Mammalian Cell Culture Technology

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Cell Culture Technology

- Introduction
- Techniques in cell culture technology
- Applications-Cell culture systems

Sources of cell cultures

- Mammalian cells/tissues
- Insect cells
- Plant cells/tissues
- Fish cells
- Hybridoma cell lines (man made)

- *We could generate cell culture from any life forms !!!!*

Date	Scientist	Event
1907	Harrison	Frog embryos in clots of frog lymph, survived few weeks.
1943	Earle	First rodent continuous cell line (rat)
1952	Gey	Hela – First human tumour cell line, from ***He*nrietta *La*ck** Cervical Cancer, most notorious cell line – contaminates other lines.
1961	Heyflick & Moorhead	Normal Cells – finite lifespan
1950 to present		Explosion in cell culture due to disposable plastic wear, laminar flow cabinets etc..

Cell/tissue culture-Terminology

- The *in vitro* growth of cells (animal; including human)
  Mimic internal environment outside the body).

- Widely used technique in cell and molecular biology, genetic and toxicology.

- Cell lines: Cells grown in culture
  Cell lines can be grown indefinitely.

- Static culture: cells grown on solid surface (mostly plastics)-*anchorage dependent*
  (tumour/haemopoietic)

- Suspension culture: cells grown as suspension in culture media-*anchorage independent (normal cells)*

Mammalian cell culture -

Types of cells that may be cultured.

Connective tissue	fibroblast/muscle
Epithelial Cells	organs/skin
Neural Cells	neurones
Endocrine Cells	pancreatic islet
Tumour Cells	various types

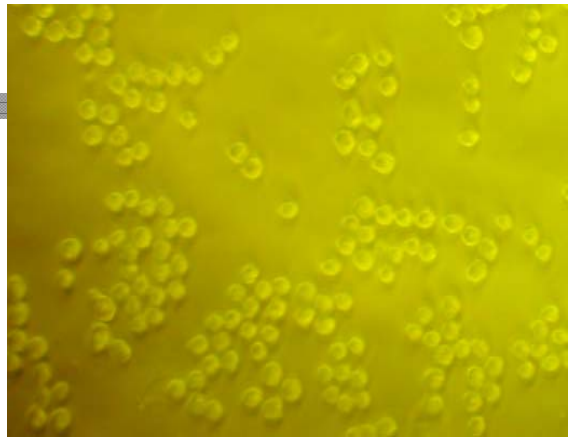
Mammalian cell culture-

Types of Tissue Cultures

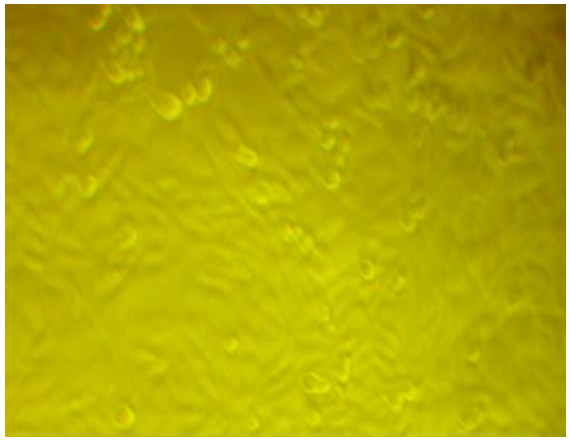
Organ Culture	Piece of tissue cultured on grid or raft at liquid-gas interface Maintains tissue architecture No new growth can occur Lasts several days to weeks. Each experiment requires new sample
Primary Explant Culture	Fragment of tissue (explant) cultured at plastic-liquid interface Cells migrate outwards Major method of initiating cultures forming continuous cell lines
Cell Culture	Tissue or outgrowth from primary explant Broken in cell suspension by enzymatic or mechanical means Primary culture is culture direct from tissue Once subcultures, referred to as cell line

Mammalian cell lines

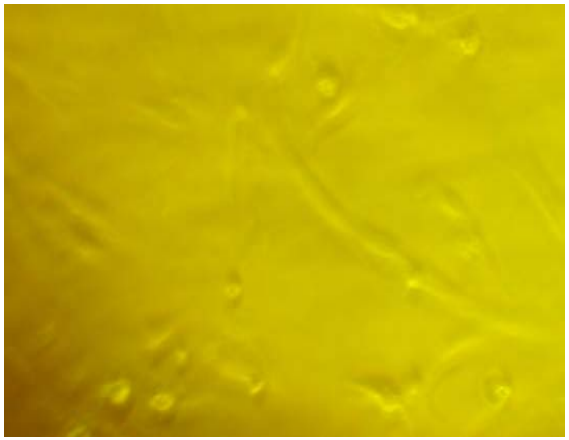
Producer cell lines	**Medical (cancer) research**	**Hybrid cell lines**	**Primary cell lines**
BHK-21	MRC-5, HEK, MDBK, LLC-MK_2, BS-C-1, CV-1, AGMK, WI-38, Vero, Hela, **3T3-L1**	**NSO**	Fibroblast cells
CHO-K1		NS1	Skin cells
Namalwa		SP2/0-Ag14	Liver cells
(therapeutic protein production)	(commonly used for viral propagation in vaccine production)	P3-X63 Ag8.653	Lung cells
		(protein production for therapeutic & diagnostic)	Retina cells etc
			(to make tissue equivalents- mortal cell lines recovered from tissue)



