Introduction to Upstream Bioprocessing

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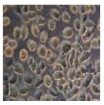
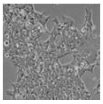
ISPE®

Outline

- Overview of Upstream Bioprocessing
- E. coli
  - Norman Garceau, PhD
- Insect/Baculovirus
  - William Hermans, BS
- Mammalian
  - Scott Gridley, PhD





BioProcess

A system that uses complete living cells or their components to manufacture biomolecular products.

Expression System Selection

- What will be produced?
  - Acids, Alcohols, secondary Metabolites, Recombinant proteins
- Intended Use?
  - Pharmaceutical or industrial product
- Post-translational modifications
- Quantity Needed?
  - On-going production
  - Short-term
- Current expertise & capabilities

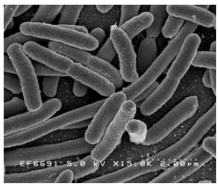
Stages of Expression Optimization

- Expression Testing & Optimization (Scout)
  - Expression host systems (E. coli, yeast, insect, mammalian)
  - Expression mode (intracellular vs. secreted)
  - Time in culture
  - Cell density
  - Feed strategies
  - Temperature
  - Induction System
- Pilot Expression
- Scale-up

Expression Systems

Microbial	Multicellular
Bacterial	Mammalian
Fungi	Insect
Algae	Plant

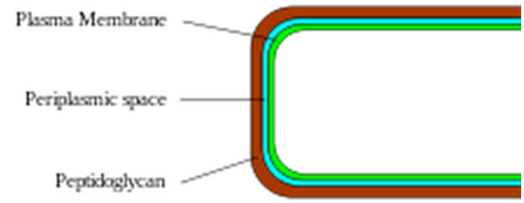
Escherichia coli



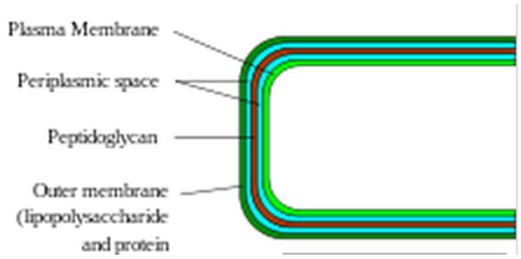
- Gram-negative
- Rod-shaped (2 microns long)
- Facultative anaerobe
- Non-sporulating
- Named after Theodor Escherich (German physician ca 1885)

Escherichia coli

- Normal flora of the mouth and intestine
- Normal gut flora that aids with digestion
- >700 serotypes distinguished by different surface proteins and polysaccharides
  - Virulent strains exist: O157:H7
- Protects the intestinal tract from bacterial infection
- Produces small amounts of vitamins B_{12} and K
- Produces vitamin B_{12} & K
- Prokaryotic model organism studied extenstively
- Divides every 20 minutes under favorable conditions

