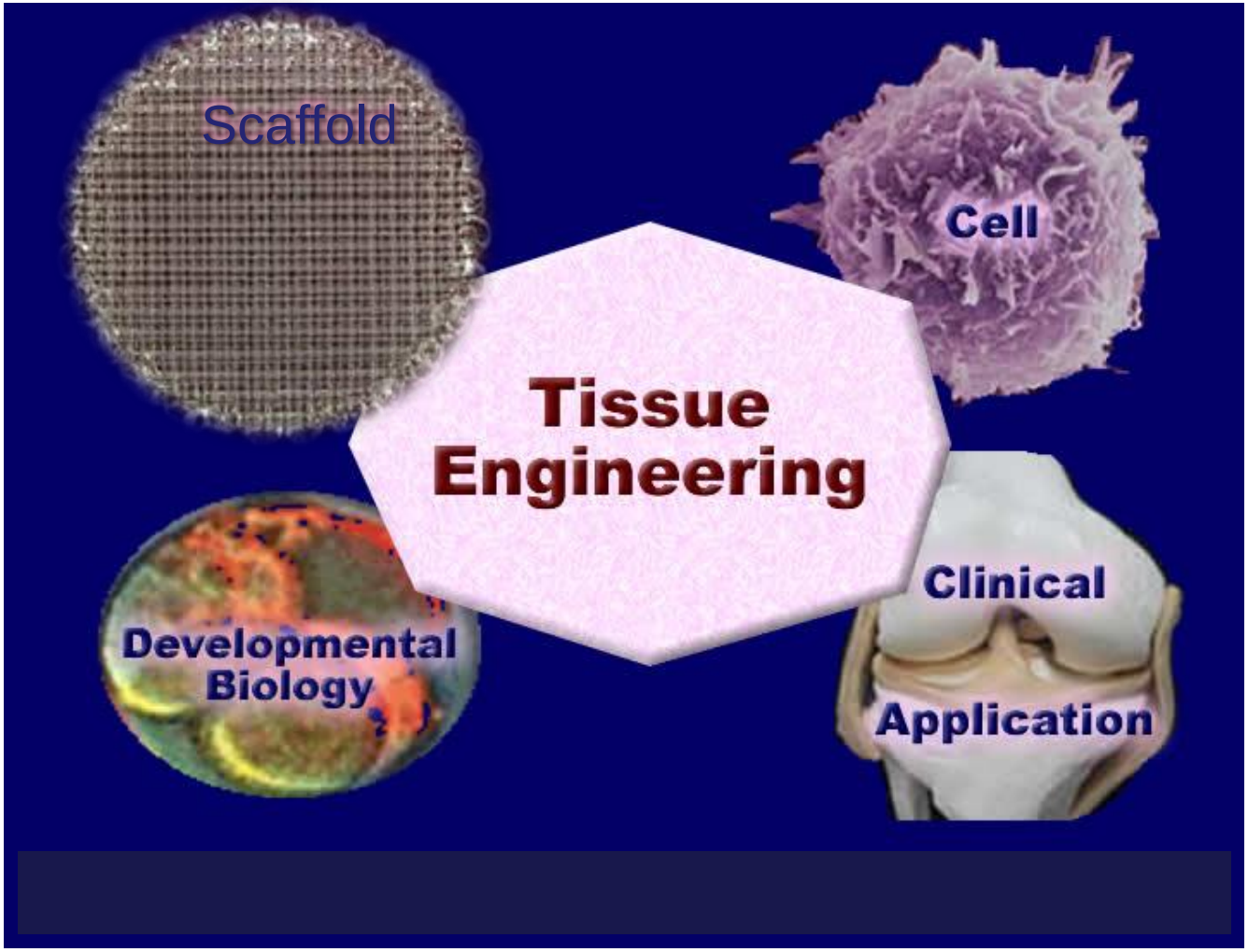
Tissue Engineering

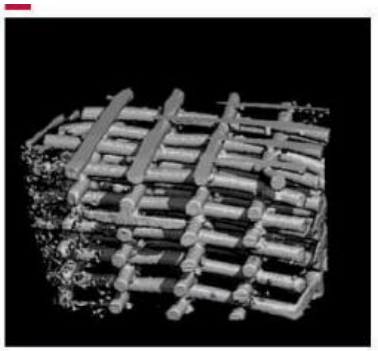
Interdisciplinary field addressing the improvement, repair, or replacement of tissue/organ function.

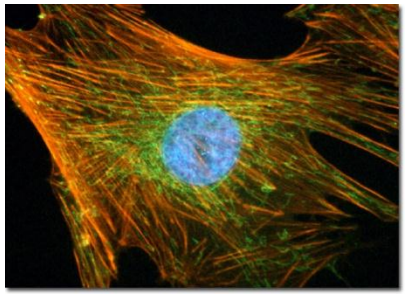


http://www.datlof.com/8Axamal/docs/Marketing/jhu/JE/index.htm

Tissue Engineering

- **Scaffolds**
  - Biomaterials, which may be natural or artificially derived, providing a platform for cell function, adhesion and transplantation
- **Cells**
  - Any class of cell, such as stem or mesenchymal cell
- **Signals**
  - Proteins and growth factors driving the cellular functions of interest
- **Bioreactor**
  - System that supports a biologically active environment





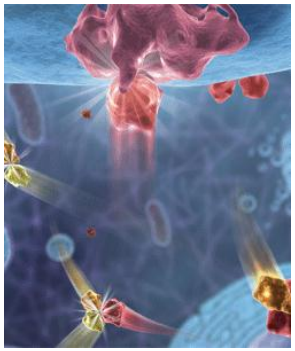


Image sourse: Stke.sciencemag.org, Nature.com

2D Cell Culture

More than 100 years history

Adhering Cell Culture

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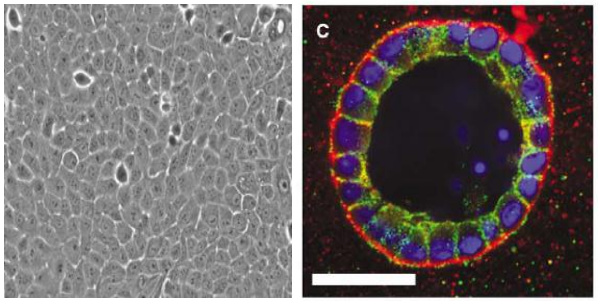
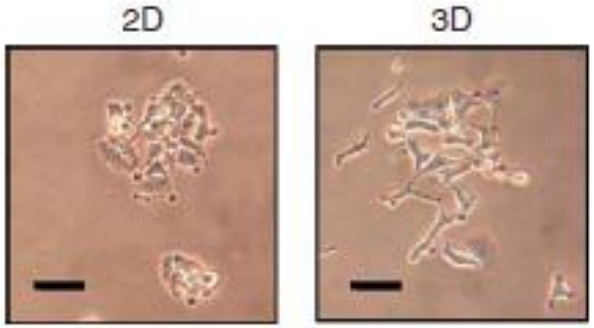
Limitations of 2D Cell Culture

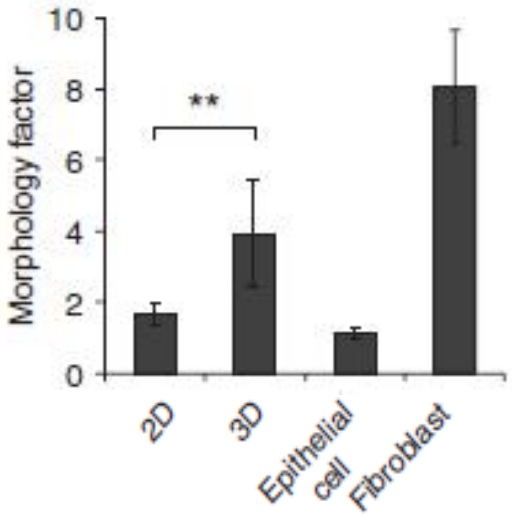
- Limited cell-cell interaction
- Disrupted cellular organization and polarity
- Inaccurate representation of the cellular environment experienced by cells *in vivo*
- Disconnect between cellular behavior *in vitro* and *in vivo*



Debnath J, et al. 2003



Fishbach C, et al. 2007



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Why 3D Cell Culture?

3-D tissue “organoid” tissue models can replicate:

- Cell-cell interactions
- Cell-matrix interactions
- Complex extracellular matrix
- Diffusion characteristics
- Multicellular organizations like in vivo tissues.

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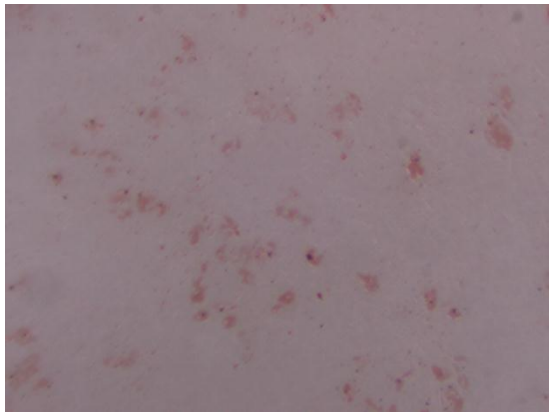
3D PS Scaffolds For Stem Cell Research

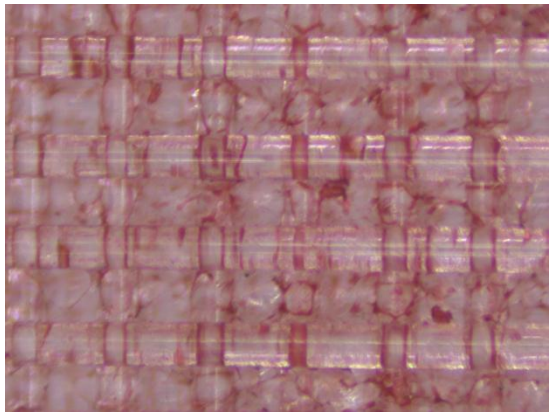
Fat: adipocytes

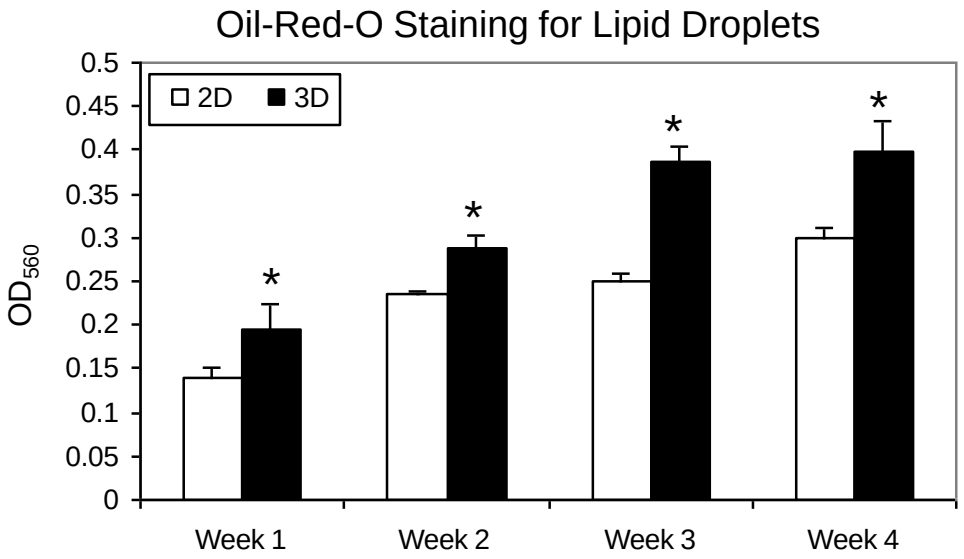
Lipid Droplets

2D



3D





p≤0.05

Human mesenchymal stem cells (hMSCs) on PS scaffolds cultured under adipogenic conditions and stained for lipid droplet formation using Oil-Red-O staining.