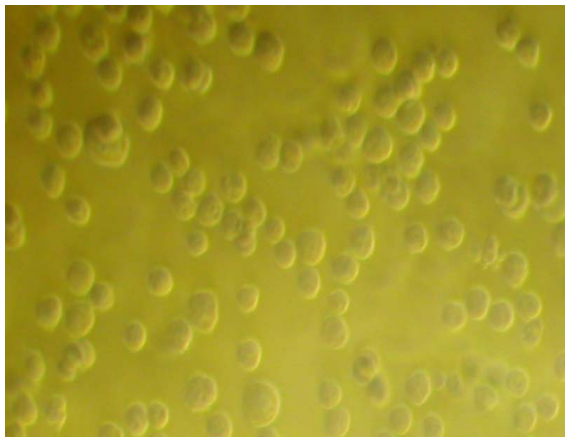
Hybridoma, NS0



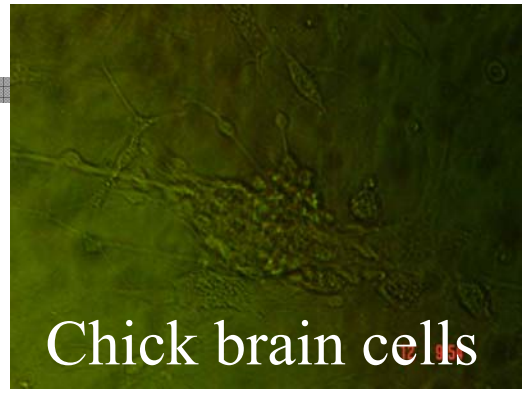
CHO-K1

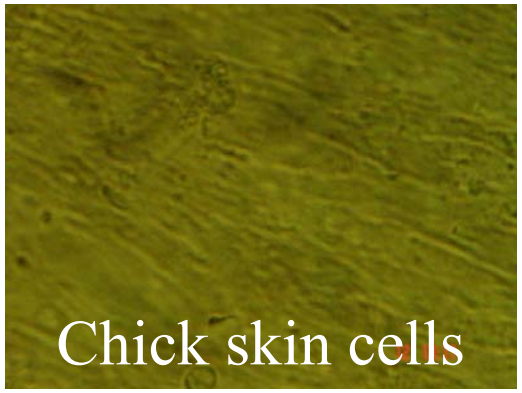


BHK-21



Hybridoma TBC3.bcl-2, producing mouse IgG1


Chick brain cells


Chick skin cells

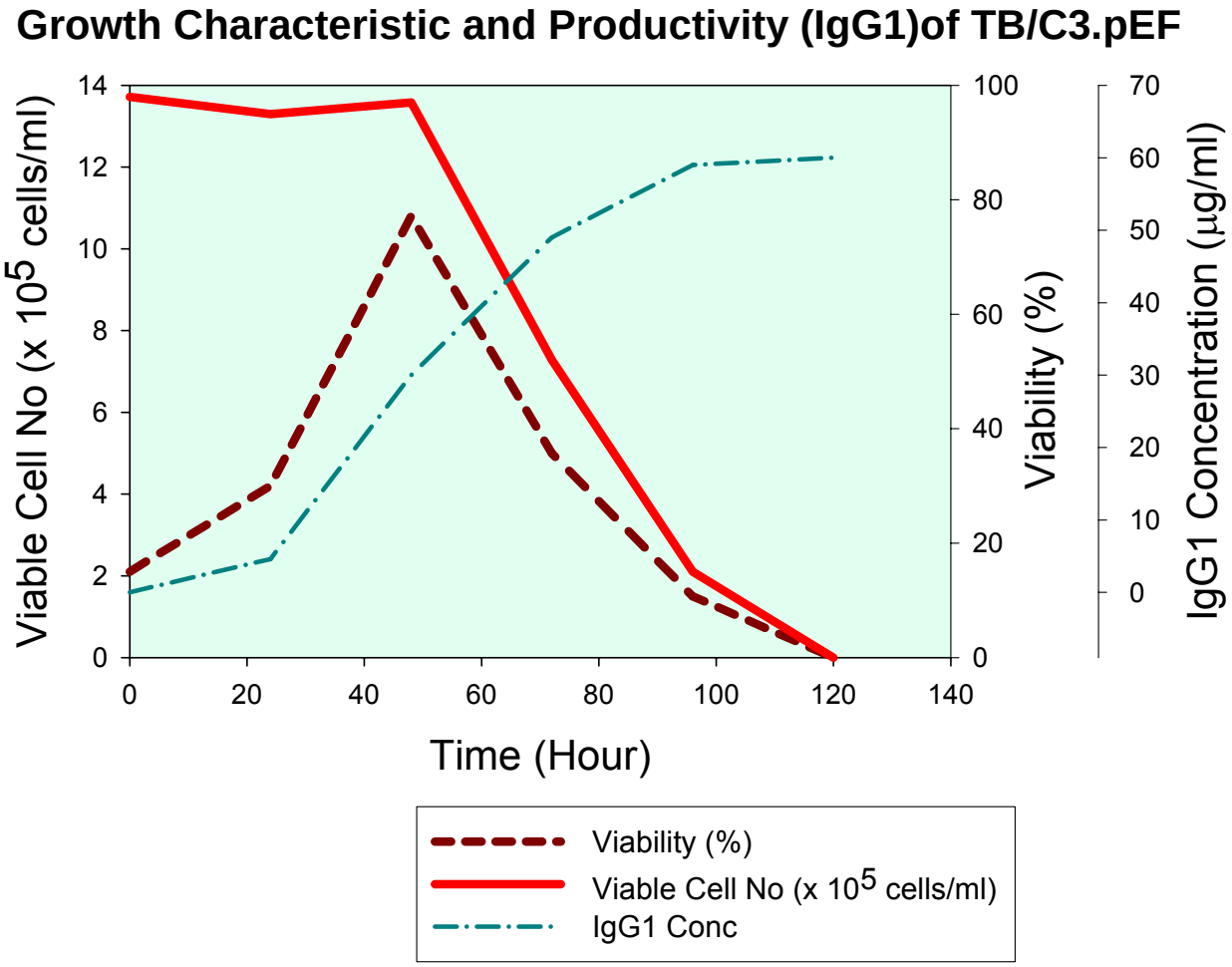

Chick heart cells


Chick muscle cells

Mammalian cell cultures-properties

AVERAGE SIZE	5 TO 5 MICRONS
AVERAGE COMPOSITION (DRY WEIGHT BASIS)	PROTEIN40% CARBOHYDRATE 25% LIPID: 20% DNA: 2.5% RNA: 6.9% WATER: 80-85%
SENSITIVITY TO ENVIRONMENT	OSMOLARITY WINDOW:- 280-350 MILLIOSMOLE/Kg PH RANGE: 6.5 TO 7.2 TEMPERATURE: 35 TO 370C ± 0.20C LACK OF PROTECTIVE CELL WALL (ADVERSELY AFFECTED BY LIQUID SHEAR FORCES, GAS BUBBLES)
LOW RATE OF GROWTH	NUMBER DOUBLING TIMES: 18 TO 36 HOURS LOW CELL DENSITY (TYPICALLY 1 X 106 CELLS/ML 0.6 grams/Liter) FASTIDIOUS NUTRITIONAL REQUIREMENTS EASE OF CONTAMINATION

Cell Growth Characteristics-



Cell Growth Kinetics

Growth rate: $dN/dt = \mu N$

μ (typical): 0015 to 0.03/Hr
T_D = 24 to 48 Hrs

Product formation rate: $dp/dt = \alpha \mu N + \beta N$

α = growth associated
β= non growth associated

Specific product synthesis rate (q_p)

$Dp/Ndt = q_p = \alpha \mu + \beta$ = Pg/ Cell-Day

q_p (typical) = 5 to 20 Pg/Cell-Day

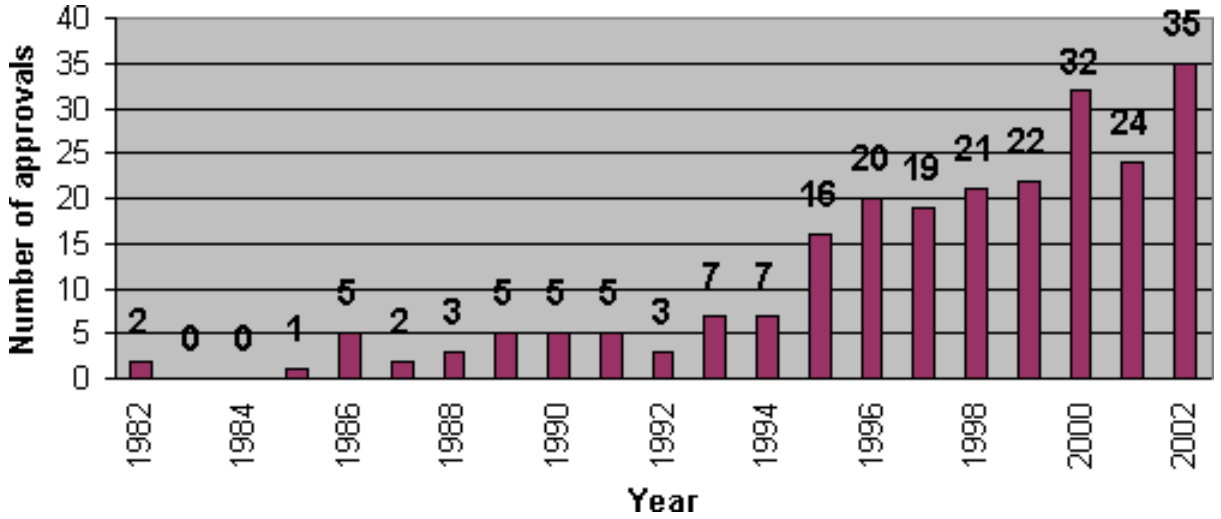
Mammalian cell culture-
Nutritional requirements (COMPLEX MEDIUM)

Minimal essential medium	ANIMAL CELLS UNABLE TO SYNTHESIZE AMINO ACIDS: (13 AMINO ACIDS: 0.05 mM TO 4 mM) ALL VITAMINS MUST BE SUPPLEMENTED (8 VITAMINS: 0.3 TO l1цM) INORGANIC ION SUPPLEMENTATION (6 INORGANIC IONS: 1.1 TO 116 mM) CARBOHYDRATE: GLUCOSE MOST COMMON (5 TO 8 mM)
Notable nutrients in high concentrations	GLUTAMINE (4 mM) SERVES AS ENERGY SOURCE INCORPORATED AS AMINO ACID AMINO GROUP: TRANSAMINATION NaCl (116 mM) MAINTAINING PROPER OSMOLARITY GLUCOSE (5 TO 8 mM) PARTIAL SOURCE OF ENERGY
Antibiotics (STREPTOMYCIN, GENTAMICIN, PENICILLIN)	REDUCE CONTAMINATION•
Animal serum	DIALYZED FETAL CALF SERUM (5 TO 10%)
Serum free medium	FORMULATED PRODUCTS-ADD HORMONES AND GROWTH FACTORS PROPRIOTARY FORMULATIONS

Mammalian cell culture-
Application of Cell Cultures

1. Cells Themselves	Skin Cells – Grafting in burn treatment Blood Cells – Transfusions/ New Veins Glandular Tissue – Replacements
2. Viral Vaccine Production	Industry is largest producer of animal cells
3. Production of cellular macromolecules.	Pharmaceuticals: interferons, monoclonal antibodies, hormones and enzymes
4. In vitro toxicology	Testing drugs on cell cultures instead of animals
5. Research tool	Cancer research-compare normal and tumour cells Predict chemosensitivity to cancer cells

New Biotech Drug and Vaccine Approvals/New Indication Approvals by Year



List of biopharmaceutical products (some examples)

Herceptin® (Trastuzumab)
Anti-HER2 antibody
For metastatic breast cancer in HER2 overexpressed tumors

Rituxan® (Rituximab)
Anti-CD20 antibody
For relapsed or refractory low-grade or follicular, CD20 positive, B-cell non-Hodgkin's lymphoma

Thrombolytics **Activase®** (Alteplase, recombinant)
A tissue-plasminogen activator
For acute myocardial infarction, acute ischemic stroke and acute massive pulmonary embolism

Cathflo® Activase® (Alteplase)
Thrombolytic agent
For the restoration of function to central venous access devices as assessed by the ability to withdraw blood

TNKase™ (Tenecteplase)
Single-bolus thrombolytic agent
For the treatment of acute myocardial infarction (AMI)

Nutropin AQ® [somatropin (rDNA origin) injection]
Liquid formulation growth hormone
For GHD in children and adults; growth failure associated with chronic renal insufficiency (CRI) prior to kidney transplantation; short stature associated with Turner syndrome