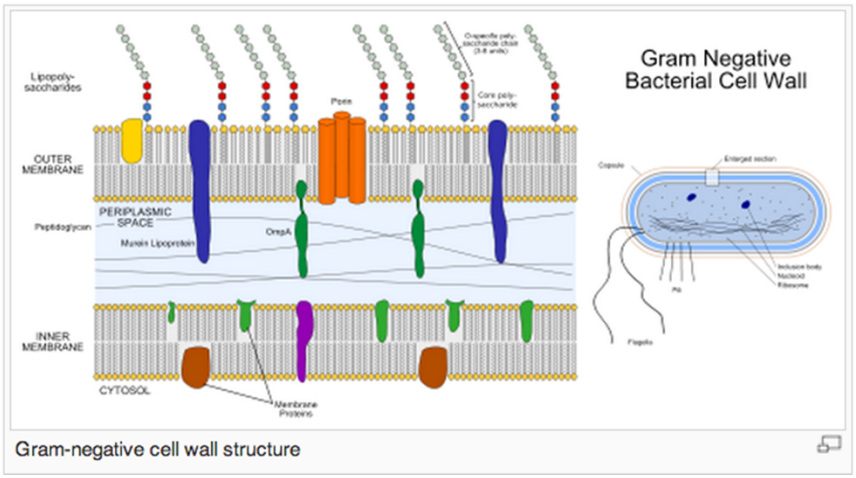


Gram Positive



Gram Negative

http://en.wikipedia.org/wiki/Gram negative_bacteria



http://en.wikipedia.org/wiki/Gram negative_bacteria

Escherichia coli in Biotechnology

- Model system for research for >60 years
- Sequence of genome published in 1997
- Circular DNA
- 4.6M bp
- 4288 proteins
- Used to manipulate DNA in molecular biology
- Common protein expression host

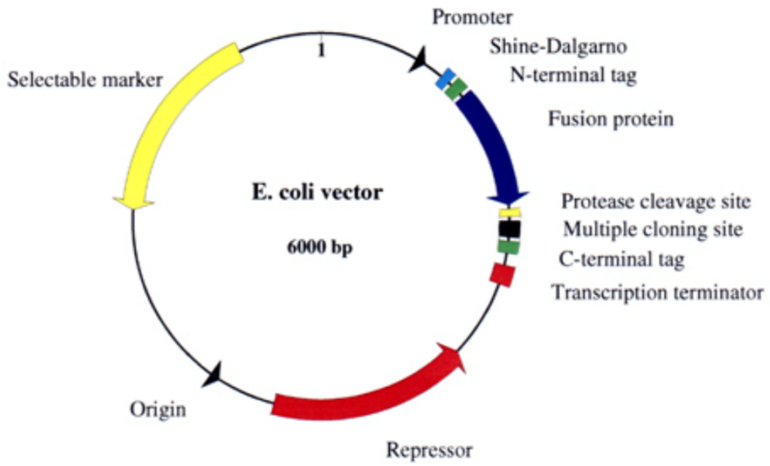
Protein Production in *E. coli:*
Desirable characteristics

- fast cell growth
- easy manipulation
- straightforward high cell density cultivation
- capacity to hold over 50% of foreign protein in total protein expression

Protein Production in *E. coli*

- 30% of therapeutic proteins are produced in E. coli
- Intracellular
  - Soluble
  - Insoluble (inclusion bodies)
- Secreted
  - Proteins directed to periplasmic space

Expression Vectors



E. coli Fermentation

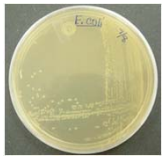
- Closed system: no supplementation to growth medium during culture
  - the exponential phase of growth remains for only few generations and then enters the stationary phase.
- Open system: nutrient supplemention during culture
  - with adequate nutrient supply and waste removal, the exponential phase can be maintained for a long time.

Factors that Affect Growth of Cultures

- Availability of nutrients
- Nutrient quality
- Temperature
- pH
- Accumulation of toxic metabolites
- Rate and nature of mixing usually change with every 10 fold increase
- Oxygen demand

Culture Systems for E. coli

Agar Plate

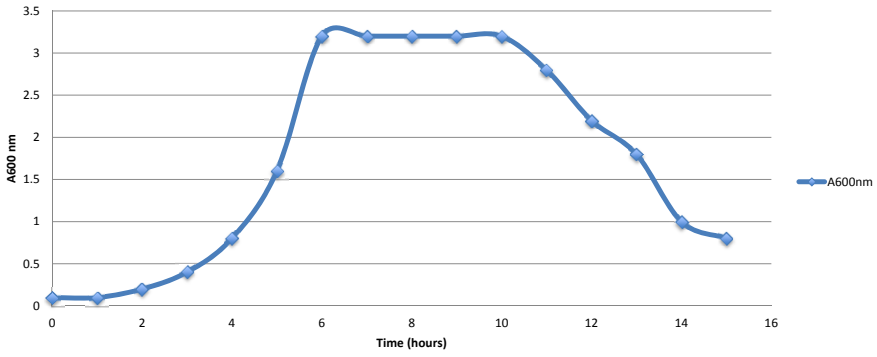


Test Tube

Flask

Fermentor

Escherichia coli Growth Curve



E. coli divides ~ every 20 minutes

Factors affecting growth:

