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Methodology in Molecular and Cellular Biology

Course designated for First Year Master Students

Applied Microbiology

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Preface

This handout is designated for Master's students in Applied Microbiology. It aims to provide students with in-depth knowledge of the experimental approach, using the concepts and techniques of modern biology: biochemistry, cell biology, molecular biology, immunology, genetics and microbiology.

The aim of this course is twofold: first, to equip the students with the technical skills needed to design and execute experiments, and second, to foster critical thinking about biological systems at the molecular level. By understanding how each technique works and recognizing its strengths and limitations, the students will be prepared to analyze data rigorously and adapt protocols to real-world challenges.

Content of the course

Chapter 1. Cloning, production and purification of a recombinant protein

Chapter 2. DNA-protein and protein-protein interaction

Chapter 3. Cell culture

Chapter 4. Reporter gene

Chapter 5. Modalities of cycle control

Chapter 6. Modern biology techniques

Chapter 7. Genetic analysis in yeast

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INTRODUCTION

Cells form the basis of all living things. They are the smallest single unit of life, from the simplest bacteria to blue whales and giant redwood trees. Differences in the structure of cells and the way that they carry out their internal mechanisms form the basis of the first major divisions of life, into the three kingdoms of Archaea ("ancient" bacteria), Eubacteria ("modern" bacteria) and Eukaryota (everything else, including us). An understanding of cells is therefore vital in any understanding of life itself.

Most of the cellular processes are conducted via complex interactions among proteins and DNA. To obtain deep insights into such functional processes of cell and modulate them in a variety of pathophysiological conditions, it is essential to understand the basic concepts underlying these interactions, which opened an emerging era of research.

Cell culture refers to the process of growing and maintaining cells outside their natural environment in a controlled laboratory setting. This technique allows researchers to study cellular behavior, function, and interactions under conditions that closely replicate those found within the human body.

Reporter genes have become an invaluable tool in studies of gene expression. Reporter gene technology is widely used to monitor the cellular events associated with signal transduction and gene expression.

More importantly, use of recombinant proteins varies widely - from functional studies *in vivo* to large-scale protein production for structural studies and therapeutics. Using the best expression system for the protein and application is key to the success.

Chapter 1. Cloning, production and purification of a recombinant protein

Learning Objectives

At the end of this chapter, you should be able to:

- Explain the principles and importance of recombinant protein expression in biotechnology.

- Describe the complete workflow from gene cloning to recombinant protein purification.

- Compare different cloning strategies, expression systems, and protein production platforms.

Introduction

Recombinant protein expression is a fundamental process in biotechnology wherein a host organism, typically a microorganism or a mammalian cell line, is engineered to produce a specific protein of interest (POI) that it would not naturally produce.

This process involves the insertion of a gene encoding the target protein into the host organism's genome or an expression vector, which is then propagated and induced to express the protein.

1. Cloning

Cloning mainly refers to the process of multiplying an organism, a stem cell or a gene, in a large number of identical copies (either *in vitro* or *in vivo*).

Molecular cloning refers to the isolation and cloning of individual genes or other segments of DNA.

The different stages go through: the construction of a DNA library, the screening of the library and the expression of the gene (Figure 1).

We can distinguish 2 methods for constructing a DNA bank:

- the first consists of fragmenting the DNA molecule using restriction enzymes;
- the second consists of purifying messenger RNA which will then be transcribed into complementary DNA (cDNA) by a reverse transcriptase.

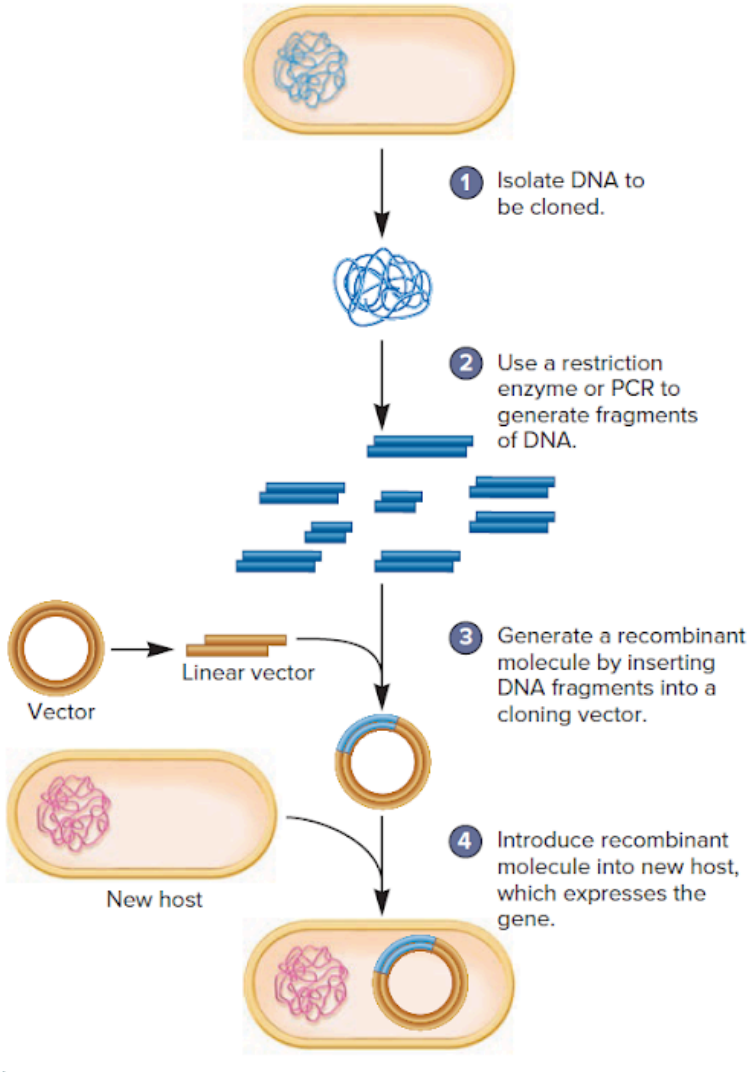

Figure 1. Steps in Cloning a Gene (Willey, 2023).

1.1. Preparation of a DNA bank using restriction enzymes: genomic DNA bank

1.1.1. DNA preparation

Restriction enzymes are nucleases purified from bacteria that cut DNA at specific sequences of 4 to 8 nucleotides, producing DNA fragments of strictly defined sizes, the restriction fragments.

Restriction enzymes are used to produce small fragments of DNA containing a particular gene.

Another property of restriction enzymes, convenient for gene cloning, is the ability, for many of them, to cause "zig-zag" cuts which leave short single-stranded ends at both ends of the DNA fragment : the sticky ends (Figure 2). These ends can form complementary base pairs with any other ends produced by the same enzyme. Thus, this makes it possible to connect 2 double helix DNA fragments coming from different genomes by complementary base pairing.

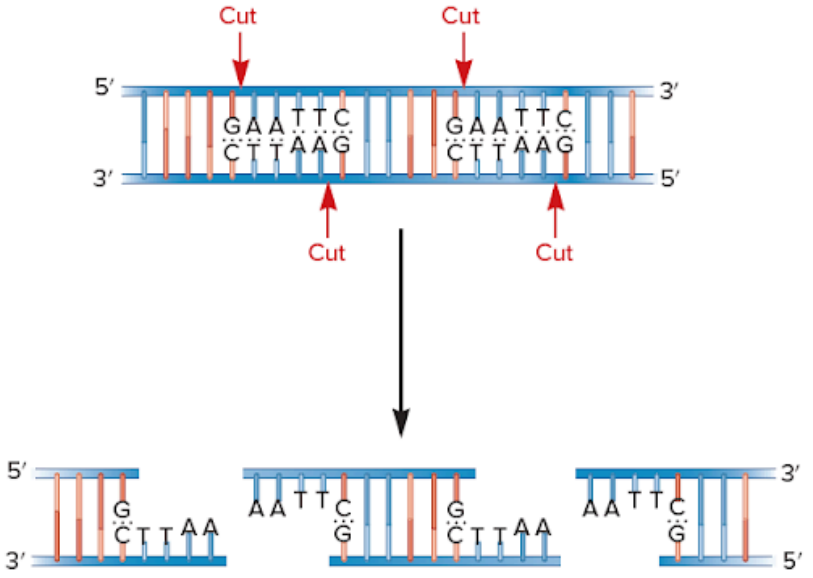

Figure 2. Restriction endonuclease action (Willey, 2023).

1.1.2. Cloning vectors

A cloning vector is a small DNA molecule which has the following properties:

- being able to replicate in a highly amplifiable bacteria,
- having restriction sites allowing the DNA fragment to be cloned,
- having 2 types of markers: markers transformation which makes it possible to distinguish between transformed bacteria (having received the vector) and other recombination markers which make it possible to differentiate between bacteria having received the vector alone from those having received the recombinant vector (i.e. with the DNA of interest).

Types of expression vectors and their features

The choice of expression vectors depends on various factors, including the host organism, desired expression level, inducibility, and compatibility with downstream applications.

Here are some common types of expression vectors and their features:

- **Plasmid vectors:**

  ➔ Plasmids make excellent cloning vectors because they replicate independently of the chromosome and are easy to extract from the host and purify.

  ➔ They can be taken up by microbes by conjugation, transformation, or electroporation.

  ➔ The first generation plasmid vectors are derived from the plasmid pBR322.

  ➔ Currently, second generation plasmid vectors are preferentially used: the pUCIS plasmid and its derivatives (Figure 3).

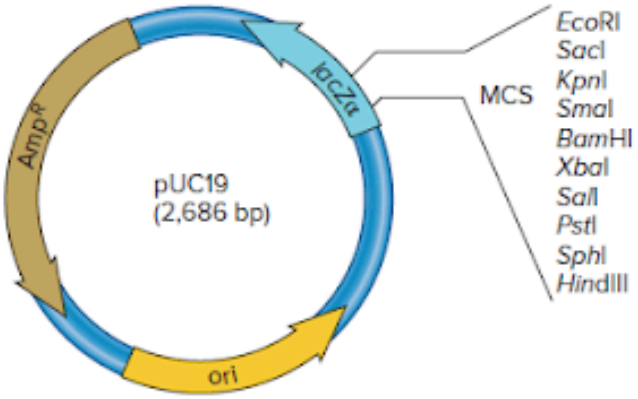

Figure 3. Cloning Vector pUC19. LacZα confers blue/white colony color (Willey, 2023).

- **Viral vectors:**

  ➔ Viral vectors are derived from viruses and can efficiently deliver genes into host cells.

  ➔ They are commonly used in mammalian expression systems. Viral vectors offer high transfection efficiency and stable gene expression.

  ➔ Examples include retroviral vectors, lentiviral vectors, adenoviral vectors, and adeno-associated viral vectors. Among the viral vectors, one of the most classic is that of the bacteriophage M13.

- **Cosmids:**

  ➔ Cosmids can be used to clone large fragments of DNA about 15,000 to 25,000 bp.

  ➔ These engineered vectors have a cos DNA packaging site from phage and a selectable marker, origin of replication, and (Multiple Cloning Site) MCS from plasmids (thus the term “cos-mid”).

  ➔ These hybrid vectors replicate as plasmids within the host cell, but the presence of the cos sites means that the vector can be packaged into phage capsids and mediated DNA transfer.

- **Bacterial artificial chromosomes (BACs):**

  ➢ BACs are large DNA vectors derived from bacterial chromosomes.

  ➢ They can accommodate large DNA inserts and are useful for cloning and expressing large genes or gene clusters.

  ➢ BACs maintain stable gene expression and are compatible with bacterial hosts.

- **Yeast expression vectors:**

  ➔ Yeast expression vectors are designed for expression in yeast species such as *Saccharomyces cerevisiae* and *Pichia pastoris*.

  ➔ They typically contain yeast-specific promoters and terminators.

  ➔ Yeast expression systems offer post-translational modifications similar to higher eukaryotes, making them suitable for certain protein expression applications.

- **Mammalian expression vectors:**

  ➔ These vectors are designated for expression in mammalian cells and often contain mammalian-specific regulatory elements such as promoters, enhancers, and polyadenylation signals.

  ➔ Mammalian expression systems allow for proper protein folding, processing, and post-translational modifications.

1.1.3. Insertion into cloning vectors

The principles underlying the methods used for cloning genes are the same for either type of cloning vector, although the details may differ.

Methods used for plasmid vectors

- When purified plasmid is available, the circular plasmid DNAs are first cut by a restriction nuclease to create linear DNA molecules.

- Furthermore, the genomic DNA used to constitute the library is also cut by the same nuclease and the resulting restriction fragments are then added to the cut and reassociated plasmids to form recombinant circular DNAs.

- These recombinant molecules containing foreign DNA insertions are then covalently sealed by DNA ligase to form intact circular DNAs (Figure 4). It must then be indicated that at this stage, certain plasmids are closed without any foreign DNA molecule.

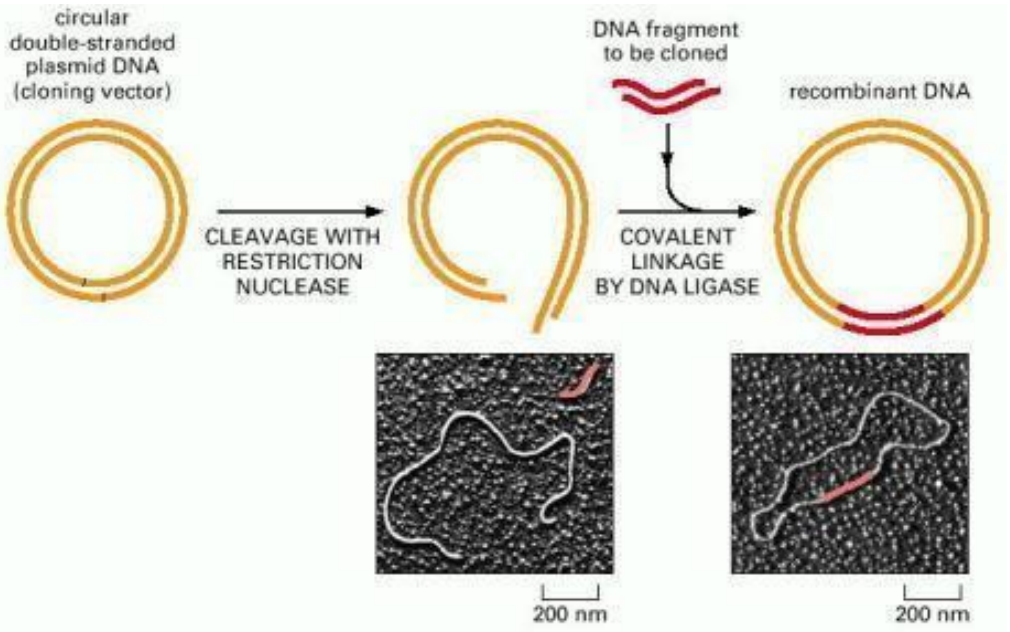

Figure 4. The insertion of a DNA fragment into a bacterial plasmid with the enzyme DNA ligase (Alberts *et al.*, 2002).

1.1.4. Transformation of cells

In the next step in preparing the library, the recombinant DNA circles are introduced into cells (usually bacteria or yeast, sometimes other eukaryotic cells) that have been made transiently permeable to DNA; such cells are said to be *transfected* with the plasmids.

To facilitate the introduction of a gene into a bacteria, the bacteria must be made competent by weakening their cell wall. This is what we do, for example, when we immerse bacteria in a cold $CaCl_2$ solution. This process allows the outside DNA to attach to the cell wall. DNA entry is then stimulated by a brief incubation at 42°C. As these cells grow and divide, doubling in number every 30 minutes, the recombinant plasmids also replicate to produce an enormous number of copies of DNA circles containing the foreign DNA (Figure 5).

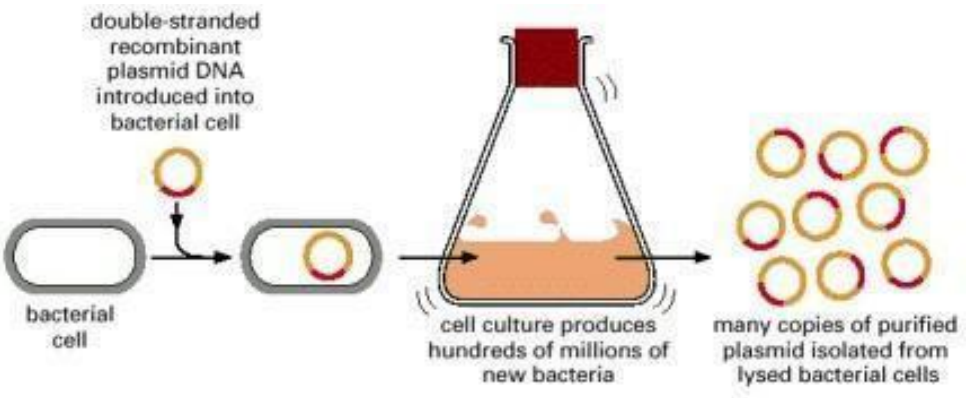

Figure 5. Purification and amplification of a specific DNA sequence by DNA cloning in a bacterium (Alberts *et al.*, 2002).

1.1.5. Selections of transformed cells

Among the transformed bacteria, some may have received the plasmid without foreign DNA, others have a recombinant plasmid and among the latter, only a tiny minority can have the recombinant plasmid which contains the gene that we want to isolate.

Many bacterial plasmids carry genes for antibiotic resistance, a property that can be exploited to select those cells that have been successfully transfected.

After transformation, *E. coli* cells are plated on medium containing ampicillin and X-Gal (Figure 6). Ampicillin ensures that only ampicillin-resistant transformants grow; B-galactosidase and X-gal are used to produce bacterial colonies that change color when insert is present within the vector.

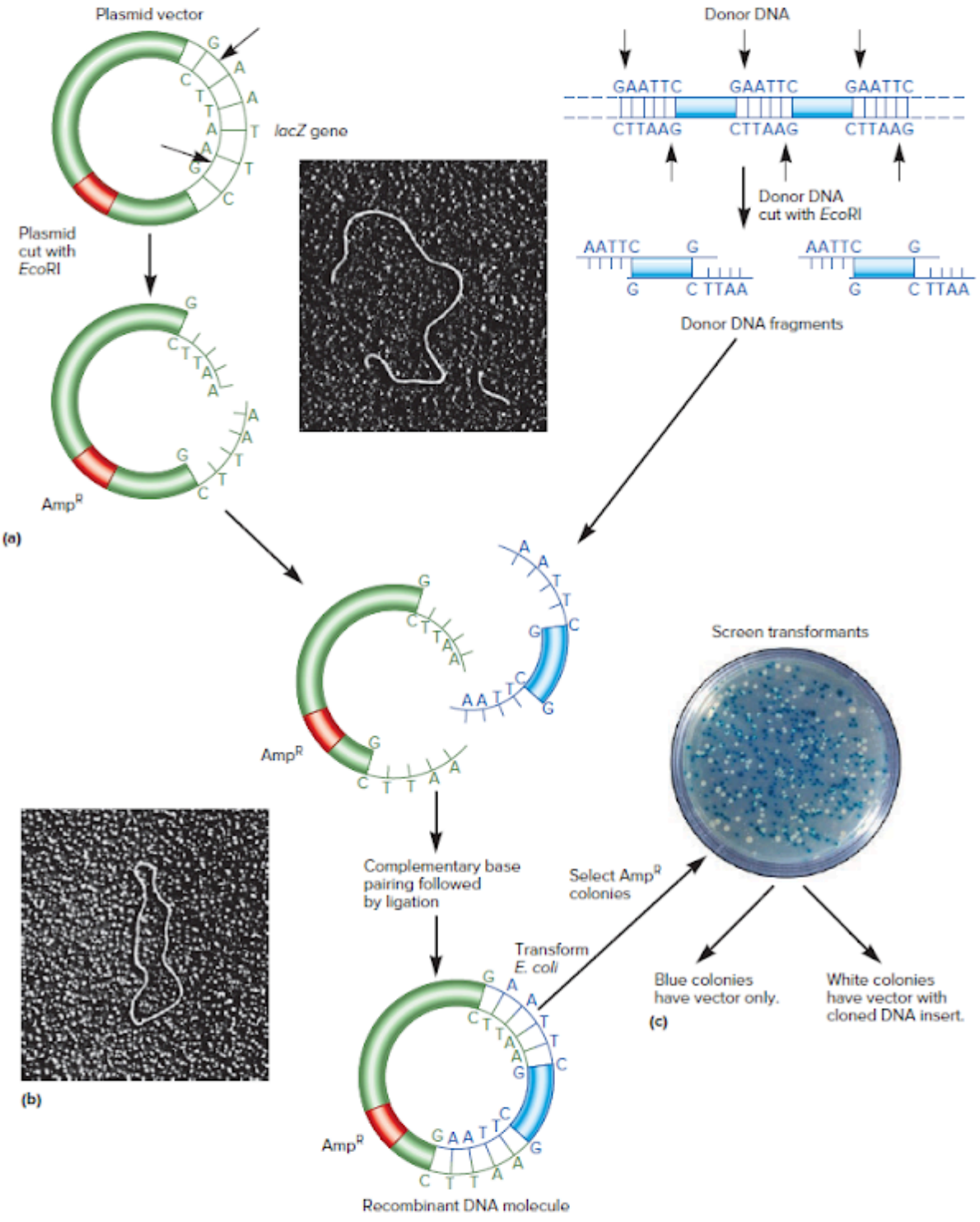

Figure 6. Construction of a recombinant plasmid (Willey, 2023).