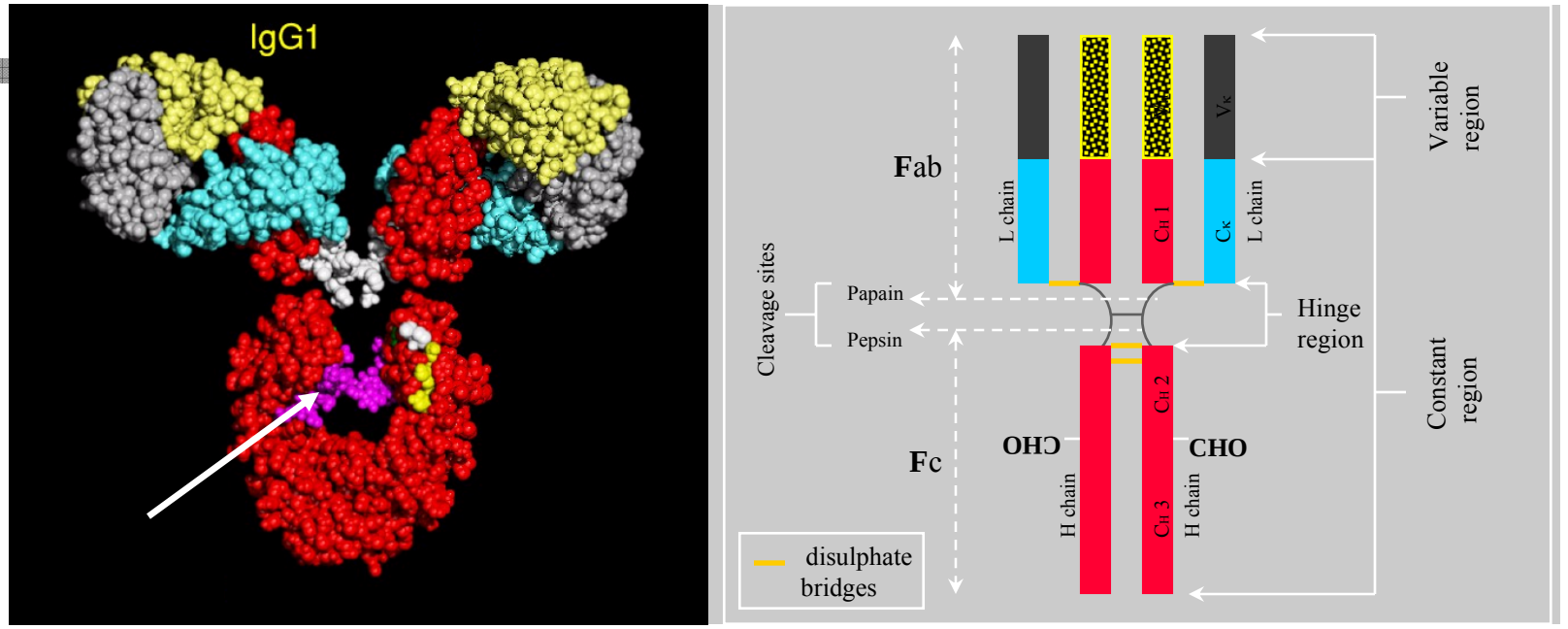
Nutropin® [somatropin (rDNA origin) for injection]
Growth hormone
For GHD in children and adults; growth failure associated with CRI prior to kidney transplantation; short stature associated with Turner syndrome

Protropin® (somatrem for injection)
Growth hormone
For growth hormone deficiency (GHD) in children

Pulmozyme® (dornase alfa)
Inhalation Solution
For management of cystic fibrosis (including patients under age 5)

Rationale for using mammalian cell cultures

POST-TRANSLATIONAL MODIFICATIONS	BIOSYNTHESIS OF CARBOHYDRATE MOITIES (SIALIC ACID, GLUCOSAMINES, GALACTOSE)
	SULFATION, PHOSPHORYLATION, ETC.
	GLYCOSYLATION ONTO PROPER SITES
OTHER PROTEIN PROCESSING CAPABILITIES	PROPER DISULFIDE FORMATION
	CORRECT FOLDING OF ACTIVE MOLECULE
	SECRETION OF ACTIVE MOLECULE
BIOSYNTHESIS AND SECRETION OF COMPLEX POLYPEPTIDES	(NO OTHER CHOICE: e.g. MONOCLONAL ANTIBODIES)
GENETIC SYSTEM FOR HETEROLOGOUS PROTEIN	RAPID METHOD FOR EXPRESSION OF NEW PROTEINS
	STABILITY OF EXPRESSION FOR MANUFACTURING
	FDA ACCEPTANCE FOR THERAPEUTIC PROTEINS
AIDING ANALYTICAL AND PRODUCT PURIFICATION.	HYBRIDOMA FOR ANTIBODY PRODUCTION
	RAPID ISOLATION AND PURIFICATION FOR ANALYTICAL USAGE



(a) (b)

Figure 1.3 : (a) 3-D structure of human IgG1 molecule (Clark, M: http://www.path.cam.ac.uk/~mrc7/lectures/models.html) and (b) The schematic model of an IgG1 (κ) antibody molecule showing the basic 4-chain structure and domains (VH,CH1,Vk, Ck, CH2 and CH3). The sites for enzymatic cleavage (papain and pepsin) are shown.

Mammalian cell culture-
Advantages of tissue culture

Environment Control	Defined physiochemical environment (growth media) - pH, temp, O_2 etc
Sample Homogenicity	Tissue has mixture of cell types Homogenous sample identical over many generations Indefinite if stored in liquid nitrogen (cryo-preserved -196^{0}C)
Economy	Less cell culture required as directly exposed to reagents Avoid moral, legal and ethical issues with animal testing

Mammalian cell culture
Disadvantages of tissue cultures

Expertise	Strict aseptic conditions required Operator requires level of skill and knowledge
Facilities	Require specialised equipment – laminar flow cabinets, CO2 incubators etc
Differentation/instability	Some continuous cell lines loose differentiated function Result in unstable chromosome number
Difference *in vivo-in vitro*	Major consideration in toxicology and research *In vivo* cells have 3D geometry, *in vitro* have 2D *In vitro* lose regulation of nervous & endocrine systems

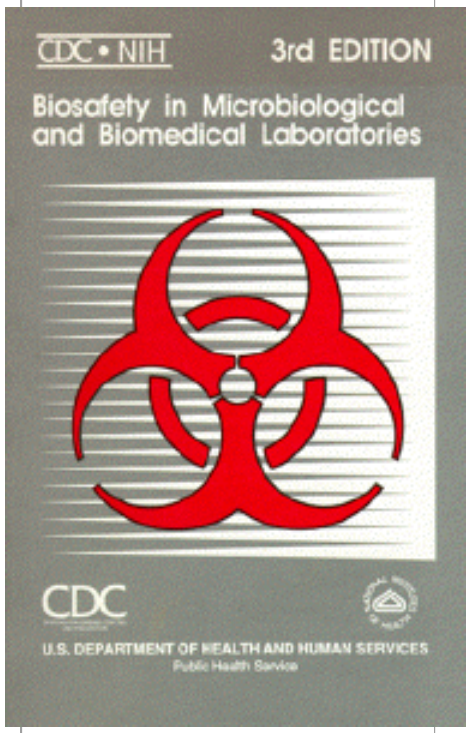
Working with cell culture-

Safety

- Safe practice need to be established due to number of possible risks
- Biohazards need to be categorised correctly
  - Risk assessment
    - How the materials or equipment is used
    - Who uses it
    - The frequency of use
    - Training
    - General environmental conditions
- Handling hazardous substances, equipment and condition should have SOP (standard operating procedure)

Safety regulations

- Advice that might help in complying safety regulations
  - USA Dept of Health and Human Services, 1993
    - E.g: Biosafety in Microbiological and Biomedical Laboratories)
    - E.g: General Laboratory Health and Safety (26 pages)
  - NIOSH, CDC, OhASIS



HHS Publication No. (CDC) 93-8395

Biosafety in Microbiological and Biomedical Laboratories

U.S. Department of Health and Human Services
Public Health Service
Centers for Disease Control and Prevention
and
National Institutes of Health
3rd Edition March 1993
U.S. GOVERNMENT PRINTING OFFICE
WASHINGTON: 1993

Working with cell culture-
Equipment

Essential	Beneficial / additional	consumable
Laminar flow cabinet	pH meter	Pipettes (glass or disposable)
Incubator/Incubator (CO_2)	Cell counter	Culture vessels (T-flasks)
Autoclave/steriliser	Magnetic stirrer	Pipette tips
Refrigerator/freezer	Fluid aspirator	Eppendorf tubes
Inverted microscope	Fluid despenser	etc
Upright microscope	Glassware washing unit	
Purified water (deionised double distilled)	Video camera and monitor	
Centrifuge		
Liquid N_2 tank		
Balance		
Hemocytometer		
Media filter unit		

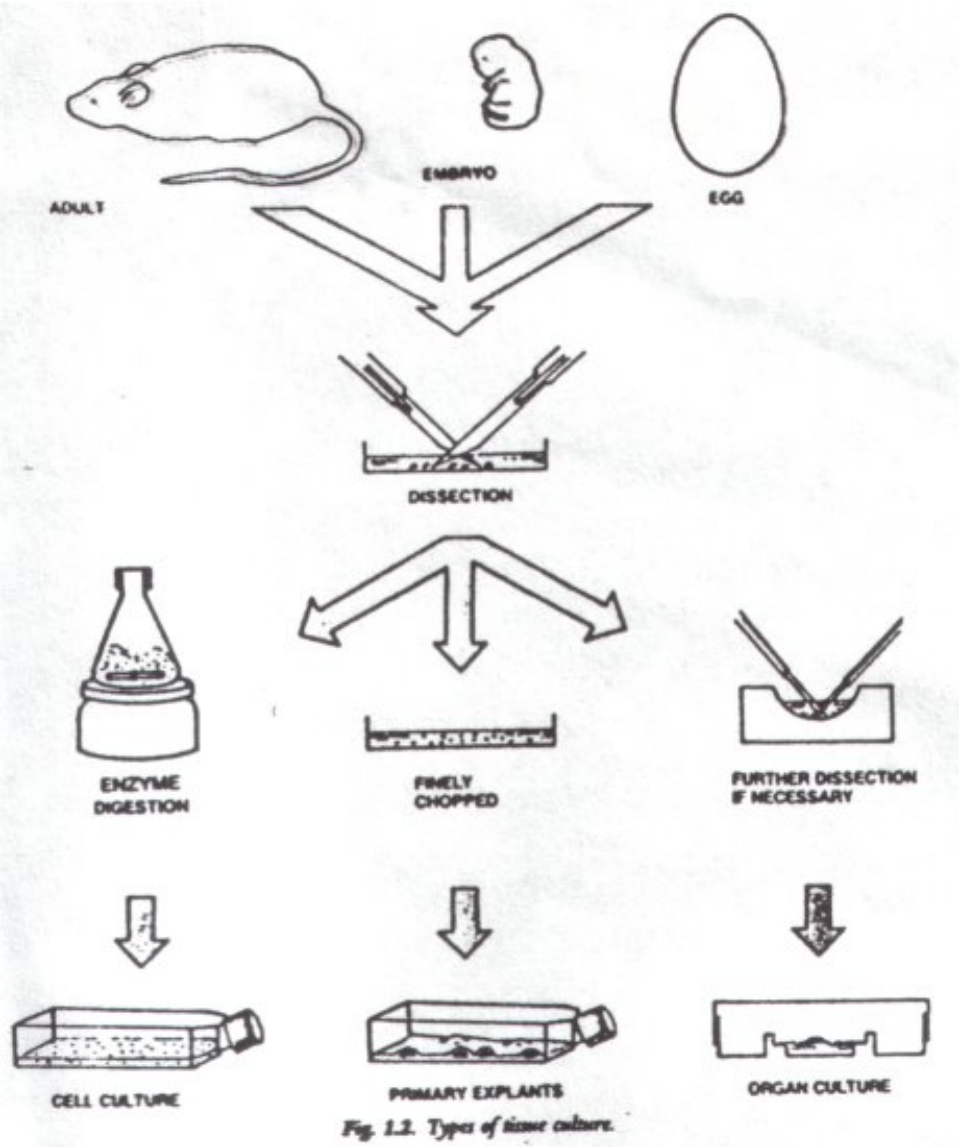


Fig. 1.2. Types of tissue culture.