- Availability of nutrients
- Nutrient quality
- Temperature
- pH
- Accumulation of toxic metabolites
- Rate and nature of mixing usually change with every 10 fold increase
- Oxygen demand

Fermentation

Allows control over key chemical, physical, and biological parameters that affect cell growth & recombinant protein production

- Control
  - Agitation
  - Temperature
  - pH
  - Dissolved Oxygen (DO)
  - Nutrients (Fed Batch)
- Laboratory Scale: 5-65L
- Pilot Scale: 200-600L
- Manufacturing Scale: >2000L

Batch & Fed-Batch Cultures

- Batch fermentation (Closed System):
  - Simpler than Fed-batch
  - Lower productivity
- Fed-Batch (Open System):
  - more complicated than Batch
  - Continuous supply of nutrients to achieve much higher cell densities & protein production
  - Monitor & Control pH and Dissolved oxygen

Fermentation for Biotherapeutics

Table 1 Recent approved protein therapeutics using *E. coli* as an expression host

Generic name/protein (brand name)	Indications	Approved date, place and company
Ranibizumab (Lucentis)	Wet type age-related macular degeneration	2006 US, 2007 EU, Genentech
Somatropin (Accretropin)	Growth hormone deficiency; Turner syndrome	2008 US, Cangene
Certolizumab pegol (Cimzia)	Crohn's disease	2008 US, 2009 EU, UCB[a]
PEG interferon alfa-2b (PegIntron)	Chronic hepatitis C infection	2008 US, Schering-Plough
Romiplostim (Nplate)	Chronic immune thrombocytopenic purpura	2008 US, Amgen
Interferon beta 1b (Extavia)	Multiple sclerosis	2008 EU, 2009 US, Novartis
Pegloticase (Krystexxa)	Chronic gout	2010 US, Savient Pharms

Data were collected from http://www.fda.gov and http://www.ema.europa.eu; [a] Union Chimique Belge

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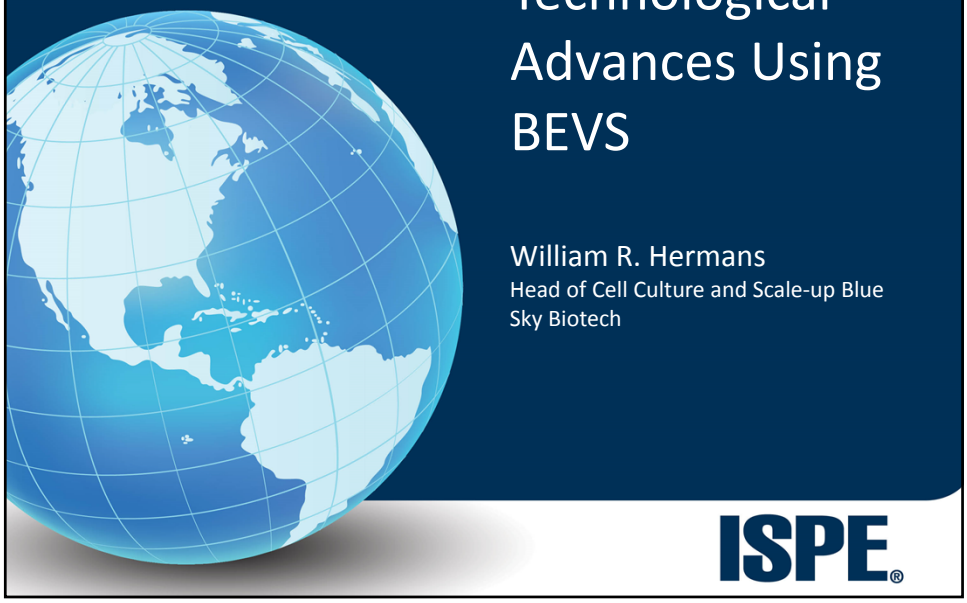
Summary: E. coli

- E. coli is a rapid, cost-effective system for protein production
- Specifications of the protein to be produced determines suitability of E. coli as a host
- E. coli can be grown in a several culture systems, but fermenters are used for GMP production.
- 30% of all therapeutic proteins are produced in E. coli



Why use BEVS?