Development Of Novel 3D Scaffolds

- Non-toxic
- Well-defined pore size and fiber diameter
- Free of animal-derived material
- Reproducible from batch to batch
- Compatible with current 2D assays

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Scaffold materials

Definition: - Biomaterials, which may be natural or artificially derived, providing a platform for cell function, adhesion and transplantation

- **Polymers**
- **Natural Polymers (Alginate, Collagen, Silk)**
- **Synthetic Polymers (PCL, PEG, PLGA, PPF, PS)**
- **Ceramics**
  - Calcium Phosphates (HA, TCP)
  - Bioactive Glasses (Bioglass)
- **Metals (Mg, Ta, Ti)**
- **Composites (Nanomaterials)**

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Core Technology: 3D Insert™ Platform

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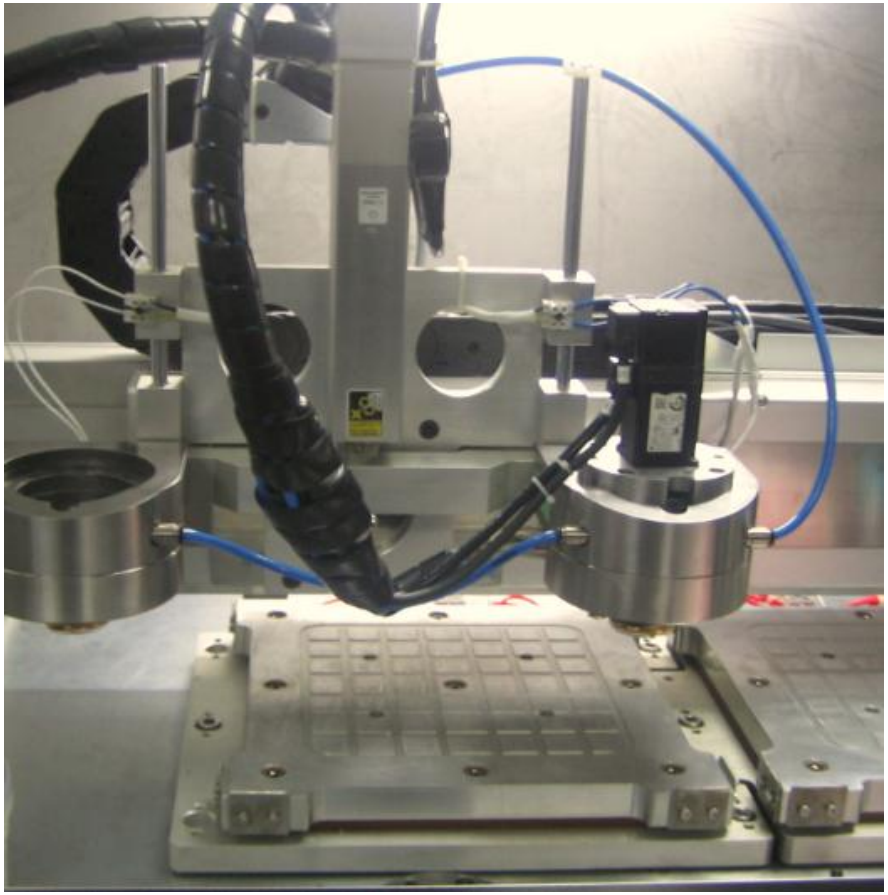
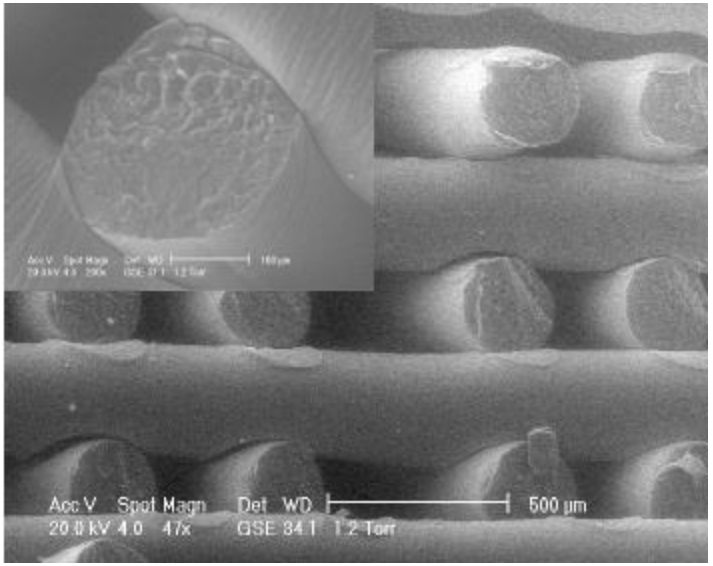
Add Extra Dimension to Your Innovation

Precision 3D Micro-Fabrication Technology





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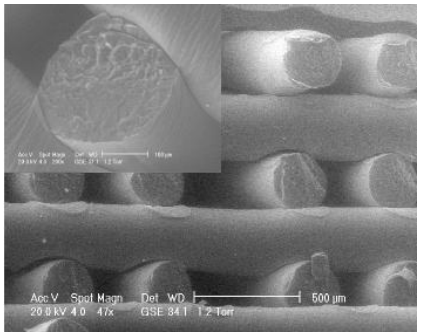
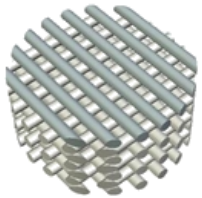
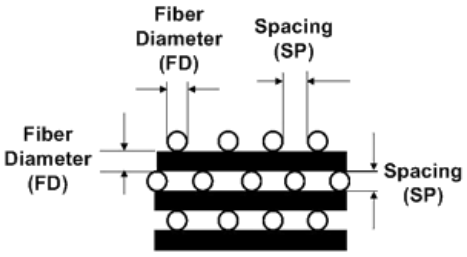
3D Insert™ Advantages in Cell Culture

- Well-defined pore size and porous structure
- 100% Pore Interconnectivity
- Solvent-Free and Non-Toxic
- Compatible with Current 2D Assays
- Batch-to-Batch Reproducibility
- Free of Animal-derived Material
- Custom Designed Fabrication

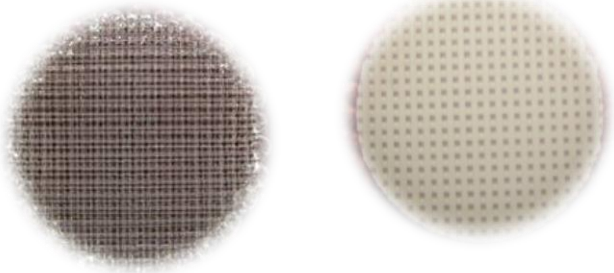




3D Insert™ Structure



3D Insert™ Structure Parameters



3D Insert™-PS 3D Insert™-PCL

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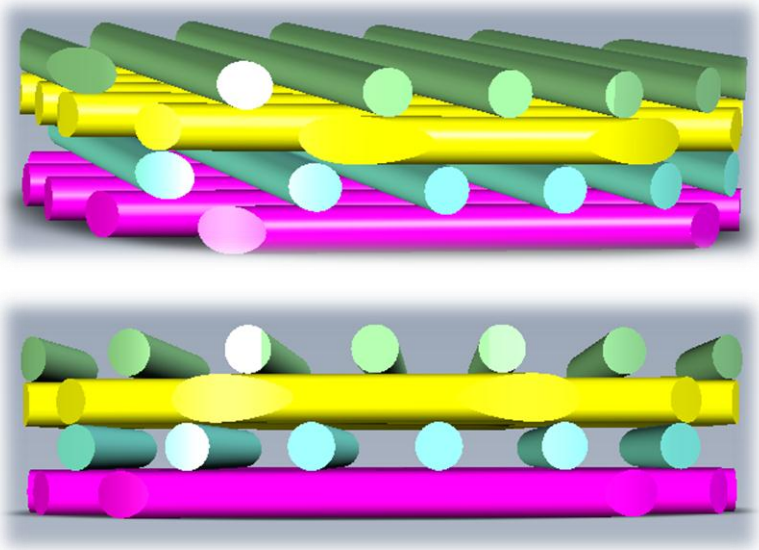
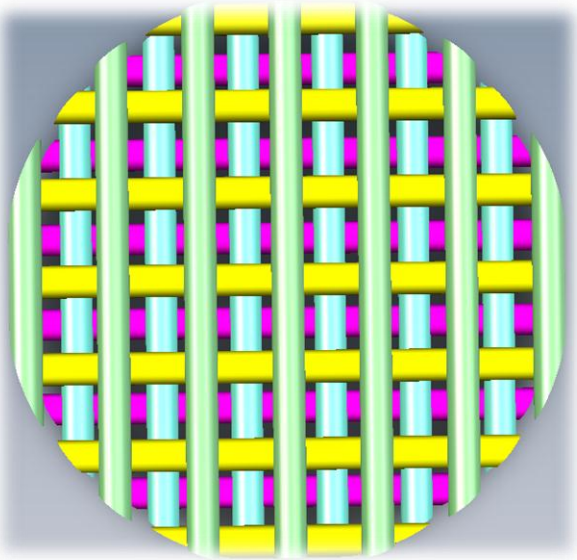
3D Insert™ PS Scaffolds