Origin of Hybridoma cell lines

Myeloma cell types (tumour cells) X Spleen cells (B-lymphocytes-antibody producing cells)

Cell fusions

Hybridoma cells

Hybridoma	**NSO**	NS1/1	SP2/0-Ag14	P3-X63 Ag8.653	P3-X63 Ag UI
properties	No IgG	Light chain	No IgG	No IgG	Light chain

Cell banking-

Liquid Nitrogen Tank (-196^{0}C)

Cell culture (10 $^{6-7}$ cell/ml)

↓

Cryo-preserved liquid
(DMSO or Glycerol, serum)

↓

Deep freezing
(LN_2)

World Cell Culture Collection Centers

American Type Cell Collection
http://www.atcc.org/
The European Collection of Cell Cultures
http://www.ecacc.org.uk/
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)
http://www.dsmz.de/dsmzhome.htm
Japanese Collection of Research Bioresources (JCRB)
http://cellbank.nihs.go.jp/

Mammalian Cell culture systems

Applications

- Production of various proteins
  - Widely used producer cell lines and its products (handout 1)
- Tissue equivalents
- Organ repair/transplant
- Alternative toxicity testing
- Cloned babies!!!!!!!

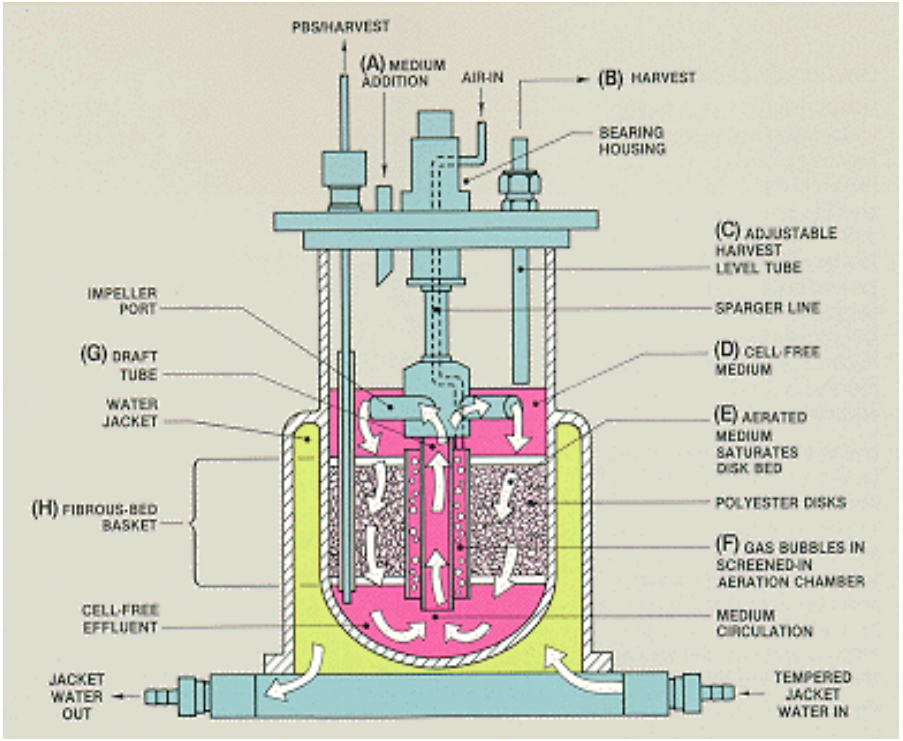
Mammalian Cell cultivation systems

1. Batch cultures
2. High density cultures
3. Continuous cultures
4. Type of bioreactors
    1. stirred tank bioreactor
    2. Airlift bioreactor
    3. Hollow fibre bioreactor
5. Microcarrier systems
6. microencapsulation, packed bed etc.

System	Maximum Concentration mg/l	Productivity mg/week	Required for the production of 1 g
In vivo	2,200	2	180 mice
In vitro			
T-flask	42	7	400 T-flasks
Stirred-tank bioreactor, batch	47	180	39 days
Stirred-tank bioreactor, fed-batch	120	250 [a]	28 days
Hollow fiber reactor	1,600	1,400 [a]	26; 5 days[b]

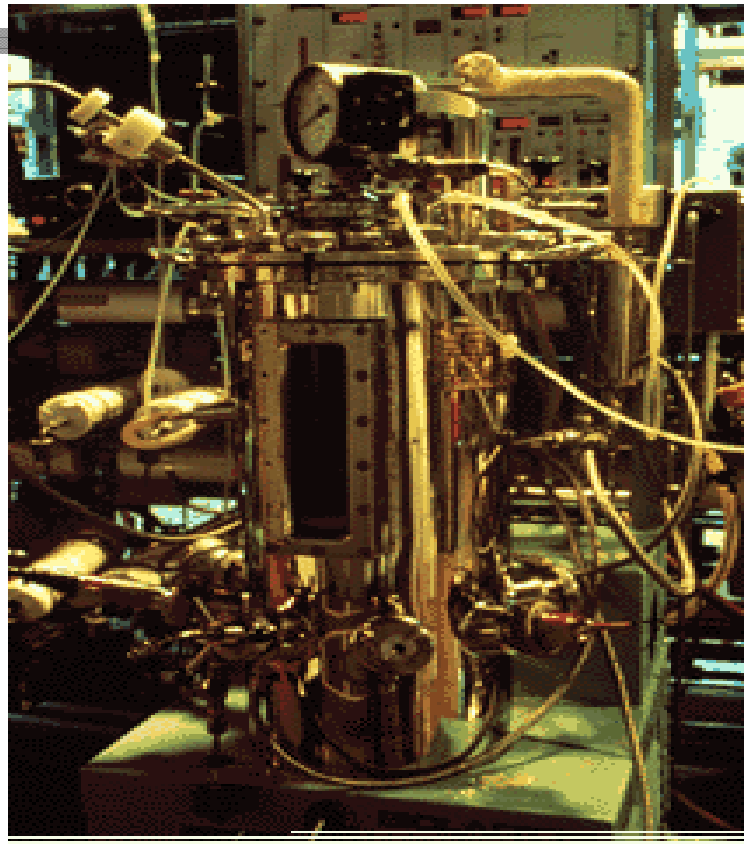
Table 1. Antibody production in different bioreactor systems

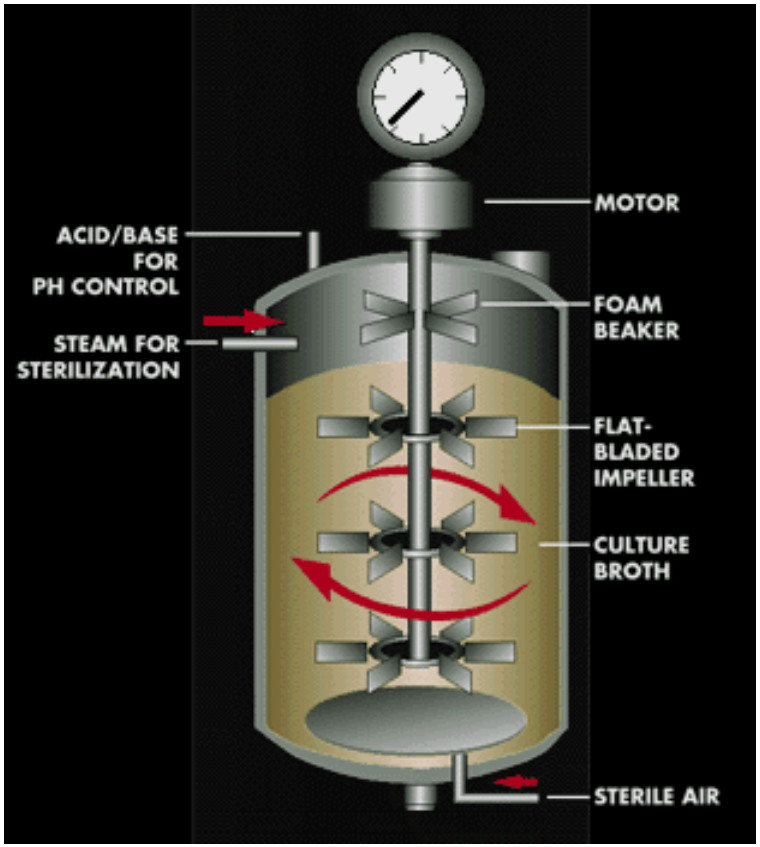
(Adapted from Stoll, T., Perregaux, C., Stockar, U. V., and I. W. Marison (1995). Production of immunoglobulin A in different reactor configurations. *Cytotechnology* 17:53-63.)