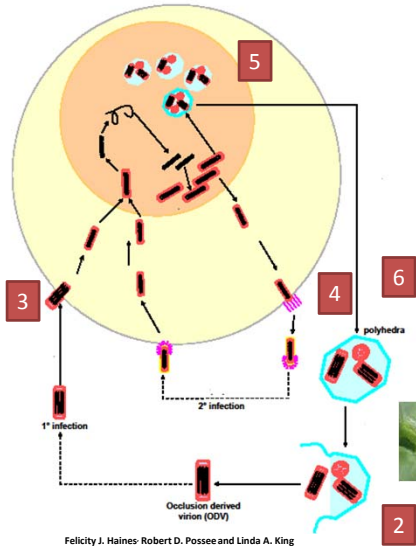
	Bacterial	BEVS	Mammalian
Ease of culture	√	√	
Cost Effectiveness	√	√	
Accuracy (protein folding, post-translational modifications, oligomerization)		√	√

Baculovirus Life Cycle

1-Insect host eats plant contaminated by baculovirus

2- Protein capsule dissolved by alkaline midgut

3- ODV enter cell and viral transcription begins



4- BV are released from cell, resulting in secondary infections

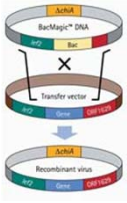
5- New ODV are formed in very late stage of the replication cycle

6- Cell lysis occurs, ODVs are released

Evolution of BEVS

Site Specific Transposition

Co-transfection



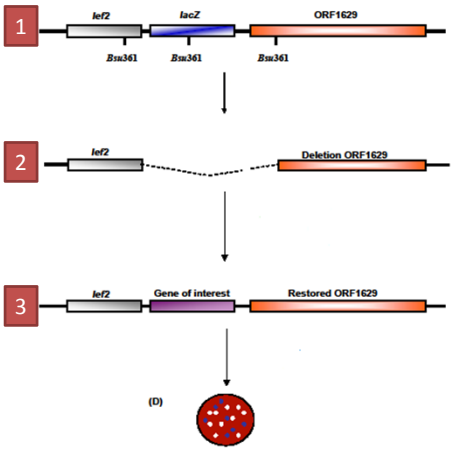
Homologous Recombination

Co-transfection Method

1- Multiple Restriction sites are added to the baculovirus DNA, including within the *lacZ* gene and an important replication gene

2- Baculovirus DNA is co-transfected with transfer vector containing GOI

3-Insertion of the GOI allows restoration of ORF, allowing DNA to replicate within cell



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Bac to Bac® Method

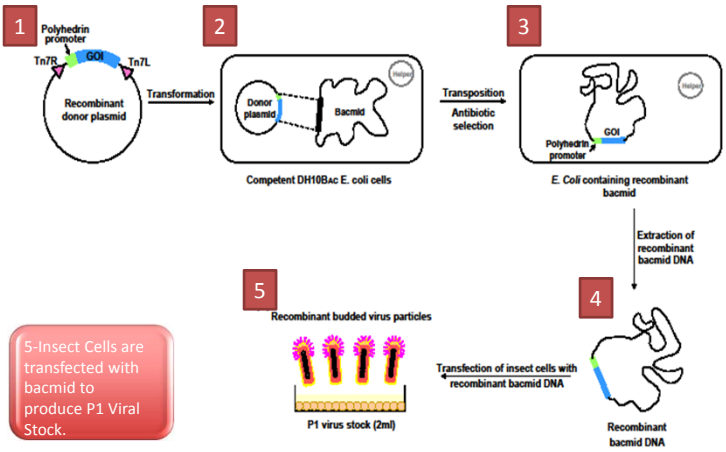
1-GOI cloned into donor plasmid.

