Level 8 Cell Culture Processing (BIO08045)
Lecture 4 – "Kinetics of Cell Growth & Productivity"

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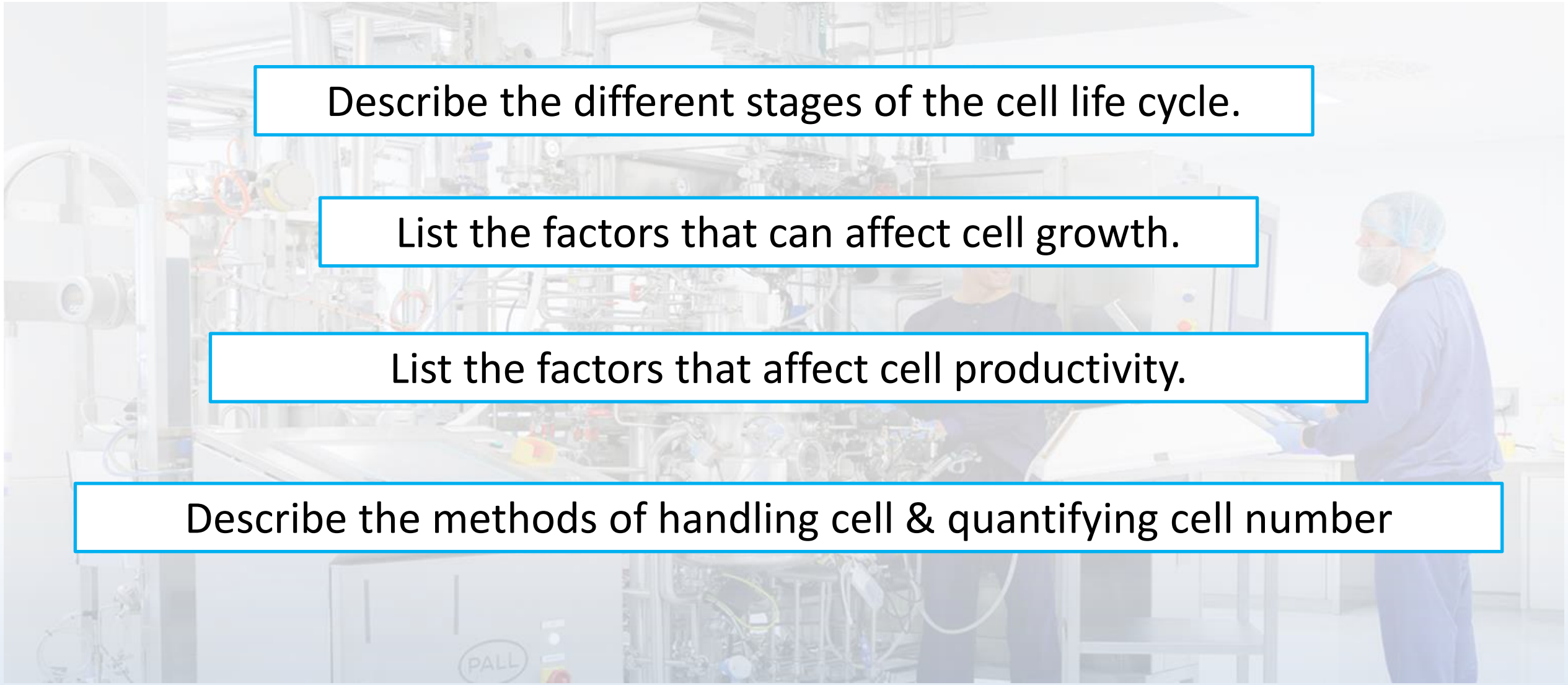
Learning Objectives



Describe the different stages of the cell life cycle.

List the factors that can affect cell growth.

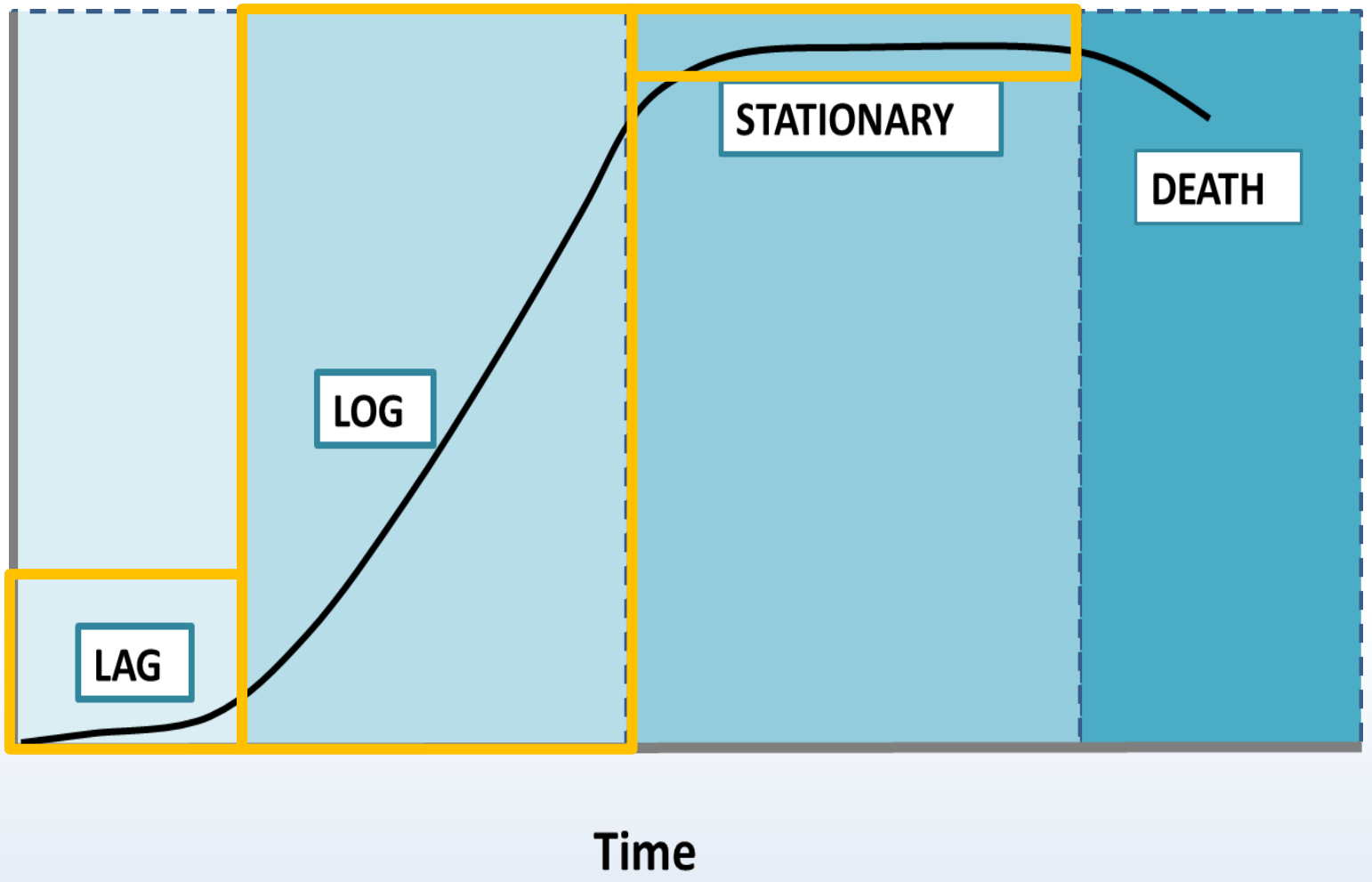
List the factors that affect cell productivity.

Describe the methods of handling cell & quantifying cell number

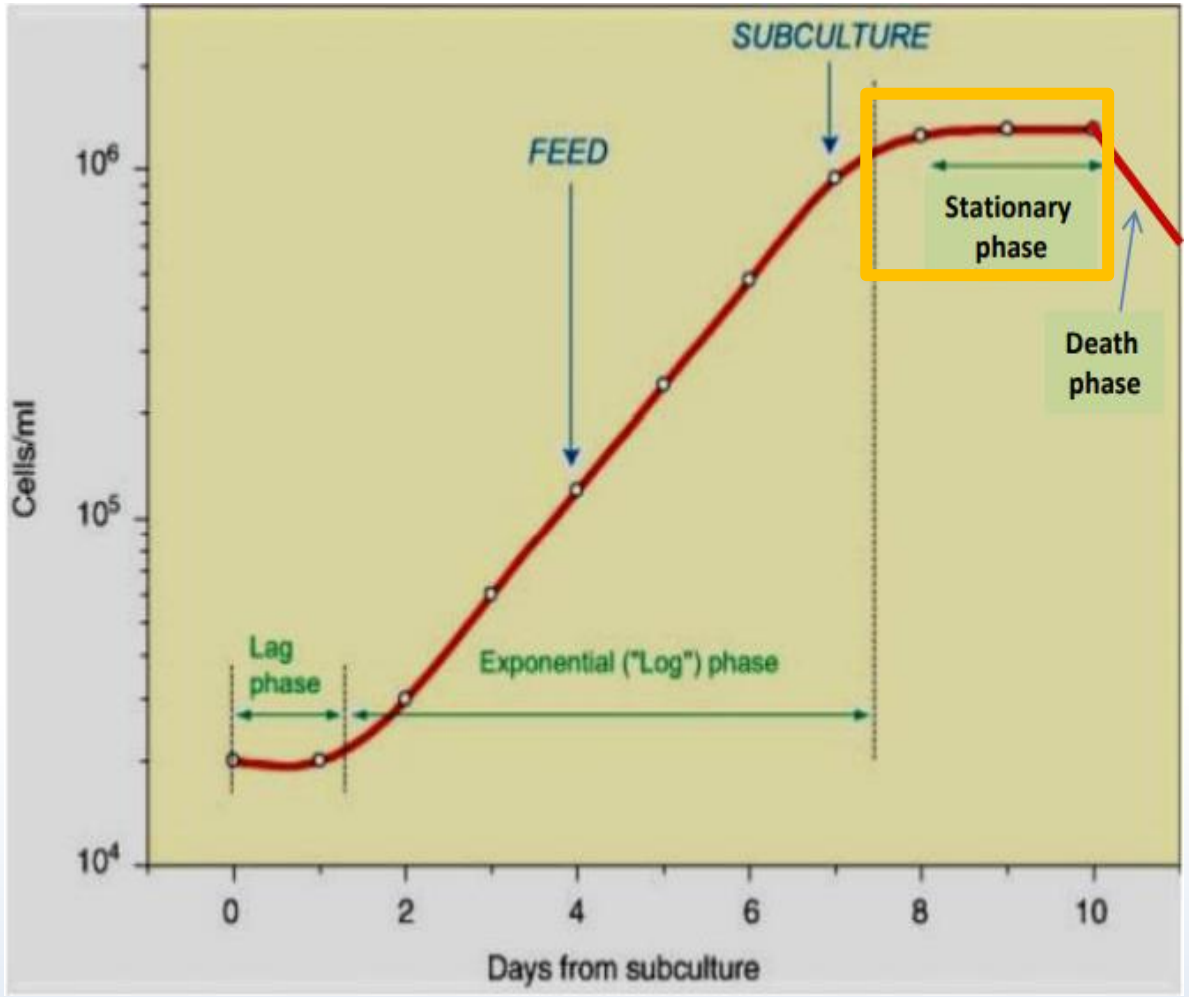
Lecture Topics

- Cell Growth
- Factors Effecting Rate of Cell Growth
- Cell Productivity Factors
- Handling & Manipulation of Cell Culture
- Measurement of Cell Growth