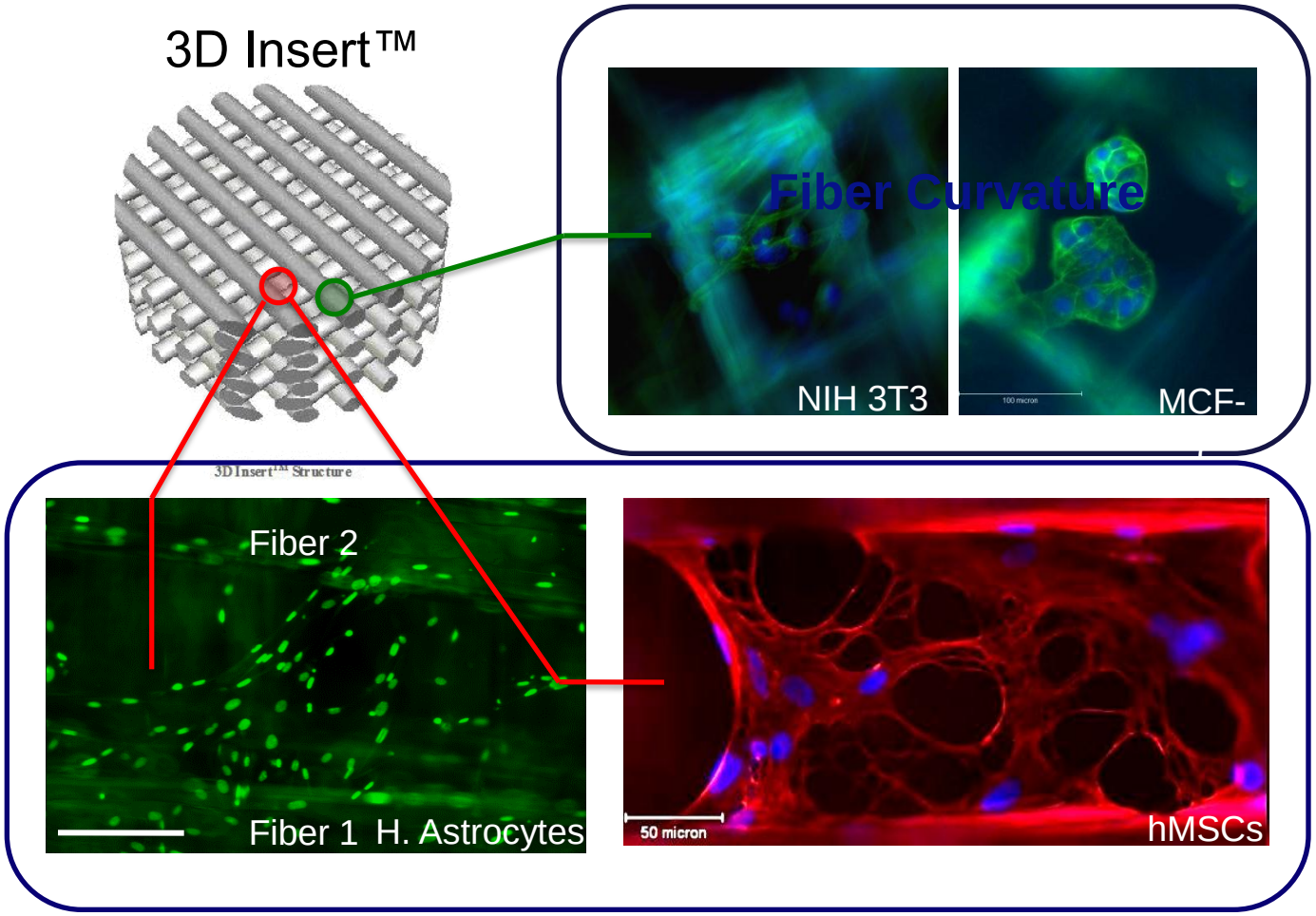
Unique Easy Monitoring/Imaging Structure Design





- **Fiber Size:** micron-scale curvature
- **Fiber Spacing:** enhancement of 3D cell infiltration
- **Layering:** 3D structure interconnectivity

3D Tissue like Structure/ Morphology



Pore Structure

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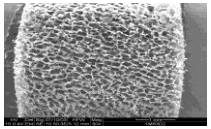
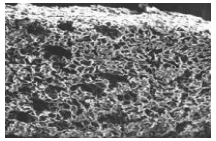
3D Cell Culture: The Ideal Scaffold

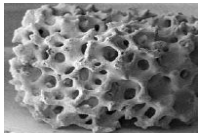
Matrigel / PuraMatrix /Coatings



AlgiMatrix

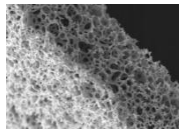


3D Calcium Phosphate Scaffold

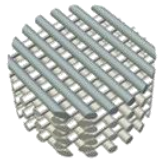


3D Collagen Scaffold

3D OPLA Scaffold



Alvatex



3D Insert Structure

	Ready to use	100% interconnected pores	High surface to volume ratio	Variable configurations (customizable)	Easy cell recovery	Plate reader compatible	Transparency (direct observation with light microscope)
3D Insert™	★	★	★	★	★	★	★
Gel Matrices	☆	☆	☆	★	☆	☆	★
PLA foam	☆/★	☆	★	☆	☆	☆	☆
CaP foam	★	☆	★	☆	☆	☆	☆
Alginate Foam	★	☆	★	☆	☆	☆	☆
Alvetex®	★	☆	★	★	☆	★	☆

★ **Compatible**

☆ **Not Compatible**

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Wide range of research applications with 3D PS Scaffolds

- In Vitro Tumor Models
- Drug Discovery
- Stem Cell Research
- Tissue Engineering
- Cell Biology

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Add Extra Dimension to Your Innovation

Dynamic Perfusion Bioreactor

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Dynamic Perfusion Bioreactors: *3D Insert™ Inside*

A system that support a biologically active environment

- ***3DKUBE for Small Scale Dynamic Cell Culture***
- ***3D Perfusion Bioreactor for Large Scale Cell Culture***

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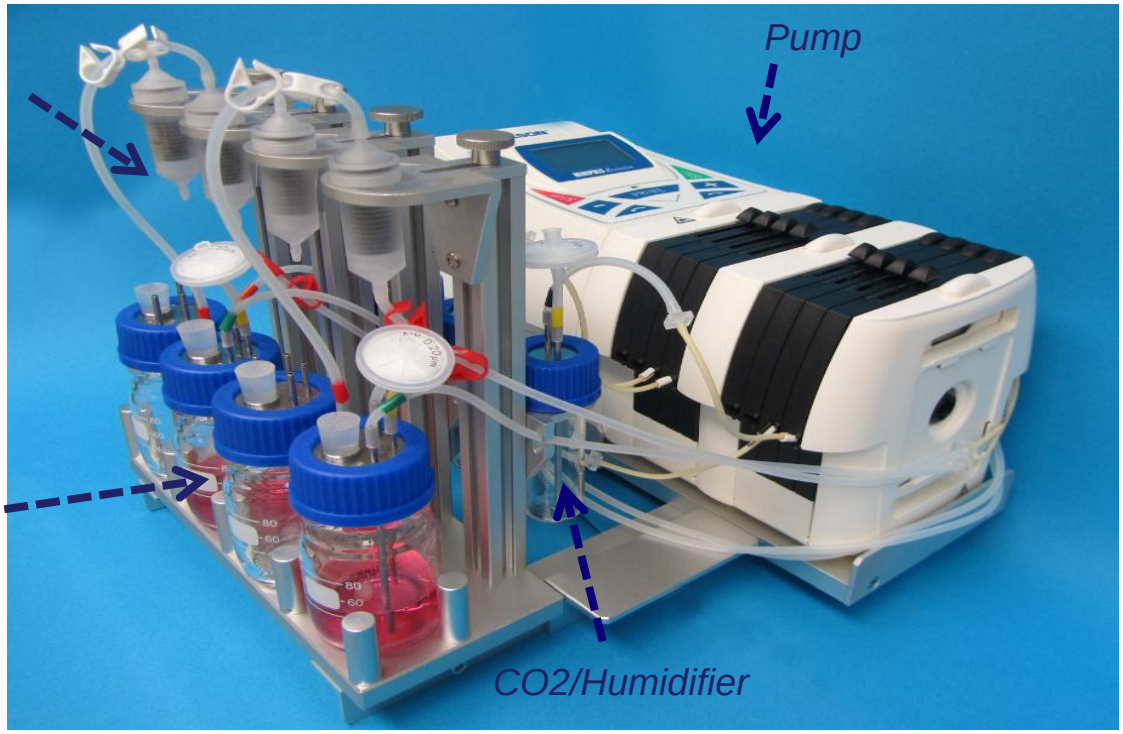
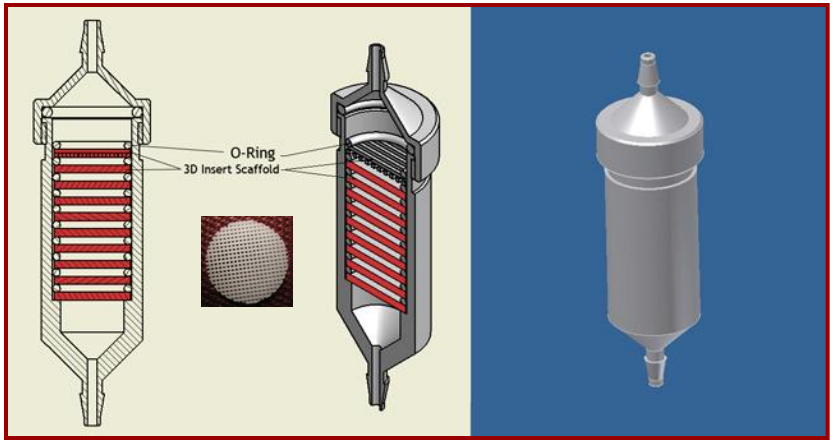
Small 3D Perfusion Bioreactor for R&D





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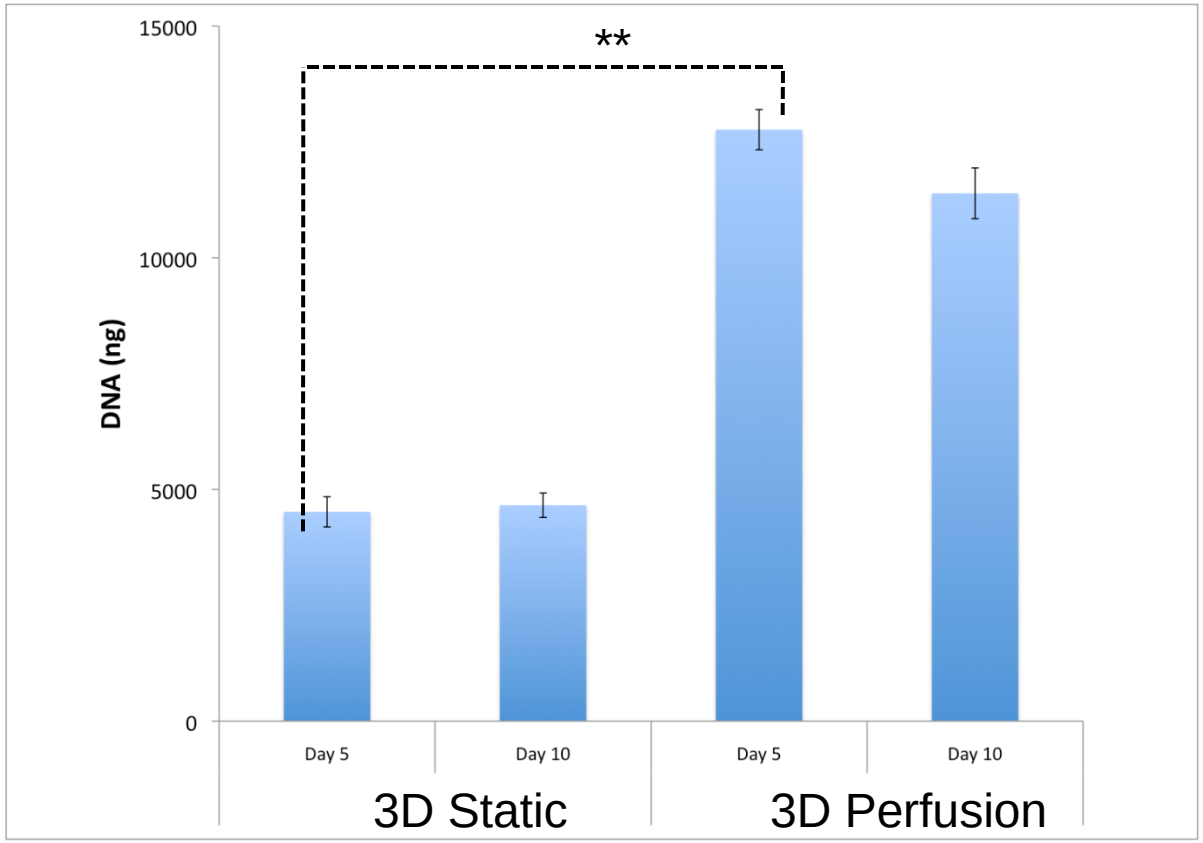
3D Perfusion Bioreactor: 3D Insert™ Inside



n = 3
(STDEV)