2-Recombinant donor plasmid is transformed into competent E. coli cells.

3-Transposition occurs, resulting in insertion of the GOI into bacmid DNA.

4-Recombinant virus DNA is extracted and purified.

5-Insect Cells are transfected with bacmid to produce P1 Viral Stock.



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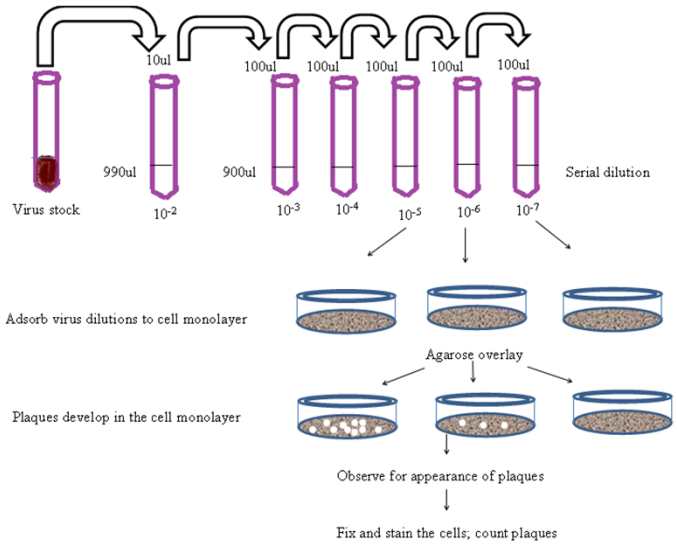
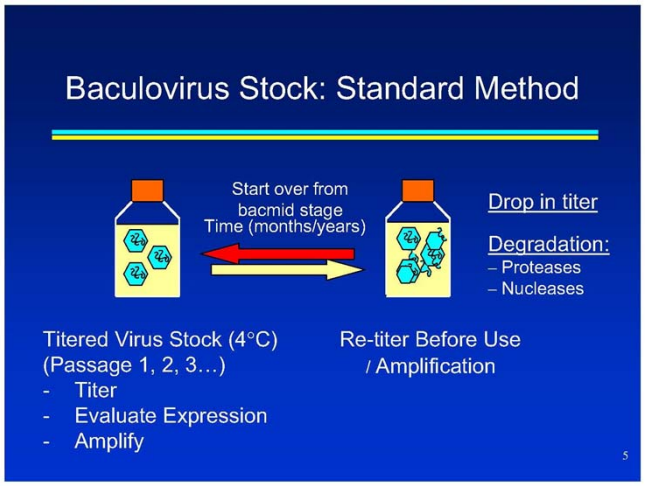
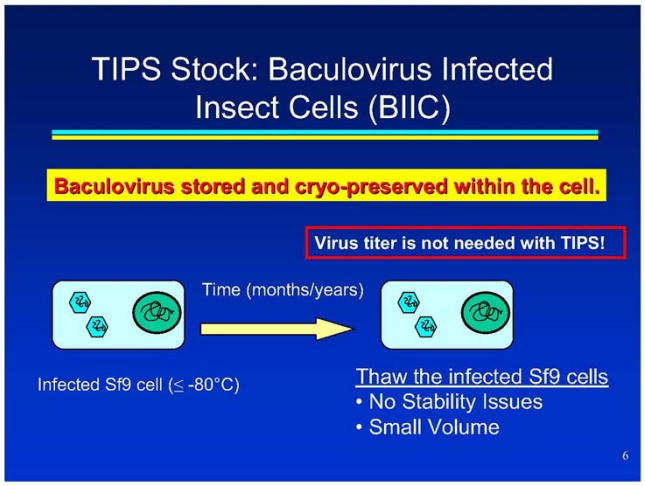
Plaque Assay to Determine Titer