Typical Cell Growth Curve



Cell Growth Curve



- Establish growth characteristics of cells:
  - Graph shows cell number –v- days in culture
  - 4 major phases: **Lag, Log, Stationary and Death**
- Establish seeding density and saturation density
- Establish when cells need to be fed (3-4 days) or when they need to be subcultured (1-2 week)

Phases of Growth Kinetics

- *Lag Phase:*
  - This is the first phase in the cellular growth process.
  - The cells have just been injected into a new environment and they need time to adjust accordingly.
  - **Cell growth is minimal in this phase.**
- *Exponential Phase:*
  - This is the second phase in the cellular growth process.
  - The cells have adjusted to their new environment and rapid growth takes place.
  - **The cell growth rate is the highest in this phase.**
  - At some point the cell growth rate will level off and become constant.
  - The primary cause of this levelling off is substrate limited inhibition i.e. the cells do not have enough nutrients in the media to continue multiplying

Specific Growth Rate (μ)

- Cell growth can be described by the equation:

$$dN/dt = \mu N - \alpha N$$

- Where:
  - N = no. of cells.
  - t = time.
  - μ = specific growth rate (h^{-1}).
  - α = specific death rate (h^{-1}).
- Rearranged as follows:

$$1/N \times dN/dt = \mu - \alpha$$

- *Growth is the balance of growth and death usually with $\mu >> \alpha$*

Exponential Growth Phase

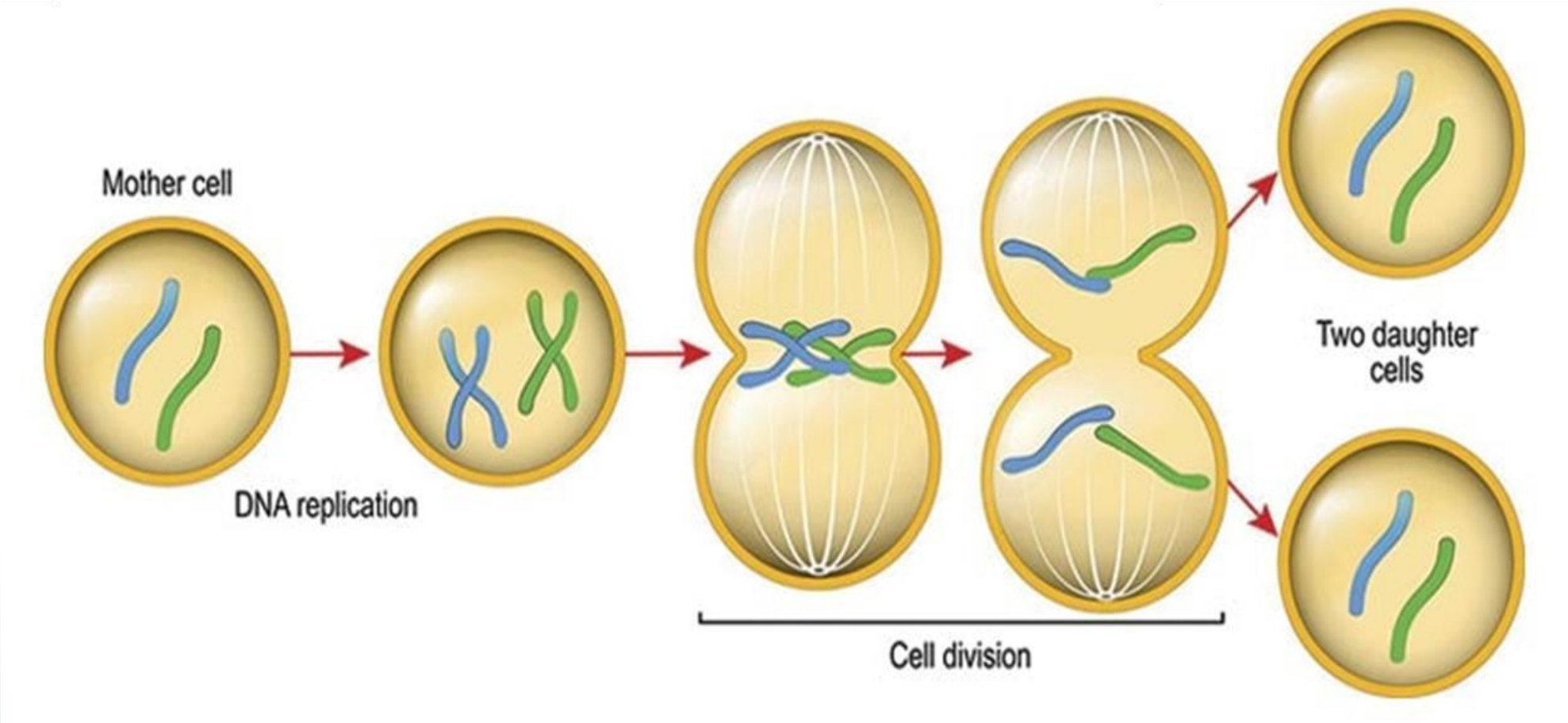
$$dN/dt = \mu N - \alpha N$$

- Log phase growth is first order kinetics where N is the number of cells produced.
- **Growth rate is proportional to population size.**
- So ln N vs. t is linear and slope = μ where μ is the specific growth rate for the cell line.
  - **μ units are 1/t (i.e. hr-1).**
- Can also be shown as $N = N_o e^{\mu t}$ i.e., the classic exponential equation.
- *Note: This first-order kinetics equation applies to many situations in biology and chemistry*

Mitosis

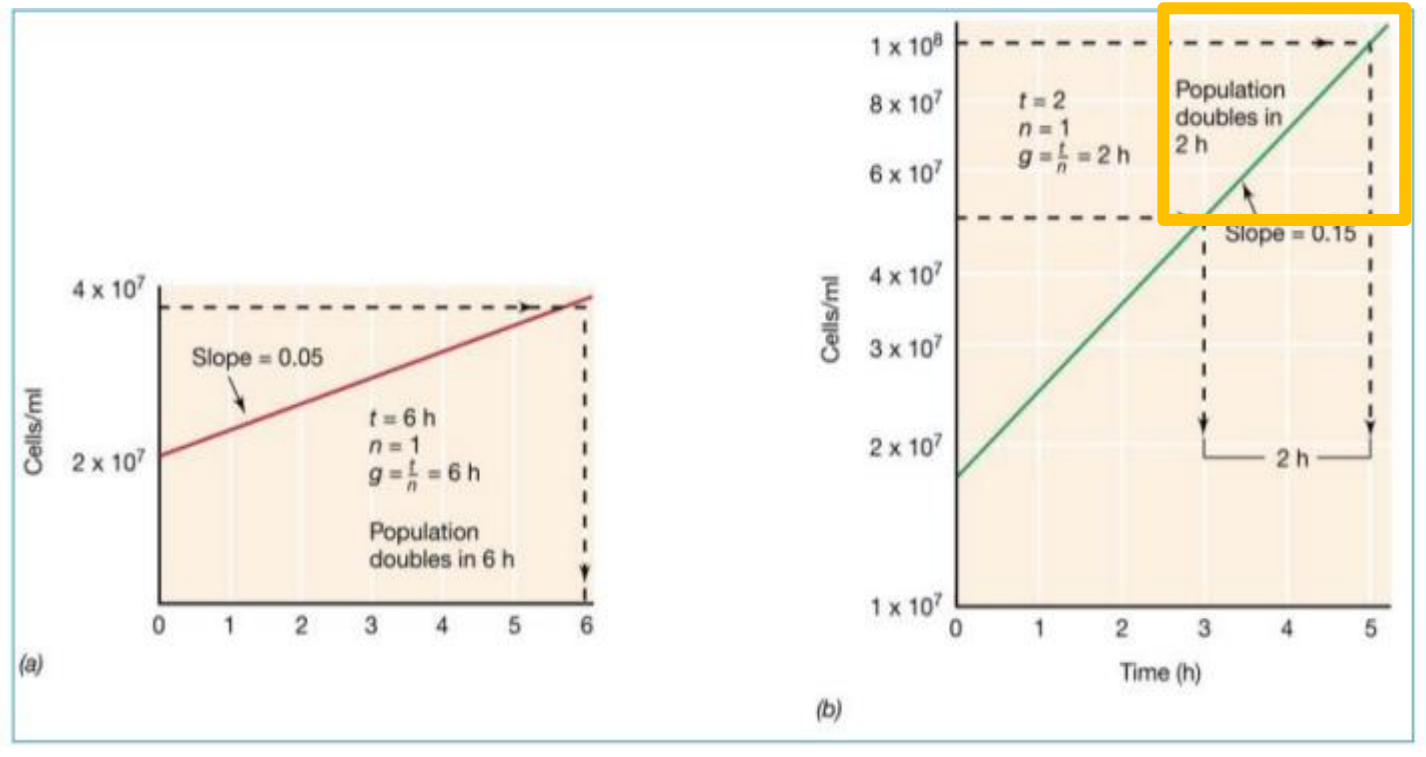
- Mitosis is divided into distinct stages: interphase, prophase, metaphase, anaphase and telophase
- Cells are mostly in interphase, once mitosis starts chromosome become distinct
- Each chromosome replicates to form pair of sister chromatids, held together by centromere
- Centromeres divide and sister chromatids separate, one of each kind goes to a daughter cell
- Mitosis produces 2 identical daughter cells with the same DNA content as parent cells, referred to as 2n or diploid
  - http://www.bioweb.uncc.edu/biol1110/Stages.htm - pictures of cells in mitosis

Overview of Mitosis



Cell Generation Time

- Generation time or doubling time, is the time required for the cell population to double.
- The calculation is: $t_d = \ln(2)/\mu$



Calculation of Generation Times

- Increase in cell number is a geometric progression of the number 2

$$N = N_0 2^n$$

Where N = final cell number, N_0 = initial cell number and n = number of generations. This can be expressed in term of n

$$n = 3.3 (\log N - \log N_0)$$

$g = t/n$

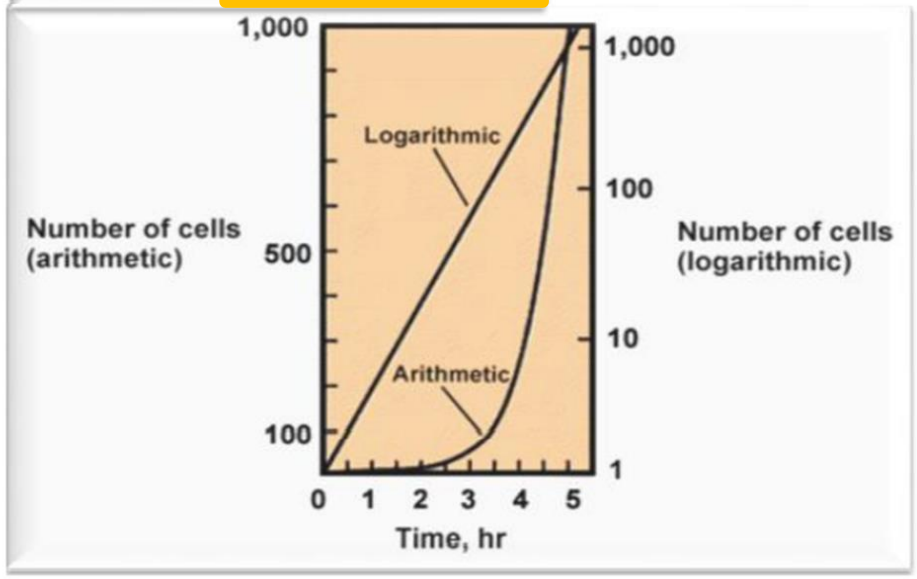
g: generation time

t: time

n: number of generations

Calculation of Generation Times

Time (hours)	Number of cells
0	1
0.5	2
1	4
1.5	8
2	16
2.5	32
3	64
3.5	128
4	256
4.5	512
5	1024
5.5	2048
6	4096
.	.
.	.
10	1,048,576



$n = 3.3 (\log 1 \times 10^6 - \log 1)$

$= 3.3 (6 - 0)$

$= 20$

In 10 hours, we have a total of 20 generations

$g = 10\ h / 20$

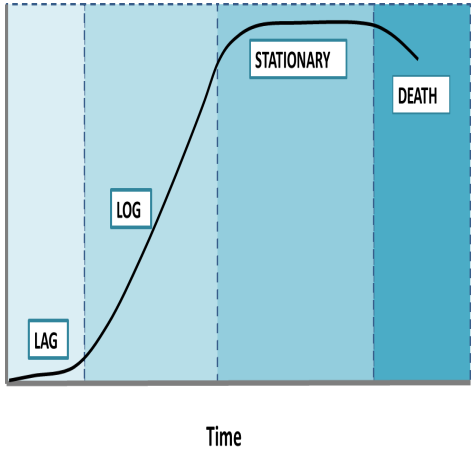
$= 0.5\ h$

Doubling time here is 0.5h

– *typical for bacteria*

Phases of Growth Kinetics

- **Stationary Phase:**
  - This is the third phase in the cellular growth process.
  - **The cell growth rate has levelled off and become constant.**
  - The number of cells multiplying is equal to the number of cells dying.
  - This is the most productive phase for protein expression.