3D Perfusion Causes Early Proliferation of Osteosarcoma

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3D Perfusion Bioreactor for Large Scale Cell Culture

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3D Stem Cell Bioreactor

- Integrated Bioreactor/Incubator System for production use
- Optimized for Adipose-Derived Stem Cell (ADSC) Expansion
- Cells are seeded and grown on 3D Biotek's Polystyrene Scaffolds
- Targeted cell expansion up to ~200 million cells in 10—14 days
- Suitable for use in stem cell therapy and bio-banking
- Disposable components for convenience
- Significantly reduce intense labor as required in 2D culture system (Flasks and CellStack)
- System can be easily modified for protein production
- Patent Pending (US Serial# 62/380,414)

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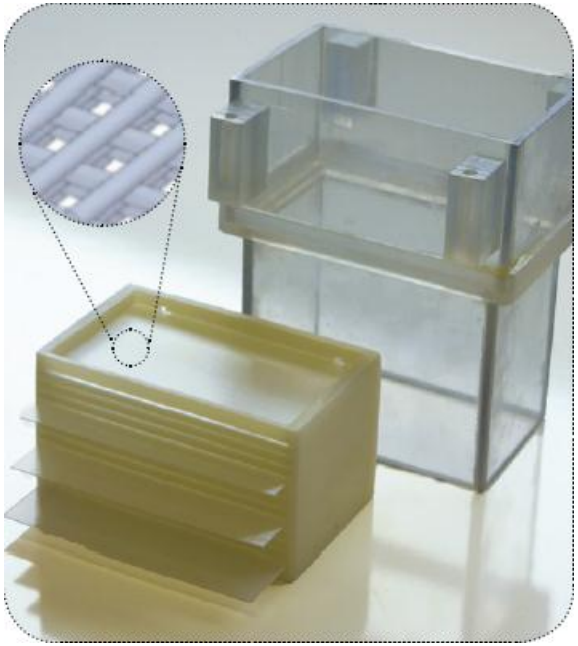
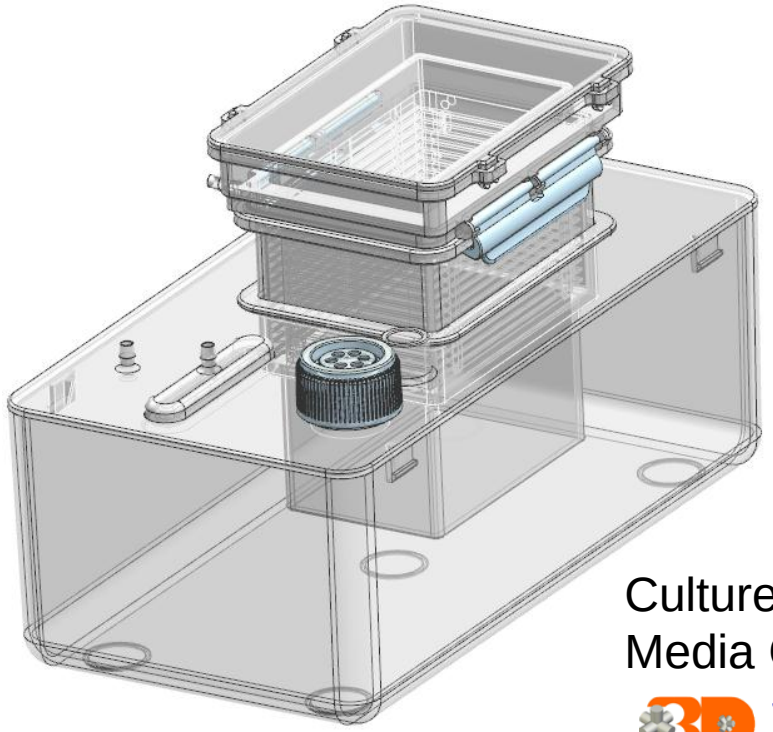
3D Stem Cell Expansion System



Scaffolds, Holder & Chamber



Culture Chamber &
Media Container

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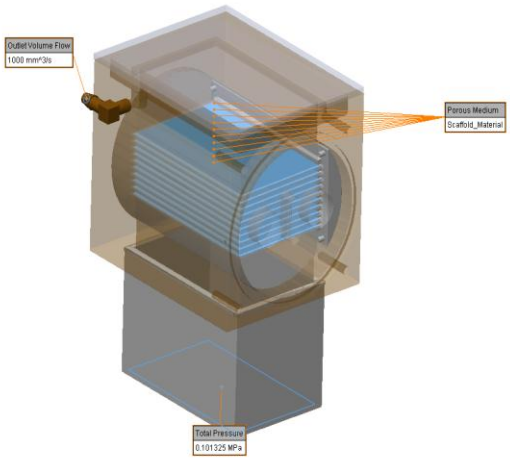
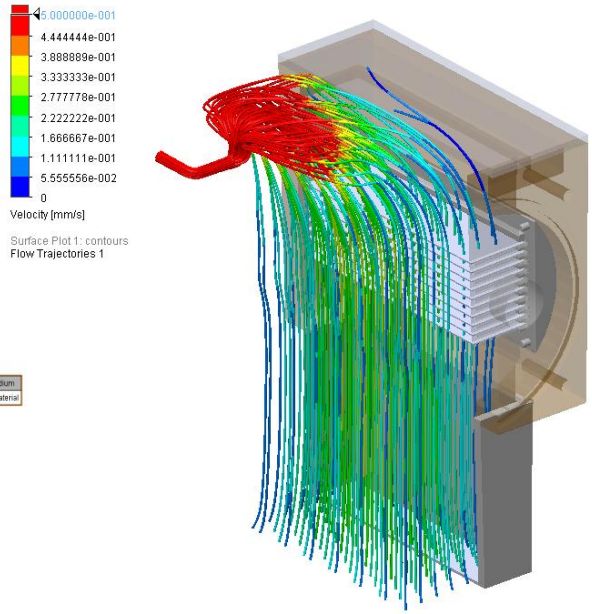
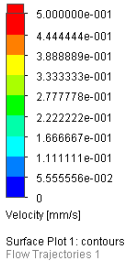
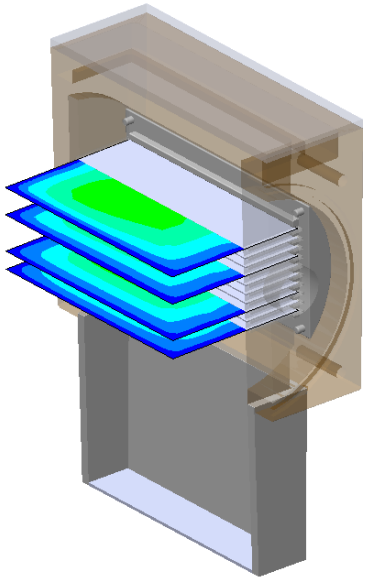
Overall Velocity Flow Field on Scaffold with Flow Rates, Q=60ml/min









Flow finds easiest path to go from entry to exit port. Uniform flow throughout the scaffold. The right figures shows consistent flow velocity magnitudes for 4 scaffold surfaces. Even at 60ml/min, the flow velocities do not cause any significant wall shear.

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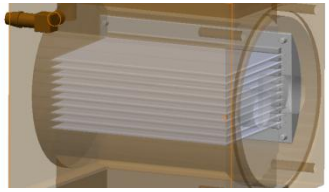
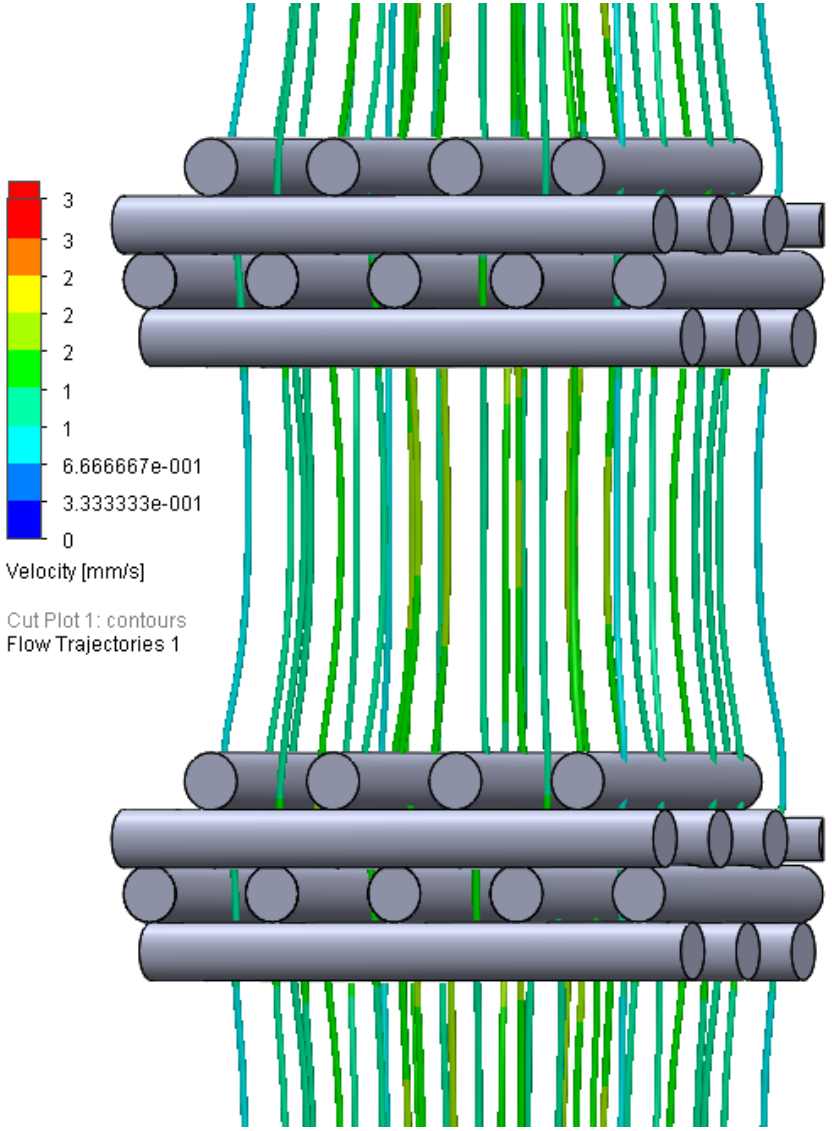
Fluid Flow Around Scaffold in