Standard Methodology



BIIC and TIPS Technology



BIIC and TIPS Technology

Virus Stock Comparison

	Standard Virus Stocks	*TIPS Infected Cell Stocks*
Volume	>100 mL	≤1 mL
Environment	Spent media	Native host cells
Storage Temp.	4-8 °C	≤ -80 °C
Stability	Questionable	No issues
Titering	Necessary	Eliminated

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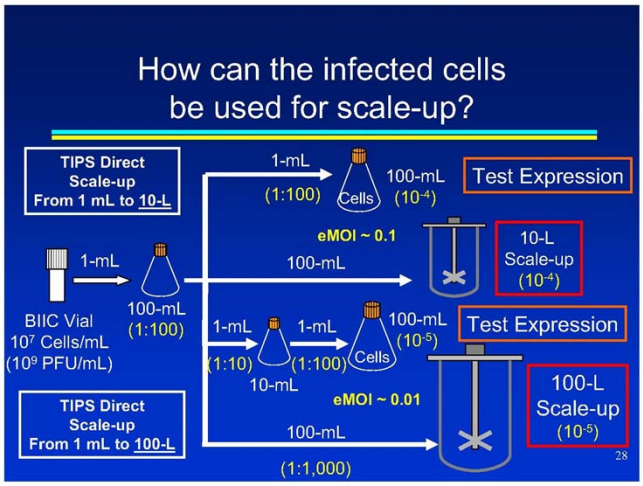
BIIC and TIPS Technology



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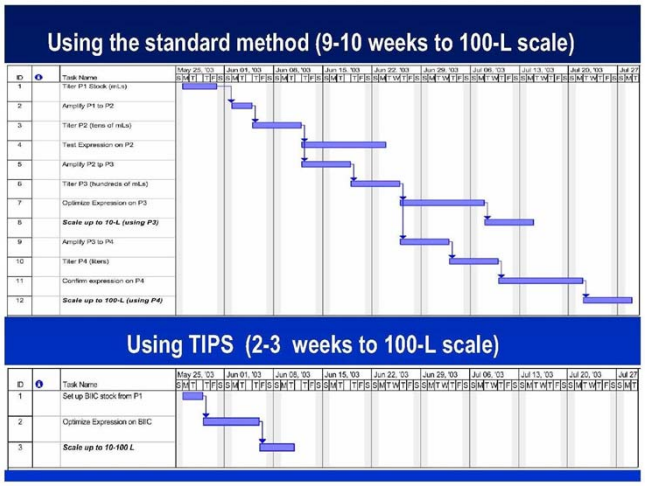
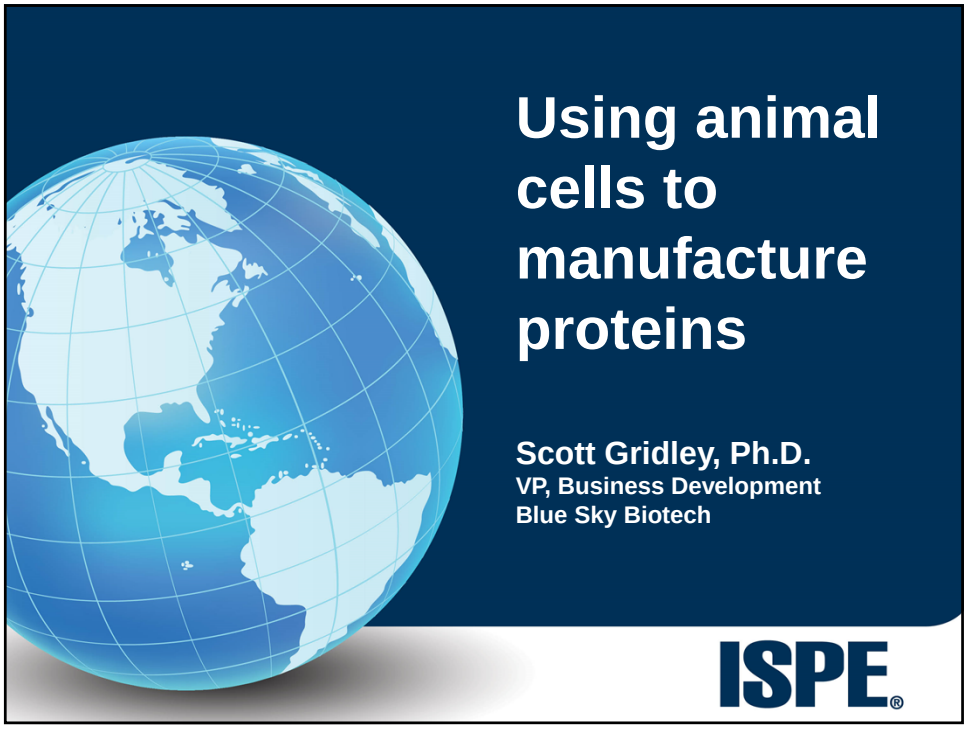