1.1.6. Selection of interesting clones in a DNA bank

One of the frequently used techniques is a form of *in situ* hybridization which makes use of the extreme specificity of the pairing interactions between 2 complementary nucleic acid molecules (Figure 7).

- Culture dishes containing the growing bacterial colonies are transferred with a piece of filter paper, to which a few bacteria from each colony adhere.
- These bacteria are then treated to burst the cells and denature the DNA of the plasmid then hybridize it with a radioactive probe containing part of the DNA sequence of the gene of interest.
- Bacterial colonies that have attached the probe are identified by autoradiography.
- It is then possible to take the corresponding bacterial colony, inoculate it for large-scale culture and purify the recombinant plasmid.

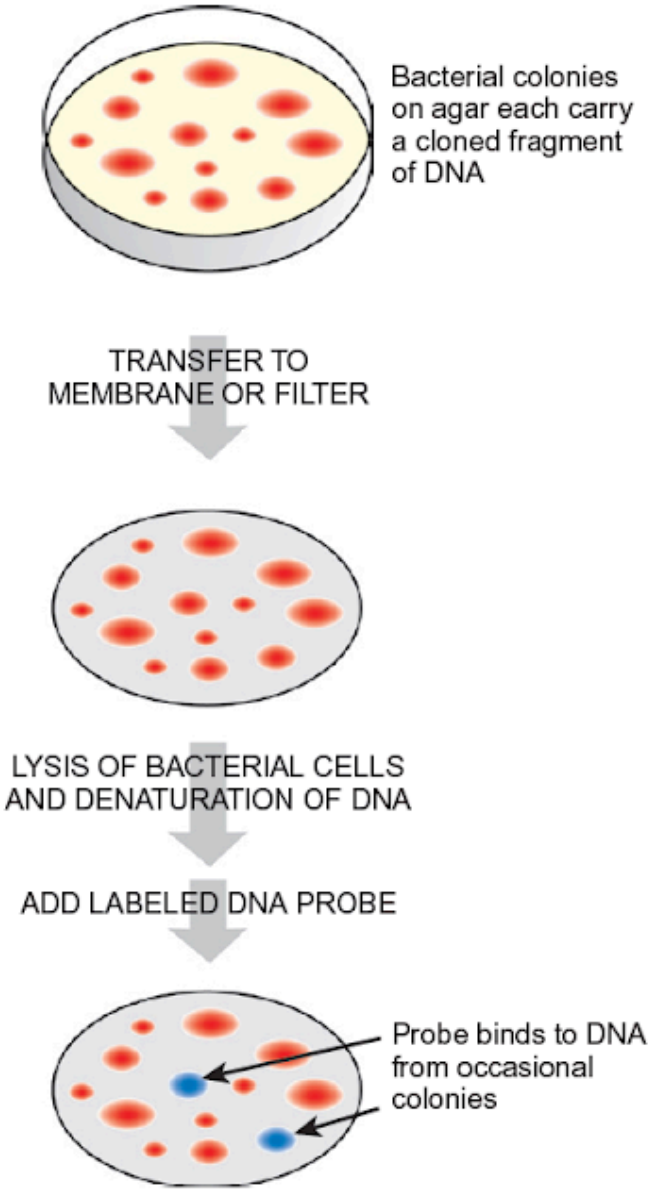

Figure 7. Screening a DNA library by probing (Clark and Pazdernik, 2013).

1.2. Preparation of a DNA bank from mRNA: cDNA bank

Another possible strategy is to begin the cloning process by selecting only those DNA sequences which are transcribed into RNA and which are therefore assumed to correspond to genes: messenger RNAs.

This method involves extracting mRNA from cells and then making a complementary DNA (cDNA) copy of each mRNA molecule present using a reverse transcriptase.

Reverse transcriptase isolated from retroviruses is capable of catalyzing the synthesis of a DNA chain from an RNA template.

The single-stranded DNA molecules synthesized by this enzyme are then converted into double-stranded DNA molecules by DNA polymerase. These cDNA molecules are then inserted into plasmids and cloned as before. We then obtain a cDNA library (Figure 8).

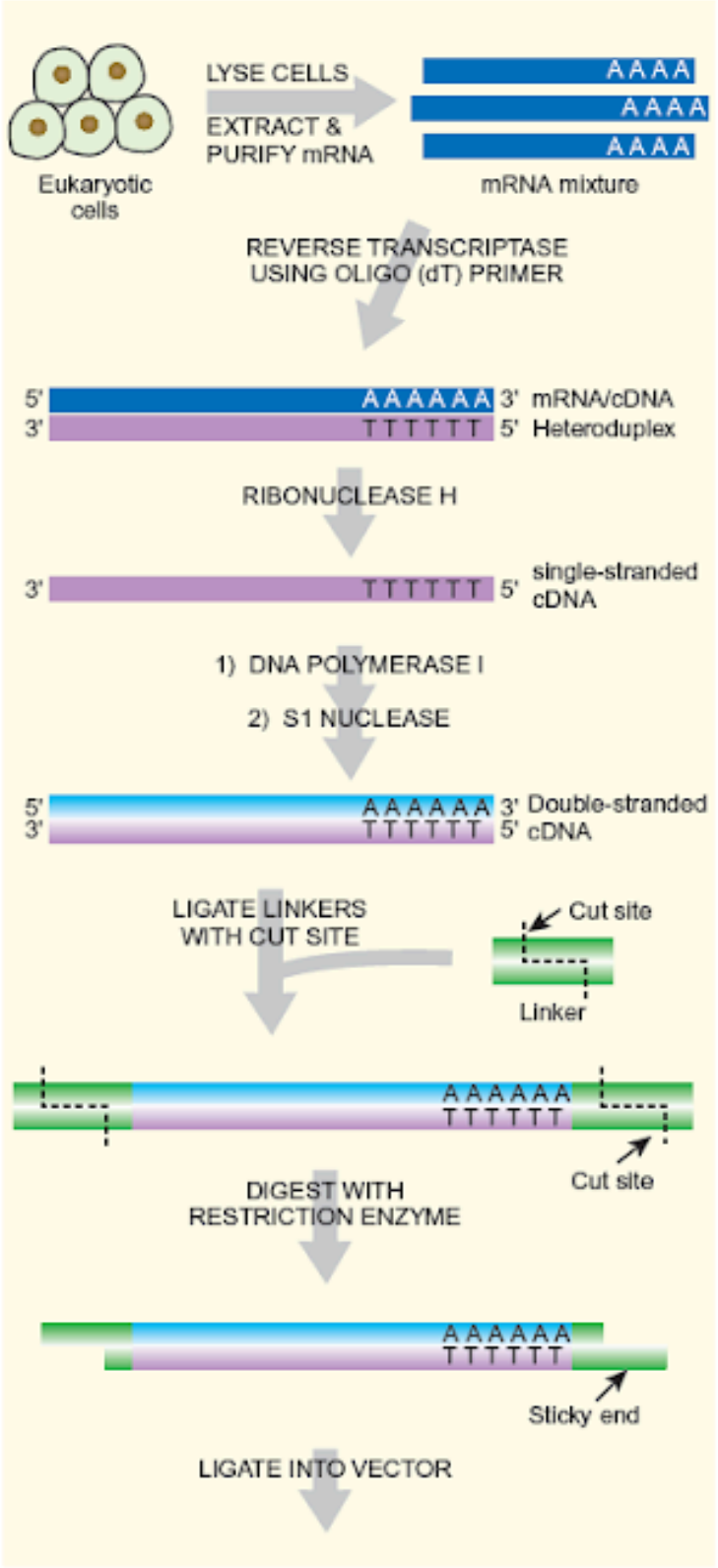

Figure 8. Making a cDNA Library from Messenger RNA (Clark and Pazdernik, 2013).

2. *Escherichia coli* – A working horse for protein production

The *E. coli* expression system continues to dominate the bacterial expression systems. The first recombinant therapeutic products were produced in *E. coli* 30 years ago.

To date, a large number of recombinant proteins have been produced using *E. coli* and many more are currently under development. The list of some industrial products produced using *E. coli* is given in Table 1.

Table 1. Examples of industrial products produced in *E. coli* and their manufacturers (Tripathi, 2016).

Product	**Manufacturer**
Insulin	Eli Lilly, Aventis
Interferon beta-1b	Interferon beta-1b Novartis
Calcitonin	Unigene
Asparaginase	Merck
Cholera toxin B subunit	SBLVaccine
Human growth hormone	Genentech, Eli Lilly, Pfizer, Novo Nordisk
Interleukin-2	Chiron
Outer surface protein A	Smithkline
Tumor necrosis factor	Boehringer

The availability of alternative expression systems like yeast and mammalian systems has experienced a major boost.

The advantages and disadvantages of the *E. coli* system from an industrial point of view compared to yeast and mammalian expression systems are given in Table 2.

Table 2. Advantages and disadvantages of the *E. coli* system for the industrial production of bioproducts compared to other systems (Tripathi, 2016).

Expression system	Advantages	Disadvantages
E. coli	- Inexpensive culture media, - Easy to cultivate, - Rapid growth rate, - High cell density cultivation, - Ease of genome modifications, - High productivity and product yield, - Easy process scale-up, - Cost effective, - Virus free	- Inability to form disulfide bonds, - Non-glycosylated proteins, - Endotoxin
Yeast (*P. pastoris* and *S. cerevisiae*)	- Inexpensive culture media, - Easy to cultivate, - Rapid growth rate, - High cell density cultivation, - Ease of genome modifications, - Easy process scale-up	- Hyperglycosylation
Mammalian cells	- Humanized glycosylation pattern, - Properly folded	- Difficult to cultivate, - Low yield, - Expensive culture media, - Viral contamination

The comparison of yields of recombinant proteins produced in *E. coli* and other expression systems is given in Table 3.

Table 3. Comparison of the yield of some recombinant products produced using *E. coli* and other expression systems (Tripathi, 2016).

Recombinant product	Expression system	Maximum product concentration
Insulin	*E. coli* *P. pastoris* *S. cerevisiae* *B. subtilis*	4.34 g L^{-1} 3.07 g L^{-1} 0.075 g L^{-1} 1 g L^{-1}
Interleukin	*E. coli* *P. pastoris* *Spodoptera frugiperda* cells	7.5 mg mL^{-1} 0.28 mg mL^{-1} 0.001 mg mL^{-1}
Glutamic acid decarboxylase	*E. coli* *P. pastoris* *S. cerevisiae* *Spodoptera frugiperda* cells	12.5 mg mL^{-1} 0.42 mg mL^{-1} 0.46 mg mL^{-1} 0.02 mg mL^{-1}

3. Recombinant protein production in *E. coli*

3.1. Small-Scale expression of recombinant proteins

Generally small scale cultures are carried out in microtiter plates, test tubes, or shake flasks for expression check and optimization purposes. Sometimes, shake flask cultures are also used to produce recombinant proteins for initial characterization of the biological activity.

3.2. Influences on protein expression

Process optimization at small scale is essential before proceeding to large scale production.

Various criteria must be considered for optimization of conditions for the high-level expression of a recombinant protein in *E. coli*.

This optimization may help in achieving high product yields and cost effective production of recombinant proteins.

- The first step to enhance the expression of recombinant proteins is to optimize the media composition.

  Generally, complex media such as Luria broth (LB) is used for the expression of recombinant proteins at small scale cultures. However, various researchers used SOB, SOC, 2YT, GYT, MBL, Terrific broth (TB), Super broth (SB), M9 salt medium, and Enbase Flo media for the enhancement of protein expressions.

- High temperature can promote cell growth and favor the aggregation reaction due to hydrophobic interactions.

- Aeration and agitation to provide oxygen may also play an important role in protein production.
  The simple way to increase the aeration effect in shake flasks is to increase the agitation speed of the shaker.
  Most of the protein expression is carried out at a shaking speed range from 150 rpm to 220 rpm.

3.3. Large-Scale processes using bioreactors for protein production

After successful expression of recombinant proteins at small scale, development of large scale cultivation processes (Figure 9) is necessary.

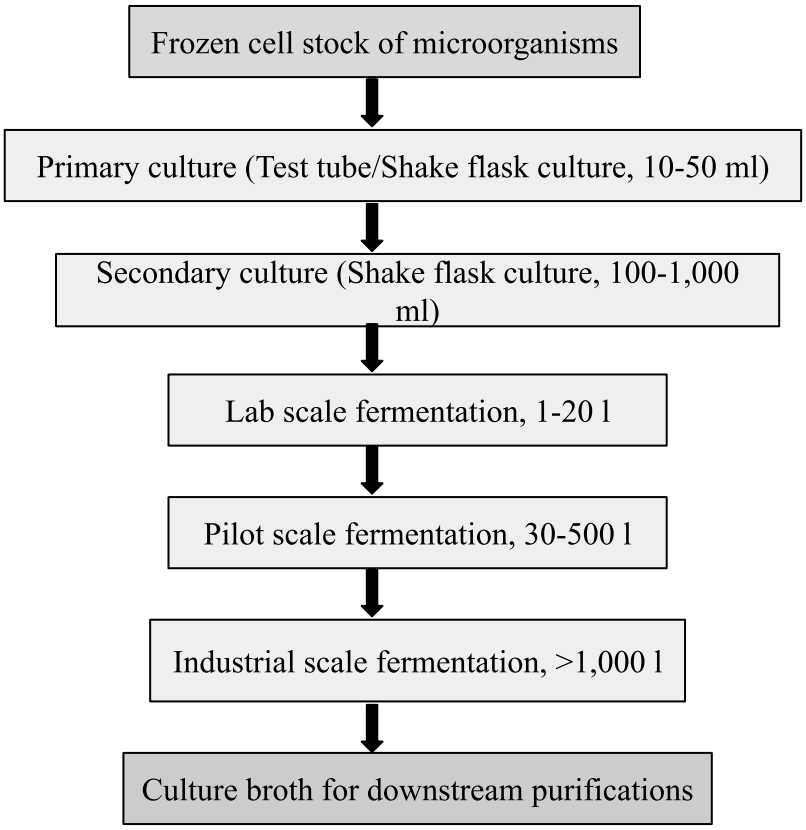

Figure 9. Flow chart showing large scale fermentation processes for production of recombinant products (Tripathi, 2016).

Fermentation processes

Bioreactors provide the facility to control various parameters which affect the cells growth as well as protein expression. These parameters include pH, temperature, agitation speed, dissolved oxygen concentration, and most importantly, nutrient addition.

Batch, fed-batch, and continuous cultures are the modes of fermentation which are employed for the cultivation of bacteria (Table 4).

Batch are commonly used for *E. coli* cultures. Using batch cultivation of *E. coli*, large amounts of biomass can be obtained in a short duration. However, batch processes are only used when small quantities of recombinant proteins are needed for detection purposes or laboratory trial studies of vaccines or therapeutics.

Fed-batch processes are used when large amounts of biomass are needed for purification purposes.

Table 4. Some recently produced recombinant products in *E. coli* using fermentation processes (Tripathi, 2016).

Product	Cultivation conditions
Aldolase	Fed-batch process (50 L)
Antibody fragment (Fab)	Fed-batch process (10 L)
Glutathione S-transferase (GST)	Batch process (4 L, 30 L)
Lysostaphin	Batch process (5 L)
Maltose-binding protein-NAP	Batch process (10 L)

4. Recovery and purification of recombinant proteins

The aim of purification process development is to achieve higher levels of purity with minimum time and less cost. The various steps involved in the purification of recombinant proteins expressed as inclusion bodies (IBs) are shown in Figure 10.

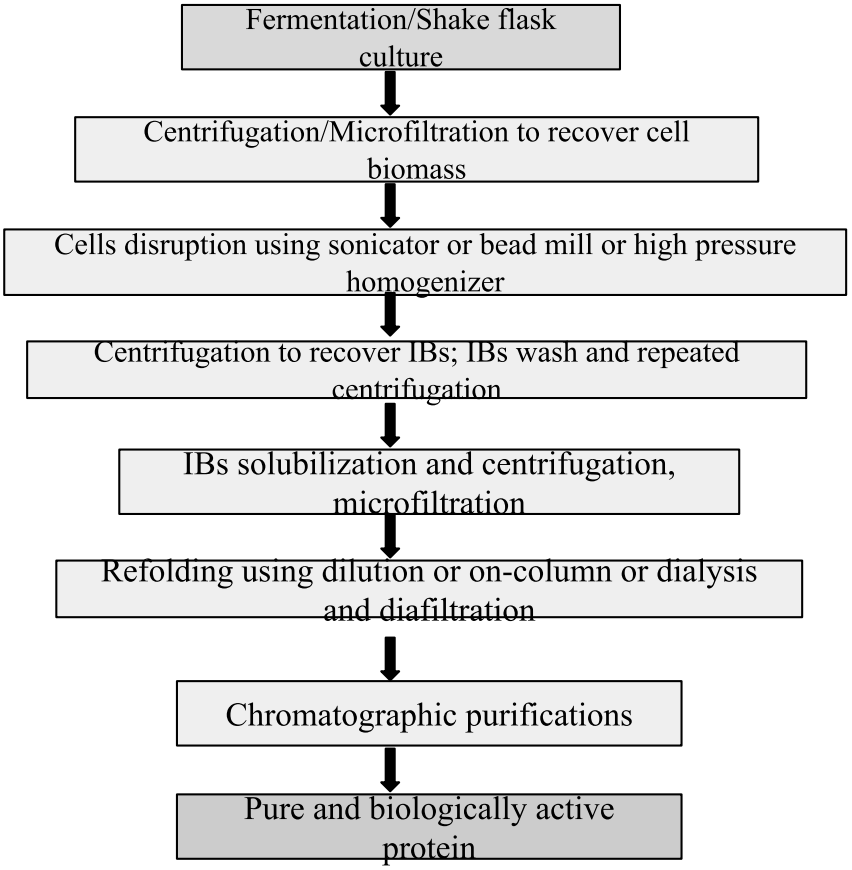

Figure 10. Process steps for purification of recombinant products expressed as IBs in *E. coli* (Tripathi, 2016).

4.1. Cell harvesting

Centrifugation is used to separate the cell biomass from the fermentation broth. Batch centrifuge is used for biomass recovery from culture broth obtained in shake flasks or laboratory fermentors. In case of large scale production, continuous centrifuges are used for cell removal.

4.2. Cell disruption/Cell lysis

Recombinant proteins expressed in *E. coli* resulted in either intracellular (cytoplasmic space or periplasmic space) or extracellular localization of the proteins as analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Cell disruption is a process used for disrupting or lysing the cells in order to recover inclusion bodies in case of intracellular expression of proteins.

The most commonly used techniques for cell disruptions are high pressure homogenization, bead milling, ultrasonication, osmotic shock, freeze thaw, enzymatic lysis, and chemical lysis.

For large scale processes, the high pressure homogenizer and the bead mill are commonly used for cell disruption.

In bead mills, the cell suspension is pumped in a horizontal grinding chamber filled with about 80 % beads. High shearing and impact forces from beads break the cell walls.

The bead mill is mostly used for the disruption of bacterial cells, yeast, algae, and filamentous fungi.

Ultrasonicators are used to disrupt the bacterial cells. Ultrasonication is generally used at the laboratory scale for cell disruption.

4.3. Solubilization of proteins expressed in inclusion bodies

High-yield expression of recombinant proteins in *E. coli* often accumulates large amounts of unfolded and misfolded proteins in the cytoplasm as insoluble aggregates and inclusion bodies (IBs).

To recover biologically active protein, inclusion bodies must be solubilized and refolded. Many commercial recombinant products such as insulin, interferons, interleukins, streptokinase, and Fc fusion proteins are produced in IBs due to high yield.