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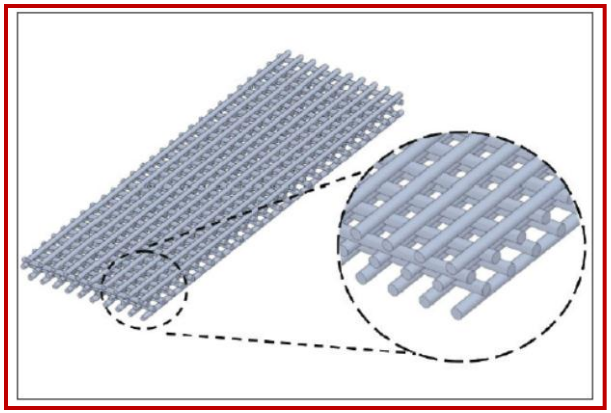
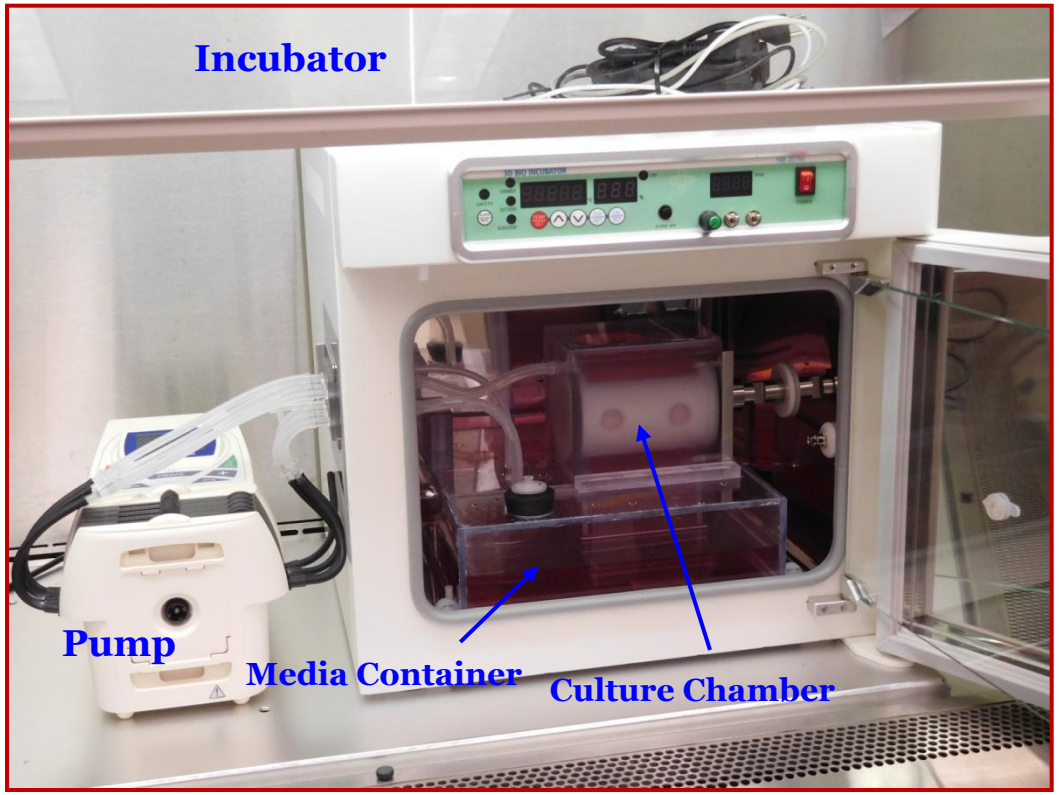
3D Stem Cell Bioreactor





Integrated Bioreactor/Incubator System

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Substantial Equivalence (SE) Comparison

Characteristics/Attributes	Proposed Device: 3D Cell Expansion System (3D Biotek)	Predicate Device: Xpansion® (PALL Corporation)	Predicate Device: U-CUP (CELLEC BIOTEK)
Intended Use	Intended for cell expansion	Intended for cell expansion	Intended for cell expansion
Tissue culture environment	3D tissue culture	2D tissue culture	3D tissue culture
Design	Large-Scale 3D Dynamic perfusion	Large-Scale 2D Dynamic perfusion	Small-Scale 3D Dynamic perfusion
Materials	Polystyrene 3D porous scaffolds	Polystyrene tissue culture flask	3D porous scaffolds made of ceramic and synthetic or natural polymer based
Media and reagent usage/Labor	Does not require addition or change of media during the experimental process	Require addition or change of media during the experimental process	Minimum hands on after setup
Sterility	Gamma-irradiation	Gamma-irradiation	Gamma-irradiation

Substantial Equivalence (SE) Comparison

Characteristics/ Attributes	Proposed Device: 3D Cell Expansion System (3D Biotek)	Predicate Device: Vitafiber FLO-PATH Bioreactor-Amicon Inc.	Predicate Device: Lifecell Recovery Container-Baxter Healthcare Corp.
Intended Use	Intended for cell expansion	used to produce large quantities of proteins such as monoclonal antibodies	Cell culture bag used for expansion and transportation of suspension cells
Tissue culture environment	3D tissue culture	3D tissue culture	2D tissue culture
Design	Dynamic perfusion	Hollow fiber FLO-PATH bioreactor	Cell Culture Bags
Materials	Polystyrene 3D porous scaffolds	The hollow fiber permeable membranes made of substances such as cellulose acetate or polypropylene	made of proprietary polyolefin blends
Media and reagent usage/Labor	Does not require addition or change of media during the experimental process	Circulating media environment-Does not require change of media during experiment	Static tissue culture (not circulating)
Sterility	Gamma-irradiation	Not known	Not known
Where Used:	Research & Clinical tissue culture laboratories	Research & Clinical tissue culture laboratories	Research & Clinical tissue culture laboratories

Expansion schedule for ASC

Days	0	2	4	6	8	10	12	14	16	18	20	22	24
Doubling time (x1000)	Starting cell number	1	2	3	4	5	6	7	8	9	10	11	12
	55	110	220	440	880	1760	3520	7040	14080	28160	56320	112640	225280
	110	220	440	880	1760	3520	7040	14080	28160	56160	112640	225280	
	220	440	880	1760	3520	7040	14080	28160	56160	112640	225280		
	440	880	1760	3520	7040	14080	28160	56160	112640	225280			
	880	1760	3520	7040	14080	28160	56160	112640	225280				
	1760	3520	7040	14080	28160	56160	112640	225280					
	3520	7040	14080	28160	56160	112640	225280						
	7040	14080	28160	56160	112640	225280							
	14080	28160	56160	112640	225280								
	28160	56160	112640	225280									
Paasage	1				2			3			4		

- Seeding: 2500/cm2
- Assuming doubling time is 48 hours
- Maximum passage before differentiation is 4
- Each passage is 3 doubling time

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present of MSC on different tissue

The tissues that contain MSC:

- bone marrow (BM) is the most widely recognized source of MSCs,
- alternative sources of MSC-like cells has been identified
  - including adipose tissue
  - placenta
  - dental pulp
  - synovial membrane
  - peripheral blood
  - periodontal ligament
  - endometrium
  - umbilical cord (UC)
  - umbilical cord blood (UCB)

In fact, evidence has suggested that MSCs may be present virtually in any vascularized tissues throughout the whole body

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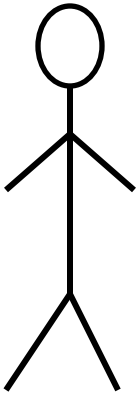
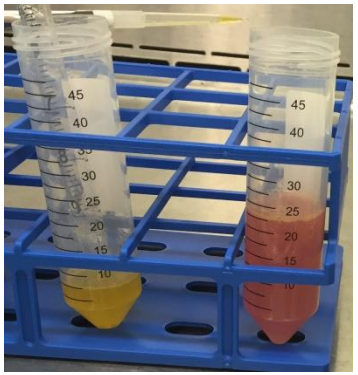
Isolation of Adipose stem cells from fat



liposuction

Adipose tissue/
lipoaspirate



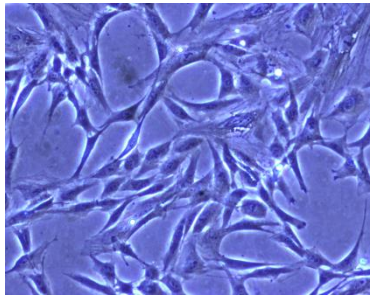
Aspirate fat and blood

Digest tissue

50 mls lipoaspirate- got 2 million SVF
after 1 week 21 million SVF

Markers	Cultured SVF-passage 0
CD44/CD73/CD90/CD105	> 97%
CD45/CD34/CD19/CD11b/HLA-DR	< 23%

Flow Cytometry



Pellet cells/SVF
And plate

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Stem cell expansion using Bioreactor

- Static cell seeding of Adipose-derived stem cells (ASC)
  - 12 x 10^6 cells on 12 scaffolds
  - Perform Flow cytometry for stem cell markers (Before bioreactor)
    - CD44, CD73, CD90, CD105 are positive markers and
    - CD11b, CD19, CD34, CD45, HLA-DR are negative markers

- 48 hrs later place scaffolds in Bioreactor
  - measure pH, glucose, lactate and dissolve oxygen
  - Collected media every other day to measure pH, glucose and lactate up to Day 15

- Stop Bioreactor 15 days later (17 days total)
  - Recover 342 x 10^6 stem cells from scaffolds (images in next slide)

  - Perform Flow cytometry for stem cell markers (After bioreactor)

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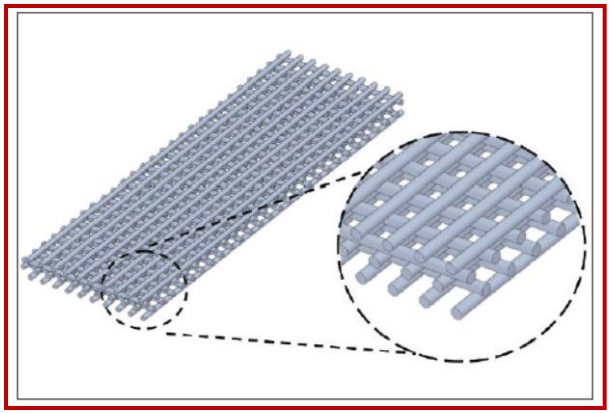
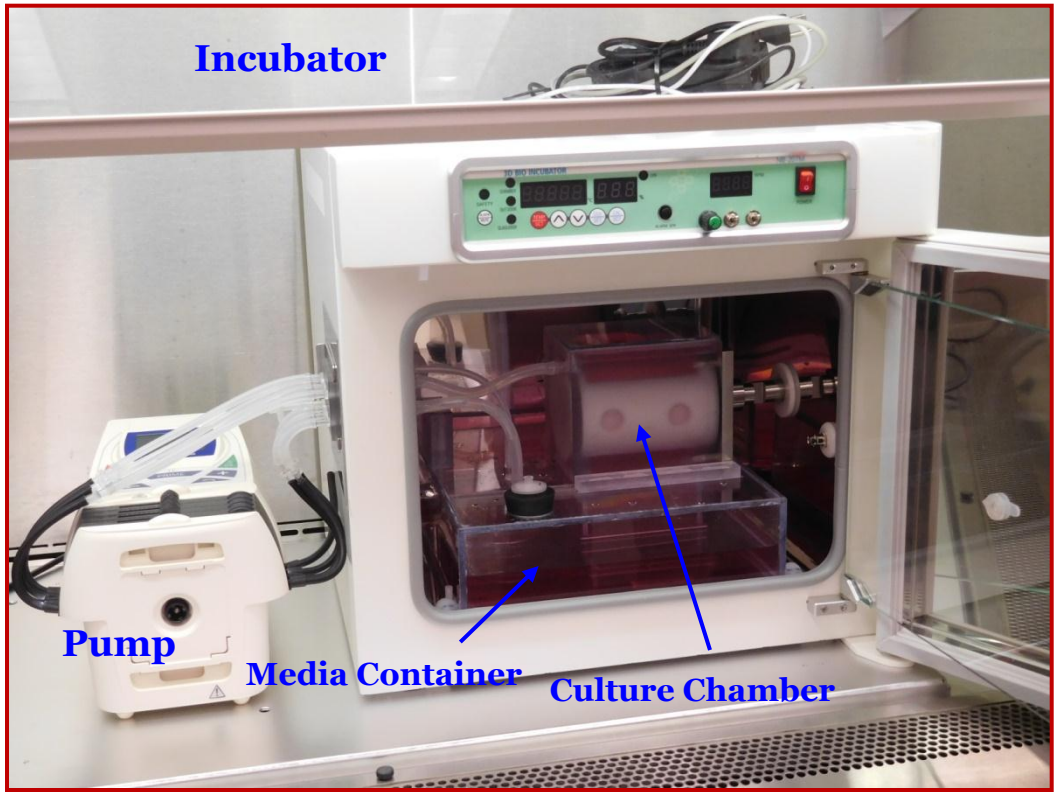
3D Stem Cell Bioreactor