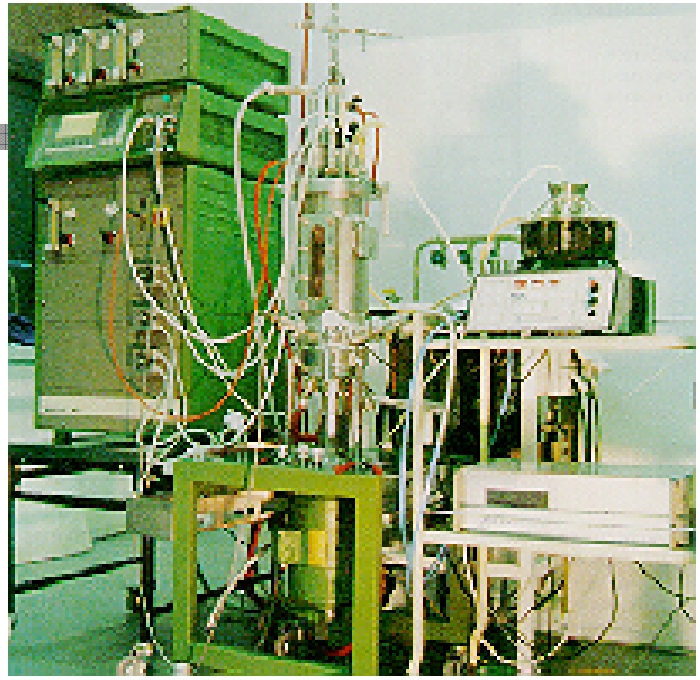
Eg: CelliGen Plus, an all-purpose stirred-tank bioreactor

CSTR (continuous stirred tank reactor)





http://www.accessexcellence.org/LC/SS/ferm_graphics/reactor.html



A 5 litre stirred tank fermenter, equipped with 3 Rushton turbines or with 3 Intermig impellers (B.Braun)

Lonza Biologics, Slough, UK. The hall containing three airlift bioreactor with a a total fermentation capacity of 6,500 liters in a range of process scales from 100 to 2,000 liters.

Air lift fermenter

-The usual height to diameter ration is 10/1 to 40/1

-no mechanical means of agitation.

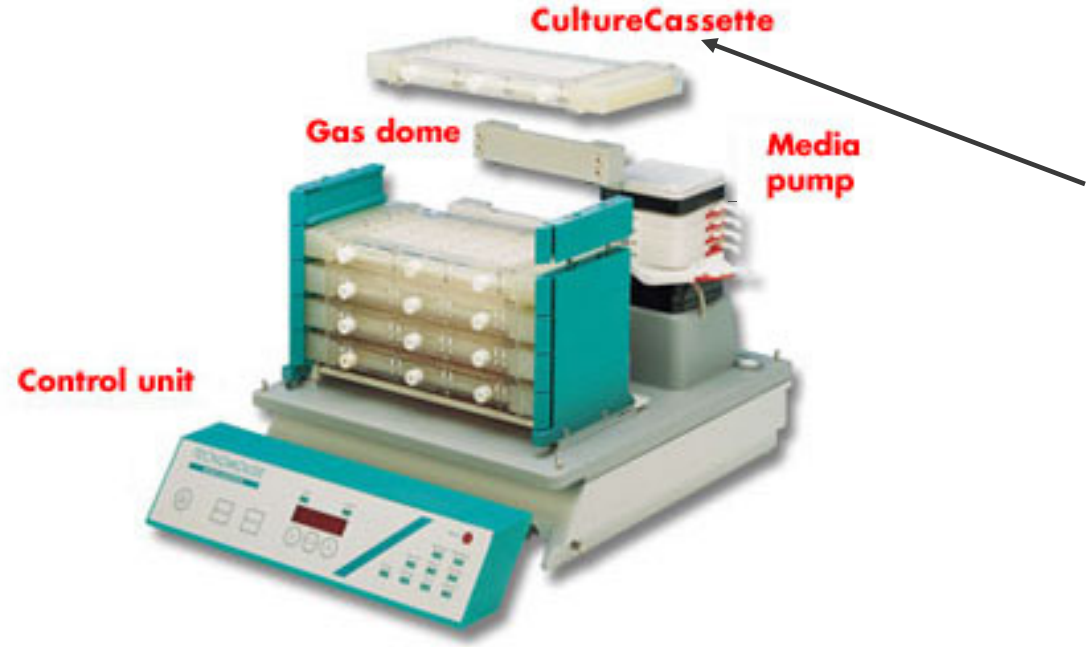
(turbulence within the culture is brought about by injection of pressurized air mainly from the bottom. To enable efficient circulation the design operates with loops either as external loop or as internal loop (so called 'draught tube').

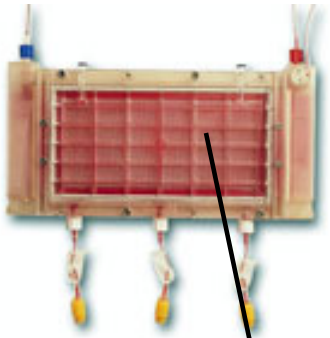
-also known as the 'pressure cycle fermenter'.

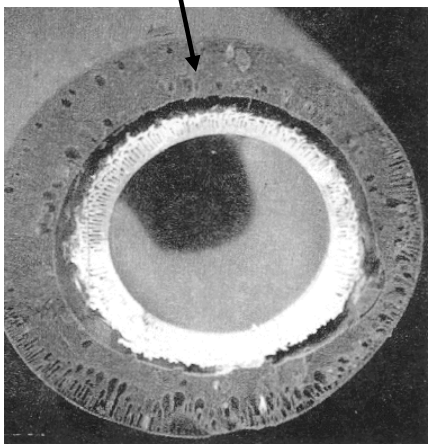
(since the growth medium undergoes successive cycles of high and low pressure) main disadvantage lies in the ascending air bubbles will increase in size in the top area of the fermenter (due to the decrease in hydrostatic pressure). Since large bubbles transfer oxygen less efficient than small bubbles this results in the generation of a sub-optimal oxygen supply in the top area.

TECNOMOUSE - perfusion system for the long-term cultivation of cells

The heart of TECNOMOUSE is the exchangeable CultureCassette - a miniature bioreactor. Ideal cultivation conditions are guaranteed by optimal gas supply and media utilisation.





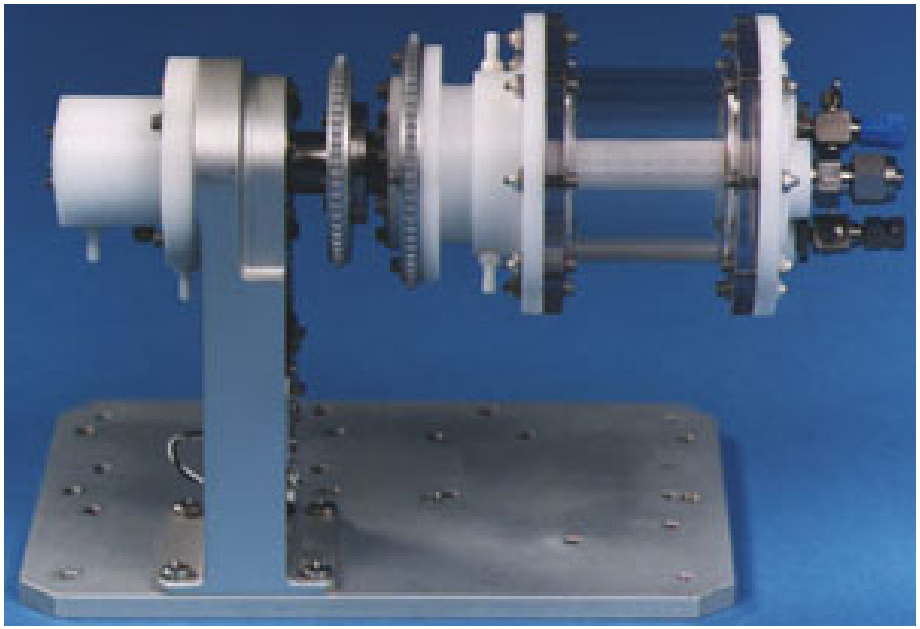


TECNOMOUSE is a modular design and comprises:

A removable, programmable control unit for ease of operation. A modular portable rack for up to 5 Culture Cassettes. A media pump. An integrated gas supply unit that guarantees adequate gas flow to the membranes.

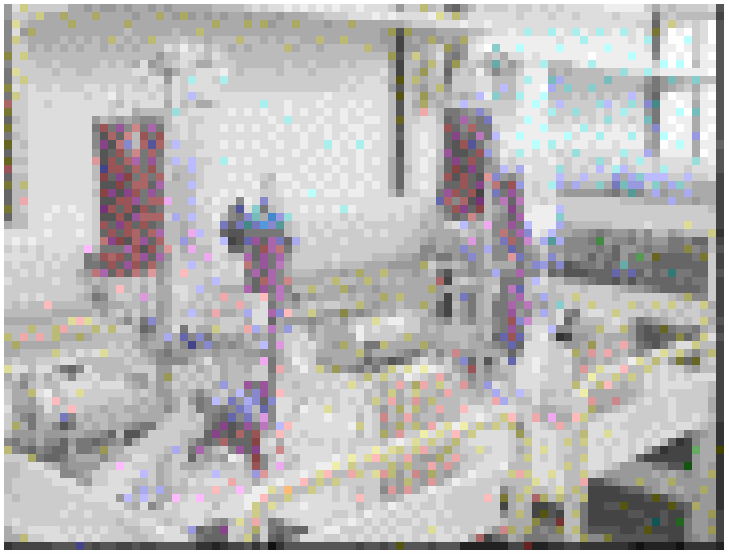
SEM graph of the cross-sectional structure of annular PSf/PESf hollow fiber.