Refolding of recombinant proteins requires in-depth knowledge of the refolding processes.
Generally, the recombinant proteins expressed as IBs are solubilized by the use of high concentrations (6–8 M) of denaturing or chaotropic agents such as urea, guanidine hydrochloride (Gd-HCl), and SDS (sodium dodecyl sulfate) along with dithiothreitol (DTT), b-mercaptoethanol, or cysteine as reducing agents.

4.4. Refolding processes to recover biologically active proteins

To recover biologically active protein, it is necessary to remove the chaotropic agent to attain its native state. For this purpose, the solubilized proteins are refolded to their original state by removing the denaturing agents.

Different methods are used (Figure 11):

(A) In dilution methods, the denaturant concentration around proteins rapidly declines through diffusion before the protein concentration has been attenuated adequately, leading to the aggregation-promoting environment where the local concentration of the refolding intermediate is high.

(B) In dialysis methods, the denaturant concentration decreases gradually and uniformly.

In a stepwise protocol, at the middle denaturant concentration, where the pathways to productive refolding or unproductive aggregation or misfolding are definitely selected, proteins can achieve equilibrium, whereas they transiently go through in a one-step protocol.

(C) In solid-phase methods, various kinds of matrices aid refolding in each different mode:

- in SEC-based methods, denaturants are captured with porous matrices;
- in immobilization methods, ligand-modified matrices trap tag-fusion proteins under denaturing conditions, and then assist in refolding by inhibiting aggregation through isolation of proteins on their surfaces;
- in standard adsorption methods and a zeolite-based method, denaturants are washed out by adsorbing denatured proteins on matrices and proteins are subsequently refolded after release from matrices with eluents.

A, U, M, I, and N represent the state of protein structures, as described in Figure 11.

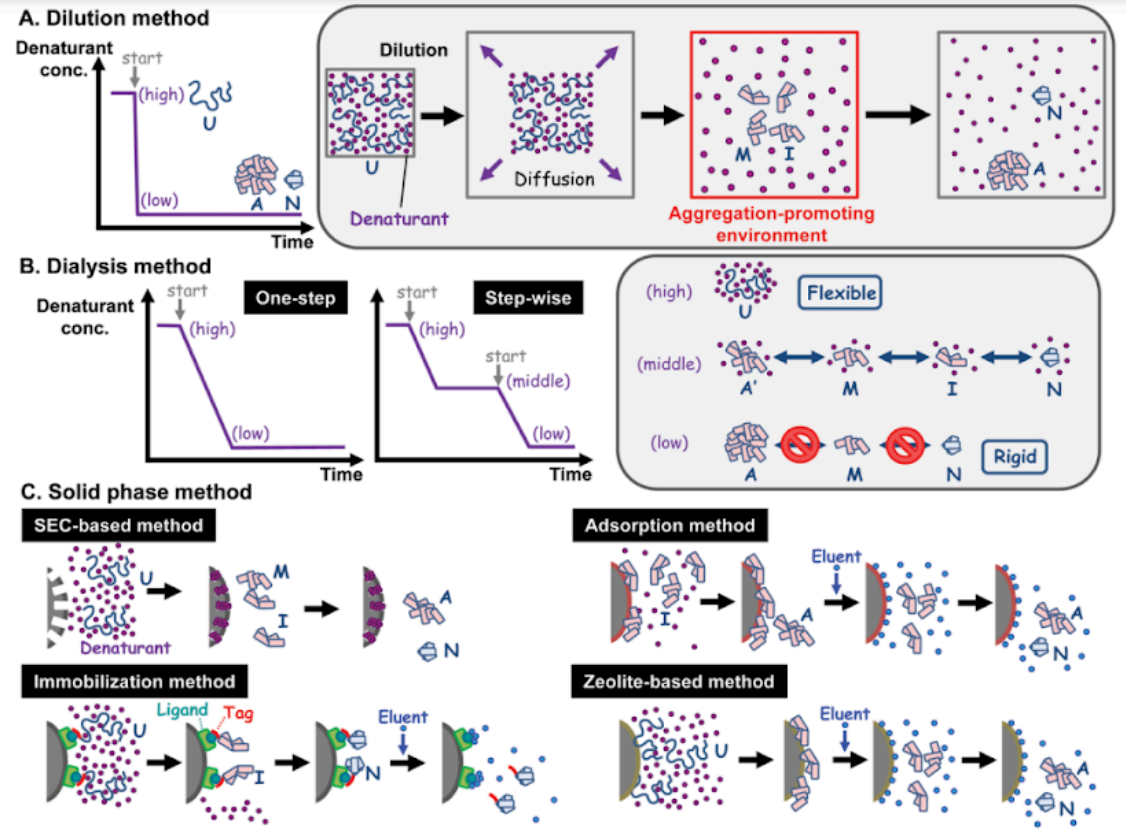

Figure 11. Schematic illustration of various methods for removing denaturants from solubilized protein solutions (Yamaguchi *et al.*, 2013).

4.5. Purification of recombinant proteins using chromatographic processes

Purification is an important step in the bioprocessing of recombinant proteins to achieve the desired level of purity. The three main types are affinity chromatography, ion-exchange chromatography, and size exclusion chromatography (Table 5).

Table 5. Chromatography techniques used for purifying recombinant proteins (Yadav, 2025).

Chromatographic method	Principle of operation	Applications
Affinity chromatography	Exploits specific interaction between a ligand attached to a chromatography matrix and target protein	- Isolation of recombinant proteins tagged with affinity tags (e.g., His-tag, GST-tag) - Purification of antibodies using protein A or protein G affinity chromatography. - Separation of enzymes based on substrate specificity
Ion-exchange chromatography	Separates proteins based on their net charge using a stationary phase with charged groups (cations or anion exchange)	- Separation of enzymes based on their isoelectric point (pI) - Separation of protein with different charges, such as enzymes or antibodies - Removal of recombinants like DNA or RNA
Size exclusion chromatography	Separates proteins based on size and shape using a porous stationary phase where larger molecules elute first and smaller molecules enter the beads and elute later.	- Purification of proteins from complex mixtures without prior knowledge of specific interactions - Removal of aggregates and large molecular weight contaminants - Estimation of molecular weight and oligomeric state of proteins

Chapter 2. DNA-protein and protein-protein interaction

Learning Objectives

At the end of this chapter, you should be able to:

- Explain the biological significance of DNA–protein and protein–protein interactions in cellular regulation and signaling.

- Describe experimental strategies used to detect, characterize, and analyze molecular interactions.

- Compare in vitro and in vivo approaches for studying biomolecular interactions.

1. Interactions DNA -protein

1.1. Interest

DNA–protein interactions are essential for several molecular and cellular mechanisms, such as transcription, transcriptional regulation, DNA modifications, among others.

The studies of interactions are centered on a specific protein, somehow related to the phenotype being investigated. The aim of each technique may be to :

- recognise DNA-binding proteins in a cell extract,
- identify the DNA-binding site,
- analyze the specificity of the binding, or
- simply confirm if a given protein does bind to the respective alleged target genes and determine the effects of the binding in these genes' expression, envisioning to reveal novel insights into gene regulatory systems.

1.2. Techniques studying DNA–protein interactions

a. Nitrocellulose filter binding assay

This assay was developed in the early stages of molecular biology in the 1970s.
The process is based on the fact that proteins bind to nitrocellulose without losing their DNAbinding capacity, while double-stranded DNA alone is not retained (Figure 12).

For analysis:

- DNA and proteins are mixed and incubated under appropriate conditions.
- The mixture is then separated by electrophoresis and subsequently blotted onto nitrocellulose.
- Since only proteins bind, DNA remains on the membrane only if in complex with a protein.

The amount of information obtained from this kind of assay is limited:

- Only the mere retention of labelled nucleic acid is detected and not the identity of the proteins involved, or the proportion of binding activity attributable to an individual protein if more than one protein in the mixture exhibits DNA-binding capacity.
- Also, the actual DNA binding site cannot be localised with this assay unless defined DNA fragments are used in the analysis.
- Moreover, as a technical complication, single stranded nucleic acids are retained at nitrocellulose filters under particular conditions, resulting in background that can obscure the measurement.

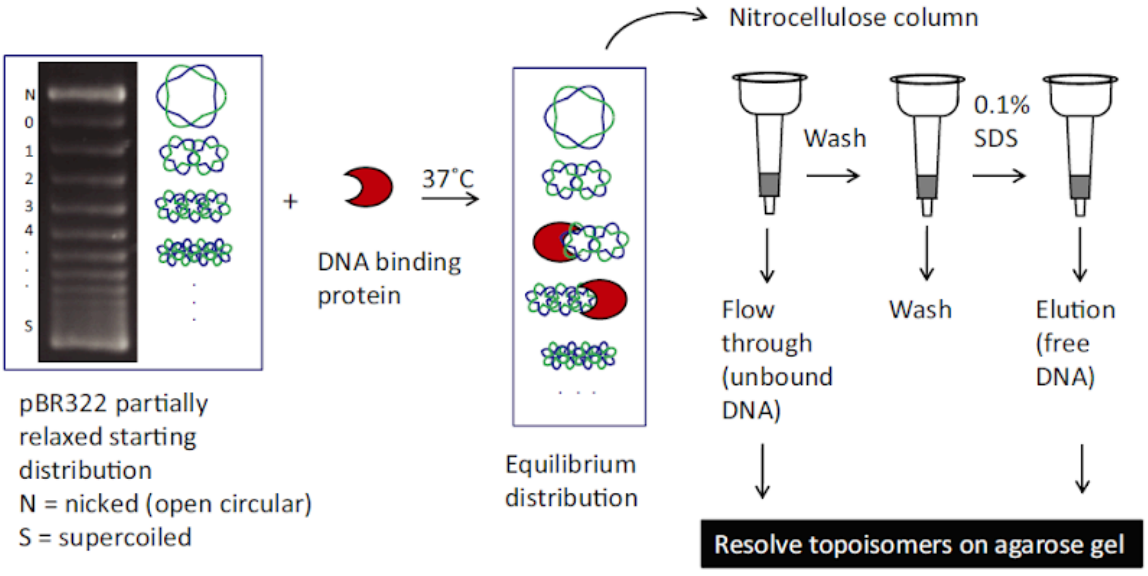

Figure 12. Topology-dependent binding assay (Litwin *et al.*, 2015).

b. Footprinting assays

Footprinting assays exploit the fact that a protein, which is bound to a specific nucleic acid, will interfere with a chemical or enzymatic modification of that DNA fragment. Thus, the modification can be used to localize the contact area between protein and DNA.

For the DNase I footprinting assay (Figure 13):

- A particular DNA fragment is labelled at one end and mixed with the protein of interest.
- Following binding, the DNA is treated with the enzyme deoxyribonuclease I (DNase I), which digests DNA that is not in close contact with a protein and thus not protected from digestion.
- Performing a partial cleavage without protein produces labelled DNA fragments that—because of the random nature of cleavage—cover the entire size range of the original DNA.
- In the presence of a binding protein, however, protection occurs in a particular region and labelled DNA fragments of the respective length are not produced, while all longer or shorter ones are still present.
- Resolving the two samples on a polyacrylamide gel side by side, the differences in the resulting ladders of DNA bands is visualized via the incorporated label.
- Gaps in the band ladder of the sample, in which protein had been present, indicate binding sites.
- Comparison of the patterns with sequencing reactions allows the identification of protected sequences with single-nucleotide resolution.

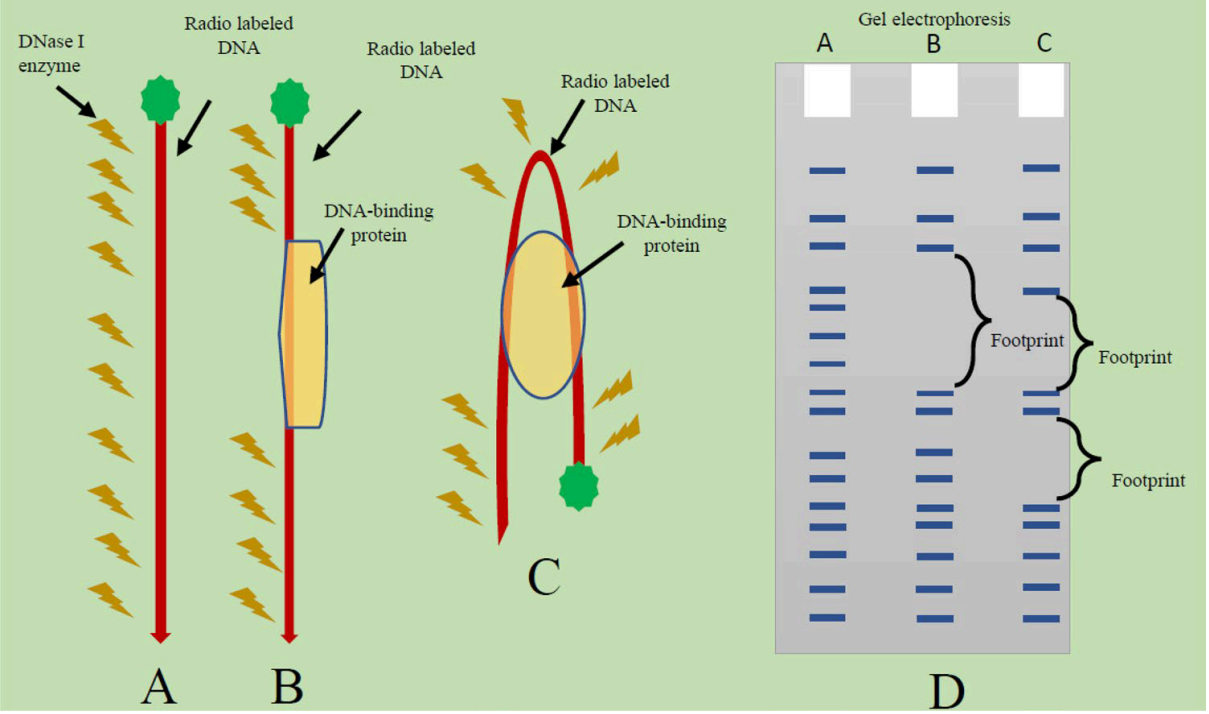

Figure 13. A schematic picture represents Deoxyribonuclease I (DNase I) footprinting method. A) DNA in the absence of the DNA-binding protein, B) Bind the protein to DNA probe, C) The protein bound to two sites forming a DNA curvature, D) Limited digestion of the end-labeled DNA fragments yields fragments terminating everywhere except in the footprint region, which is protected from digestion. **(Emamjomeh *et al.*, 2019)**.

c. Chromatin ImmunoPrecipitation

Chromatin ImmunoPrecipitation (ChIP) is one of the efficient experimental techniques used for accurately characterizing bound for the functional protein to DNA molecule.

ChIP is based on the combination of different techniques, including covalent bridging, immunoprecipitation, and PCR.

ChIP is used to determine whether a given protein binds a specific fragment of DNA.

The major advantage of ChIP is that it is carried out *in vivo*, that is to say that the information drawn from this experiment comes directly from an analysis carried out on living cells.

Thus, after the growth of the cells under the desired conditions, all the proteins which touch the DNA are immobilized there by covalent bonds at the precise location of their interaction with a treatment with formaldehyde. Subsequently, the cells are lysed, then the DNA-protein complexes are fragmented into short segments before being immunoprecipitated.

This technique can be broken down into several steps as follows (Figure 14):

A) Covalent binding of DNA to proteins *in vivo* (bridging)

Several methods are practiced such as the use of UV, formaldehyde or methylene blue.

B) The DNA-protein complex (bridged chromatin) is fragmented

For this, sonication or digestion by restriction enzymes are used. These techniques are standardized to produce bridged DNA fragments of the order of 500 base pairs minimum.

C) Immunoprecipitation of bridged chromatin

- This is one of the key points of the method and it is necessary to have excellent quality antibodies for the technique to work correctly.
- To recover the DNA fragments on which the protein of interest is bridged, we use a specific antibody against the latter.
- The antibody will be biotinylated, which will allow it to be retained against a magnetic bead coupled to streptavidin.
- The magnetic bead-streptavidin-antibody-bridged protein-DNA complex is held at the bottom of a tube by a magnet while all other DNA-protein complexes are removed by washing.
- After the washes, the magnetic bead made it possible to retain all the DNA fragments with which the protein of interest has a significant enough interaction to allow formaldehyde bridging.

D) DNA purification

The DNA/Protein complexes are dissociated by heat and the proteins are digested by Proteinase K. The protein studied was associated with the region that interests us, DNA fragments containing this sequence will have been retained during the washes (They are in small quantities and they must then be amplified by PCR to be able to establish their presence).

E) DNA analysis

This is carried out either by Slot blot or by Southern blot after PCR amplification of the immunoprecipitated DNA: the DNA to which the protein was bound is recovered and amplified by PCR.

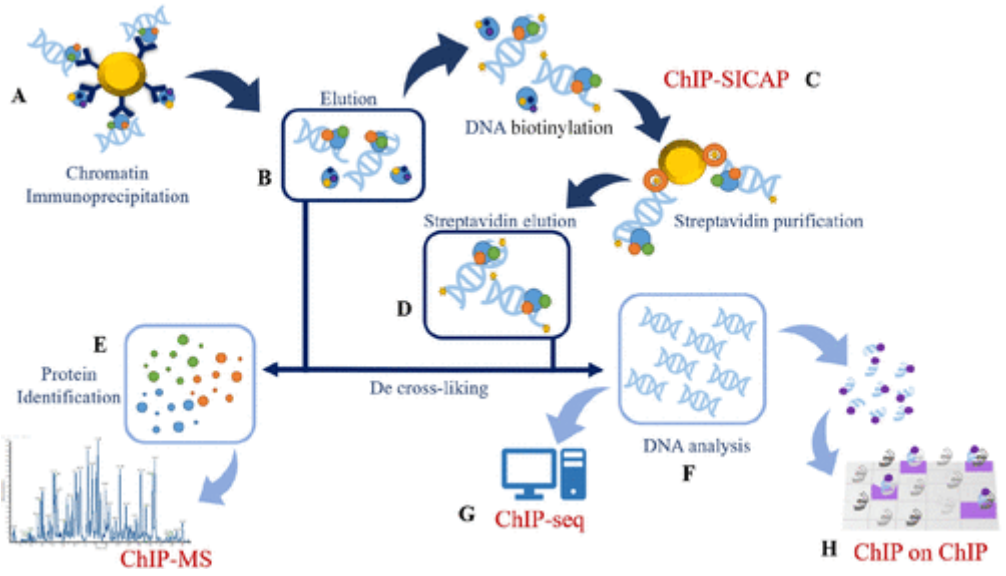

Figure 14. A generic representation of ChIP experiments. (A) After chromatin immunoprecipitation, (B) DNA−protein complexes are eluted. For only DNA−protein complex isolation, ChIP-SICAP experiment is possible by DNA biotinylation and streptavidin purification, and then (D) elution and de-cross-linking. Finally, protein identification (E) was carried out by mass spectrometry approach (ChIP-MS) and DNA analysis (F), by (G) sequencing (ChIP-seq) or (H) by hybridization with a pull of fluorescence probe (ChIP on ChIP) **(Cozzolino *et al.*, 2021).**

d. Electrophoresis Mobility Shift Assay Mass Spectrometry (EMSA-MS)

Since 1981, the electrophoresis mobility shift assay (EMSA) on either polyacrylamide or agarose gel has constituted the most largely employed biochemical procedure to detect *in vitro* DNA−protein interactions, for the simplicity of the procedure, its low cost, and the speed of execution.

More recently, an unbiased approach was developed by coupling the classic EMSA assay with advanced mass spectrometry methodology for the identification of DNA-binding proteins (EMSA/MS).

The EMSA/MS procedure combined the simplicity and effectiveness of the EMSA experiments with the ability of high sensitivity, high resolution mass spectrometry to identify all the proteins interacting with the probe in an unbiased operative mode.

This technique is based on the observation that the segments of binding nucleic acid to protein cause a decrease in the segment's electrophoretic mobility compared with the free nucleic acid in an agarose gel under a native condition or non-denaturing polyacrylamide gel.

In this technique crude proteins' mixture or purified proteins are mixed with the nucleic acid sequence in a suitable buffer and specific binding is allowed to occur, stable complexes of nucleic acid and protein were separated by non-denaturing gel electrophoresis.

Following the EMSA experiment, the shifted band containing the probe-protein complex is excised from the gel and the protein components are identified by tryptic digest and nanoLC-MS/MS analysis of the resulting peptide mixture (Figure 15).