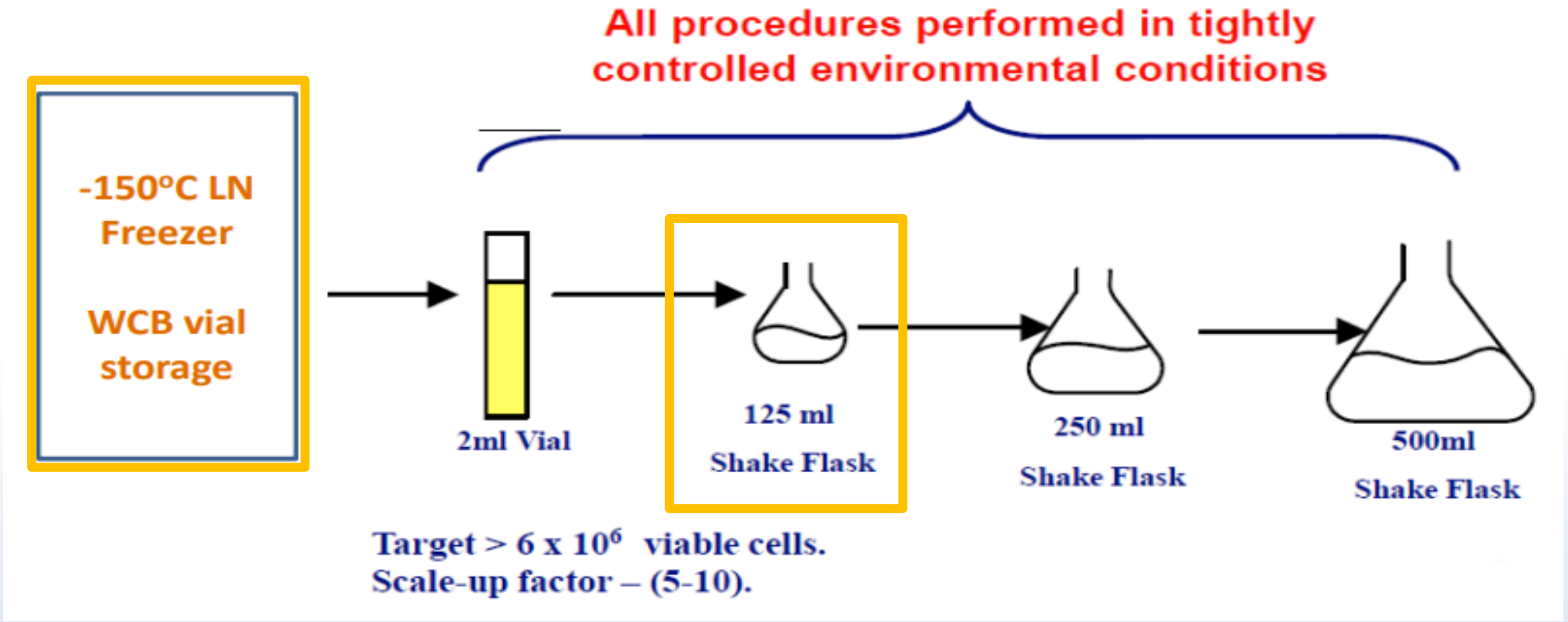
- **Death Phase:**
  - This is the fourth and final phase in the cellular growth process.
  - **The number of cells dying is greater than the number of cells multiplying.**
  - The primary cause of the death phase is that the cells have consumed most of the nutrients in the media and there is not enough left for cell sustainability.
  - Hence the media feed system for a bioreactor has a major bearing on the cell growth and decay process i.e. fed-batch vs. continuous culture

Impact of Growth Kinetics on Operations

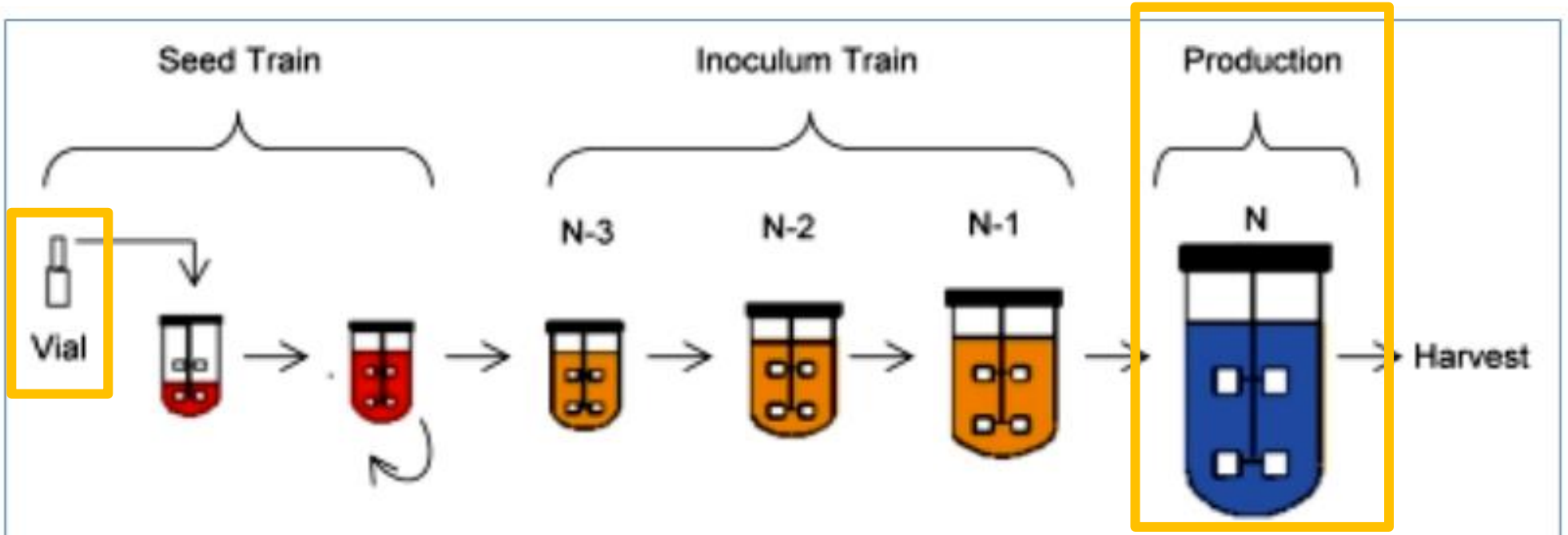
- The doubling rate for mammalian lines can vary from 24 – 30 hrs.
  - This rate determines the duration of scale-up from vial thaw to bioreactor seeding.
- It will also determine the strategy for scale-up in terms of the intermediate scale-up steps required e.g. from vial to shaker flasks and from shaker flasks to wave bags etc.
  - Operational plans such as the changeover of a bioreactor tank and starting of a new run are made based on a knowledge of the scale-up rate which in turn is based on the doubling time.

Vial Thaw and Pre-Culture Scale-up

- Start of Cell Culture Operation begins with cracking a WCB 2ml vial
- **Objective: Grow Cells to inoculate a WAVE bioreactor**



From Cryovial to Production



Manufacturing Scale	20L	80L	400L	2,000L	12,000L
Scale-Down Model	2L	2L	2L	2L	2L
Key Performance Parameter	Growth rate, final viability				Titer, Product quality

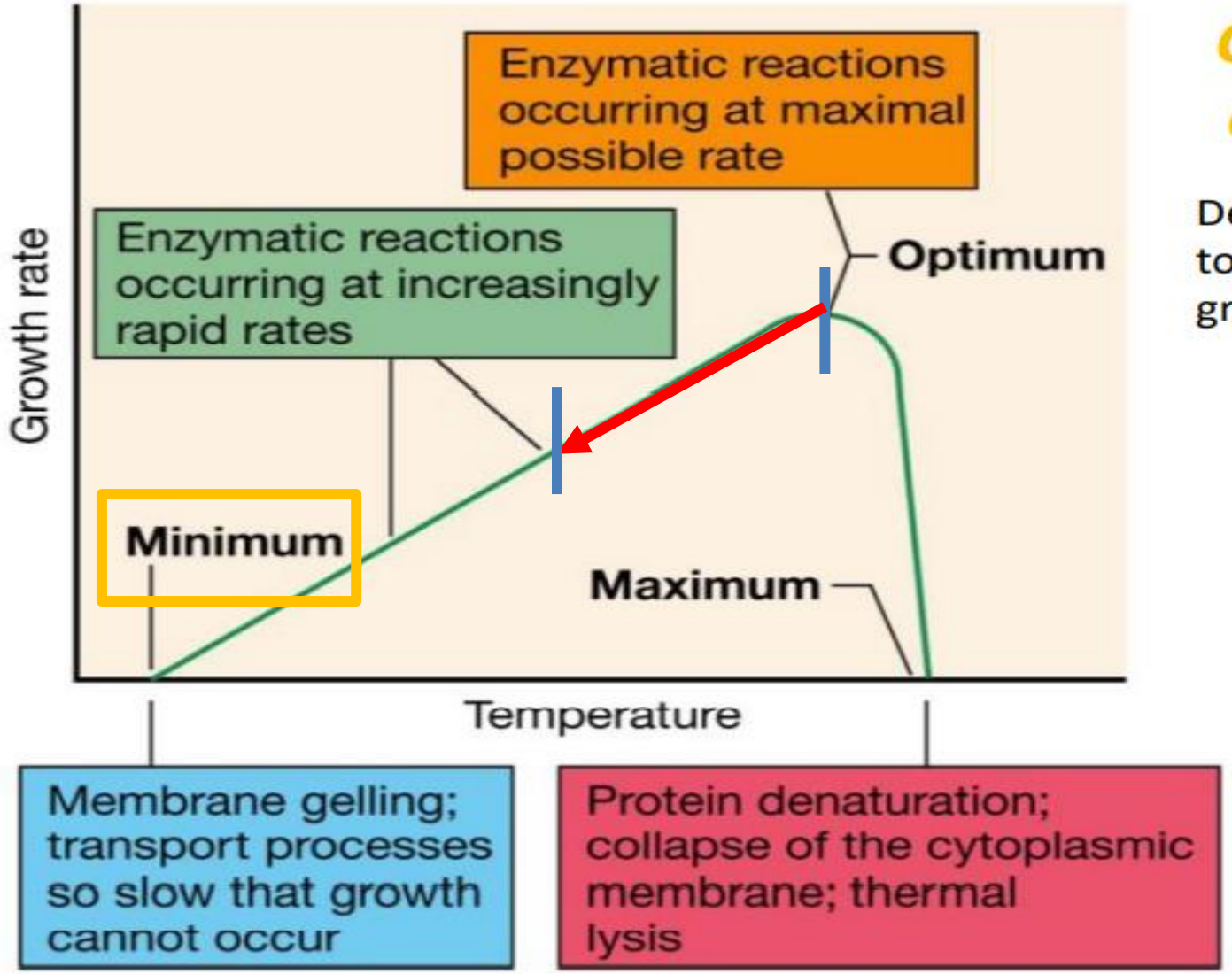
Lecture Topics

- Cell Growth
- Factors Effecting Rate of Cell Growth
- Cell Productivity Factors
- Handling & Manipulation of Cell Culture
- Measurement of Cell Growth

What Factors Affect Cell Growth Rates?