Acknowledgements

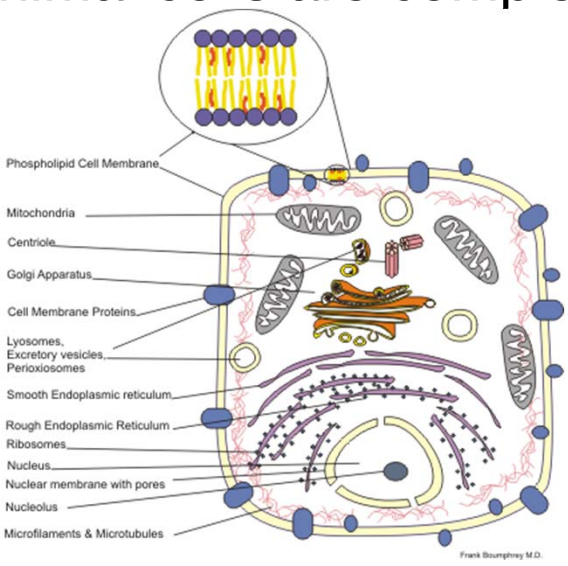
- Dr. S. Edward Lee
- David J. Wasilko



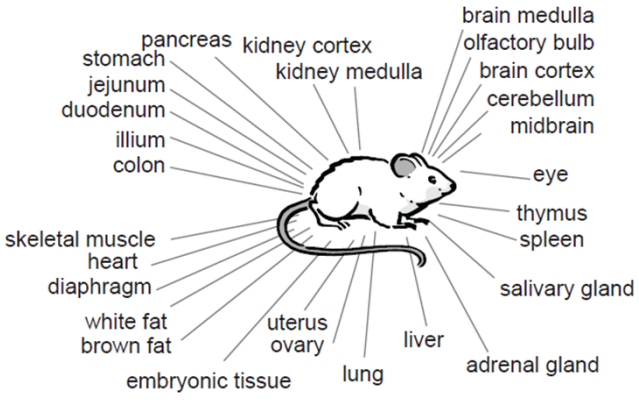
Why use Mammalian cells?

	Bacterial	BEVS	Mammalian
Ease of culture	√	√	
Cost Effectiveness	√	√	
Accuracy (protein folding, post-translational modifications, oligomerization)		√	√