A convincing negative control can be obtained by loading the same amount of protein extract on a separate gel lane in the absence of the probe. For each band excised from the sample lane, an analogous band with the same electrophoretic mobility is picked up from the control lane.

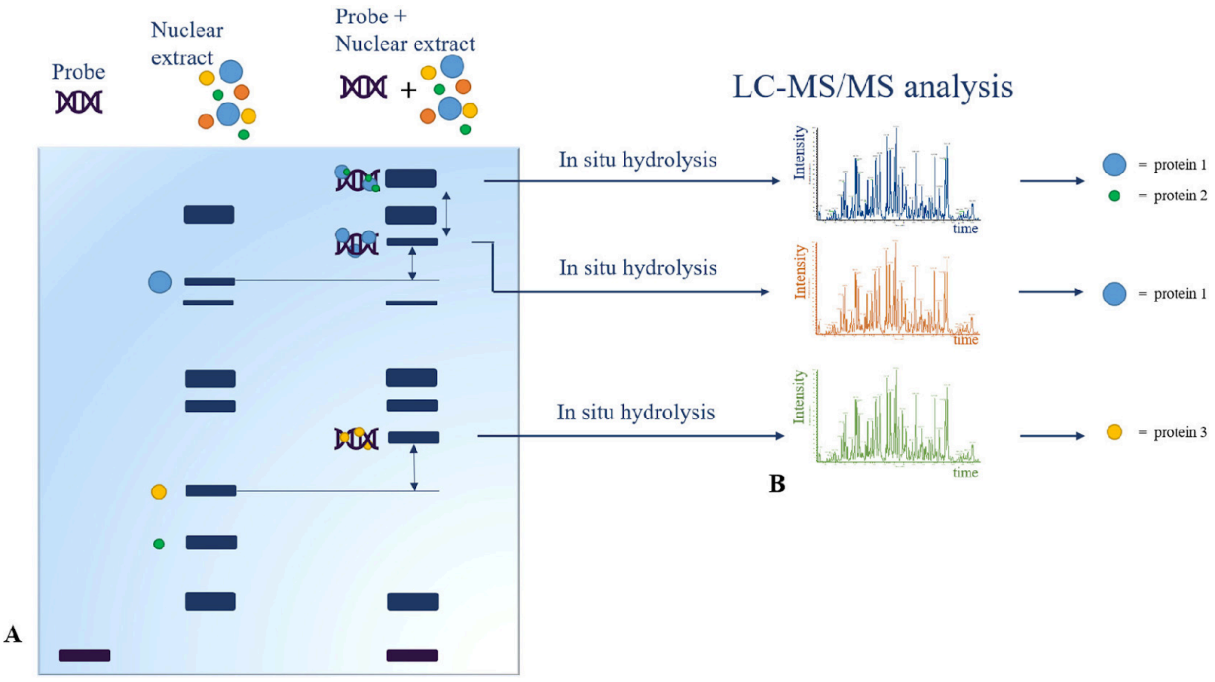

Figure 15. Schematic representation of EMSA-MS experiment.

(A) Nuclear proteins are incubated with an oligonucleotide probe and bands showing an electrophoretic mobility shift in comparison to the control are *in situ* hydrolyzed by trypsin and (B) proteins identified by LC-MS/MS approach. The control is the nuclear extract alone (second lane) **(Cozzolino *et al.*, 2021)**.

2. Protein–Protein interactions

2.1. Interest

Protein-protein interactions (PPIs) are an essential aspect of biological processes.
They are strongly involved :

- in the formation of macromolecular structures,
- in signaling,
- in regulation and in different metabolic pathways.

Their study is therefore crucial for understanding protein interaction networks, a major goal in the study of biological systems.

Protein-protein interactions have a significant role in the induction of many pathological conditions and in processes important for the pathogenesis of bacterial and viral infections.

2.2. Determination of protein–protein interactions

a. Coimmunoprecipitation

Co-immunoprecipitation (Co-IP) is one of the most utilized techniques aimed to identify PPIs.

Based on the fundamental principle of interaction between an antigen and antibody, this method utilizes a bait-specific antibody that enables the precipitation of the bait protein along with its interacting partners from the sample. In essence, it is an extension of affinity chromatography. Co-IP is either followed by Western blot analysis or mass-spectrometry to identify the interactors (Figure 16).

- Co-IP has the advantage of reflecting physiological interactions of proteins expressed at endogenous levels since it is carried out in a tissue or whole cell lysates.
- The technique is non-denaturing in nature and does not induce any artificial effects such as modification or over-expression of the interacting proteins.
- Elaborated complexes that otherwise face difficulty *in vitro* assembly can be easily co-precipitated using this technique.

However, since the concentration of the bait protein is much lower in its native conditions, this technique suffers from limitations of sensitivity.

Moreover, Co-IP requires a sufficient quantity of protein (100–1000 mg).

Lastly, Co-IP does not always denote direct PPIs since co-immunoprecipitated proteins can in essence be a part of larger complexes.

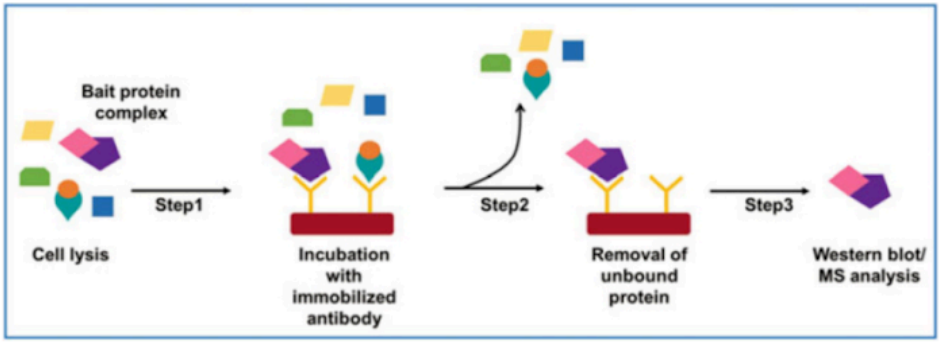

Figure 16. working principle of co-immunoprecipitation (Poluri *et al.*, 2021).

b. Protein-fragment Complementation Assay (PCA)

A family of assays, known as protein-fragment complementation assays (PCAs) (Figure 17) are designed to detect (PPIs) *in vitro*, *in vivo* or in living cells.

Procedure :

- In these assays, the two target proteins (the bait and the prey) are fused to C- or N-terminal fragments of a reporter protein that are complementary in nature and rationally dissected.
- On interaction of the prey and the bait proteins, the fragments of the reporter protein are brought into close vicinity that leads to the proper folding of the protein, ultimately reconstituting its activity .
- The reporter proteins that are most commonly used include beta-galactosidase, GFP and its variants, ubiquitin, etc.

There are several PCA-based techniques and the principle of each of them relies on the detectable effects that are produced by the reporter protein on reconstitution.

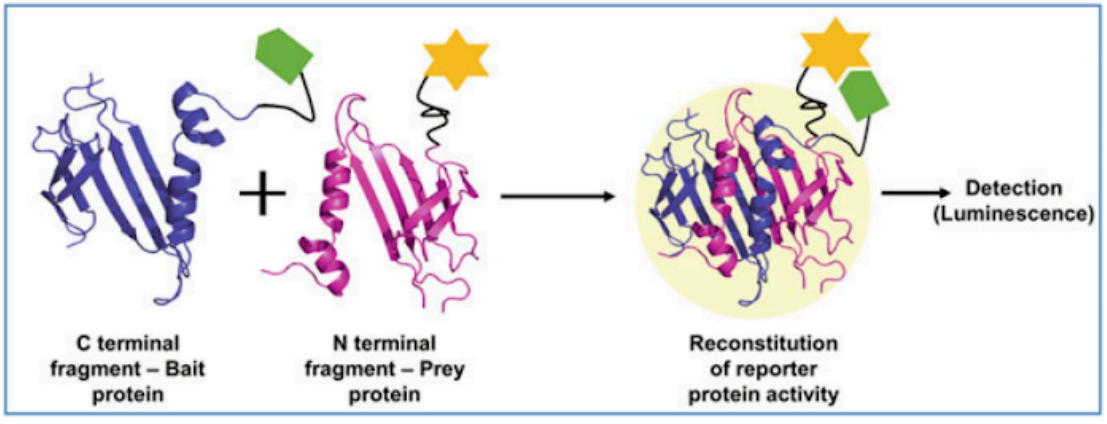

Figure 17. Principle of protein fragment complementation assay (PFCA) (Poluri *et al.*, 2021).

c. Forster/Bioluminescence Resonance Energy Transfer (FRET/ BRET)

Fluorescence resonance energy transfer (FRET) is a process of non-radiative energy transfer from an excited donor fluorophore to an acceptor fluorophore in close proximity (1–10 nm) via dipole–dipole interactions. Apart from the close proximity, a donor–acceptor FRET pair needs an overlap between the donor's emission spectrum and the acceptor's excitation spectrum (Figure 18).

The widely used FRET pairs come from GFP variants, such as cyan fluorescent protein (CFP)–yellow fluorescent protein (YFP), CyPet–Ypet, blue fluorescent protein (BFP)–GFP, MiCy–mKO, mVenus–mStrawberry, cerulean fluorescent protein (CrFP)–YFP, etc.

In a typical FRET experiment designed to elucidate PPIs:

- One of the two target proteins is fused with the donor fluorescent chromophore, while the other is fused with the acceptor fluorescent chromophore.
- Both the fused proteins are subjected to a binding assay after their expression. In case if the two proteins do not interact, the donor fluoresces upon excitation, but the acceptor does not. However, upon interaction of the two proteins, the donor and acceptor come in close proximity.
- Upon excitation of the donor in this state, non-radiative energy is transferred from the donor to the acceptor. In short, the occurrence of PPIs leads to FRET when the donor's fluorescence intensity decreases and that of the acceptor increases.

FRET has been extrapolated to *in vitro* binding assays to design PPI inhibitors via optimized FRET pairs. It has also been utilized to visualize PPIs in real-time in live cells, with high spatiotemporal resolution in combination with other imaging techniques.

Bioluminescence resonance energy transfer (BRET) is an analogous method to FRET except for the fact that a bioluminescent protein acts as the donor rather than a fluorescent protein.

The luminescent donor protein is usually Renilla luciferase (Rluc), and upon oxidation of its substrate, it emits light to excite the acceptor. Improved versions of BRET in recent times have also enabled researchers to investigate PPIs in real-time over an extended timescale.

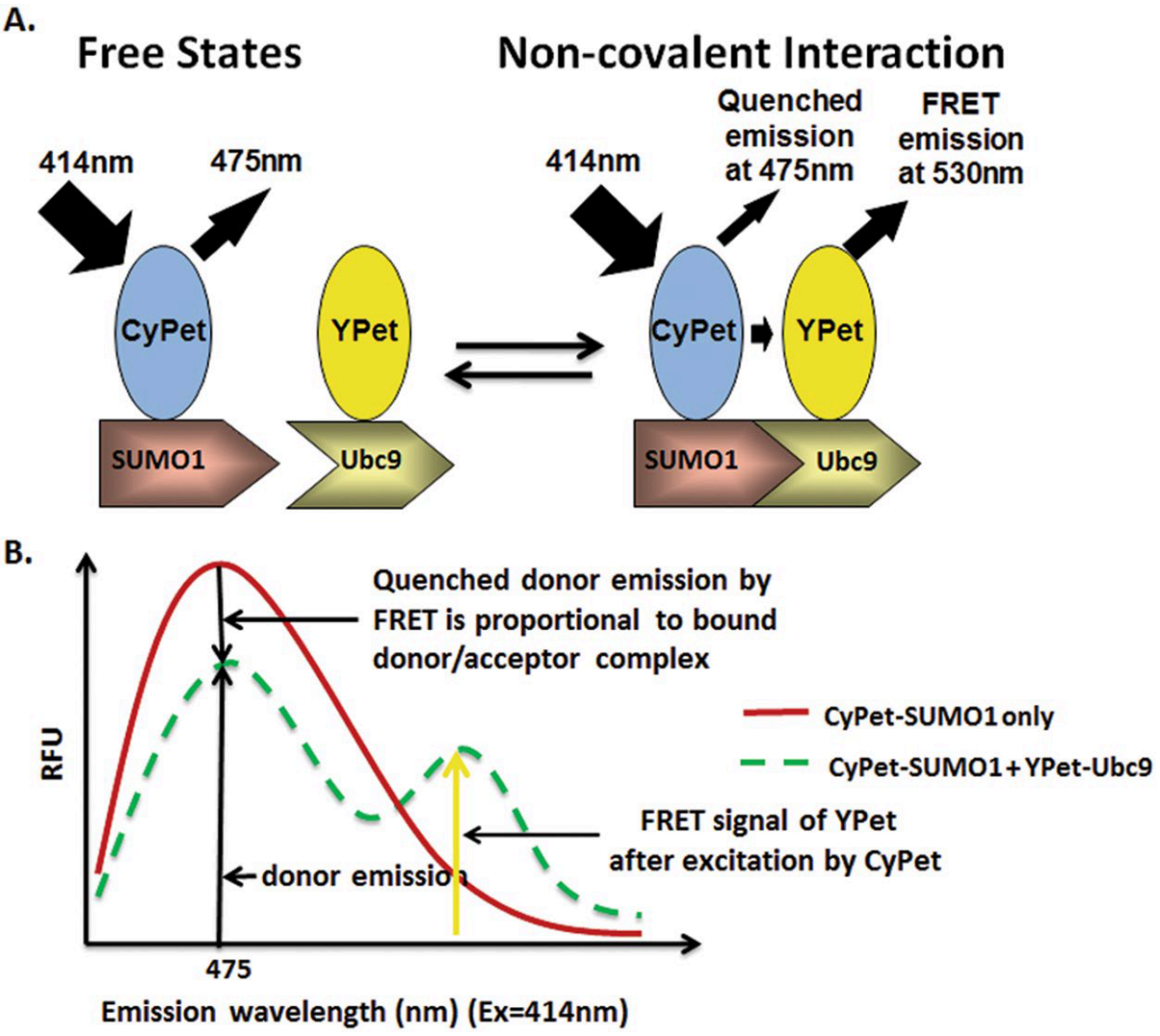

Figure 18. Revelation of the interaction between proteins A et B by FRET. CyPet : blue fluorescent protein, YPet : yellow fluorescent protein (Jiang *et al.*, 2019).

d. Gel Filtration

Gel filtration is a technique that is used for the separation of molecules in a solution on the basis of their mobility. The differences in their mobility in turn arise from the differences in their molecular weights. In other words, the process of gel filtration acts like a molecular sieve.

Procedure (Figure 19)**:**

- This method utilizes inert beads made of polymers (polyacrylamide, agarose, etc.) that contain pores of a particular dimension.
- When a solution containing a mixture of molecules is subjected to the beads in a column, the larger molecules face lesser penetration into the pores and are eluted first.
- Smaller molecules, on the other hand, are permeable through these beads and are retained for a greater time in the column, resulting in their elution at a later stage.
- Molecules that fall in the intermediate size range elute on the basis of their molecular radius. Gel filtration can be utilized in three different ways to estimate binding constants of PPIs.
- In the non-equilibrium "small-zone" gel filtration, a solution of a protein and a solution of its interactor are administered to the column in small volume and the resolution is performed in the usual way.
- The elution profile is then compared with the chromatograms of the individual proteins.
- In case of formation of a protein complex, the elution is observed earlier than either of the proteins alone.
- The protein complex can also be purified via fractionating the corresponding peak fraction.

This approach helps to evaluate the binding constant via the measurements of protein concentration that are needed to form the complex.

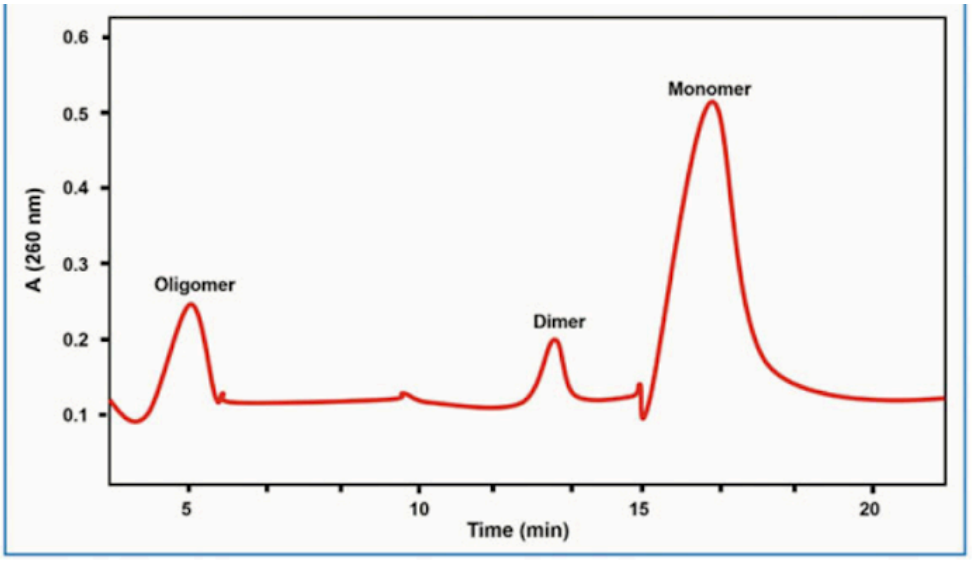

Figure 19. A gel chromatogram showing presence of oligomeric structures (Poluri *et al.*, 2021).

Chapter 3. Cell culture

Learning Objectives

At the end of this chapter, you should be able to:

- Define cell culture and explain its essential components, including *in vitro* conditions, cell viability, and metabolic activity.

- Explain the importance of controlled environmental conditions for maintaining healthy cell cultures.

- Describe the major applications of cell culture in biomedical research, drug development, and tissue engineering.

1. What is cell culture?

While there are many different definitions of cell culture, we can always find the following three components in its definition:

(1) *In vitro*, meaning "in a representation," that is, in a tube or a culture dish, and outside a living organism;

(2) Maintain cells in a viable state, indicating that cells should be alive and actively proliferating (dividing or reproducing);

(3) Maintain cells in a metabolically active state.

To accomplish all three, we need to feed oxygen, nutrients, and growth factors appropriately. Maintenance of metabolically active state is quite crucial for tissue engineering applications. Cells should produce necessary proteins and respond to environmental factors (e.g., physical and biological cues) to perform essential functions toward tissue engineering applications.

2. Applications

Cell cultures are commonly used for various applications (Figure 20).

- Cell culture is being popularly used for evaluating the efficacy and toxicity of drugs. Once a new drug is identified or developed, it is assessed for its efficacy and toxicity in three different steps, namely, (1) *in vitro* test, (2) animal test, and (3) human clinical trial.
- Cell culture has frequently been used for the first step – *in vitro* test. In such a test, varying amounts (doses) of the drugs are added to the cell culture, for example, human liver cells, human kidney cells, etc., and the cells' viability and metabolic status are assessed. The shape of cells (cell morphology) can also be assessed. Production of specific proteins (e.g., albumin from liver cells) is also frequently evaluated.

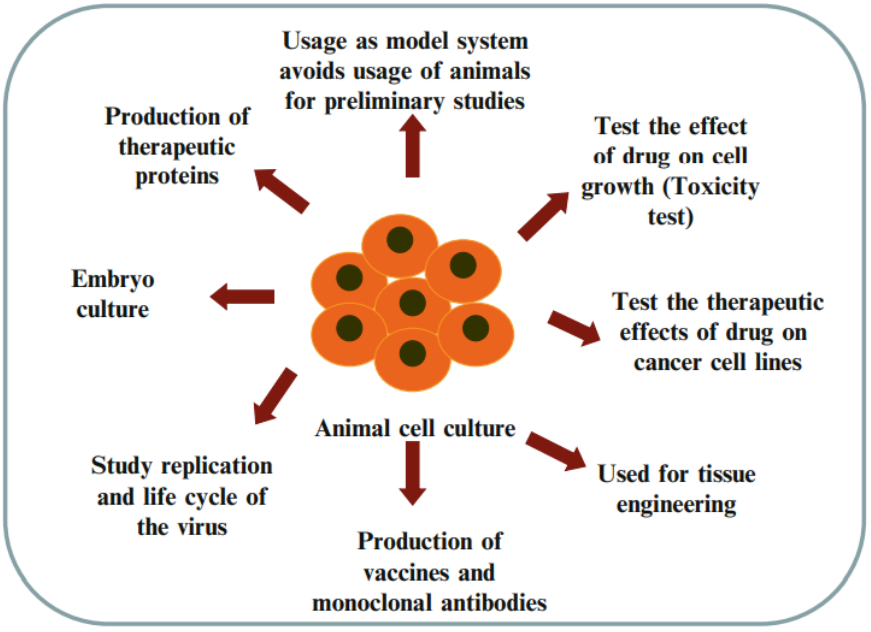

Figure 20. The important applications of animal cell culture (Gupta *et al.*, 2016).