Temperature	• Every cell line will have an optimal growth temperature
pH	• Fluctuations in pH may affect growth rate
Osmolality	• May damage plasma membrane or disrupt the water content of the cell
Dissolved Oxygen	• Insufficient oxygen supply may become the rate limiting factor affecting growth rate
Nutrients/Metabolites	• Sufficient nutrient levels required for growth • High levels of metabolites may negatively affect growth
Contamination	• Contamination may affect cell growth and induce cell death

Effect of Temperature on Growth Rate



Cell growth rate:
$$dN/dt = \mu N - \alpha N$$

Death rate (α) is more sensitive to temperature change than growth rate (μ).

Effect of Temperature on Specific Growth Rate cont/d

Cell growth rate: $dN/dt = \mu N - \alpha N$

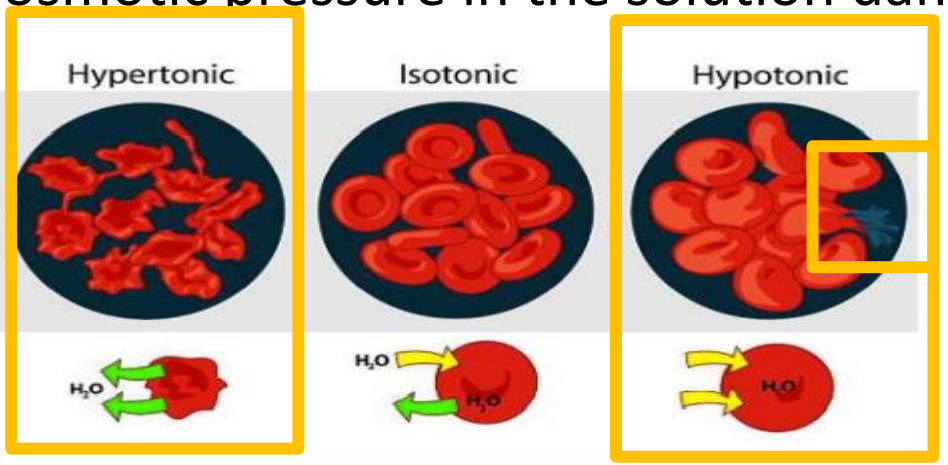
- Death rate (α) is more sensitive to temperature changes than growth rate (μ).
- Increased death rate at higher temperatures is due to thermal denaturation of proteins and increased maintenance energy required for other cells.
- At low temperatures cell regulatory mechanisms are affected by diffusional limitations e.g. substrate transport into and within cells.
- Temperature can affect enzymatic activity within cells that could affect glycosylation and product degradation and in turn bioactivity

Other factors affecting Specific Growth Rate – μ.

- pH Impact: This depends on whether the cells are:
  - Acidophiles – prefer acidic conditions.
  - Neutrophiles – prefer neutral conditions.
  - Basophiles – prefer alkaline conditions.
- **Note: The hybridoma cell line is a neutrophile with a typical optimum pH range of 6.8 – 7.3**
- pH can also affect enzymatic activity within cells that could affect glycosylation and product degradation and in turn bioactivity

Other factors affecting Specific Growth Rate – μ.

- Osmotic Strength of Cell Culture Media:
  - 330 - 360 mOsmole is optimal range.
  - Too high or too low osmotic pressure in the solution damages the individual cells.



- Product Inhibition:
  - End - product inhibition (by metabolites in medium).
  - Toxic byproducts (ammonia, lactate, pyroglutamate etc.).

Factors Affecting Growth

- **Nutrient/substrate concentration**: if cell growth is limited by a substrate source- e.g. carbon source (glucose), the growth rate is expressed as a function on the concentration of this substrate.
  - At low substrate concentrations (S), the growth rate (μ) is proportional to increasing S
  - However, there is a maximum value for the specific growth rate.
  - This is described by the Monod mathematical model:

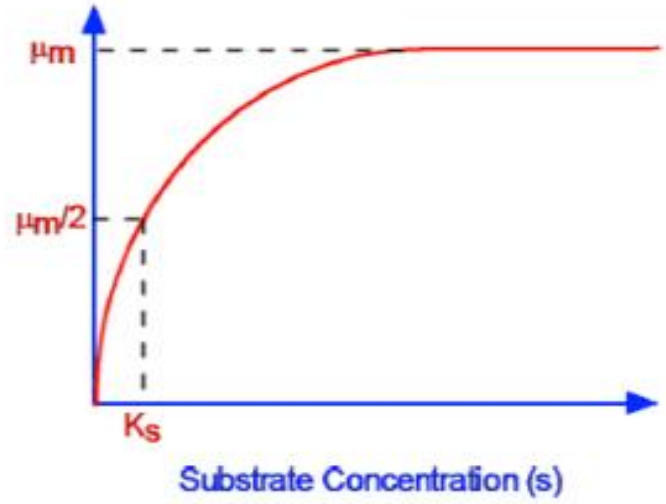
$$\mu = \frac{\mu_{max} S}{K_S + S}$$

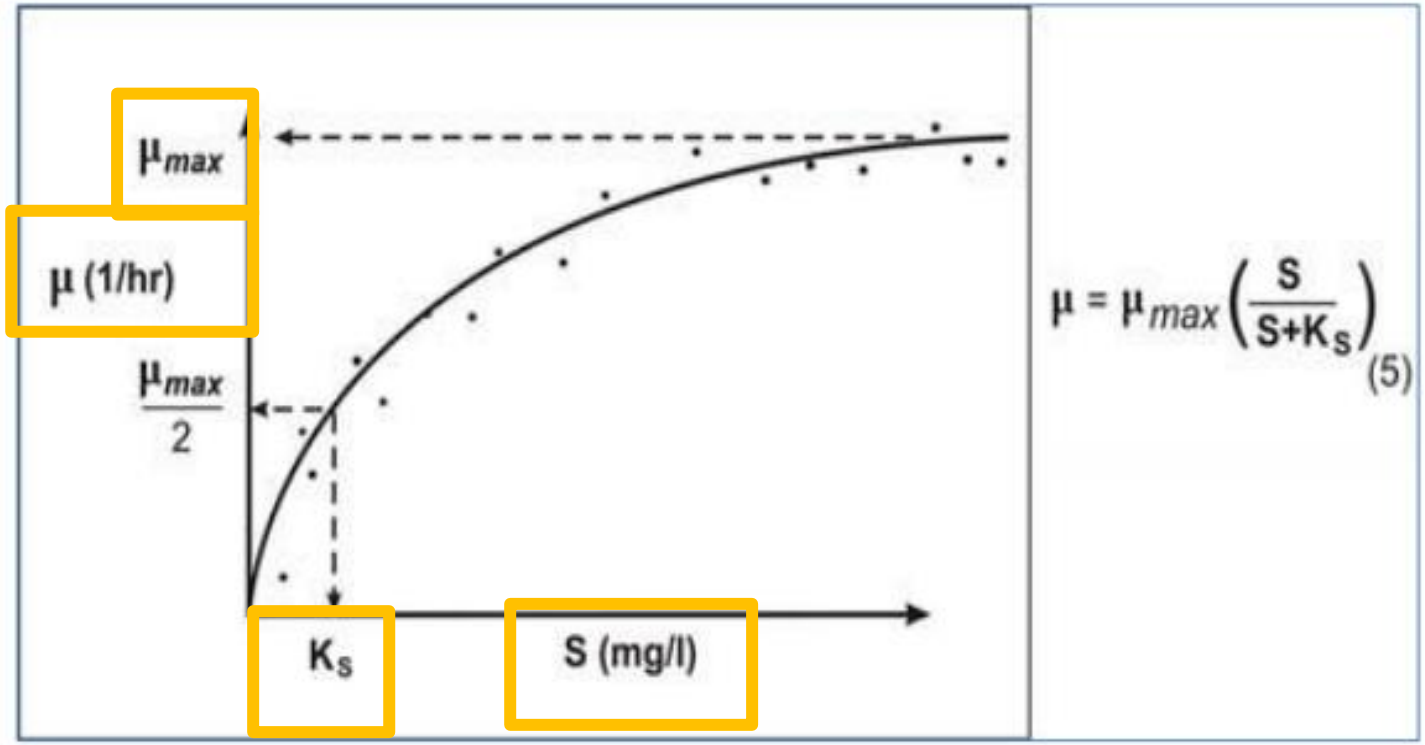
μ	= specific growth rate (h^{-1})
μ_{max}	= maximum specific growth rate achievable
S	= substrate concentration ($g\ l^{-1}$)
K_s	= substrate concentration for $\mu_{max}/2$

  - The specific growth rate of cells will increase, with an increase in substrate, only up to a certain point. Beyond this point it may be a waste of media to increase substrate concentration

Substrate Concentration and Growth Rates

The Relationship Between Growth Rate and Substrate Concentration





Monod Kinetics:

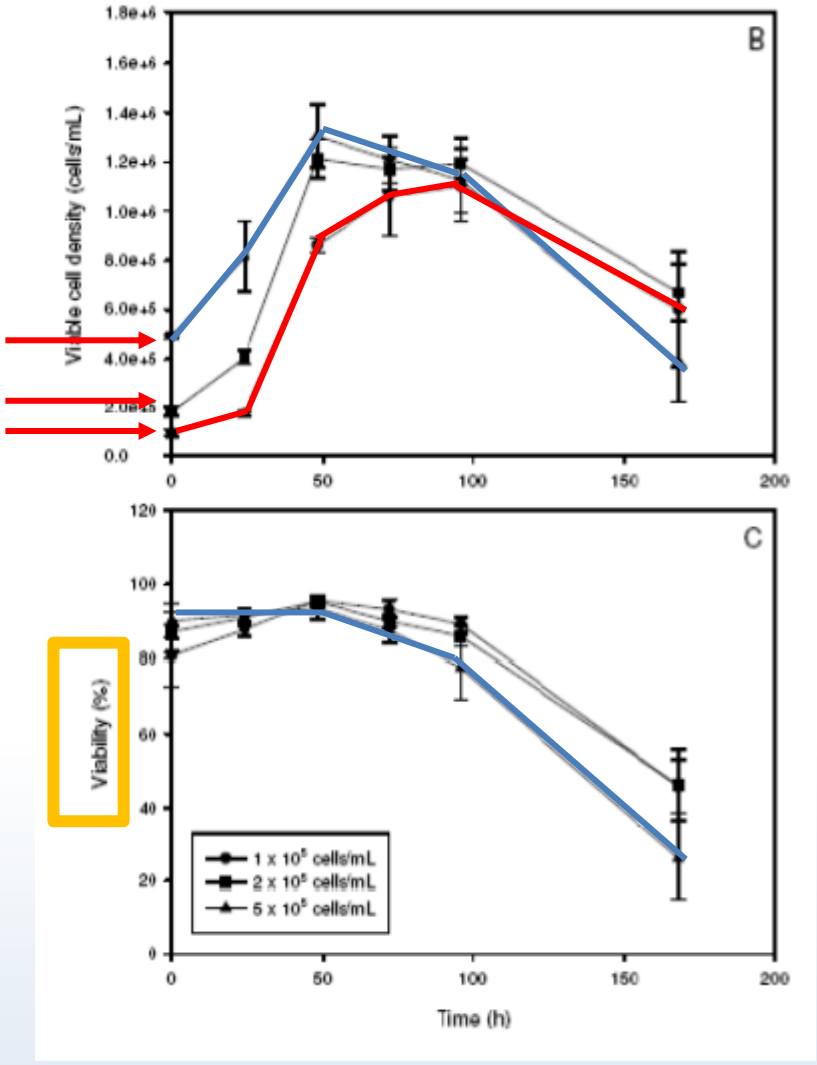
$$\mu = \frac{\mu_m \cdot s}{K_s + s}$$

If $S >> K_s$ then $\mu = \mu_{max}$

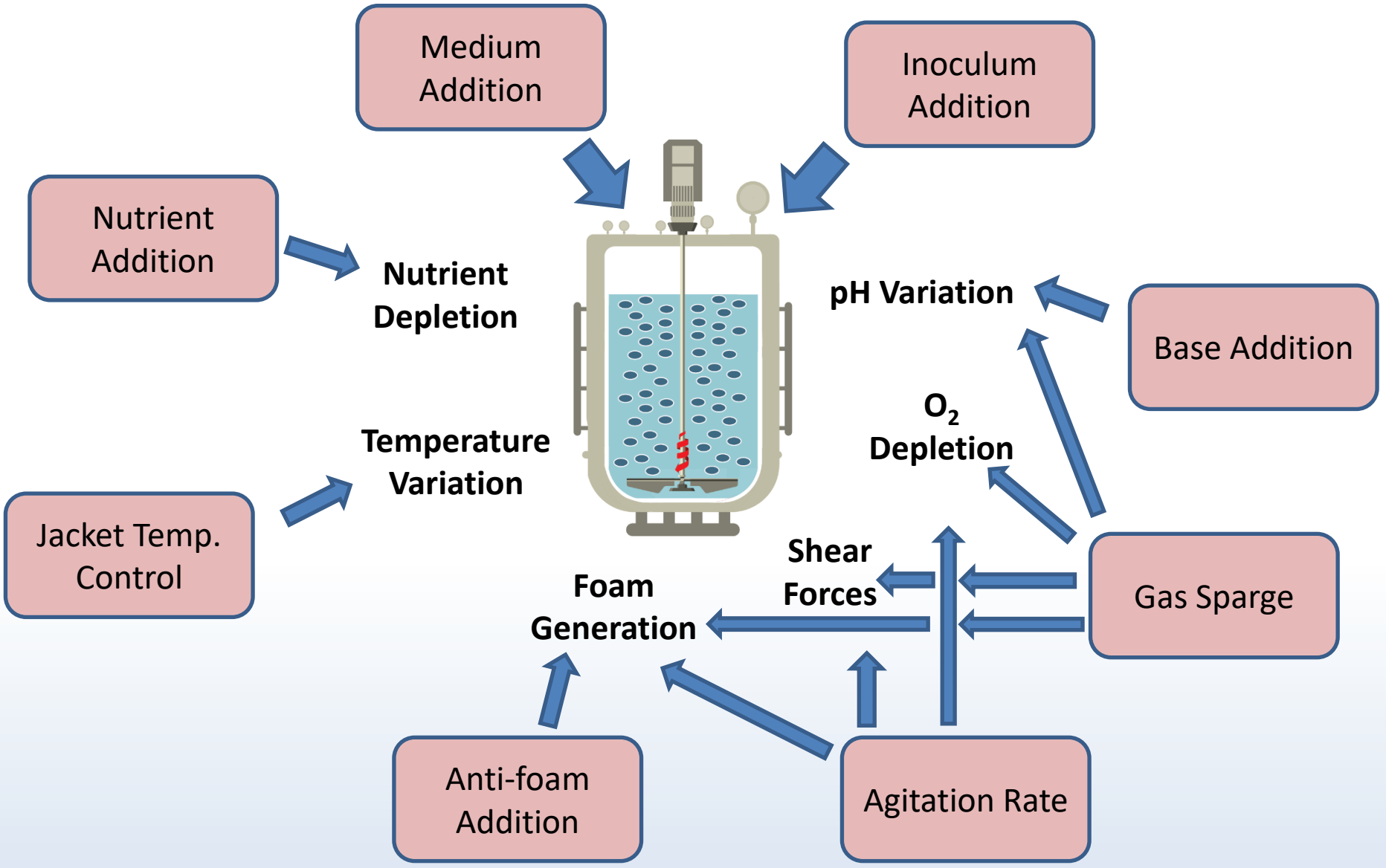
Growth Rate

- Graph show growth curves for hybridoma cell line seeded at 3 different seeding densities
- Graphs show viable cells and viability
- Cells reach similar maximal cell density
- Higher cell densities see greater death rate

Seeding density Cells/ml	Growth rate μ_{max} (/hr)	IVCD (Cell day/ml)
1 x 10^5	0.065	5.25 x 10^6
2 x 10^5	0.053	6.25 x 10^6



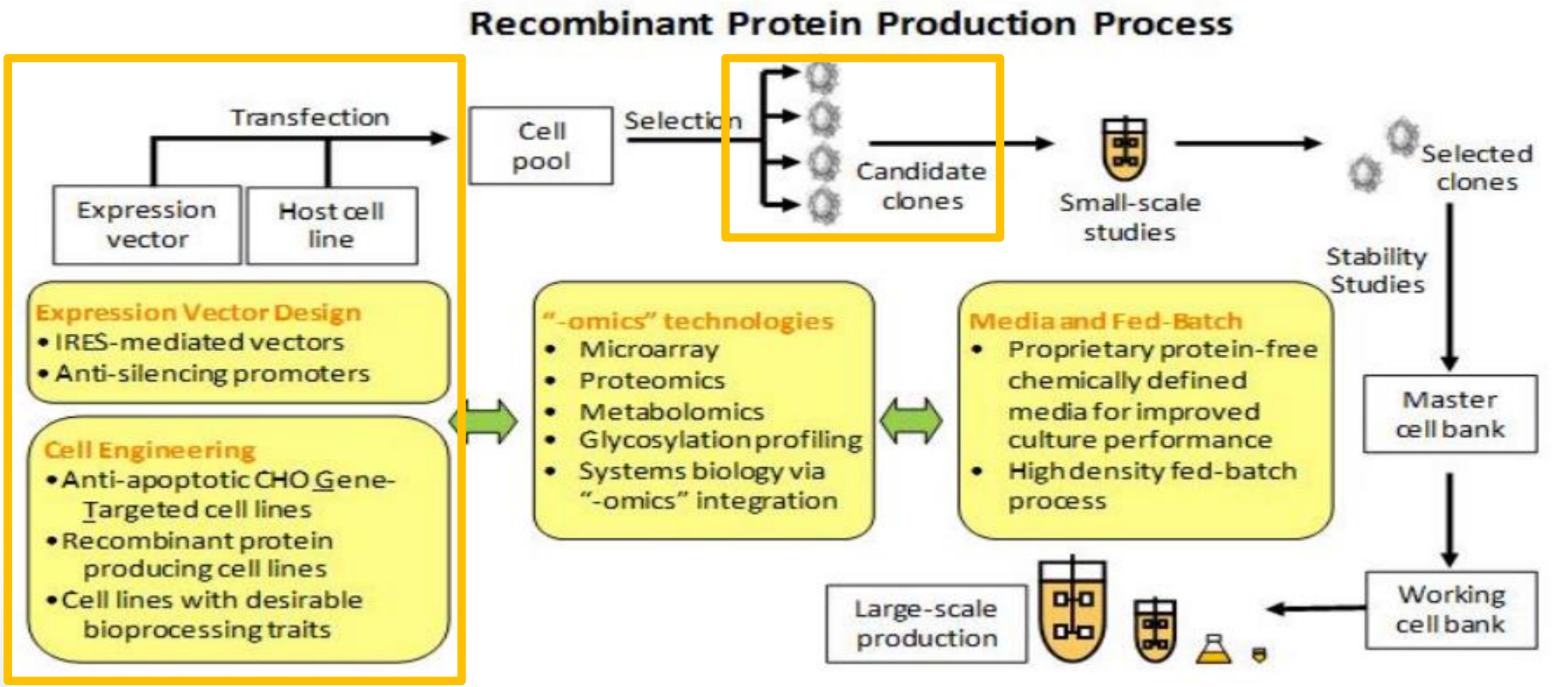
Factors Affecting Growth and Productivity



Lecture Topics

- Cell Growth
- Factors Effecting Rate of Cell Growth
- Cell Productivity Factors
- Handling & Manipulation of Cell Culture
- Measurement of Cell Growth

Mammalian Cell Productivity



From: http://www.a-star.edu.sg/bti/Research/Research-Groups/Animal-Cell-Technology.aspxreactions.

Cell Productivity & Temperature Effects

- Mild hypothermia is an increasingly popular practice to improve recombinant protein yields in CHO cells.
- Studies have attributed the enhancement of protein titres at sub-physiological temperatures to increased mRNA levels as well as extended stationary phase.
- It has been observed that reducing the culture temperature arrested cell growth, prolonged viability, and increased cell size.
- However, the reduced culture temperature had a differential effect on protein and mRNA expression of closely related antibody mutants from stable cell lines.

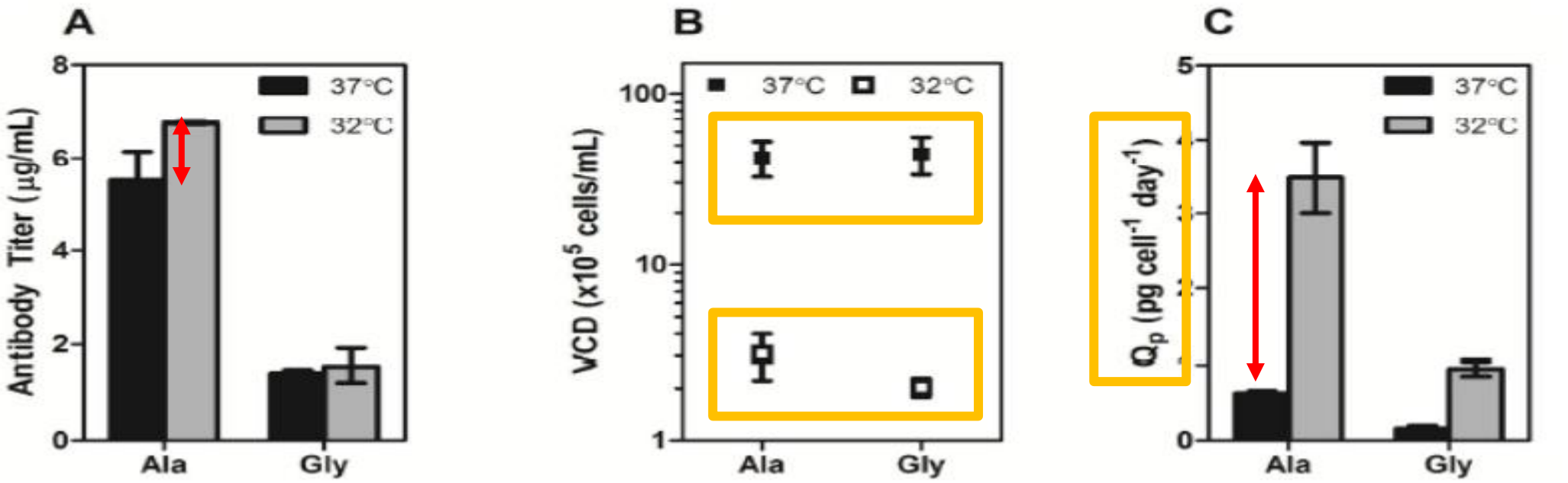
Cell Productivity & Temperature Effects

- Comparing cultures at 32°C with those at 37°C, some cell lines show changes in the specific and volumetric productivity
  - Differences in specific productivity are reflected in the amounts of heavy- and light-chain mRNA produced
- Analysis of the 2º and 3º configurations show fundamental structural differences (CD spectroscopy)
  - The effect of reduced culture temperature on expression was protein-dependent; protein folding fidelity and assembly is improved at lower temperatures, enhancing the expression of proteins that have a propensity to mis-fold.