NASA Bioreactor



- For growing cells in **zero gravity**
- A rotating chamber cells grow in suspension
- Simulated zero gravity achieved by slowly rotating the chamber, altering the sedimentation vector continuously.
- The cells remain stationary, subject to zero shear force, and tend to form three- dimensional aggregates, which, reputedly, enhances product formation.
- When the rotation stops, the aggregates sediment, and the medium can be replaced.

Home made HFBR



Example of an actual Home-made Bioreactor Run

Cell line:
YTS 177.9.6.1 rat IgG2a anti-CD4 (DA spleen x Y3/Ag1.2.3 hybridoma)
NB. This is a reliably good producer by all methods (eg 100 micrograms/ml in roller culture)!

Cartridge:
Initial seeding with 2x10**8 cells only - this took 2 weeks to reach optimal production and we would normally use more cells.

Medium:
Total usage over 6 weeks: 40L (cost 80 pounds)

Harvest:
Total harvest over last 4 weeks: 2.15L (average of 77ml/day)

Production:
Total mAb after 50% SAS purification: 3.2 g (avge. of 114mg/day at greater than 1.5mg/ml)
N.B. final mAb greater than 90% pure [native gel].

Other costs:
Estimate 40 pounds per litre of harvest for cost of nutrition bags, FCS, filters, syringes etc.

Total cost:
80 pounds; consumables + 80 pounds; medium + 12 pounds; cartridge (AltraNova 140) = 172 pounds total (for 3.2 g)
ie. approx. 50 pounds per gm (excluding labour/purification/hidden and indirect costs).

http://www.molbiol.ox.ac.uk/pathology/tig/mprod.html

Tissue cultures-organ replacement

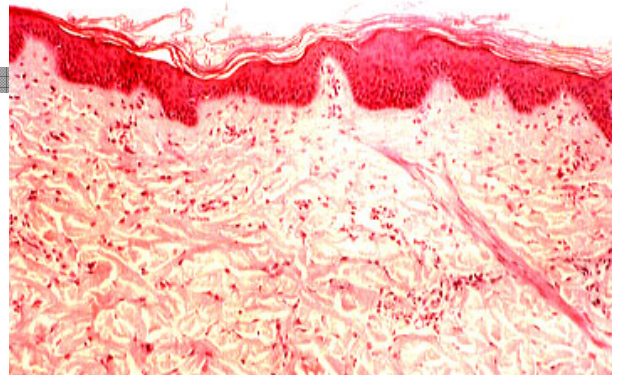
- –Skin
- –Artificial organs
- –Biomaterials
- –Cartilage
- –Blood substitutes
- –Organs
- –Vascular grafts

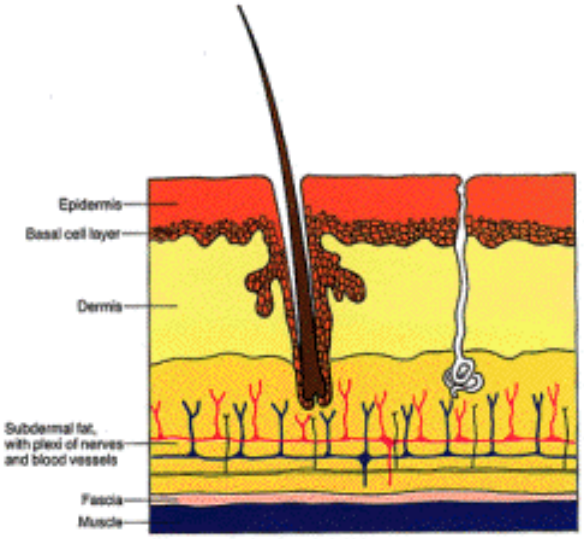
Research projects on the listed tissues. Refer handouts

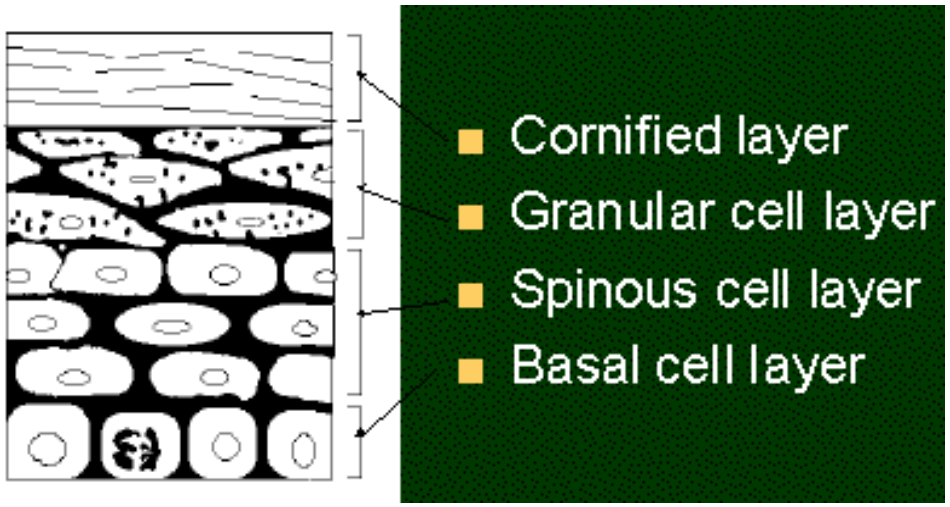
Tissue Equivalents-Skin cells

- Dermal equivalents
  - Skin repair
  - To study efficacy, metabolism and toxicity of drugs

Structure of the Skin

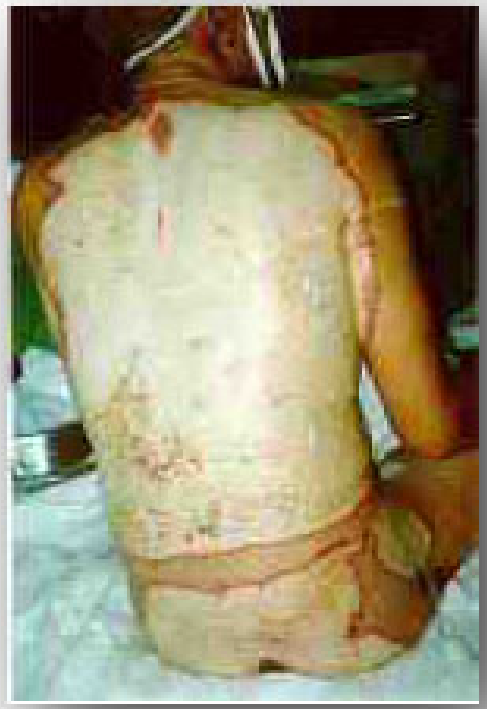






Structure of the epidermis. Cells stratify into corresponding layers according to differential stages of keratinocytes.

Autografting Technique

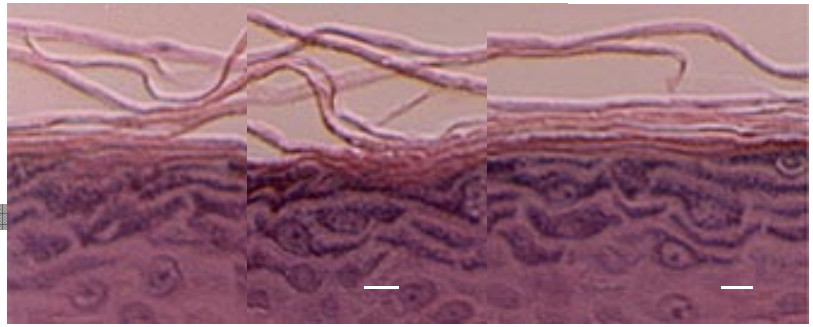


Classical

Removal of patient's own skin from donor sites for grafting. Accompanies morbidity, pain, scarring and sometimes insufficient. Skin from donor site has to be at least the same size as burn site.