Integrated Bioreactor/Incubator System

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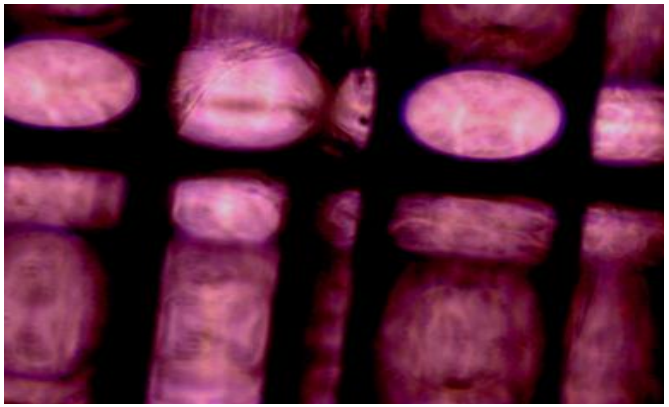
Detachment of Cells from Scaffold

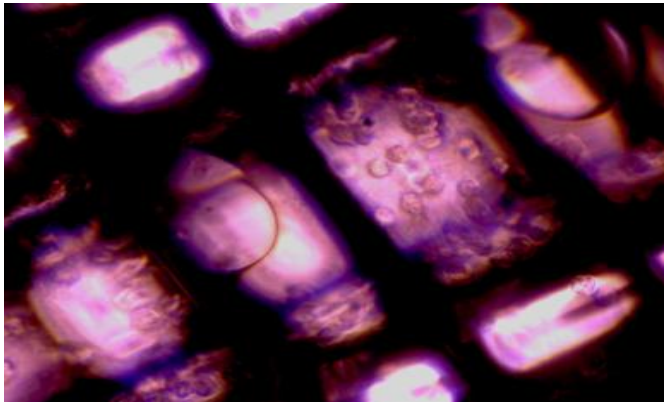
Attempts:

- **Up to 2 hrs with tryp-LE**
- **0.05 % trypsin for 30 min**
- **0.25% trypsin for 30 min**
- **Combine 0.05 % trypsin with tryp-LE**

Finally tried combination of multiple enzymes



Cells on scaffold prior to detachment



Cells entangled within ECM at fiber joints after detachment 10X

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EXTRACELLULAR MATRIX (ECM)

The proteins in the extracellular matrix and cell junctions control: the three-dimensional organization of cells in tissues and it's growth, movement, shape, and differentiation

- **Collagens:** Provides structural support to tissues (a family of over 20 different extracellular matrix proteins). They are the most abundant proteins in the animal kingdom. Collagen subunits are secreted from cells then assembled into larger fibrils and fibers in the extracellular space
- **Fibronectins:** The principal function fibronectin is to connect cells to matrices that contain fibrillar collagen. At least 20 different forms of fibronectin have been identified. Fibrin, heparan sulfate proteoglycan, and collagen: bind to distinct regions in fibronectin integrate fibronectin fibers into the extracellular matrix network. Some cells express integrin receptors that bind to the Arg-Gly-Asp (RGD) sequence of fibronectin.
- **Elastin:** The principal function of elastin is to impart elasticity to tissues.
- **Laminins:** are a family of extracellular matrix proteins are found in virtually all tissues of vertebrate and invertebrate animals. The principal functions of laminins are to provide an adhesive substrate for cells to resist tensile forces in tissues
- **Proteoglycans:** consist of a central protein "core" to which long, linear chains of disaccharides, called glycosaminoglycans (GAGs), are attached. GAG chains on proteoglycans are negatively charged.
- **Hyaluronan:** is a glycosaminoglycan. It forms enormous complexes with proteoglycans in the extracellular matrix.

Molecular biology of cell 4th edition

Proteases degrade extracellular matrix components

Cells must routinely degrade and replace their extracellular matrix as a normal part of development and wound healing.

Extracellular matrix proteins are degraded by specific proteases, which cells secrete in an inactive form. These proteases are only activated in the tissues where they are needed.

- **The matrix metalloproteinase (MMP) family: is one of the most abundant classes of these proteases. It can degrade all of the major classes of extracellular matrix proteins.**
- **ADAMs are a second class of proteases that degrade the extracellular matrix.**

Molecular biology of cell 4th edition

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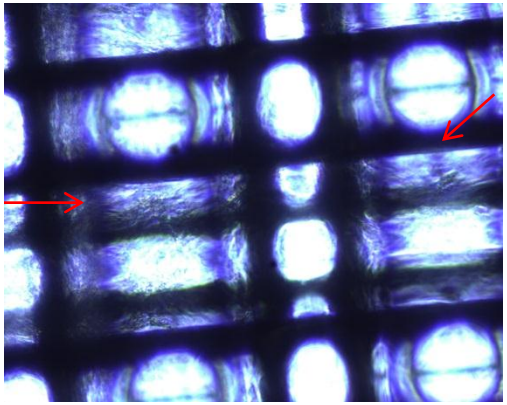
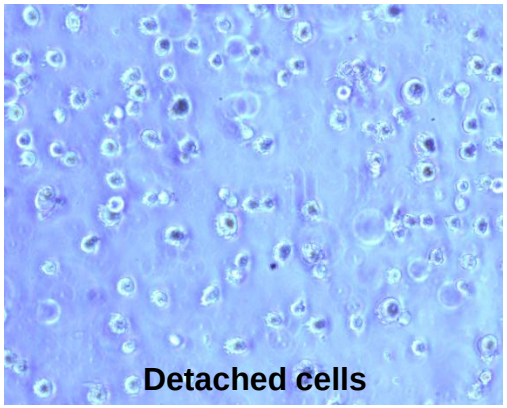
Detachment of Cells from Scaffolds