3. Cell classification

3.1. Anchorage-dependent *versus* anchorage-independent cells

Anchorage-dependent cells require a surface they can anchor. The surface is typically an extracellular matrix (ECM) *in vivo*, while an artificial surface can also be used for the cell culture *in vitro*.

Anchorage-dependent cells cannot be stacked in multilayers on a surface, that is, they can only form a monolayer.

When a surface is saturated with anchorage-dependent cells in a monolayer (caused by contact inhibition), such a condition is referred to as confluency, for example, 80% confluency represents 80% of the surface is covered by anchorage-dependent cells.

On the other hand, anchorage-independent cells do not require a surface for their proliferation and metabolism and can be cultured in a suspension. The best examples are blood cells, including red blood cells (RBCs) and white blood cells (WBCs) (Figure 21).

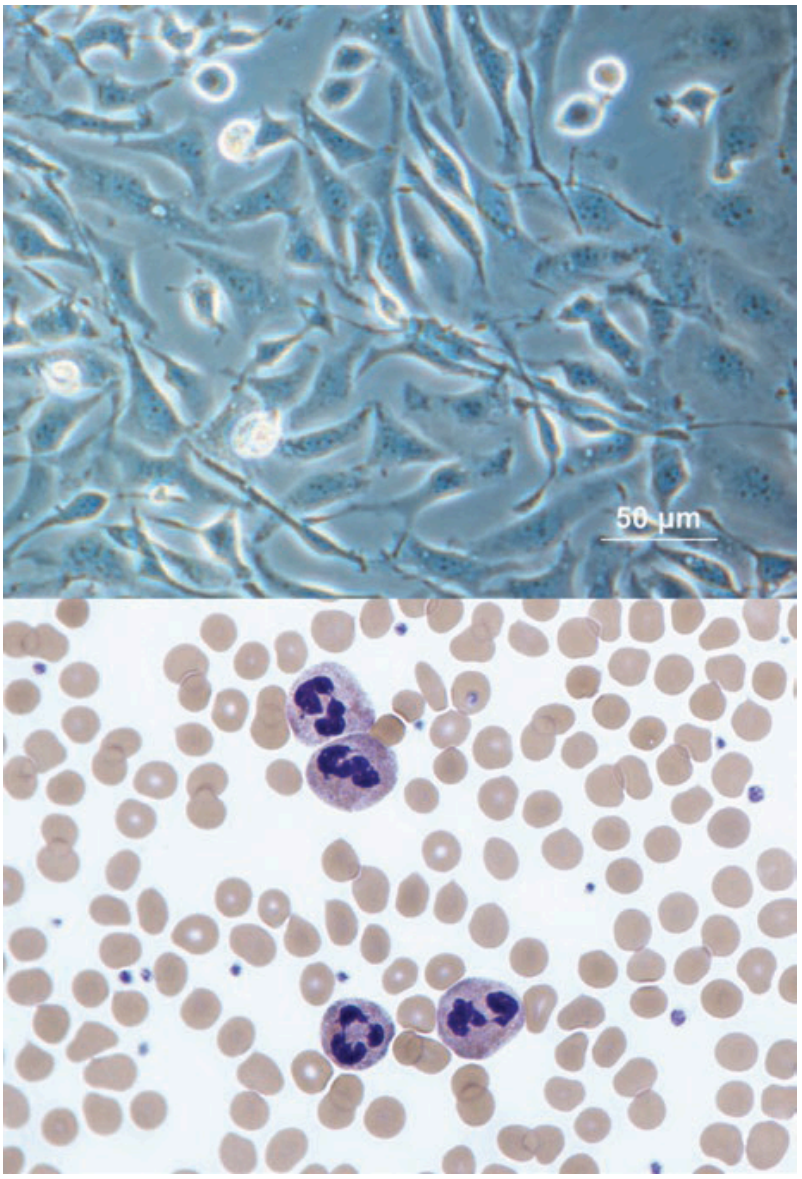

Figure 21. Top: Chinese hamster ovary (CHO) cells representing anchorage dependent cells. Bottom: human neutrophils (more giant cells with multiple nuclei within cells; a part of human white blood cells) shown together with human red blood cells (smaller, donut-like cells), representing anchorage-independent cells (Yoon, 2022).

3.2. Normal *versus* immortalized cells

Cells can also be classified based on their growth behavior, that is, normal versus immortalized.

Normal cells can proliferate (divide) for a limited number of times (typically limited by Hayflick limit) and are highly differentiated, that is, they perform specific functions.

On the other hand, immortalized cells can proliferate (divide) for an indefinite number of times, thus immortal. They are sometimes referred to as a more generic term of transformed cells, although these two terms' specific meanings may slightly differ.

Cancer cells are considered as spontaneously immortalized cells. One of the earliest human cell lines is HeLa cells, descended from the cancer cell from Henrietta Lacks in the 1960s, who died of cancer. Figure 22 shows the microscopic image of cultured HeLa cells with Hoechst staining, which stains the cell nuclei in blue.

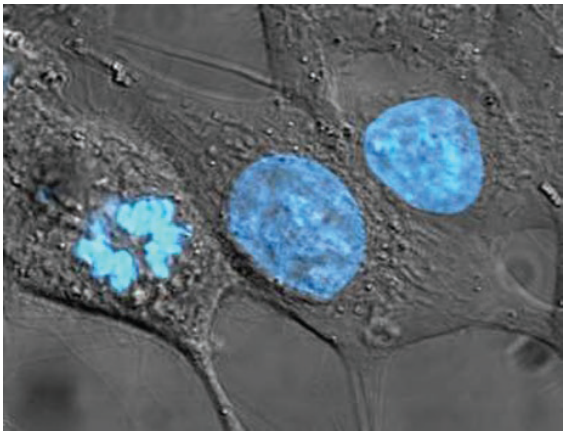

Figure 22. HeLa cells with Hoechst staining (Yoon, 2022).

Various methods can be used to immortalize normal cells.
One of the standard techniques is infecting normal mammalian cells (= human and mammal cells) with a virus that can cause cancer.
SV-40 (simian vacuolating virus 40 or simian virus 40 in short) is a good example, which is a small DNA virus found in monkeys and humans (thus "simian") and can cause cancer to them. Various mammalian cells have been infected with SV-40, causing them to partially or fully lose their serum requirement, anchorage-dependence, and thus gaining immortality.

3.3. Normal *versus* stem cells

All cells in a human body retain identical genetic information, and they originate from a single type of cell (zygote = fertilized egg). In this sense, it is a totipotent (toti = all; potent = ability) stem cell.

You can also find stem cells in an adult human body that can differentiate into a large number of cell types but not a whole organism, called pluripotent stem cells, or a limited number of cell types, called multipotent stem cells. Examples include mesenchymal stem cells and hematopoietic stem cells (Figure 23).

Stem cells are undifferentiated and cannot perform specific functions, while normal cells are fully differentiated and perform particular operations.
Stem cells can also proliferate (divide) for a small number of doublings or are close to immortal.
However, stem cells are fundamentally different from immortalized cells (including cancer cells) as they are not “crazy” cells.

In tissue engineering applications, normal cells may not be ideal as they can proliferate only for a limited number of doublings and require specific growth media like a serum.

The use of immortalized cells may cause several issues, as they are “crazy” cells and sometimes challenging to retain their functionality.

The use of stem cells is ideal as they can proliferate for a great number of doublings and are not “crazy” cells. Differentiation can be induced later to make the cells perform specific duties.

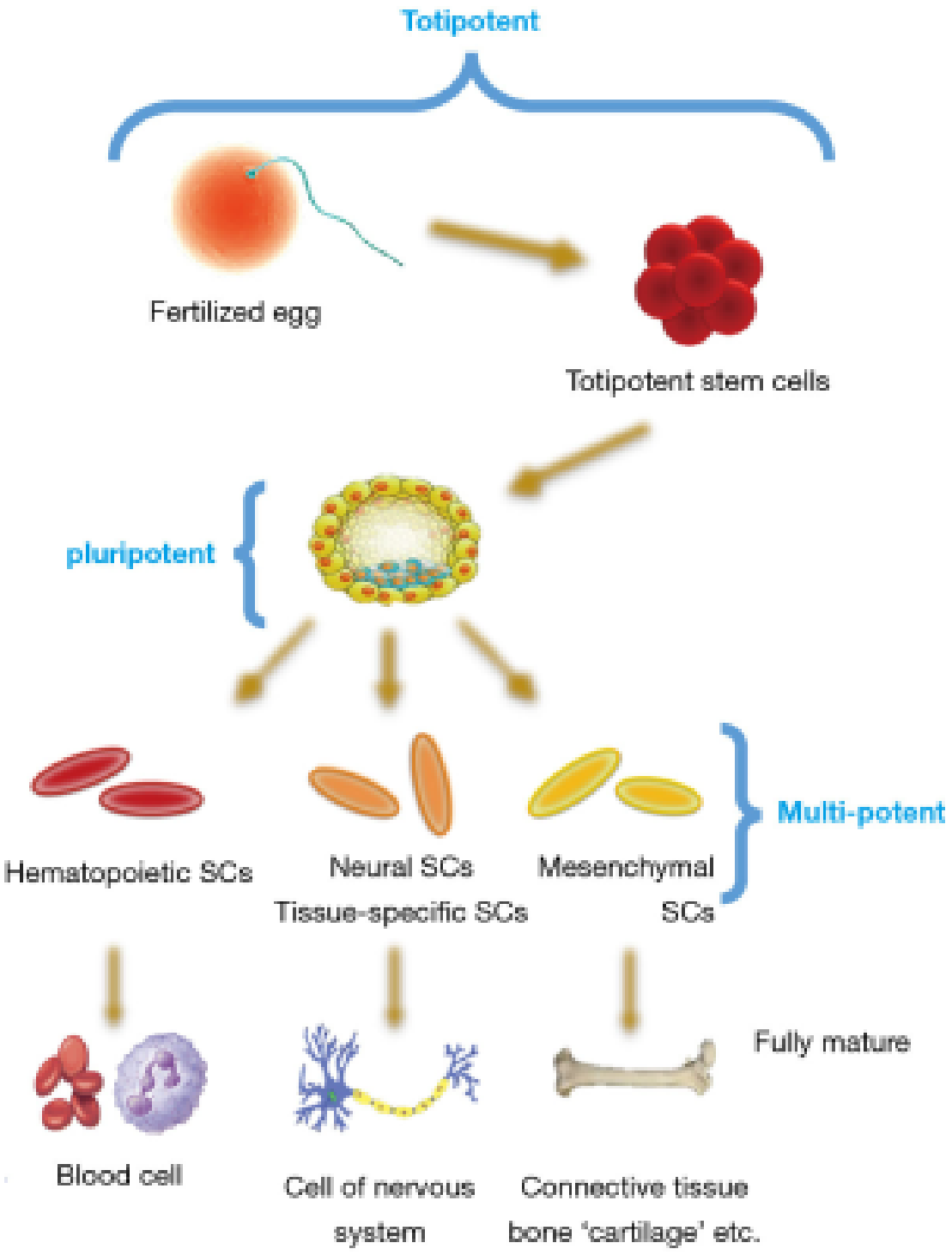

Figure 23. Totipotent, pluripotent, and multipotent stem cells (Rajabzadeh *et al.*, 2019).

4. Technical aspects of cell cultivation

4.1. Maintaining sterile environment: Biosafety Cabinet

Cell culture can be contaminated by bacteria or yeast, which is quite a common problem. In such a case, mammal and human cells (= mammalian cells) compete with bacteria and yeast for nutrients. Mammalian cells may quickly lose such competition, and the entire cell culture is overrun by bacteria or yeast.

Maintaining a sterile environment is mandatory to avoid such contamination.

Preparation of cells and culture media and in-culture practices should be conducted in a biosafety cabinet, also known as laminar flow hood.

A typical biosafety cabinet is shown in Figure 24, which is a class II biosafety cabinet.

Class II biosafety cabinet is the most common. It protects the laboratory and personnel (class I's function) and additionally provides a sterile environment within the cabinet.

Figure 24. A class II biosafety cabinet: (left) overall view and (right) inside view (Yoon, 2022).

4.2. Maintaining sterile environment: Autoclave

An autoclave is another essential piece of equipment for maintaining a sterile laboratory environment.

Tubes, transfer pipettes, pipette tips, glassware, etc., can also be contaminated with bacteria or yeast and must be disinfected. An autoclave is quite useful to disinfect them altogether. These tubes and tips can be placed inside an autoclave, and high temperature (over 120 °C) and high pressure (above 15 PSI = 0.1 MPa) are applied to disinfect (= kill) any bacteria, yeast, etc.

4.3. Cell Culture: CO_2 incubator

For most tissue engineering applications, both incubator and bioreactor are necessary.

Incubators provide an environment that is optimum for cell culture. It typically provides:

- a fixed temperature, for example, 37 °C (human's body temperature),
- optimum relative humidity (RH), for example, at 95% (preventing water evaporation), and
- HEPA-filtered air (providing oxygen but not particulates and aerosols).

For culturing mammal or human cells, that is, mammalian cells, a specific incubator type is necessary, that is, CO_2 incubator. As its name indicates, it provides an additional environmental condition of CO_2, typically at 5%. This 5% CO_2 is the physiological condition of most mammalian tissues, allowing them to maintain appropriate pH (when dissolved into water, CO_2 turns into bicarbonate and becomes acidic).

Figure 25 shows a typical CO_2 incubator. In this case, two incubators are stacked. You can see a CO_2 gas tank next to it. Within the CO_2 incubator, multiple cell culture flasks are stacked, each being cultured with different cells and varying media conditions.

Figure 25. Left: double-stacked CO_2 incubator with a CO_2 gas tank attached to it; right: multiple cell culture flasks within a CO_2 incubator (Yoon, 2022).

4.4. Cell imaging: Fluorescence Microscope

Once cells are properly cultured, you may wish to image them to count their number, check their shape (morphology), and identify subcellular components. As cells are too small to be imaged by a conventional camera, a microscope becomes necessary.

A typical microscope is shown in Figure 26 where an objective lens is located underneath the sample stage. This setup is known as an inverted microscope, which is popular in cell imaging as it provides more room to a user. When the objective lens is located on top of the sample stage, it is known as an upright microscope, which is more traditional but less popular in cell imaging as it does not provide sufficient room to a user.

In cell imaging, it is quite common to stain different subcellular components with varying fluorescent dyes. It is quite challenging to figure out subcellular parts purely based on their shapes.

- For example, nuclei can be stained with a blue-fluorescent dye, actin filaments with a green-fluorescent dye, and mitochondria with a red-fluorescent dye.
  As it is not possible to excite all three fluorescent dyes together, a user needs to excite the fluorescent dyes one by one and acquires three different fluorescent

images. These three images can be stacked together to create a single image (Figure 27).

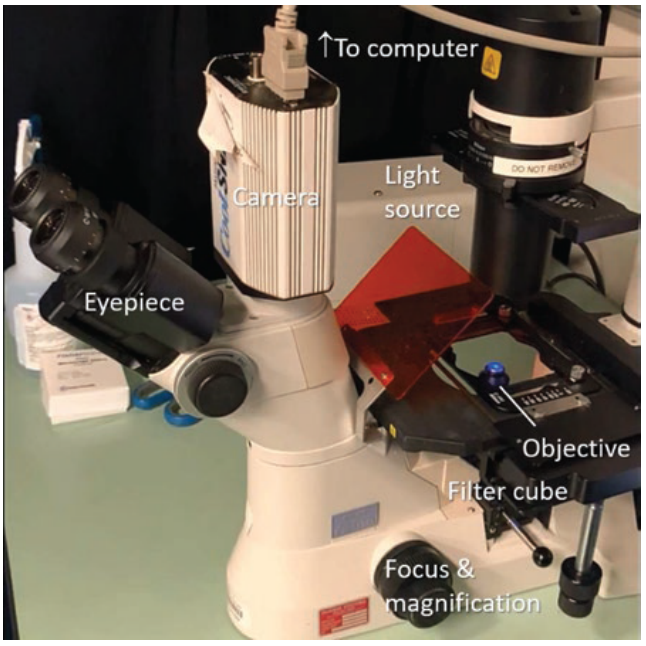

Figure 26. A fluorescence microscope (Yoon, 2022).

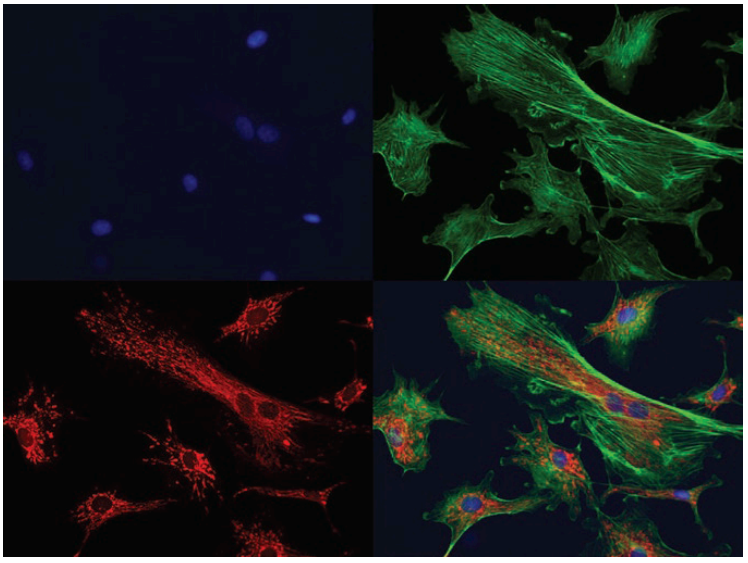

Figure 27. Images acquired by a fluorescence microscope. The blue fluorescence image shows nuclei (top left), the green fluorescence image shows actin filaments (top right), the red fluorescence image shows mitochondria (bottom left), and the stacked image is finally shown (bottom right) **(Yoon, 2022).**

4.5. Storage

Both primary cells and cell lines should be stored frozen for long-term storage.

Most refrigerators have a dedicated section for freezing, and exclusive freezers are also available. However, these freezers can only provide a temperature around −20°C and are inappropriate for storing mammalian cells for the long term. Because of this, deep freezers are available, which can provide −40 °C or −80 °C, respectively (Figure 27, left).

The −80 °C deep freezer is substantially more expensive than the −40 °C one. However, the ultimate solution is to store these cells in liquid nitrogen storage, which will provide −196 °C (the boiling temperature of liquid nitrogen) (Figure 28, right).

Figure 28. −40 °C deep freezer (left) and liquid nitrogen tank (right) for storing mammalian cells (Yoon, 2022).

4.6. Cell culture flasks

4.6.1. Monolayer cultures

After it has been recognized that practically any vessel with a flat bottom surface that can be sterilized is suitable for growing cells including coverslip cultures, a variety of flasks, bottles and dishes such as Carrel flasks, T-flasks (Figure 29), Kolle flasks, penicillin culture flasks, Erlenmeyer flasks, prescription bottles, Roux bottles, Petri dishes, etc. have been used.

The cells growing as a single layer have asymmetrical shapes and stop growing upon reaching confluency. Cells are maintained and propagated by periodic passaging.

Figure 29. T-25 tissue culture flask (Yoon, 2022).

4.6.2. Growth of suspension cultures

These cultures are grown in culture flasks under sterile conditions. This makes subculturing significantly easier than passing adherent cells. Cells are already suspended in the medium, thus enzymatic detachment is not needed, but sedimentation of cells to the bottom of the culture flasks has to be avoided.

In culture flasks the volume of the medium relative to the surface area is drastically increased, consequently proper gas exchange is limited. Adequate gas (CO_2, O_2) supply is achieved by agitation most often using a magnetic stirrer and spinner flask (Figure 30).