From: Reduced Culture Temperature Differentially Affects Expression and Biophysical Properties of Monoclonal Antibody Variants. Mason, M. Et al. (2014) Antibodies 3:253-271; doi:10.3390/antib3030253

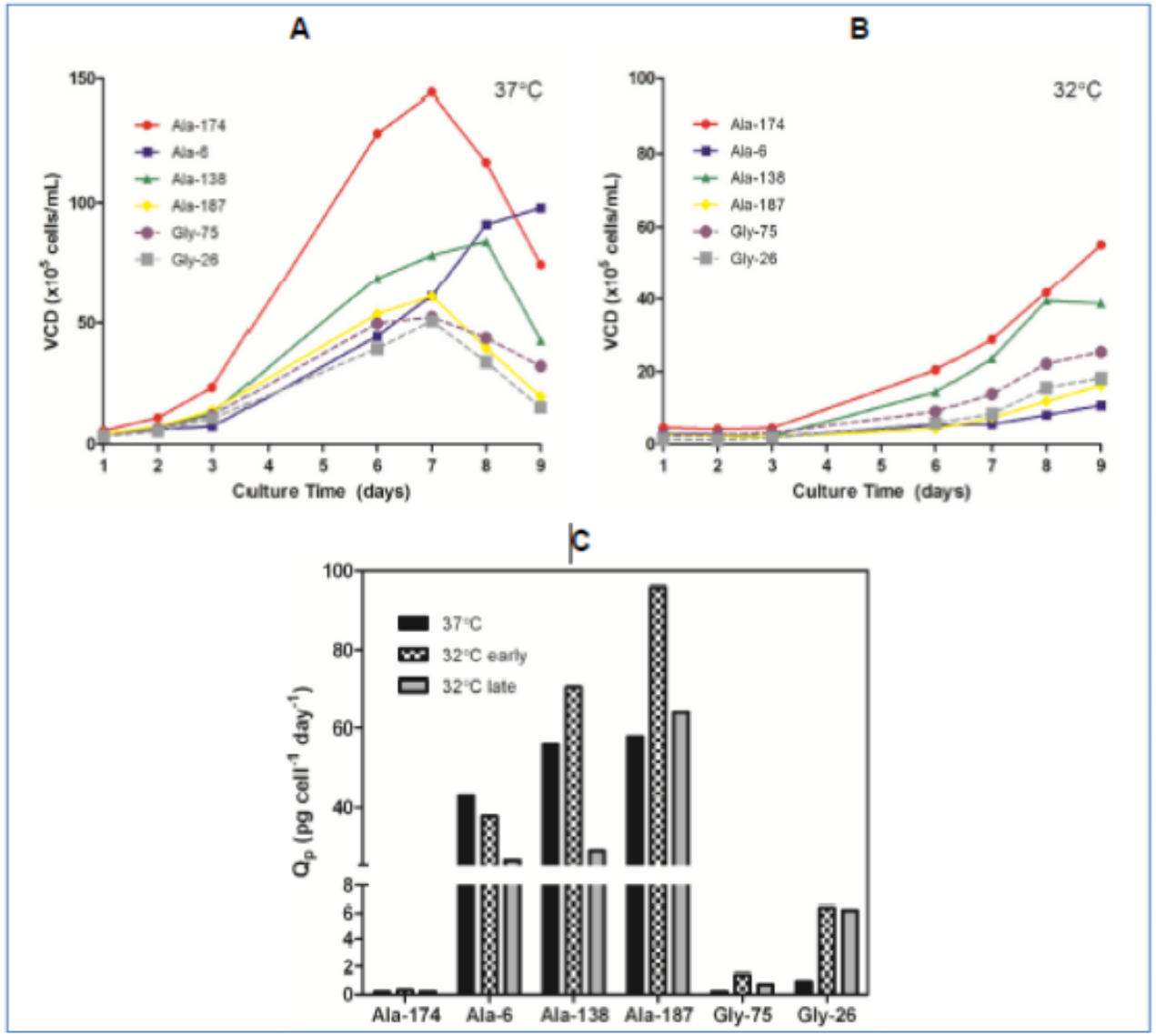
Effect of Mild Hypothermia on MAb Productivity

Figure 2. (**A**) Transient expression titers of Ala and Gly antibodies after a 5-day incubation at 37 °C (black bars) or 32 °C (gray bars). (**B**) The viable cell density (VCD) of the Ala and Gly cultures at 37 °C (■) and 32 °C (□). (**C**) Specific productivities of Ala and Gly antibodies at 37 °C (black bars) or 32 °C (gray bars). The error bars represent ± 1 standard deviation from the mean of experimental replicates (n = 2).



From: Reduced Culture Temperature Differentially Affects Expression and Biophysical Properties of Monoclonal Antibody Variants. Mason, M. Et al. (2014) Antibodies 3:253-271; doi:10.3390/antib3030253

Performance of Cell Cultures at Different Temperatures



- **(A)** Viable cell density (VCD) profiles for various cell clones at 37°C in serum-free culture.
- **(B)** VCD profiles for clones grown at 32°C in serum-free batch culture .
- **(C)** Specific productivity (Qp) of clones in mid-exponential growth at 37°C (days 3 – 6) and 32°C (early phase: days 3 to 6; late phase: days 7 – 8).

Lecture Topics

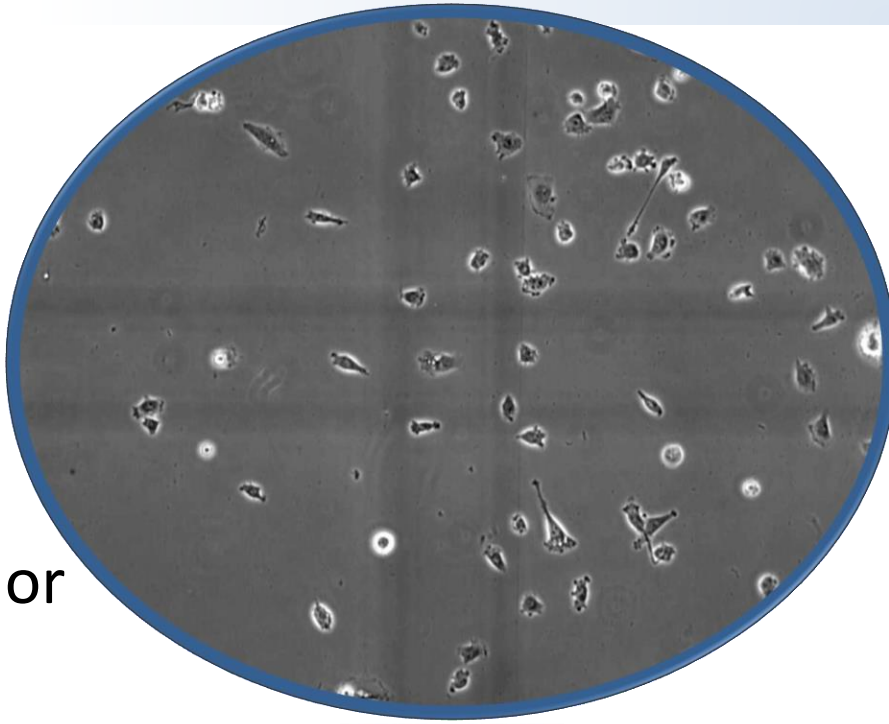
- Cell Growth
- Factors Effecting Rate of Cell Growth
- Cell Productivity Factors
- Handling & Manipulation of Cell Culture
- Measurement of Cell Growth

Routine Handling of Cell Cultures

1. ***Starting up****:* thaw quickly in water bath (~1min)
2. ***Counting:*** remove aliquot, measure viability using trypan blue
3. ***Change of medium:*** within 24hrs, frequency depends on cells
4. ***Detachment:*** generally use trypsin
5. ***Subculture:*** Cells should be 90% viable, and if attached ~ 80% confluent (record passage number)
6. ***Inoculation:*** cell density depends on cell type, will come on information sheet – hybridomas generally do not grow at concentrations below $1x10^5$ cell/ml
7. ***Growth behaviour:*** how long it takes cells to become confluent
8. ***Control for contamination:*** microbial and mycoplasma
9. ***Freezing:*** freeze slowly, set up own cell bank and store in N_2 vapour or liquid

Routine Handling of Cell Cultures

1. Check liquids (media, buffers etc) : incubate on appropriate media
   - Bacteria : Nutrient Agar
   - Fungi : Sabouraud Dextrose Agar (SDA)
   - Yeast : YPAD medium
2. Work areas are swabbed and swabs placed on plates or liquid media for specific microorganisms (above)
3. Microbial contamination is huge problem as it will kill cells
   - Bacteria and yeast contamination detected by sudden change in pH (overnight), media cloudy, see bacteria swimming if motile
   - With fungal contamination you will see hyphae



Mycoplasma: problem?

- Mycoplasma are very small bacteria (mycobacterium) 0.2 - 1µm diameter, act as a parasite inside cell
- Many different species present, can cause serious illness
- Spread from contaminated cells, aerosols (?), individuals, and reagents, bovine (serum) and porcine (trypsin) sources
- Most common sort in cells comes from human and porcine origin; some individuals are known to carry mycoplasma

Mycoplasma: problem?

- Estimated that 15-25% cells in culture are contaminated
- Can exist in cells without killing them but will alter growth rate, metabolism and productivity of cells
- Can be lethal to some hybridoma cell lines
- Once detected, usually best to get rid of cells as you don't want to spread contamination
- Can be treated by antibiotic e.g. BM cyclin developed by Roche can kill 80% mycoplasma but it can also kill cells

Mycoplasma: problem?