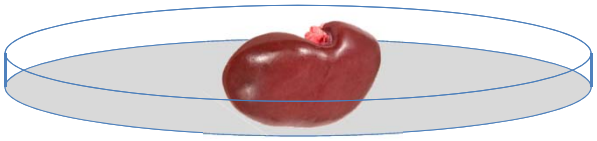
Animal cells are complex



Animal cells are specialized

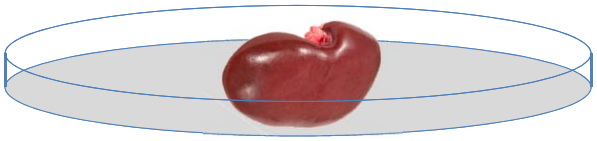


Primary cell culture



Primary cell culture

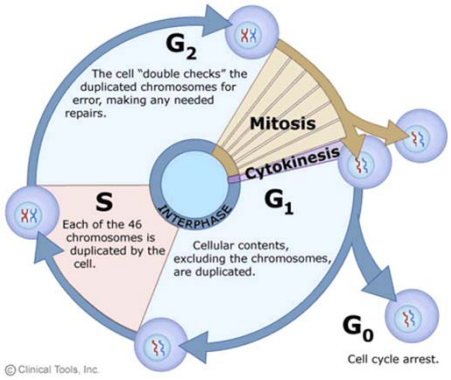
Necessary supplements:
- Blood = Media
- Growth factors = Serum
- Oxygen = Oxygen



•Cells will only survive a few doublings before dying

Transformed cell lines

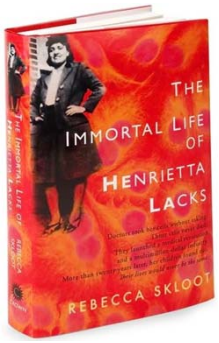
Cells that have been “deprogrammed” and lost their normal cell cycle regulation



Commonly used cell lines

- CHO = Chinese Hamster Ovary
- HEK = Human Embryonic Kidney
- BHK = Baby Hamster Kidney
- NS0 = Mouse Myeloma
- HeLa = Cervical Cancer……→

•ATCC:



Transfection and Transduction

How do we make cells manufacture specific proteins we want them to?

- **Chemical**
  - CaPO4
  - Lipid-Mediated
- **Physical**
  - Electroporation
  - Injection
  - Gene gun
- Transient vs. Stable

- **Viral-mediated**
  - Adenovirus
  - Retrovirus
  - Others

Adherent vs. Suspension

Cells per Flask

3×10^6

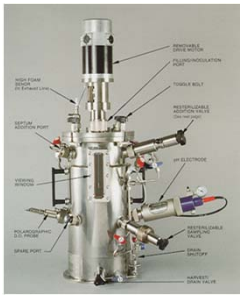
1×10^7

2×10^7

1×10^6 PER ML

Adherent Scale-Up Methods

Suspension Scale-Up Methods



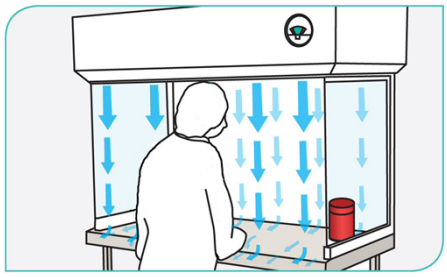
Media Formulations

- Aqueous Buffer containing nutrients and other factors necessary for growth
- First medias were tissue or embryo extracts or serum
  - Highly variable and costly
- Defined Media:
  - Replicate physiologic properties of body fluids with chemically-defined buffers
    - Eagle's Minimum Essential Medium (MEM)
    - Dulbecco's modified Eagle's Medium (dMEM)
    - Others
  - Components:
    - Buffer to maintain pH of 7.4
    - Sugars (glucose), Amino Acids and Vitamins
    - Balanced salts
    - Trace metals
    - GROWTH FACTORS PROVIDED BY ADDITION OF SERUM (~10%)
      - Serum is a variable, costly, and labile component,
      - Often a source of contamination

Media Formulations

- Serum-free Medium: Replace Serum with cocktail of known growth factors, etc.
  - What's in serum?
    - Essential nutrients (Fatty acids, vitamins, intermediate metabolites)
    - Adhesion factors (fibronectin)
    - Hormones (Insulin, hydrocortisone, estrogen)
    - Growth Factors (PDGF, TGF-beta)
- Cells may still grow slower in Serum-free medium than standard medium.

Aseptic Technique



Summary

- Mammalian cells may be the best host for producing mammalian proteins
- Recombinant genes can be transfected or transduced into mammalian cells
- Unique cell lines may exist for various tissue types (biological relevance)
- Cell lines may grow adherently or in suspension
- Cell lines may grow in serum-free media or require supplements
- Mammalian cell culture is technically challenging