D7 ASC on scaffold



Enzyme combination

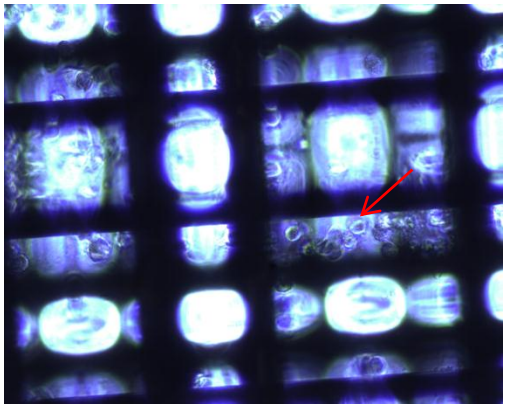
Round 1



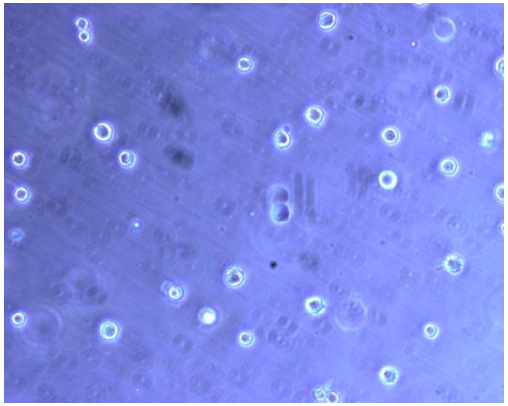
Detached cells

Enzyme combination

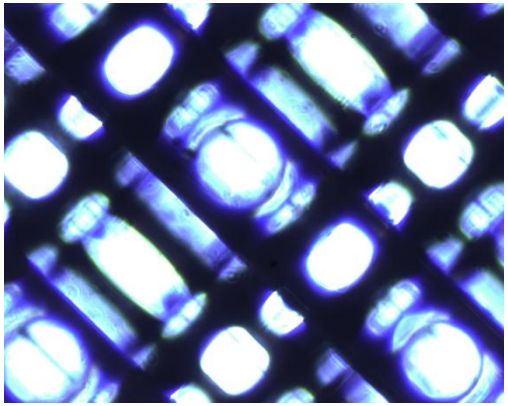
Round 2



Remaining cells



Detached cells



No cells remain

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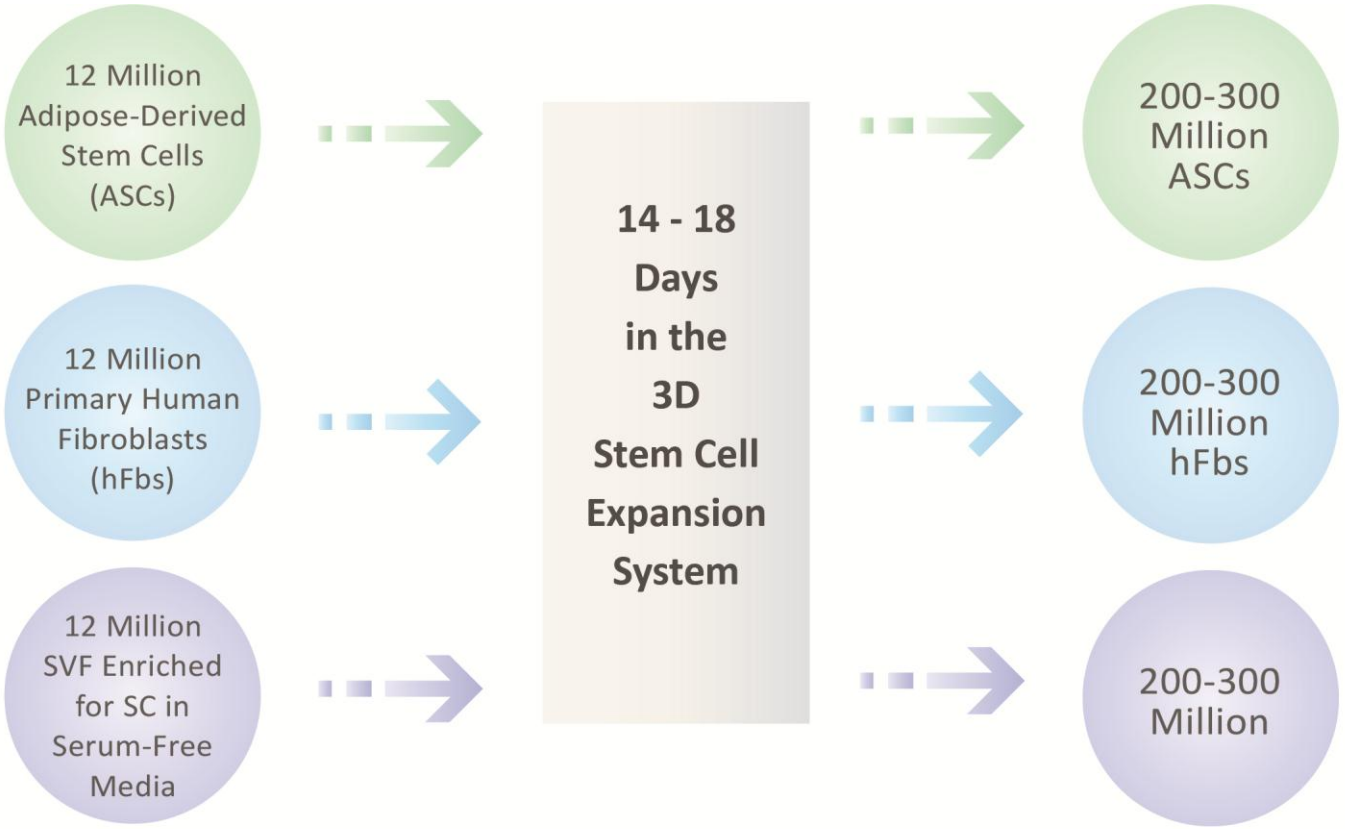
Stem cell expansion using Bioreactor



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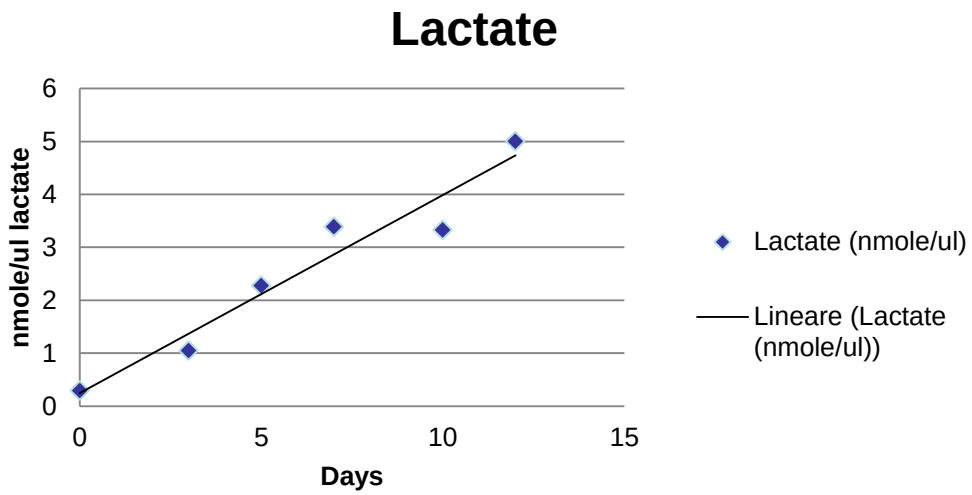
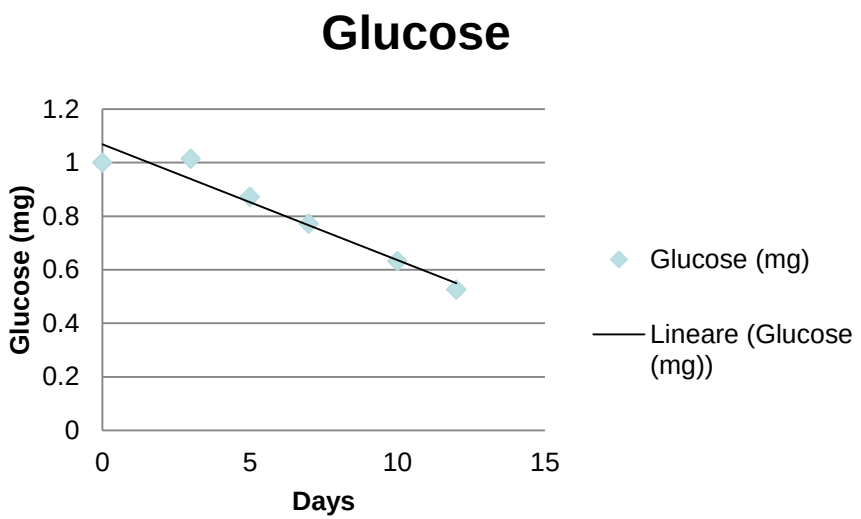
Changes in Glucose and Lactate during expansion





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Flow Cytometry analysis of ASC before and after expansion Using media with 2% FBS

	Markers	Day 0 – cells plated on scaffolds for bioreactor	Day 18– After removal from bioreactor
Positive markers	CD44/CD73/CD90/CD105	> 99%	> 95% Except for CD105 < 10%
Negative markers	CD45/CD34/CD19/CD11b/HLA-DR	< 1%	< 1%

Flow Cytometry analysis of SVF before and after expansion in SF/XF media

	Markers	Day 0 – cells plated on scaffolds for bioreactor	Day 16 – After removal from bioreactor
Positive markers	CD44/CD73/CD90/CD105	> 90%	> 90%
Negative markers	CD45/CD34/CD19/CD11b/HLA-DR	< 1%	< 1%

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Endoglin (CD105)

- Human endoglin (CD105) is a 633 amino acid, 180 kDa homodimeric disulfide-linked hypoxia-inducible transmembrane glycoprotein.
- It contains a large extracellular domain, a hydrophobic transmembrane domain, and a short intracellular domain

- CD105 is an accessory co-receptor for TGF-β, a pleiotropic cytokine regulating cellular proliferation, differentiation, migration and adhesion.
- TGF has been shown to upregulate CD105 transcription under hypoxic condition

CD105 is important in :

- **Angiogenesis**
- **In improved myocardial performance**

- Absence of CD105 expression ($CD105^-$) on mMSCs and hMSCs have been shown to identify differentiated MSCs with increased osteogenic gene expression while selection of CD105 positive ($CD105^+$) MSCs favors chondrogenesis.

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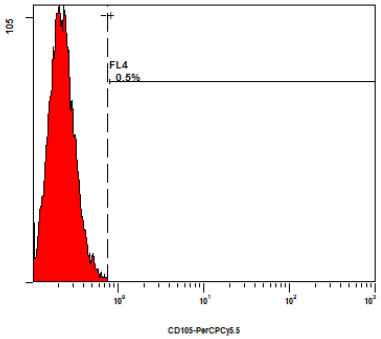
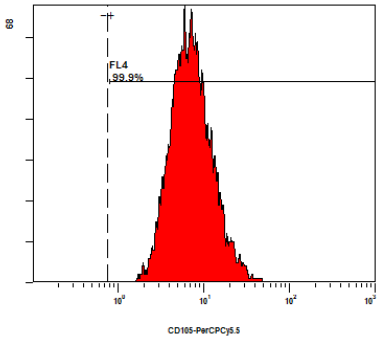
CD105 Recovery

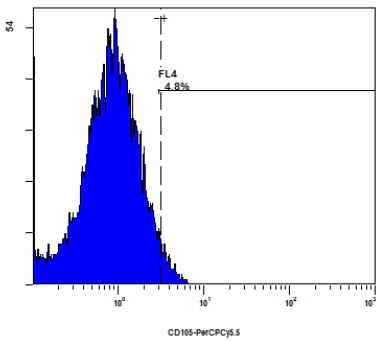
Neg control



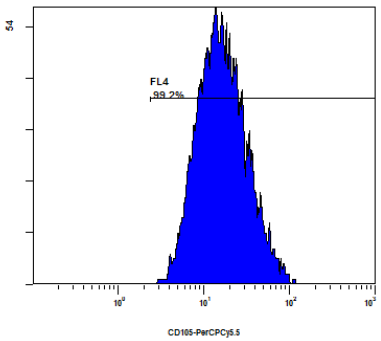
D0- before reactor



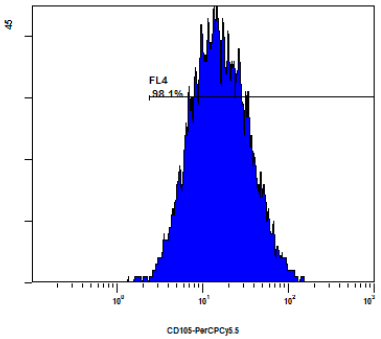
D16 – after reactor



Pos control for reactor



After reactor- 1 wk culture



Marker	Day 0	Day 18	Day 7 (after expansion)
CD105	>99%	<5%	>98%

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Prediction of Cell Number by Monitoring Secretion of Lactate

It is impossible to visualize the cells while inside the bioreactor

- Experiment was designed to measure the daily lactate concentration and Cell number in a 2D cell culture (6 well plate)
- Experimental Result: There is a correlation between lactate production and increase in cell number.
- Further experiments are needed to determine standard curve for cell number and lactate concentration

Lactate measurements for cell number perdition

Days	nmole/ul lactate	Cell count
Day 0		0
Day 1	1.708	4.75 X 104
Day 2	6.78	1.34 X 10 5
Day 3	11.92	3.34 X 10 5
Day 4	18.46	6.35 X 10 5
Day 5	15.17	4.95 X 10 5
Day 6	16.4	4.88 X 10 5