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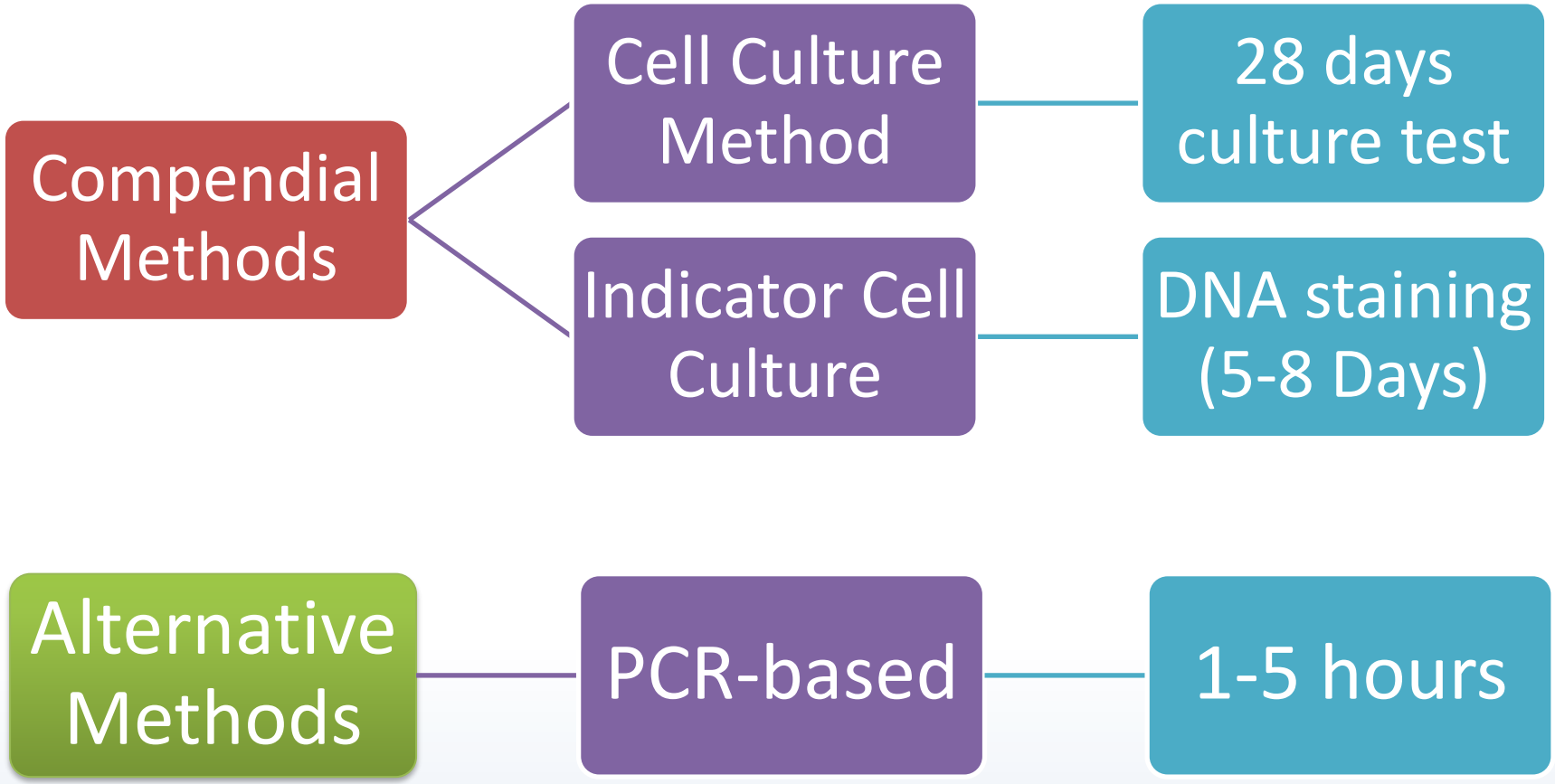
Current Mycoplasma Test Methods

Ph Eur 2.6.7

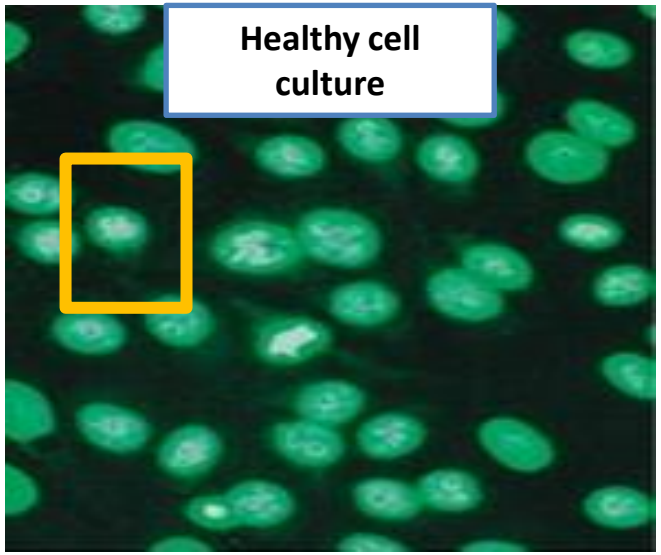
Chapter <63>

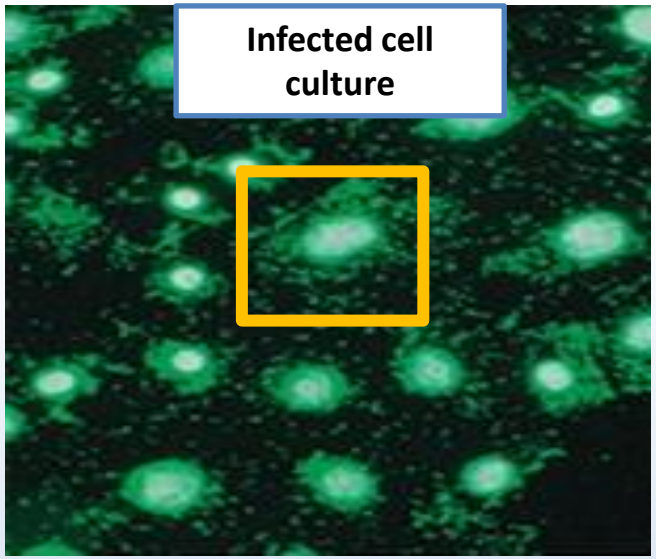
Points to Consider (PTC) – 1993



Mycoplasma Detection

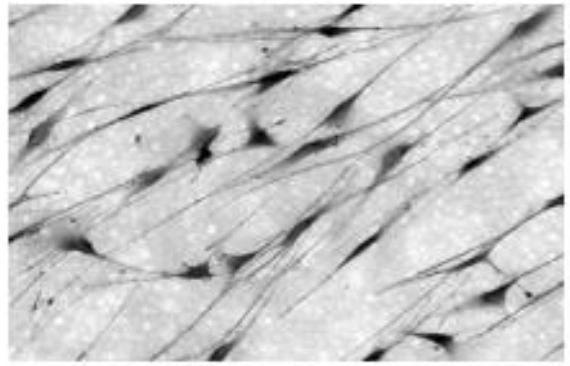
- Direct detection by growing in special agar can take up to 4 weeks
- Variety of tests have been developed by companies
- Highly regarded is DAPI stain (stains DNA in nucleus) - in picture we can see mycoplasma in cytoplasm
- **Kits using PCR – DNA fingerprint**
- Assay developed by Cambrex uses ATP generation and luminometer highly regarded (fast and sensitive)

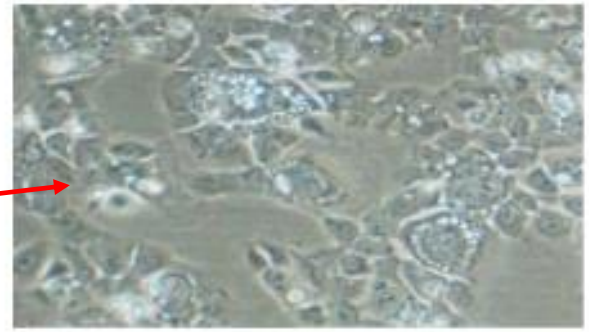




Cell Morphology

- Always check growth of cells with inverted microscope
- Get to know what your cells look like (morphology):
  - Epithelial, fibroblast
- Adherent cells have specific shape and appearance
- Should know ‘sick’ cells : cells have lots vacuoles
- Healthy cells in suspension are very round (smooth edges) and almost halo effect





Lecture Topics

- Cell Growth
- Factors Effecting Rate of Cell Growth
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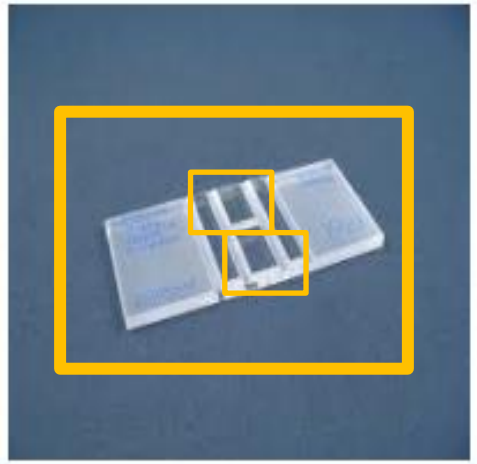
- Handling & Manipulation of Cell Culture
- Measurement of Cell Growth

Cell Counting

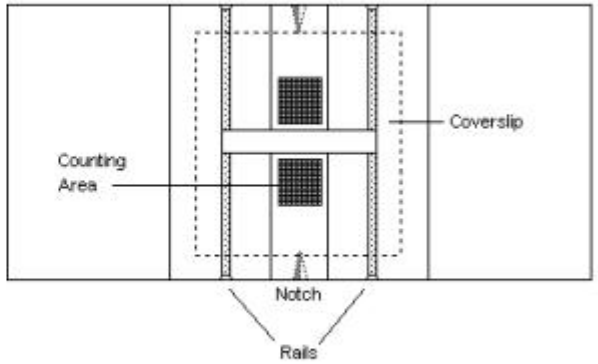
- Need to count cells to know:
  - How many cells you have? Are they alive or dead?
- Need to know this to confirm:
  - How much to feed cells? When to feed cells?
  - Are cells following expected growth patterns?
- Variety of methods
  1. ***Trypan blue***: manual methods, easiest, cheapest
  2. ***Vi-Cell and Cedex***: automated image analysis (based on trypan blue) used in industry to process large numbers
  3. ***Flow cytometer***: laser based method, expensive
  4. ***Coulter counter***: particle counter (measures number of cells but does not measure cell viability)

Manual Cell Counting

- Counting cells manually using a haemocytometer counting slide, microscope
- and Trypan Blue dye
  - Trypan Blue is a vital dye – distinguishes between live and dead cells
- Take cell culture sample (100µl)
- Add 100 µl trypan blue (i.e. 1:2 dilution)
- Allow to sit for 3-5 minutes to allow trypan blue to stain cells
- Add 10 µl onto slide and count on microscope



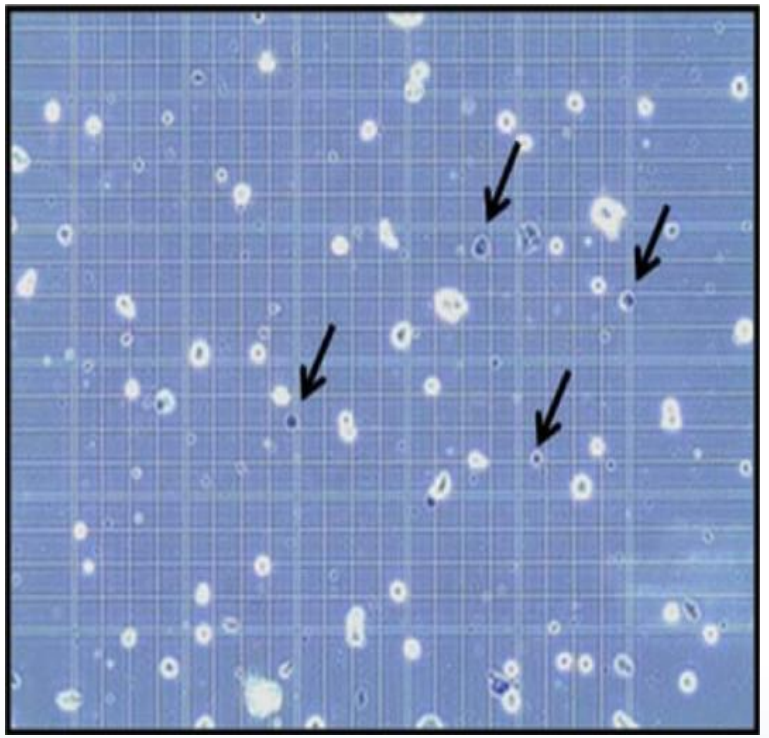
Haemocytometer



Haemocytomer: glass slide with markings (counting area), notch or well for adding sample, and covered with glass slide. All you need is a standard microscope to count number of *live and dead cells*. Most frequently used method, probably cheapest, easiest, **but not as precise** (operator error, subjective) as other methods