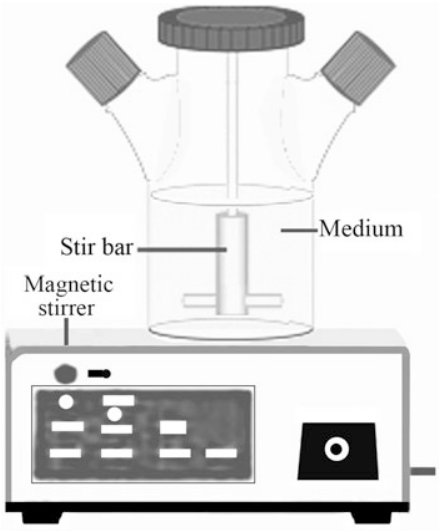

Figure 30. Spinner flask and magnetic stirrer for suspension culture (Banfalvi, 2014).

4.7. Cell culture media

We need to provide the followings to the media of cell culture:

- Sugars, preferably glucose. The minimum required concentration is about 1 mM. Typical glucose concentration is about 20 mM in media.
- Glutamine (optional). The minimum required concentration is about 0.2 mM. Typical glutamine concentration is about 2 mM in media.
- Amino acids. Required for protein synthesis.
- Buffers to maintain proper pH, typically at 7.4. The acceptable range of physiological pH is from 7.2 to 7.5. A pH indicating dye is sometimes added to the media to monitor its pH. Phenol red is frequently used in cell culture as its color is yellow under pH < 6.8 and red (bright pink) under pH > 8.2.

These are just bare minimum requirements. To maintain healthy cell culture, we additionally need growth factors, cofactors, vitamins, hormones, and minerals.

Many companies provide defined media that are mixed with growth factors, cofactors, vitamins, hormones, and minerals, as well as glucose, glutamine, amino acids, buffers, and/or pH indicator dye, all at fixed concentrations (or with defined “recipe”). A partial list of well-known defined media is shown below:

- DMEM: Dulbecco's modified Eagle's medium
- IMDM: Iscove's modified Dulbecco's medium
- RPMI 1640: Roswell Park Memorial Institute 1640 medium

All these defined media are sterilized through filtration. There exist many variations for each defined media. For example, as many as 36 different variations of DMEM are available from the Sigma-Aldrich website. The "recipe" of one such DMEM is shown in Table 6. You can notice the presence of sodium bicarbonate – note that CO_2 becomes bicarbonate (HCO_3^-) when it is dissolved in water. You can also see that glutamine is offered as optional. A pH indicator dye, phenol red, is also included to monitor the medium pH.

The addition of antibiotics (penicillin, streptomycin, gentamicin) and antifungals (fungizole) is optional, but not recommended as antibiotics may mask the microbial infections of the cell culture.

Advantages of defined media include:

(1) no batch-to-batch variation,

(2) no use of mammals (except for the possible mammal use for producing individual components),

(3) no viral contamination from mammals, and

(4) low to no proteins, leading to no foaming and easier later purification process.

Table 6. Composition of DMEM (Yoon, 2022).

Category	Component	Concentration (g/L)
Inorganic salts	Calcium chloride	0.2
	Ferric nitrate · $9H_2O$	0.0001
	Magnesium sulfate (anhydrous)	0.09767
	Potassium chloride	0.4
	Sodium bicarbonate	3.7
	Sodium chloride	6.4
	Sodium phosphate monobasic (anhydrous)	0.109
Amino acids	L-Arginine · HCl	0.084
	Glycine	0.03
	L-Histidine· HCl · H_2O	0.042
	L-Isoleucine	0.105
	L-Leucine	0.105
	L-Lysine · HCl	1.46
	L-Phenylalanine	0.066
	L-Serine	0.042
	L-Threonine	0.095
	L-Tryptophan	0.016
	L-Tyrosine · 2Na · $2H_2O$	0.12037
	L-Valine	0.094
Vitamins	Choline chloride	0.004
	Folic acid	0.004
	myo-Inositol	0.0072
	Niacinamide	0.004
	D-Pantothenic acid (hemicalcium)	0.004
	Pyridoxine · HCl	0.004
	Robiflavin	0.0004
	Thiamine · HCl	0.004
Nutrients and others	D-Glucose	4.5
	Pyruvic acid · Na	0.11
	L-Glutamine (optional)	0.584
pH indicator dye	Phenol red · Na	0.0159

4.8. Cell feeding

While the media will be provided at the beginning of the cell culture, many components, especially nutrients, will be consumed over time, and wastes will be produced.
Therefore, cells need to be fed with new media, typically every 2–3 days. This process is called cell feeding.

4.9. Cell passaging

As cells continue to proliferate (divide) in culture, they grow to fill the available surface area (for anchorage-dependent cells) or volume (for anchorage-independent cells). Such proliferation can generate multiple issues, including:

(1) nutrient depletion in the growth media,

(2) accumulation of dead cells,

(3) contact inhibition for anchorage-dependent cells, and

(4) occasionally unwanted cellular differentiation.

Both contact inhibition (for anchorage-dependent cells) and high cell density (for anchorage-independent cells) can lead the cells to senescence, that is, cells stop proliferating (dividing).

To prevent these complications, you should periodically split the cells into a new vessel, called cell passaging (Figure 31).

For anchorage-independent cells (suspension culture), cells can easily be passed by transferring a small amount of cell culture into a larger volume of fresh media in a new flask (or bioreactor).

However, for anchorage-dependent cells (adherent culture), cells first need to be detached from the surface. This detachment is commonly done with trypsin-EDTA, where trypsin is an enzyme (a protein) that can help digest proteins. However, other enzyme mixes are also available for cell passaging. A small number of detached cells can then be seed into new media in a new culture flask.

Cell passaging is mandatory when the anchorage-dependent cells reach confluency or the anchorage-independent cells reach the maximum allowable cell density.
Without it, cells will go senescence and stop proliferating.

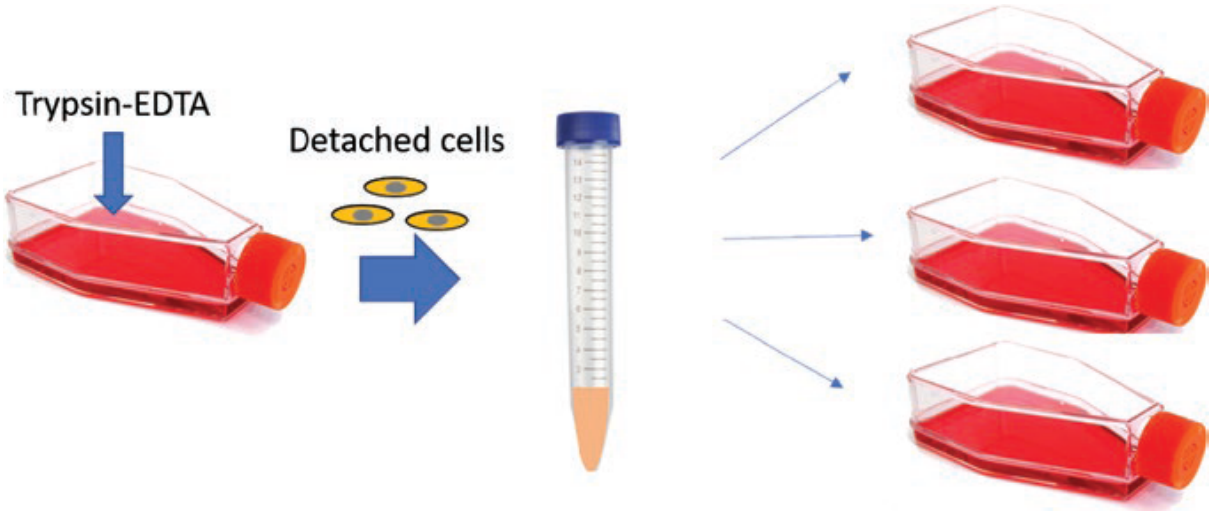

Figure 31. Schematic representation of cell passing (Yoon, 2022).

4.10. Measurement of viability and cytotoxicity

Viability of cells represents their capability to exist, survive, and develop.

Cytotoxicity refers to the metabolic alterations of cells, including cell death due to exposure of some toxic chemicals or environment.

To determine cytotoxicity of any chemicals or for screening a drug, cell viability and cytotoxicity assays are used.

There are several methods of measuring cell viability and cytotoxicity based on various cell functions like enzyme activity, cell permeability, cell adherence, ATP production, coenzyme production, or nucleotide uptake activity.

The outcome of the assay is determined by color change of a substrate, uptake of a radioactive substance, and counting the live and dead cells under the microscope or by their ability to form colonies. Some of those methods are described below.

4.10.1 Assays based on membrane integrity

The most common method for determining cell viability is to determine membrane integrity.

Due to cell disaggregation, cell separation, or freezing and thawing, cell membrane gets damaged. This can be measured by uptake of dyes, retention of dyes inside the cells, or release of radiolabeled chromium from cells. These methods are rapid but they can't distinguish the healthy cells from the cells that are losing functions but still alive.

a. Dye exclusion assay

The basis of this assay is that the viable cells are impermeable to several dyes such as trypan blue, eosin Y, nigrosin blue, erythrosine B, etc. Live cell membrane is not permeable to those dyes and is not stained. It stains only the dead cells. After staining, stained cells are counted using a hemocytometer.

b. Dye uptake assay

The viable cells take up dyes and due to enzymatic activities they are converted to a substance that is impermeable to cell membrane.

Dead cells are unable to convert them into cell impermeable substances. For example, diacetyl fluorescein is converted to fluorescein by hydrolysis inside the live cells and then the live cells emit fluorescent green color. This can be easily detected by fluorescent microscope or measured using a fluorimeter.

c. Chromium release assay

Radioisotope of chromium (^{51}Cr) binds to intracellular proteins as it binds to basic amino acids. Due to cell membrane damage, the labeled proteins leak out of the cell and the leakage is proportional to cell damage. This is a very sensitive method however; handling, storage, and disposal of radioisotopes are problematic and need special permission.

d. Enzyme release assay

When cell membranes are damaged, cellular enzymes are released from the cells. This can be assayed by measuring the activities of the released enzymes. Lactate dehydrogenase (LDH) assay is the most commonly used assay as this enzyme is stable during cell death and provides more reliable data.

4.10.2. Assays based on radioisotope incorporation

By using radiolabeled substrates or metabolites that are incorporated into cells during cell growth and division , the level of incorporation is measured using a scintillation counter. This method is used to determine drug toxicity. It is very sensitive but requires radioisotopes which cause various safety concerns.

a. Incorporation of labeled nucleotides

Tritium-labeled (^{3}H) thymidine or uridine is used in this method. Labeled ^{3}H-thymidine or ^{3}H-uridine are incorporated into cell nuclei during cell growth due to their incorporation into newly synthesized DNA and RNA. The amount of incorporated tritium can be measured using a scintillation counter.

b. Release of labeled phosphates

Cells are labeled with ^{32}P-phosphates. Due to cell damage, they release labeled phosphates that can be measured.

4.10.3. Colorimetric assays

These methods are based on the production of colored substances by live cells' activities. There is a good correlation between cell numbers and color production.
This method is far superior to previously described methods as it is easy to use and safe and has higher reproducibility.

Protein content, DNA, lysosomal activity, Golgi body activity, or other enzyme activities are used to determine cell number.

One example is XTT assay in which conversion of the water-soluble XTT (2,3-bis-(2-methoxy-4-nitro-5- sulfophenyl)- 2H-tetrazolium-5-carboxanilide) reagent to an orange formazan product by actively respiring cells is measured. The amount of water- soluble product generated from XTT is proportional to the number of living cells in the sample and can be quantified by measuring absorbance at wavelength of 490 nm.

4.10.4. Luminescence assay

This method determines the level of ATP. It is based on the reaction between ATP and luciferin in the presence of oxygen and luciferase enzyme.
The reaction produces AMP, 2Pi, carbon dioxide, and light. The production of light is measured by a luminometer. It can quantify the cell number as low as 20/ml.

3.9.5 Apoptosis assay

Apoptosis can be used to determine cytotoxicity. It can be measured by detecting cellular morphology changes, DNA laddering, or detection of phosphatidyl serine in the membrane by using annexin V conjugated to fluorescein isothiocyanate or biotin.

Chapter 4. Reporter gene

Learning Objectives

At the end of this chapter, you should be able to:

- Define a reporter gene and explain its role in studying gene expression.

- Explain how reporter gene expression reflects the spatial and temporal activity of a gene of interest.

- Describe the basic principle of reporter gene assays using recombinant DNA technology.

1. Definition

A reporter gene is a non endogenous gene encoding an enzyme or fluorescent protein whose expression is controlled by a promoter for a separate gene of interest.
Therefore, it is possible to examine the spatial and temporal expression of the gene by measuring the expression of the reporter.

For example, imagine that a scientist is interested in studying the expression pattern of Gene X. The scientist can create a reporter construct (using recombinant DNA technology methods) in which the reporter gene is placed under the control of the promoter for Gene X (Figure 32).

Reporter genes are chosen on the basis of being easy to assay, and some of the most widely used reporter genes are :

- those coding for the firefly luciferase (LUC) gene,
- the bacterial chloramphenicol acetyltransferase (CAT) gene,
- the bacterial β-galactosidase (β-GAL) (LacZ) gene, and
- the jellyfish green fluorescent protein (GFP) gene.

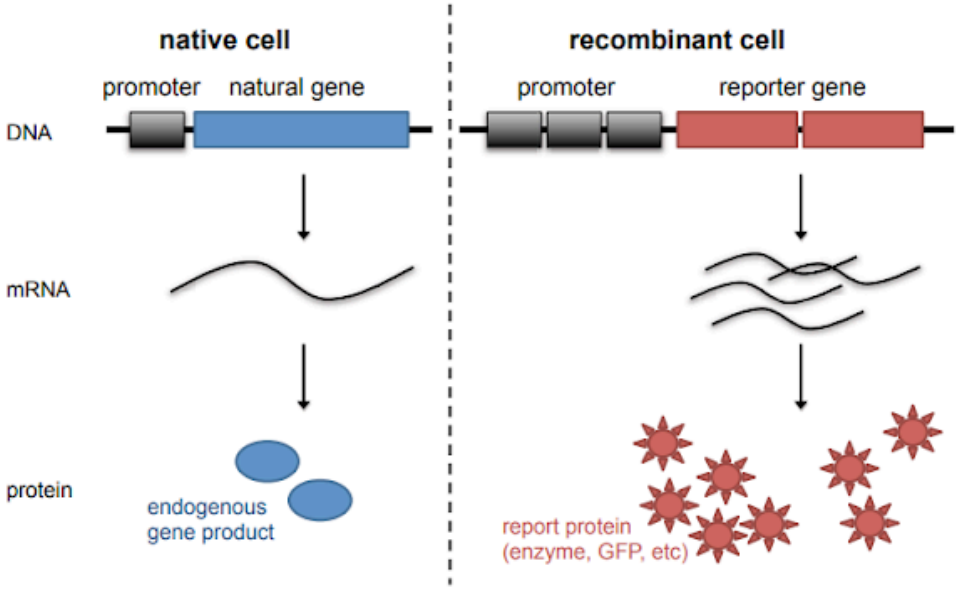

Figure 32. Principle of the reporter gene assay (Escher and Leusch, 2011).

2. Examples of reporter genes

2.1. Luciferase

Luciferases comprise a group of enzymes that emit light in the presence of oxygen and a substrate (luciferin). Such a luciferin–luciferase system is found in nature, for example, in bacteria (*Vibrio harveyi*), dinoflagellates, and the firefly (*Photinus pyralis*) (Figures 33-35).

These luciferases, in particular the eukaryotic firefly luciferase (Luc), have been *In vivo* imaging allows the visualization of the spatial and temporal behavior of Luc-expressing cells in living animals; for example, growth of tumor cells (Figure 36), and migration of transplanted cells.

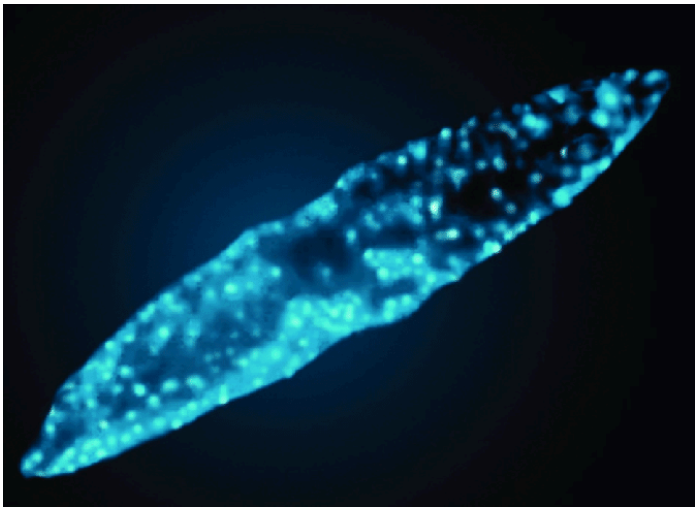

Figure 33. Bioluminescence in the dinoflagellate *Pyrocystis fusiformis* originates from microsources in the cytoplasm. Cell length = 1 mm (Widder, 2022).

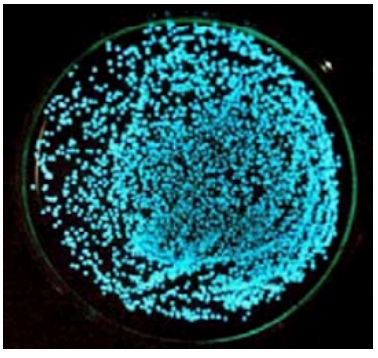

Figure 34. *Vibrio fischeri* (Medvedeva e*t al.*, 2009).

Figure 35. *Photinus pyralis* (Rénoult and Valeur, 2015).

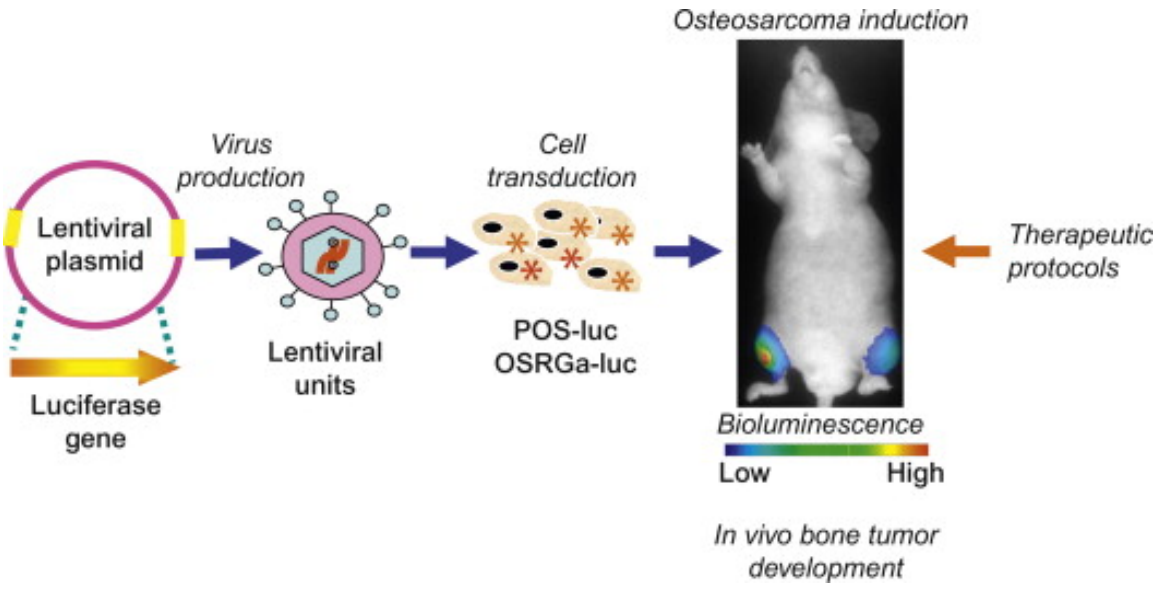

Figure 36. *In vivo* bioluminescence imaging to follow and quantify the progression or regression of bone tumor in response to therapeutic protocols (Rousseau *et al.*, 2010).

2.2. β-Galactosidase

The lacZ gene encoding b-galactosidase from *E. coli* was the most widely used reporter in both prokaryotic and eukaryotic model systems (Figures 37-38). While proving to be a robust and reliable reporter protein, LacZ had several limitations:

- Many bacteria have endogenous b-galactosidase activity.
- The LacZ protomer is very large (120 kDa) requiring more than 3 kb of DNA to encode it so that plasmids encoding lacZ fusions are correspondingly large and often prone to deletion.
- While LacZ is a good qualitative indicator of expression, quantitation requires cell harvesting and lysate preparation.