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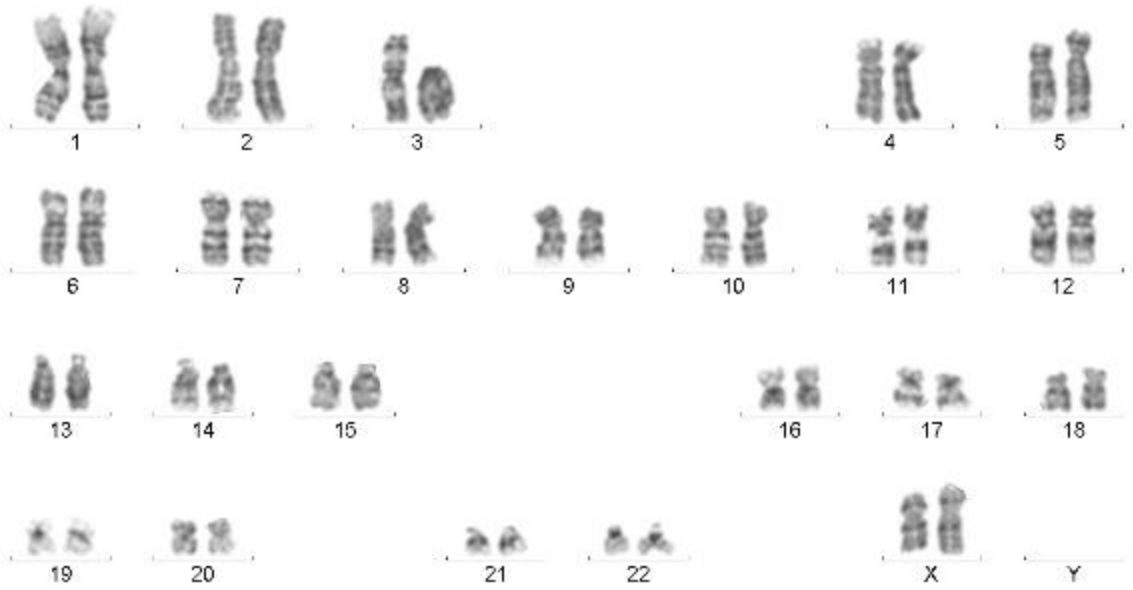
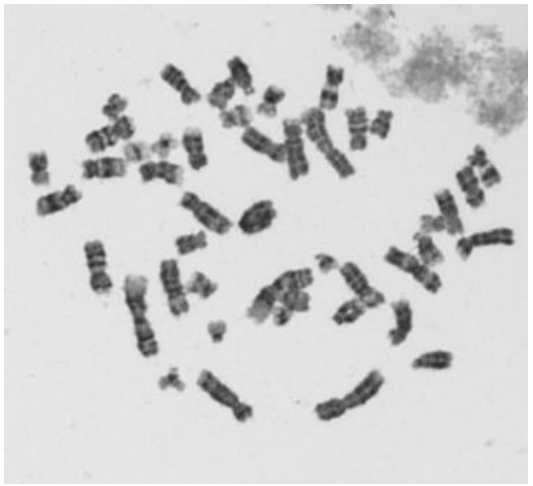
Karyotyping results from bioreactor 12





Specimen type: stem cell
Procedure: GTG-banding
Chromosome band-resolution: 350-400
Results: 46, XX, Normal Karyotype

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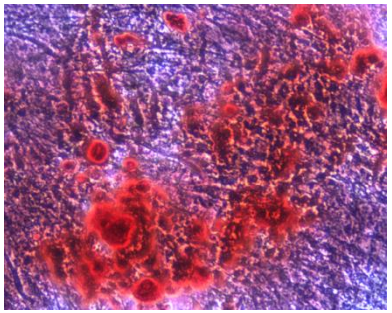
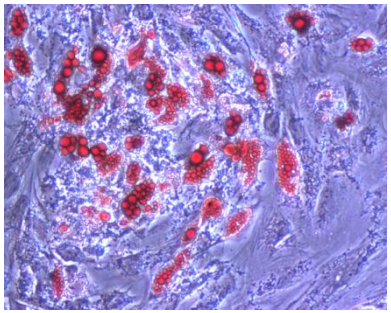
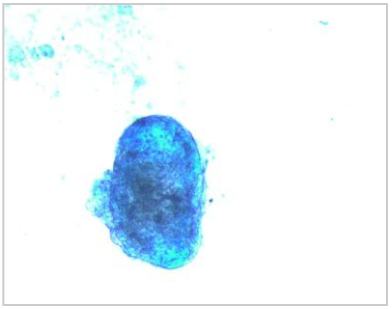
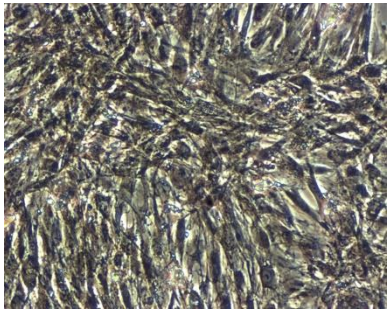
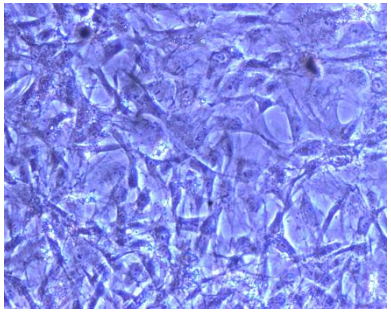
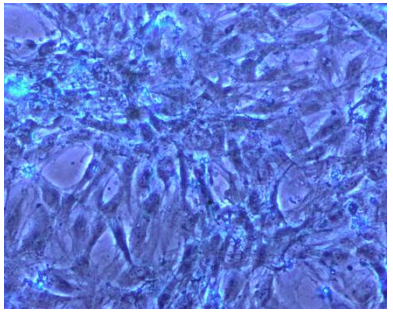
Differentiation of ASC after expansion

	Osteogenic Differentiation	Adipogenic Differentiation	Chondrogenic Differentiation
Induced			
Control	Alizarin Red 	Oil Red O 	Alcian Blue 

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Conclusions

- ASCs can attach and grow on polystyrene (PS) scaffold
- Cell detachment was major problem (resolved using combination enzymes)
- ASCs maintain their stem cell characteristics after expansion
- ASCs were able to differentiate into osteocytes, adipocytes and chondrocytes after expansion
- The 3D Stem Cell Expansion System is a unique product capable of large scale stem cell expansion in 3D
- Great potential for use in stem cell therapy & bio-banking
- Also suitable for use in protein production for cosmetic industry

Acknowledgements

- **This presentation is based on the work of following people**
  - Azadeh Jadali, PhD
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  - David Richards, BS
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- **Collaborator**
  Dr. Binil Starly, PhD (North Carolina State University)

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Thank you

Questions?

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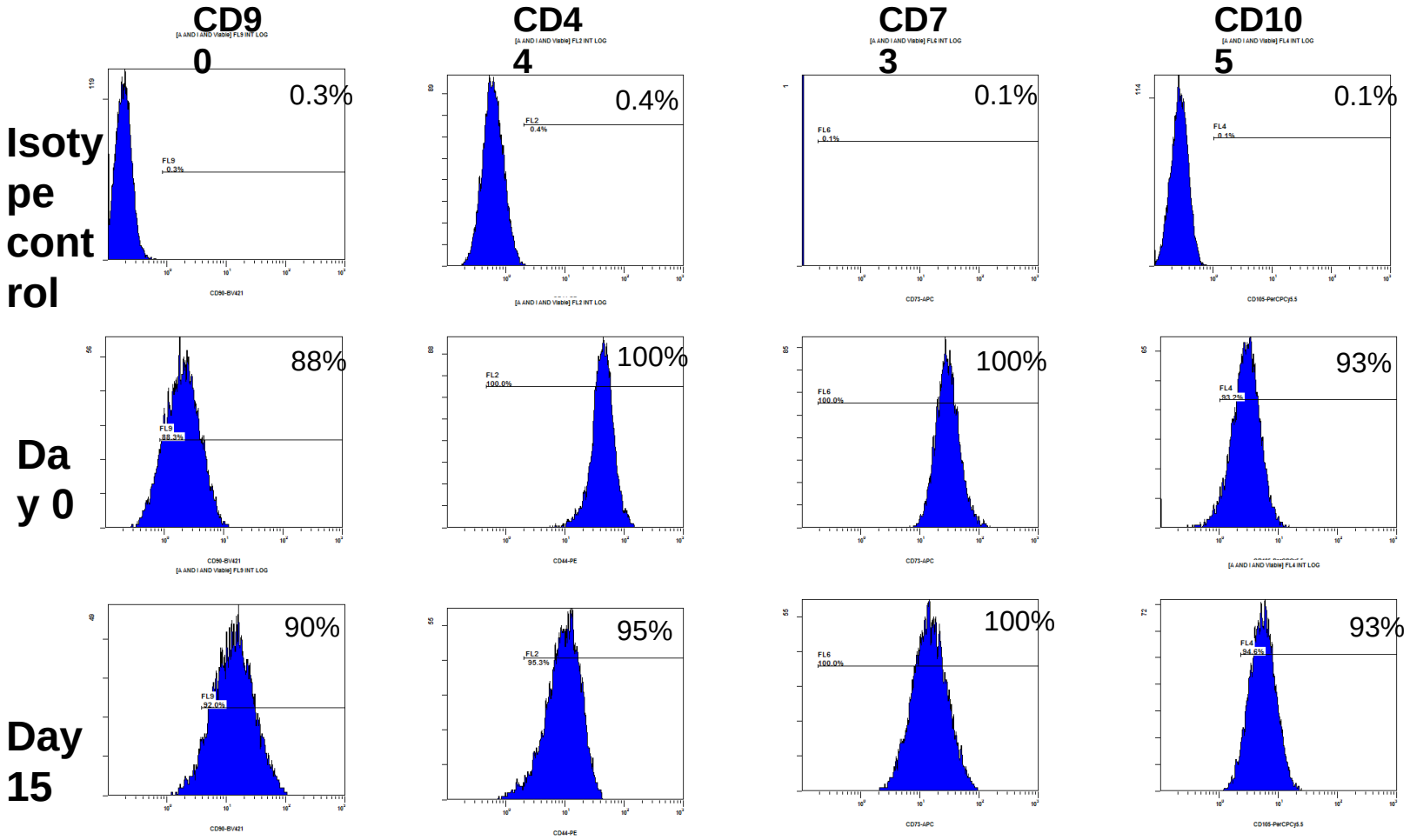
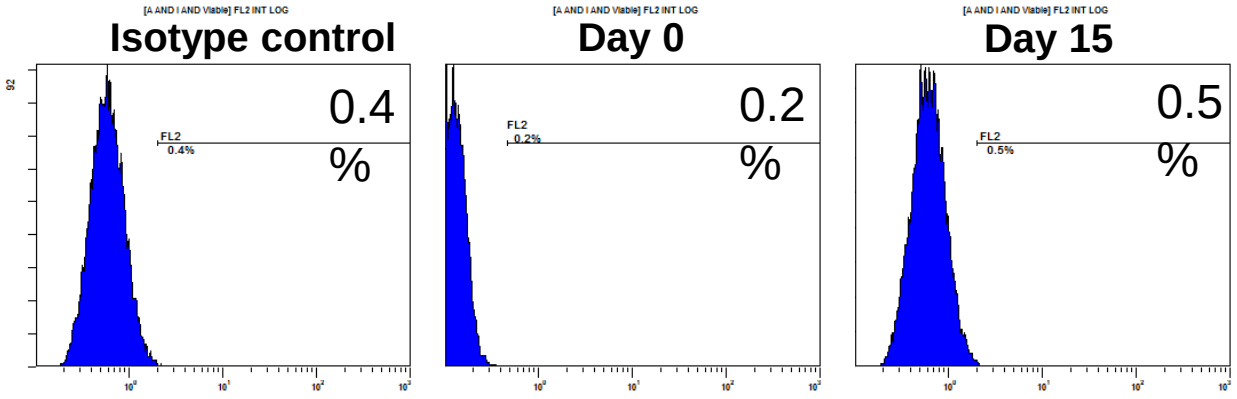
Flow Cytometry analysis of SVF before and after expansion in SF/XF media



Flow Cytometry analysis of SVF before and after expansion in SF/XF media



Isotype control

CD11b, CD19, CD34, CD45, HLA-DR-PE

Day 0

CD11b, CD19, CD34, CD45, HLA-DR-PE

Day 15

CD11b, CD19, CD34, CD45, HLA-DR-PE