Cultured Keratinocyte Sheet

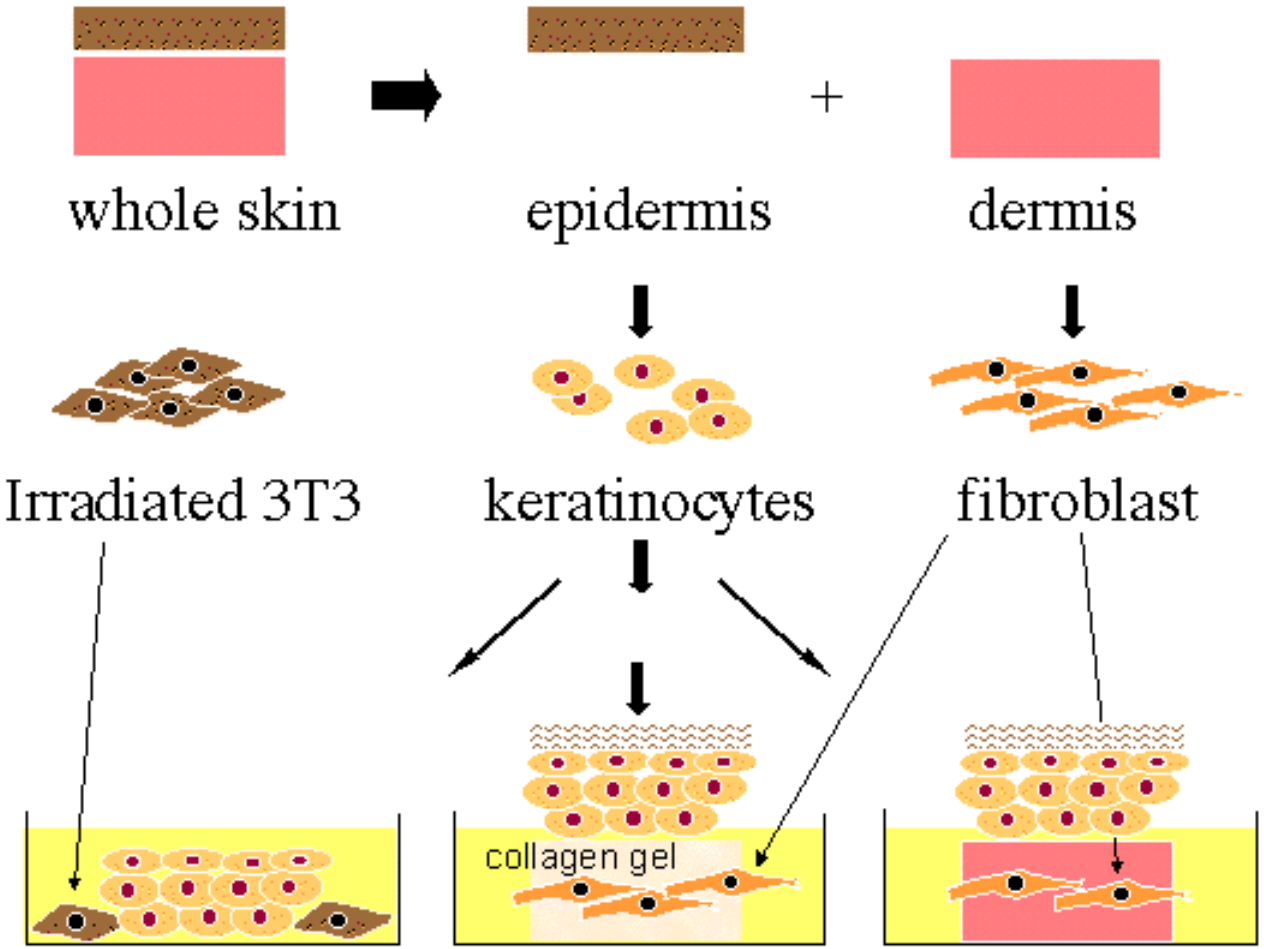
Large number of sheets can be grown from a small piece of patient's normal skin.



EpiDerm™ Skin Model (EPI-200)

1.Type: Normal human epidermal keratinocytes (NHEK).
2.Genetic make-up: Single donor.
3.Derived from: Neonatal-foreskin tissue.
4.Alternatives: NHEK from adult breast tissue.

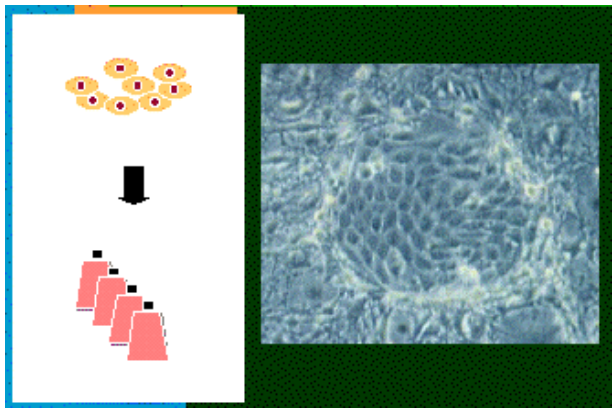
From Whole Skin to Cultured Skin: Possible Methods



Processing of Initial Skin Biopsy into Single Cells

1. Separation of epidermis from dermis

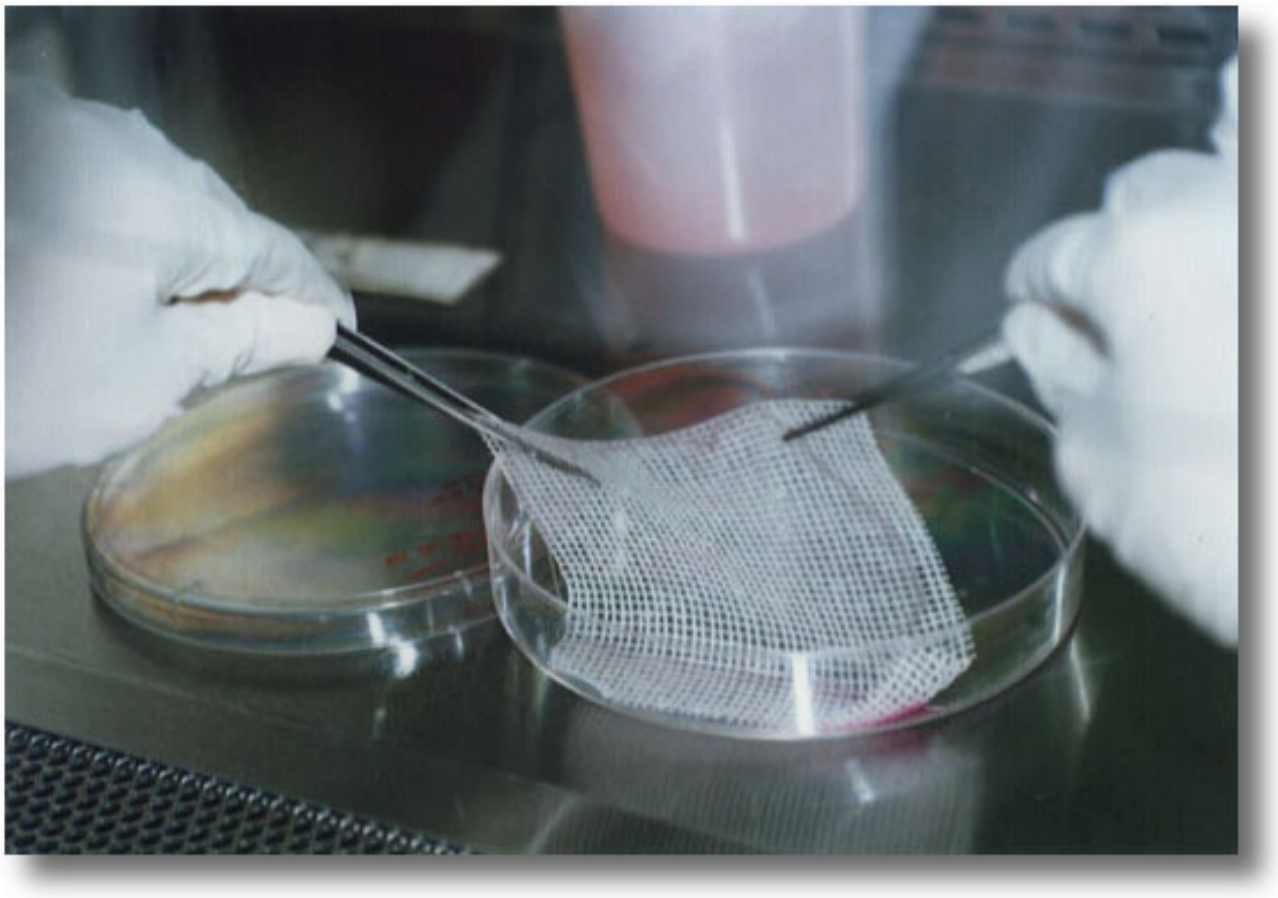
3. Filtering to exclude crude tissues, followed by centrifugation and cell count.



4. About $1x10^6$ cells from $1cm^2$ skin biopsy => 4 to 5 T-75 tissue culture flasks (3000-5000 times total area expansion).

Formation of skin tissue-

Detachment of Skin Graft from Culture Dish



Fully grown keratinocytes will form a transparent skin sheet in the culture dish. Using the enzyme dispase, the graft sheet is detached from plastic within 30-45 minutes. At this stage, a petrolatum guaze is used as backing dressing to support the graft and preserve the orientation. The graft is detached and then transferred to the operating room under sterile conditions.

Procedures for In Vitro Production of Bovine Embryos

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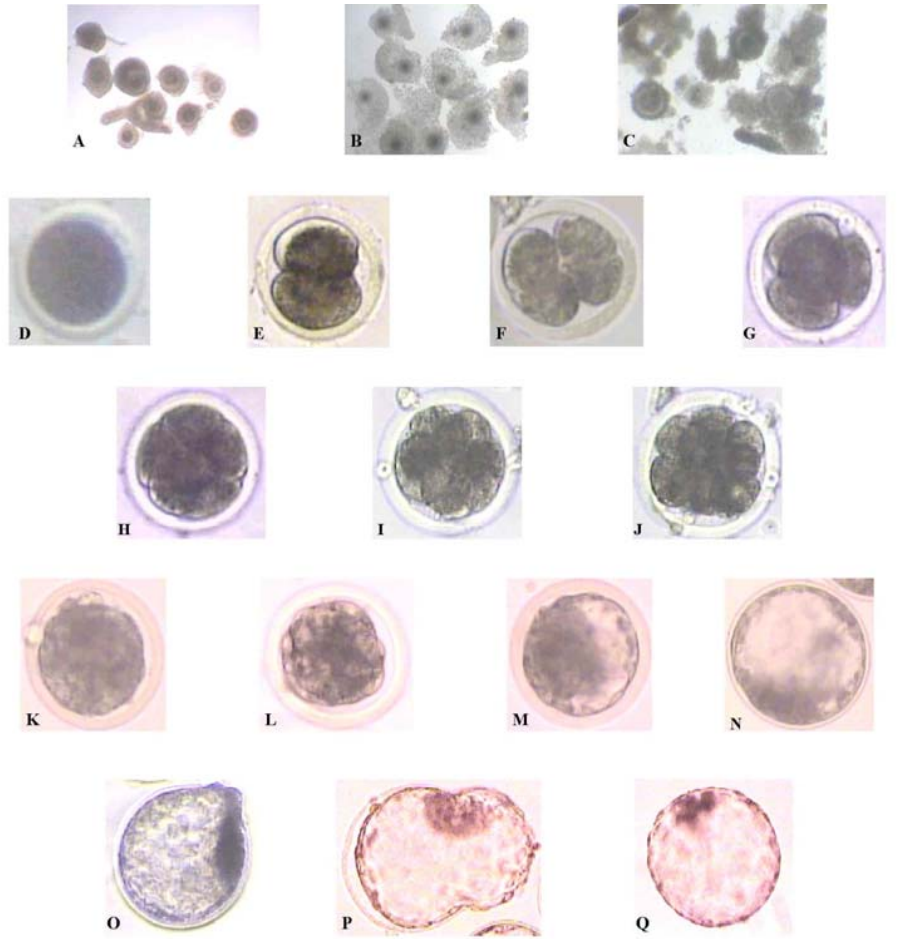
The procedures for in vitro production [IVP; i.e. in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC)] of embryos described here are based on procedures developed in other laboratories at the University of Wisconsin (Parrish et al., 1986), University of Guelph (Xu et al., 1992), and University of Missouri (Hernandez-Ledezma et al., 1993). These procedures as used by our group have been published previously (Edwards et al., 1997; Paula-Lopes et al., 1998). Keep in mind that the protocols described here are not fixed but rather constantly evolve as new developments take place. Therefore, practitioners of IVP will be well advised to experiment with the procedures used, especially after reading of improvements made by other laboratories. This protocol is organized by day of the protocol with d 0 being the day of fertilization.