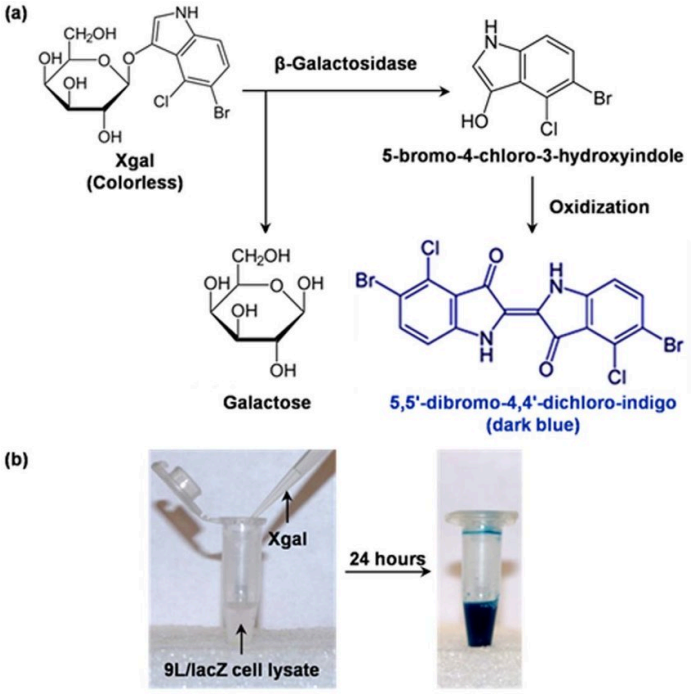

Figure 37. Detecting the expression of the lacZ reporter gene using the chromogenic X-gal probe. (a) The hydrolysis of X-Gal catalyzed by b-galactosidase. (b) Photographs showing the chromogenic change after addition of X-gal solution into the native lysate cells **(Cai *et al.*, 2012)**.

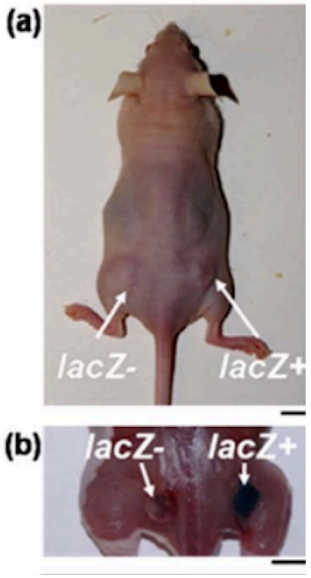

Figure 38. Imaging 9 L tumors with and without the lacZ reporter gene by an array-based photoacoustic and ultrasonic imaging system *in vivo*. (a) Photograph of a tumor-bearing mouse before imaging. (b) Post-euthanasia photograph of the lacZ- and lacZ+ tumors after removing the skin above **(Cai *et al.*, 2012).**

2.3. Green Fluorescent Protein (GFP)

Characteristics:

- Green fluorescent protein (GFP) is a naturally occurring protein found in jellyfish *Aequorea victoria* (Figure 39), which exhibits a green fluorescence when exposed to light.
- This fluorescent protein has transformed biomedical research because cellular biological processes can now be observed in real time.
- This protein is located in the jellyfish's umbrella where it interacts with another protein called aequorin. This specific interaction between GFP and aequorin emits blue light when calcium ions are added. Furthermore, this chemical reaction supplies the energy to produce the green fluorescence.
- GFP itself is non-toxic, stable and spontaneously conforms into its functional shape without the need for outside chemicals or enzymes. Also, GFP doesn't interfere with any of the biological processes within an organism.

Applications:

- Scientists added this harmless fluorescent gene into insulin producing cells in the pancreas of mice. The researchers were able to observe the biological process and reform existing diabetes treatments.
- GFP can indicate the presence of viruses and microorganisms.
- In molecular biology research, the labeling of viral proteins makes GFP a useful transfection marker. GFP can also be used to monitor infectious processes in plants and animals.

Figure 39. Jellyfish *Aequorea victoria*, which exhibits a green fluorescence when exposed to light (Andrei, 2016).

Chapter 5. Modalities of cycle control

Learning Objectives

At the end of this chapter, you should be able to:

- Describe the organization and phases of the eukaryotic cell cycle.
- Explain the molecular mechanisms that control cell cycle progression.
- Explain the importance of experimental model organisms in the study of cell cycle regulation.

1. Cell cycle

The cell cycle of cells of higher eukaryotes includes four phases.

- During two of these phases, S phase and M phase, the cells carry out the two fundamental events of the cycle: DNA replication (S phase, for synthesis) and strictly equal sharing of chromosomes between the 2 daughter cells (M phase, for mitosis).
- The two other phases of the cycle, G1 and G2, represent intervals (Gap): during the G1 phase, the cell grows, integrates mitogenic or anti-mitogenic signals and prepares to correctly carry out the S phases; During the G2 phase, the cell prepares for the M phase (Figure 40).

In a cycle, the four phases follow one another in an immutable order: G1, S, G2 and M.

The first three phases (G1, S, G2) constitute the interphase, during which the nucleus of the cell is limited by an envelope nuclear, while mitosis (M) is characterized by the disappearance of this envelope and the appearance of chromosomes. The latter then become visible under a light microscope because they are compact. After mitosis, cells can either pass into G1 or enter G0, the quiescent stage of non-division.

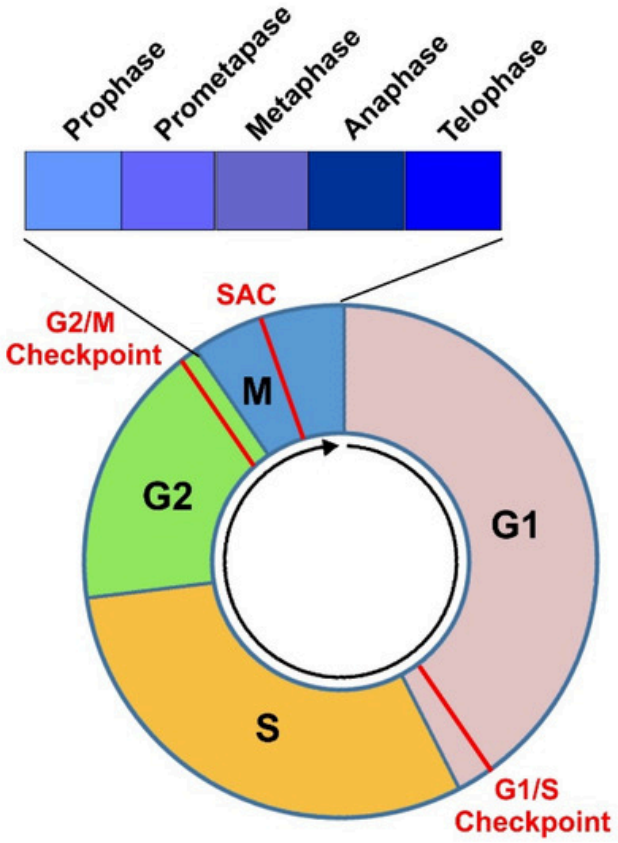

Figure 40. Different phases of the cell-cycle (Wang, 2021).

2. Control of the cell cycle

To ensure, on the one hand, the immutable order of the succession of the four phases of the cycle (regulation of the cycle), and on the other hand, the obtaining of two strictly identical daughter cells (DNA monitoring), the cell has highly sophisticated regulation systems:

- In the first case (regulation of the cycle), it is essentially cyclin-dependent kinases, the Cdks, which intervene.
- In the second case, other molecules intervene in different cycle monitoring mechanisms to inhibit cycle regulation Cdks and stop the cycle, if the previous step is not completed, or if "repair" is necessary .

3. Biological models

Cell cycle control mechanisms are similar in all eukaryotes.
All eukaryotic cells have a similar regulatory system controlling the progress and coordination of cell cycle events.
The most commonly used model organisms are: yeast, Xenopus and mice.

3.1. Yeast

Some yeast genes have a high degree of conservation with cell genes higher eukaryotes, both in terms of sequence and in terms of function in the cell: this is how yeast can serve as a study model to understand the mode of functioning of a cell.

Cyclin-dependent kinases and other proteins involved in the regulation of the cell cycle of mammalian cells have been identified thanks to studies carried out on yeast (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) by a systematic search for mutations in the genes coding for molecules involved in in the cell cycle, cdc (cell division cycle).

Temperature-sensitive cdc mutant strains were used. For these strains, the mutant proteins are functional at normal temperature but not at elevated temperature.

All proteins identified in yeast are proteins that have been conserved during evolution and they also exist in mammals.

3.1.1. Interest in using yeast

- Among yeast genes, some are clearly of biomedical interest. Indeed, some yeast genes have a high degree of conservation with cell genes higher eukaryotes, both in terms of sequence and in terms of function in the cell: this is how yeast can serve as a study model to understand the mode of functioning of a cell.
- In addition, yeast is the organism that presents the most technical possibilities in the field of genetic and molecular manipulation.
- The same goes for the study of the molecular causes of certain diseases for which models have been developed.

For example, we can cite the study of aging cellular, prion diseases, mitochondrial diseases, cystic fibrosis or still have Parkinson's disease.

In these cases, yeast is a simple system used to study the mechanisms of a disease but it can also serve as a “test tube biological" to characterize molecules with high therapeutic potential that can be used in Man due to the high degree of conservation of the mechanisms involved in these pathologies.

- Other arguments in favor of using these models lie in the level of ease of manipulation of this organism: unlike cells higher eukaryotes, they are easy to use experimentally due to their speed of dividing (in liquid culture as well as on agar medium) as well as low cost and of the simplicity of the culture environments.

3.1.2. Ease of genetic manipulation

- Because of the growth modes that are easy to implement in laboratories, *S. cerevisiae* has become a reference tool for genetic manipulation.

- In using various techniques based on the use of PCR and transformation of yeast, it is very easy to generate a mutation on one of the sixteen chromosomes, or even to delete an entire gene.

- Yeast is the only eukaryotic organism where mutagenesis directed, down to the nucleotide, is possible over the entire nuclear genome and mitochondrial. Such a change generated on the DNA of a cell will be transmitted from generation to generation.

- In addition, it is possible to integrate a DNA sequence, most often a tag, also called a tag, at a specific location in the genome. This allows, among other things, to follow the expression of the tagged protein in the cell or even its location when the tag is visible in microscopy: this is for example the case with a GFP tag (Green Fluorescence Protein).

- On the other hand, still using molecular biology techniques, the construction of cloning vectors, in order to express a gene in yeast, can be done quickly.

3.2. *Xenopus laevis*

The embryonic cycles of *Xenopus laevis* have been important for the discovery of the essential components and behavior of cell cycle control.

A key advantage of eggs (Figure 41) is their large size; the fertilized Xenopus egg measures approximately one millimeter in diameter and it is easy to inject test substances into it to determine their effect on progress in the cell cycle.

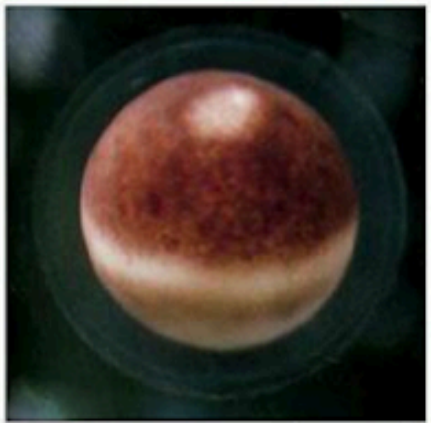

Figure 41. A mature Xenopus egg ready for fertilization. The pale spot near the top indicates the location of the nucleus (Alberts *et al.*, 2008).

3.3. Mice

Since the 19th century, the mouse has been the most widely used model for genetic research in mammals. This is mainly due to its close genetic (Figure 42), physiological and pathological similarities with humans.

- The mouse genome is about 14% smaller than the human genome (2.5 gigabases (Gb) compared to 2.9 Gb) but the two genomes each contain about 24,000 to 25,000 protein-coding genes. Indeed, 75-80% of mouse genes have a unique and identifiable ortholog in humans and less than 1% of genes appear to have no homolog between the two genomes.

- Technological developments, and in particular genetic engineering, have made it possible to manipulate the mouse genome to produce all types of mutations and thus facilitate its analysis.

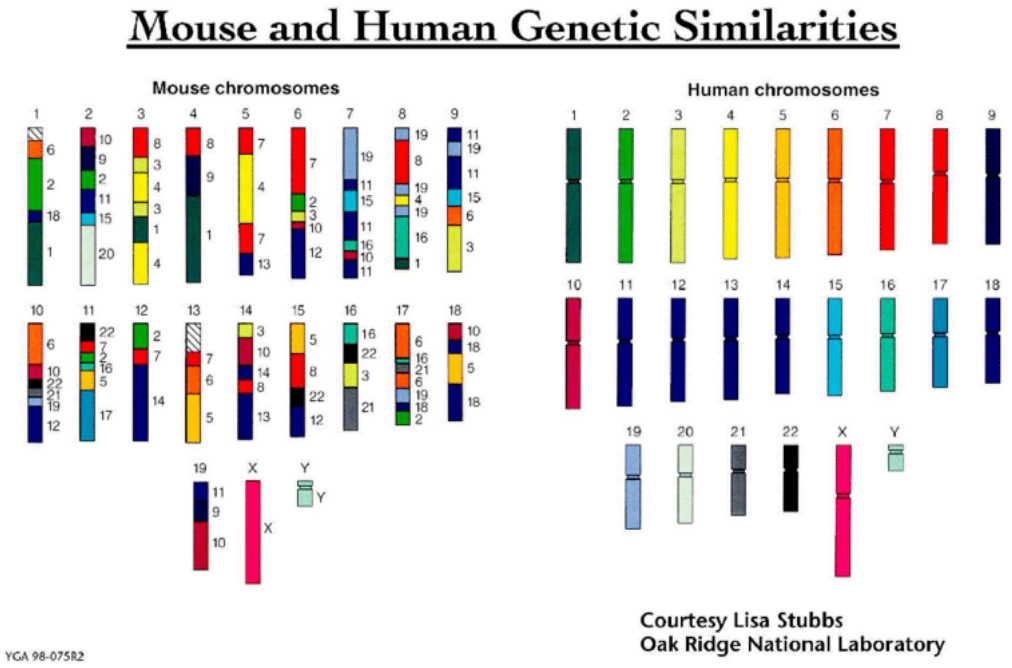

Figure 42. Preserved segments between mouse and human genomes showing long stretches of conserved DNA from their common ancestor. More than ninety percent of the mouse genome consists of shuffled pieces of the human genome (Brisbin *et al.*, 2019).

Chapter 6. Modern biology techniques

Learning Objectives

At the end of this chapter, you should be able to:

- Understand the principles underlying major modern biological techniques used for protein detection and cellular analysis.

- Explain the methodological steps, applications, and limitations of immunoblotting, immunofluorescence, and flow cytometry.

- Select appropriate techniques to investigate protein expression, localization, and cellular characteristics in biological samples.

1. Immunoblot

A Western blot is a technique used to determine the presence or absence of selected proteins in a sample. The technique takes place in several stages (Figure 43):

- The first step of a Western blot protocol is protein extraction from cells or tissue.
- protein samples are placed on an electrophoresis gel and are separated according to their molecular weight. To do this, an electric current is applied to the gel. The larger the proteins, the less quickly they migrate;
- Once the proteins have migrated, they are transferred to a membrane which can be made of nitrocellulose or polyvinylidene fluoride (PVDF). The attachment of proteins to the membrane occurs thanks to hydrophobic and ionic interactions between the membrane and the proteins;
- blocking the membrane: this step is essential to limit subsequent non-specific interactions between the antibodies and the membrane. Blocking is carried out in a concentrated protein solution;
- detection: the principle consists of applying labeled antibodies to the membrane which are specific for the proteins that we want to observe. We will thus be able to observe their position on the gel.

Western blots are widely used in research laboratories interested in the roles of proteins. In the medical field, they are used in the diagnosis of certain diseases. Confirmatory HIV tests use this technology to detect an anti-HIV antibody in a serum sample.

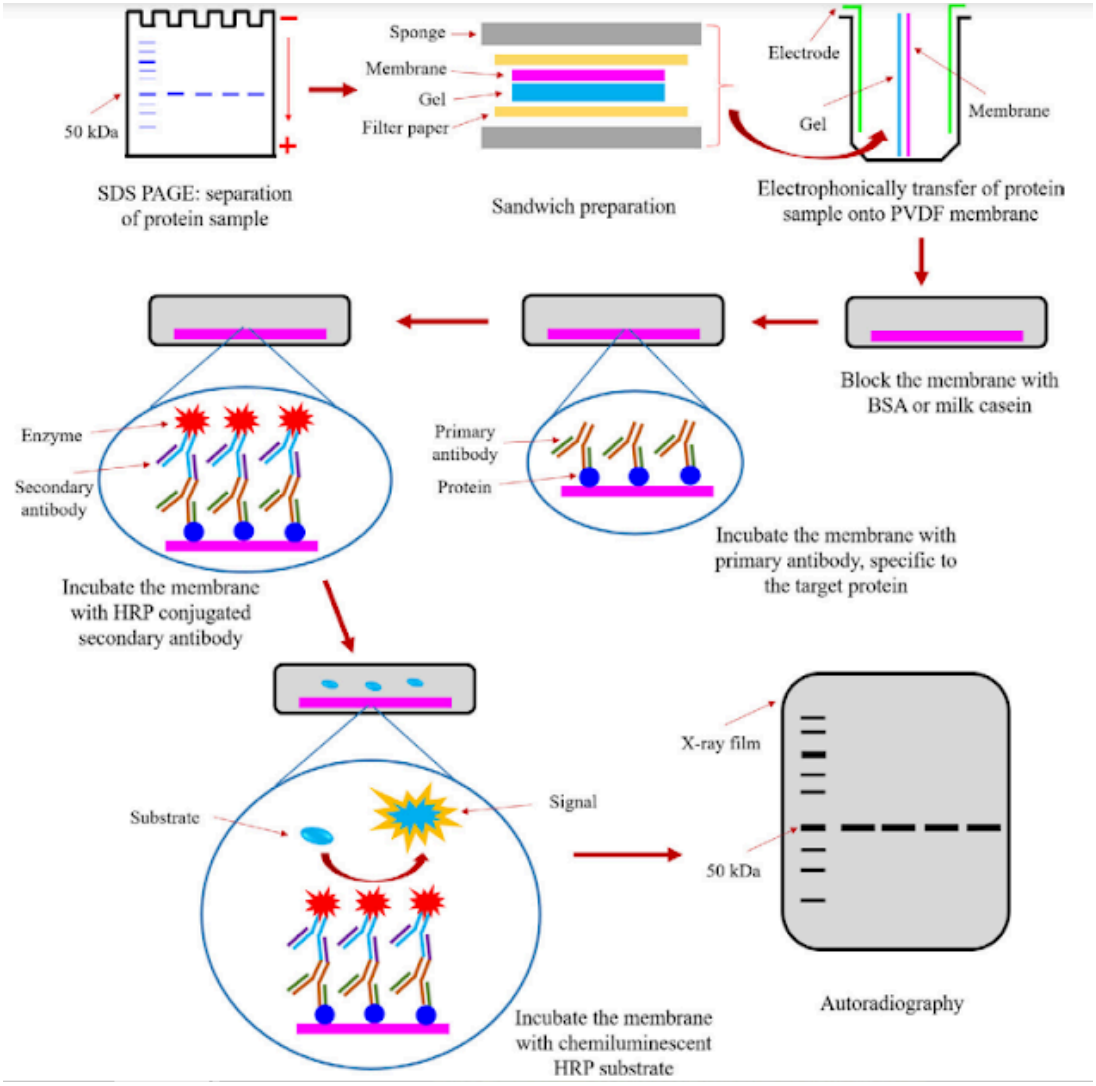

Figure 43. The steps of western blotting (Roy *et al.*, 2019).

2. Immunofluorescence

Immunofluorescence (IF) is a histochemical laboratory staining technique used for demonstrating the presence of antibodies bound to antigens in tissues or serum.

2.1. Principle

In IF techniques, antigens, antibodies or their complexes are viewed under a fluorescence microscope, using the corresponding antibodies complexed to a fluorochrome.

The combination of antibody with its specific antigen does not lead to a visible change and therefore a readily identifiable label (fluorochrome) must be irreversibly bound to the antibody so that its localization can be recognized.

2.2. Collection and handling of specimens for immunofluorescent studies

Two types of specimens can be examined by IF techniques, notably biopsy specimens of skin or mucous membranes and serum samples.