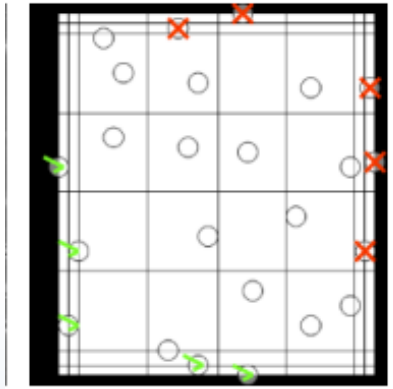
Cell Counting

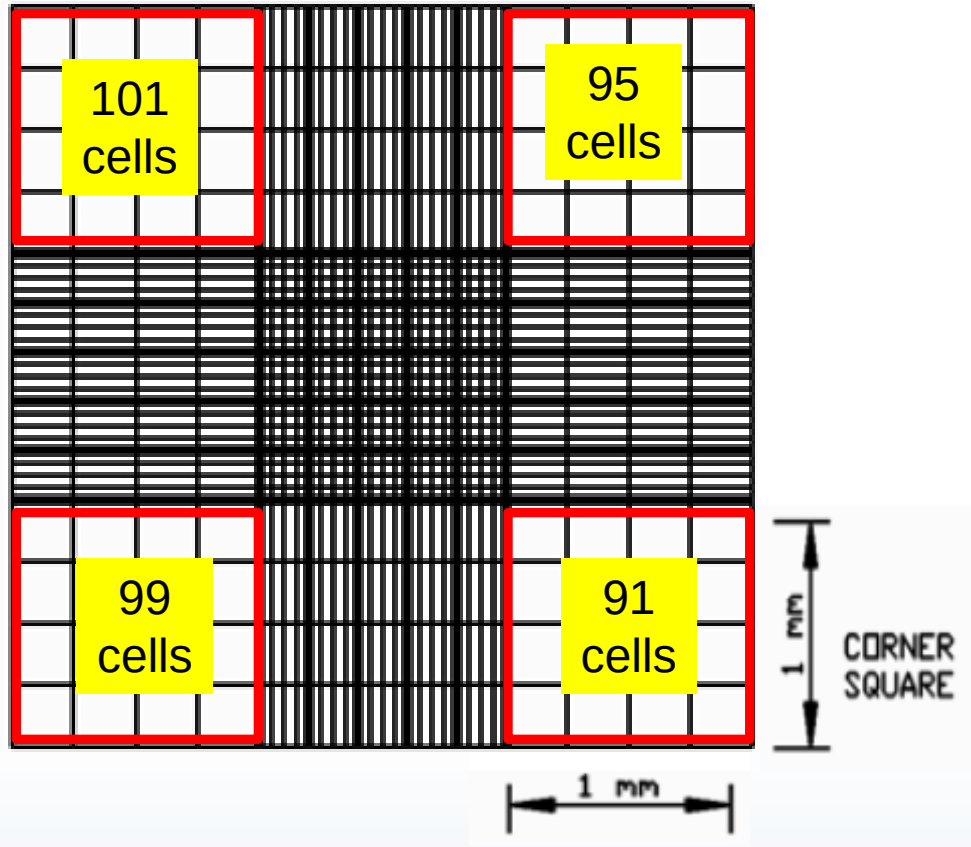
- Mammalian cells have a cell membrane
- If cells are healthy and membrane is intact and trypan blue cannot enter cells
- If cells are dying/dead, trypan blue enters cells and stains them blue
- Hence trypan blue staining can be used to determine a VCD (Viable Cell Density)



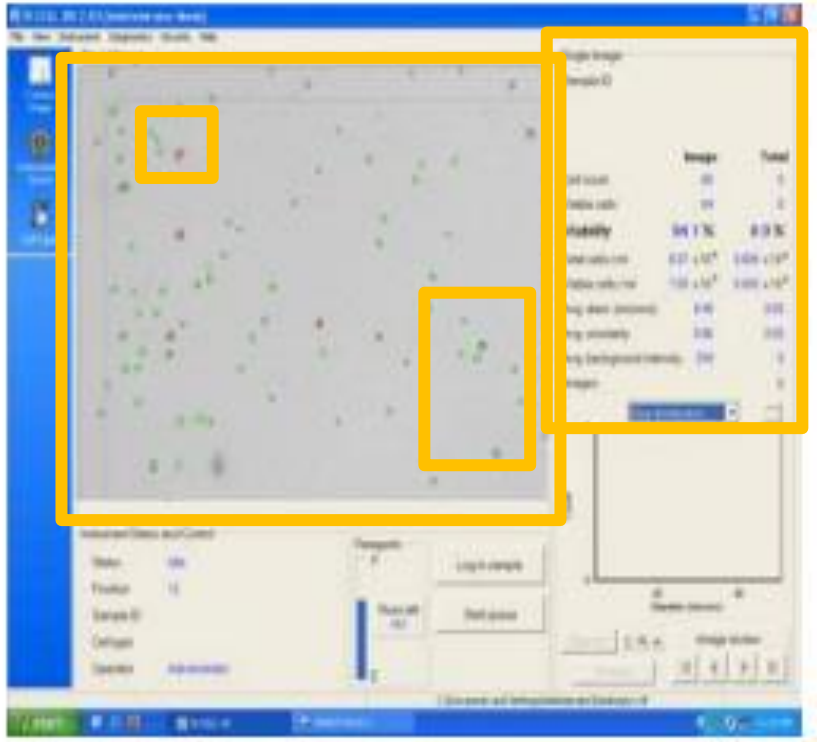
Cell Counting

- Total cell count:
  101 + 95 + 99 +91 =386
- Dilution factor:
  500µL cells +100µL trypan blue = 1.2
- Average cells per quadrant:
  386 ÷ 4 = 96.5
- Dilution factor accounted for:
  96.5 x 1.2 = 115.8
- Size of counting square is 0.0001 of a millilitre:
  115.8 x 10^4 = 1.158 x 10^6 cells/mL



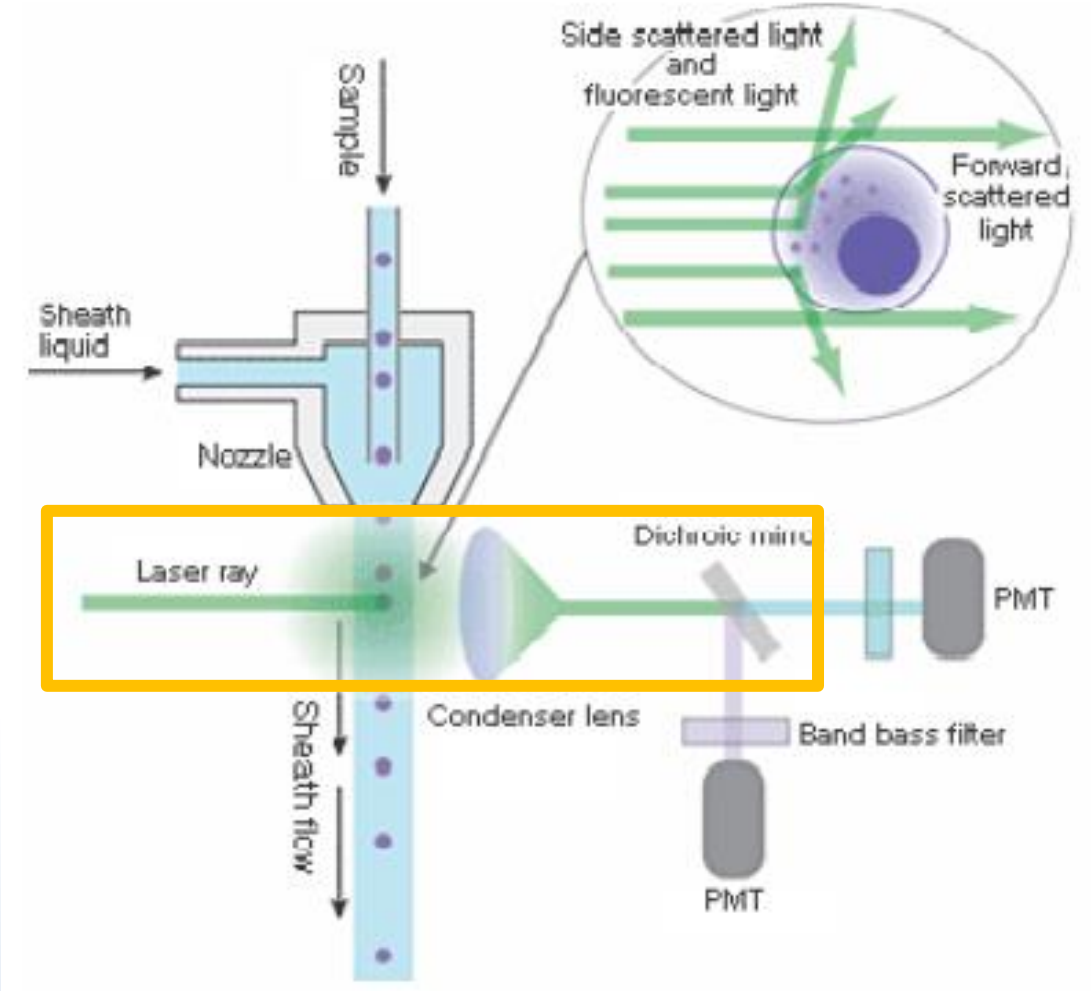


Automated cell counting



- Vi-Cell™ & Cedex: automated system that measures cell number, % viability and cell size
- Based on trypan blue assay and image analysis

Automated cell counting



- **Flow cytometer**: *laser based method of measuring cell number and viability*
- Cells must first be fixed in alcohol and are then mixed with fluorescent dyes
- Can be used to measure cell number, viability
- Also used to measure cell cycle and apoptosis
- Powerful and expensive: generally more research tool

Summary

- Cell growth kinetics describes how cells grow in a bioreactor. Understanding allows us to determine optimal batch performance
- The main growth phase is exponential: $dN/dt = \mu N$ and the cell generation or doubling time is $td = \ln(2)/\mu$
- Factors affecting growth rate include: substrate concentration, temperature, adequate CO^2 levels during scale-up, ph, osmotic strength of media solution, substrate toxicity, and any inhibitory factors
- We can measure productivity in different ways e.g. Specific ($g/10^6$ cells/h); Volumetric (g/l/h) or Global (g/h)
- Product titres from cells have increased over time from 1 g/l currently and with 10 g/l envisaged in the future. Titres of 5.5 g/l have been reported for CHO cell line and 5.1 g/l for NS0 cell line

Sample Questions

- How is cell viability measured? Outline 2 approaches that might be used and comment on any pros and cons of the chosen methods.
- Why is it so important to monitor cell number during process scale-up? Comment on the expected pattern of cell growth and identify some factors likely to impact negatively upon it if not managed correctly.

Questions?