A. Immature oocytes (d -1)
B. Matured oocytes (d -0)
C. Fertilized oocytes (d 0)
D. 1-cell embryo (d 0-1)
E. 2-cell embryo (d 1)
F. Cleaving 2-cell embryo (d 1-2)
G. 4-cell (d 2)
H. 8-cell embryo (d 2-3)
I. 8-16 cell embryo (d 3)
J. >16 cell embryo (d 3-4)
K. Compact morula (d 4-6)
L. Compact morula-early blastocyst (d 5-7)
M. Blastocyst (d 6-8)
N. Expanded blastocyst (d 7-9)
O. Hatching blastocyst (d 8-9)
P. Hatching blastocyst (d 8-9)
Q. Hatched blastocyst (d 8-9)

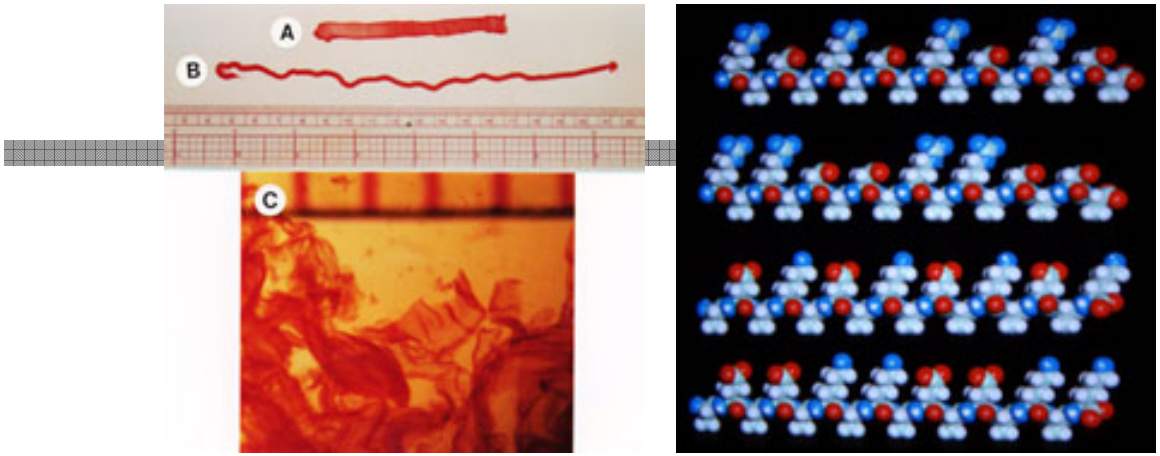
DAY-BY-DAY PROCEDURE

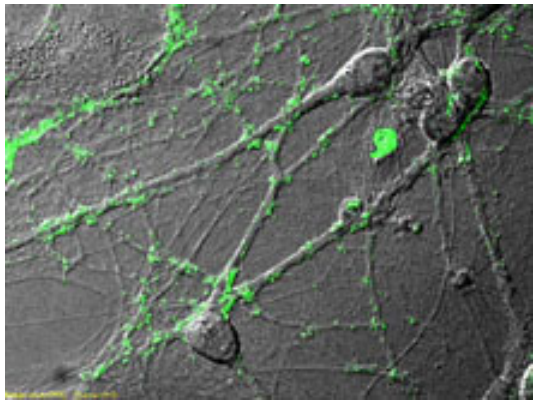
Biomaterial-
Artery, knee cartilage replacement up for FDA approval (Salubria)

A unique biomaterial developed for patients needing artery or knee cartilage replacement. It may also be used to speed repair of damaged nerves in patients with spinal cord injuries and as the basis for an implantable drug delivery system. It is biocompatible with body tissue because of

- its attraction to water,
- mechanical strength can be adjusted as needed,
- it is compliant like normal body tissue
- it is made from an organic polymer (Hydrogel), rather than silicone.

Biomaterials-





Left image: fabrication of various geometric shapes of sapeptide scaffolds. (A) The tape is approximately 8cm long, 0.5cm wide and 0.3mm thick. (B) The rope is about 2mm in diameter. (C) Membrane form.
Right image: molecular models of the ionic self-complementary peptides that form distinct polar and nonpolar sides. These peptides undergo molecular self-assembly to form the nanofibers that further assemble into scaffold.
Images courtesy Nature

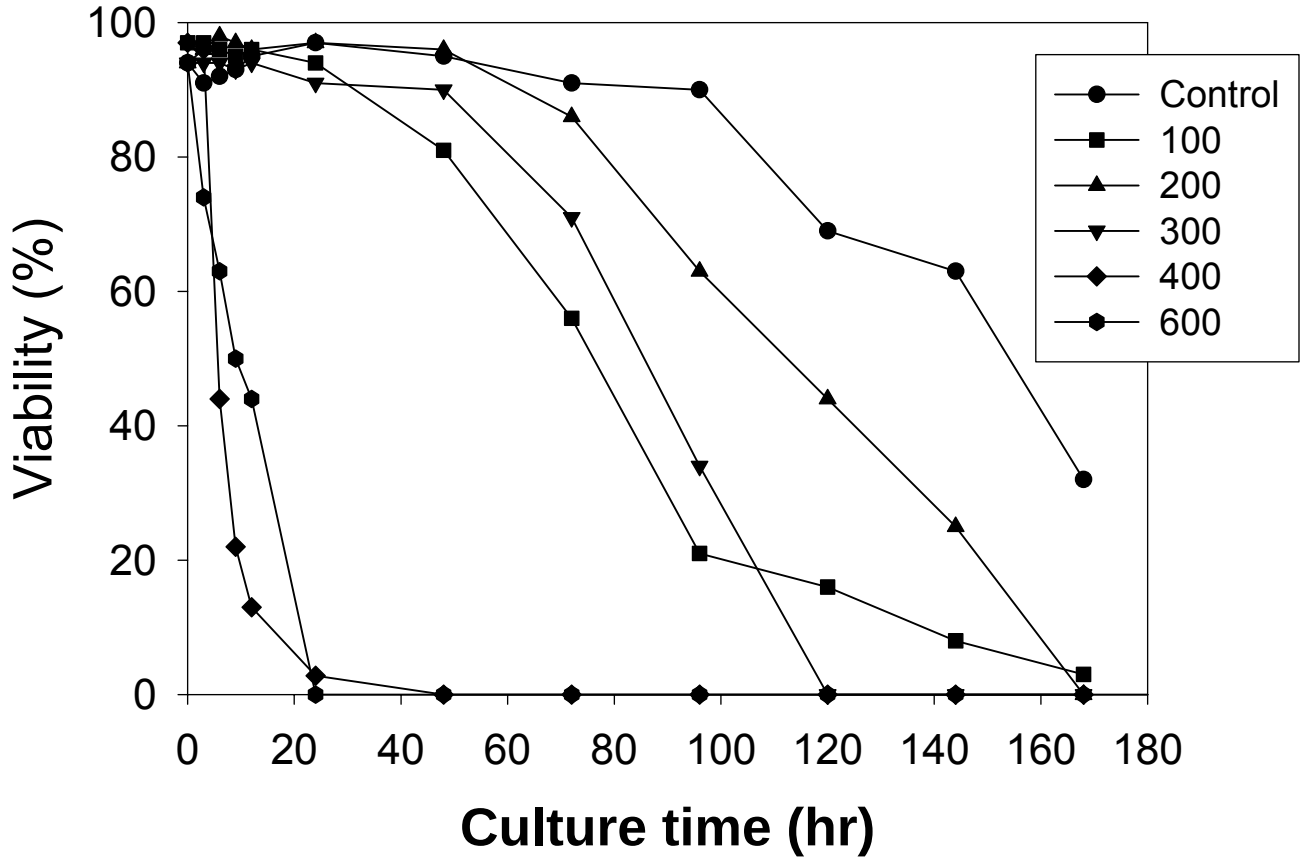
The peptide scaffold structure is analyzed by scanning electron microscopy (magnification 60,000X). Higher magnification reveals interwoven sapeptide nanofibers that are approximately 10-20nm in diameter.
Image courtesy Nature

This peptide-based scaffold, on which neurons grow fibers to communicate with each other and establish functional synapses, ideal medium for growing replacement nerve cells

CAMBRIDGE, Mass. -- Researchers at the Massachusetts Institute of Technology and New York University report in the June 6 issue of the Proceedings of the National Academy of Sciences (PNAS) that they have made a biomaterial that supports living nerve cells.

Cell culture-Phytochemical testing

(Dosage effects of MHCP extract on growth of TB/C3.bcl2 cell culture)



Thank You For Your Kind Attention