- **Biopsy specimens**
  Direct immunofluorescence (DIF) studies require a biopsy of the patient's skin or mucosa.
- **Serum samples**
  - Used in IIF techniques to detect circulating autoantibodies.
  - Approximately 10 ml of blood is collected in a tube without anticoagulant and transported to the laboratory within 48 hours.
  - Serum is then centrifuged and stored at –25°C until analysis is performed. Alternatively, separated serum may be sent to the laboratory within 1 month of collection.

2.3. Immunofluorescence techniques and procedures

There are two basic types of immunofluorescence techniques: direct and indirect techniques.

2.3.1. Direct Immunofluorescence Technique (DIF)

An IF protocol involving a fluorophore-conjugated antibody to the target antigen of interest is referred to as direct IF.

DIF is a one-step procedure used to detect and localize immunoreactants deposited *in vivo* in the patient's skin or mucosa.

Direct immunofluorescence microscopy is a technique that allows the detection of immunoglobulins/immune complexes and complement fractions (IgG, IgA, IgM and C3) in tissues, using specific fluorescein-conjugated antibodies and a fluorescence microscope.

This technique is essential in dermato-pathology for the diagnosis of autoimmune blistering diseases, such as bullous pemphigoid, pemphigus, dermatitis herpetiformis, and may also be useful in other indications, such as lupus and vasculitis.

2.3.2. Indirect Immunofluorescence Technique (IIF)

An IF protocol involving detection of an antigen by a fluorophore-conjugated secondary antibody that recognizes an unlabeled primary antibody bound to the antigen of interest is classified as indirect IF).

IIF is a two-step serological technique for detection of circulating antibodies in body fluids.

In parasitology, most protozoa can be searched for and identified with IIF.

- **Amoebiasis**: research and identification of *E. histolytica* in different biological fluids is facilitated by this technique.
- **Toxoplasmosis**: detection of tachyzoite in several samples: amniotic fluid, placenta, CRL.

2.4. Procedure

To detect protein expression:

- the biological sample of interest is incubated with an antibody specific to the protein of interest; the antibody may be coupled to a fluorophore (direct fluorescence, Figures 44-45) or may be detected by a secondary antibody conjugated to a fluorophore (indirect fluorescence, Figure 46).

- The proteins or antigens can then be visualized by examination under a fluorescent microscope or a confocal microscope depending on the biological question being addressed by this method.

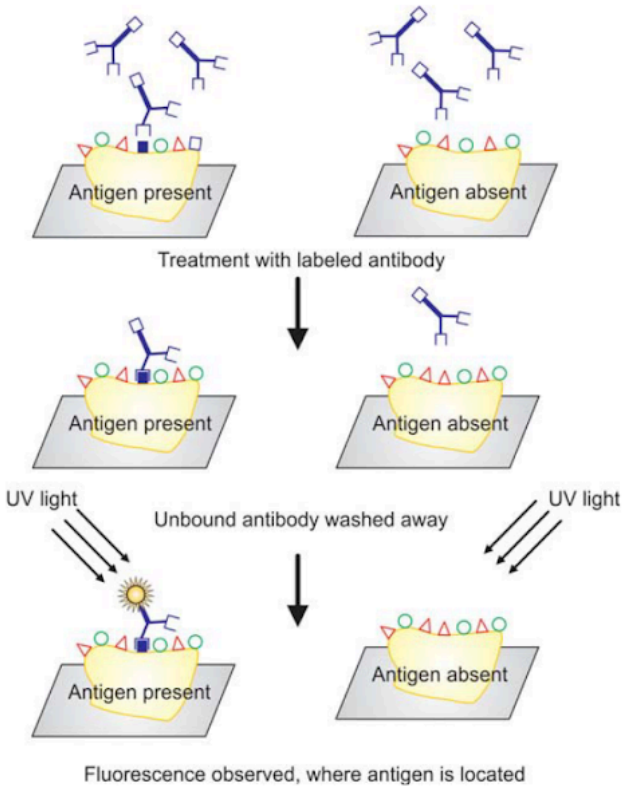

Figure 44. Direct immunofluorescence procedure (Premalatha *et al.*, 2011).

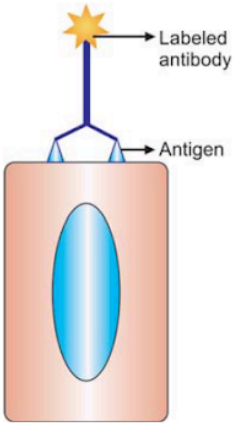

Figure 45. Direct immunofluorescence technique (Premalatha *et al.*, 2011).

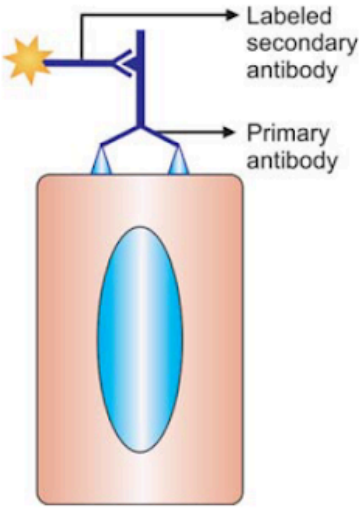

Figure 46. Indirect immunofluorescence technique (Premalatha *et al.*, 2011).

3. Flow cytometry

Flow cytometry is a powerful and flexible technique that can rapidly analyze multiple parameters of individual cells, within heterogeneous cell populations.

The flow cytometer performs this analysis by passing thousands of cells per second through a laser beam and capturing the fluorescence and scattered light that emerges from each cell. As the cells pass through, fluorescence data is collected and analyzed statistically by flow cytometry software to report cellular characteristics including size, complexity, phenotype and health (e.g. viability, proliferation and apoptotic states).

The principle behind fluorescence is that any fluorophore has a range of specific wavelengths at which it absorbs light energy.

In absorption, high energy light excites fluorophores to a higher energy level called an excited state. Once this state is achieved, fluorophores return to a low energy ground state by releasing the excess energy in the form of light. This transition of energy is called fluorescence.

Fluorescence is an important feature of fluorophores as it is used to differentiate their color, that is, the wavelength at which they emit fluorescent light. The ability to simultaneously utilize multiple fluorophore-conjugates with distinct and well-separated emission profiles permits multi-parametric analysis, which is the real power of flow cytometry.

Schematically, the flow cytometer comprises three key systems: the fluidic, the optical and electronic system (Figure 47).

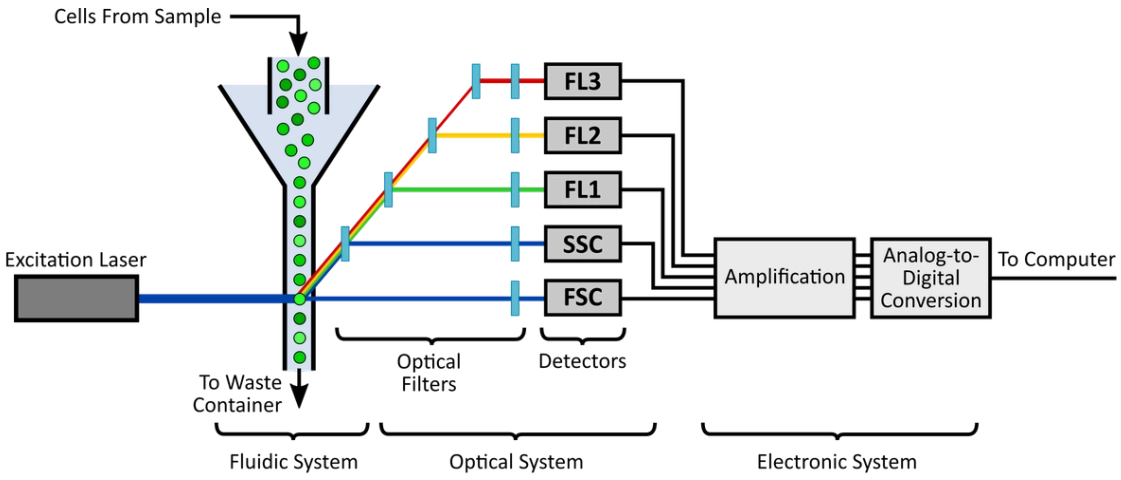

Figure 47. Schematic of a common flow cytometer, illustrating the fluidic, optical and electronic systems (Sapkota, 2022).

a. The fluidic system is responsible for transporting cells. It passes cells individually through pressurized lines containing sheath fluid to the interrogation point where the laser intersects with the sample. The sample flow rate of the fluidic system can be manipulated in order to improve analysis.

b. The optical system is responsible for illumination and light collection within the flow cytometer.

   This system consists of excitation lasers, lenses and filters.

   The lasers ensure that cells in the interrogation point are excited with uniform light of a specific wavelength. For instance, argon lasers emit light at the 488 nm wavelength and can be used to excite fluorophores with a 488 nm absorption maximum such as iFluor® 488 (Cat# 1023) and FITC (Cat# 135).

   As the cells pass through the laser it emits fluorescence and scattered laser light at all angles. The collection optics " lenses and filters " serve to separate and direct the specific wavelengths of fluorescence and scattered laser light to the appropriate detectors. These detectors capture the emitted fluorescence and scattered laser lights, convert them into a photocurrent and pass it to the electronics system to be digitized and processed for subsequent analysis.

Since its introduction in the 1970s till today, flow cytometers have evolved significantly. Early designs consisted of single-laser cytometers that could only detect size. While today's cytometers feature multiple laser and filter configurations to facilitate multicolor analysis, with some cytometers capable of detecting up to 14 parameters simultaneously.

4. Biosensors

A biosensor is an analytical device which integrates a biological recognition element with a physical transducer to generate a measurable signal proportional to the concentration of the analyses. In the general scheme of a biosensor, the biological recognition element responds to the target compound and the transducer converts the biological response to a detectable signal, which can be measured electrochemically, optically, acoustically, mechanically, calorimetrically, or electronically, and then correlated with the analyzed concentration.

Figure 48 shows the schematic representation of a biosensor.

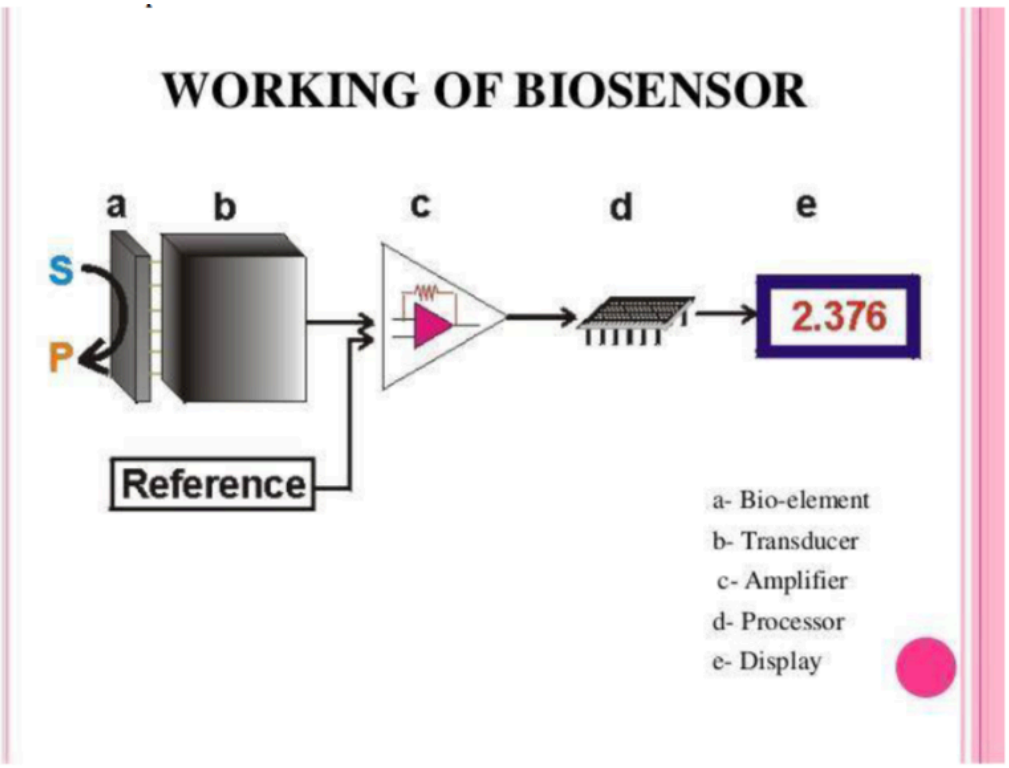

Figure 48. The schematic representation of a biosensor (Balootaki and Hassanshahian, 2014).

Microbial biosensor

A microbial biosensor consists of a transducer in conjunction with immobilized viable or non-viable microbial cells. Non-viable cells obtained after Permeable or whole cells containing periplasmic enzymes have mostly been used as an economical substitute for enzymes. Viable cells make use of the respiratory and metabolic functions of the cell, the analyze to be monitored being either a substrate or an inhibitor of these processes. Bioluminescence-based microbial biosensors have also been developed using genetically engineered microorganisms constructed by fusing the lux gene with an inducible gene promoter for toxicity and bioavailability testing. Figure 49 shows the schematic representation of a microbial biosensor.

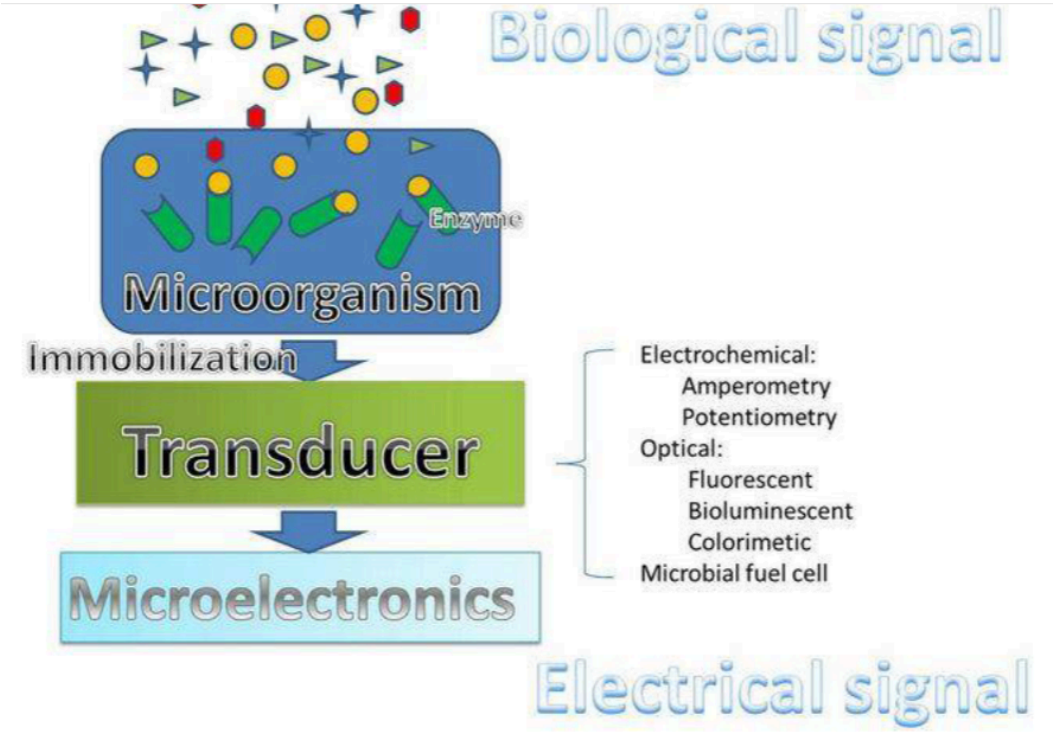

Figure 49. The schematic representation of a microbial biosensor (Balootaki and Hassanshahian, 2014).

Chapter 7. Genetic analysis in yeast

Learning Objectives

At the end of this chapter, you should be able to:

- Understand the role of yeast as a powerful model organism for genetic and genomic analysis.

- Explain how yeast-based cloning systems contribute to the study of complex genomes.

- Describe the principles and applications of yeast artificial chromosome (YAC) technology.

1. Yeast artificial chromosome

Yeast artificial chromosome (YAC) cloning systems play an important role in the analysis of a large variety of complex genomes, including those of insects, reptiles, birds, mammals, and plants. YAC cloning systems have the ability to clone DNA stretches of 50 to well over 2000 kb.

2. Construction of YAC libraries

A schematic illustration of YAC cloning is given in Figure 48, and a brief description of the principles of YAC library construction is given below.

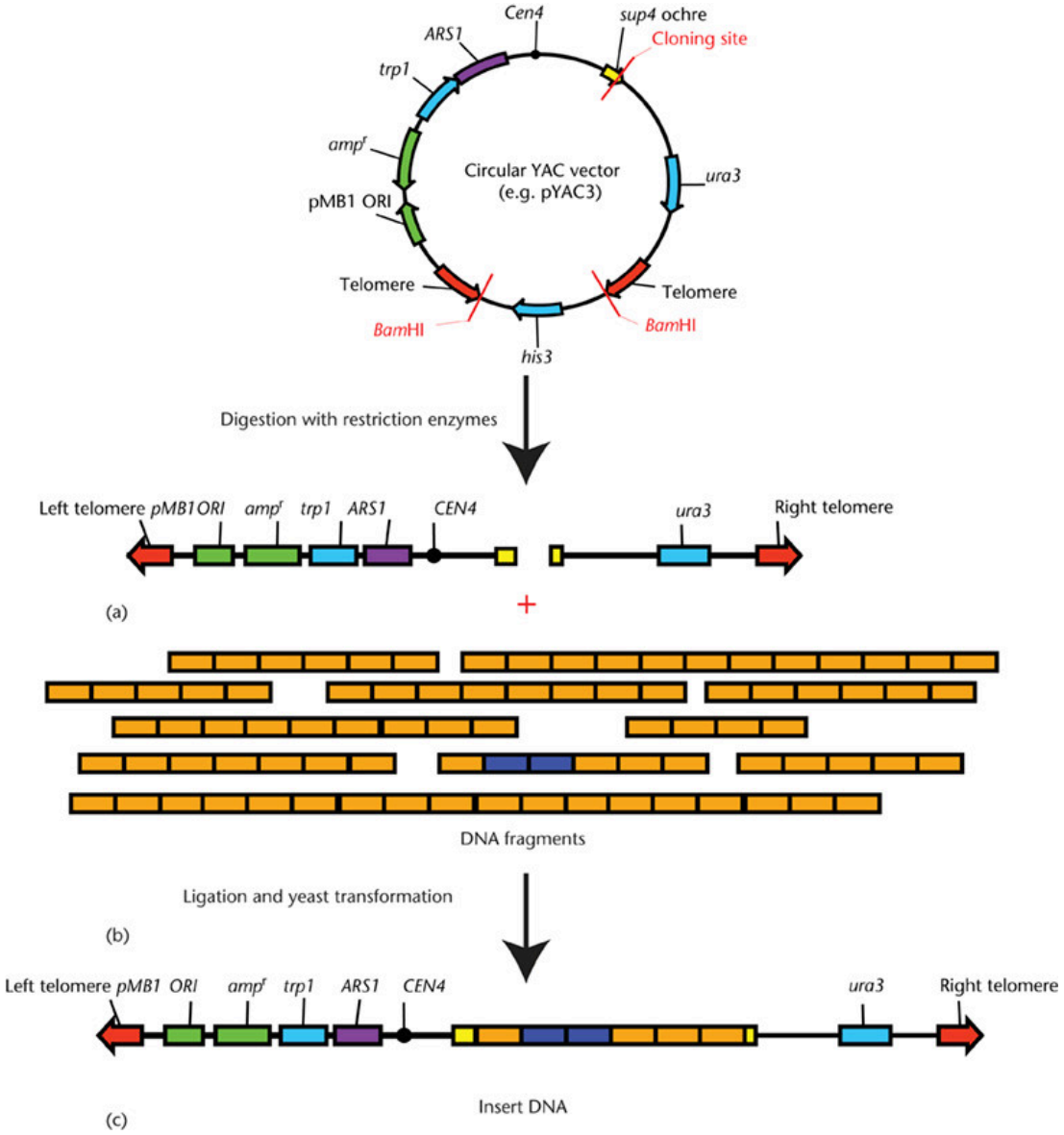

Figure 48. Cloning of very large DNA fragments into a YAC vector (Bruschi and Gjuracic, 2001).

2.1. DNA donor

DNA can consist of an entire plant or animal genome or a single chromosome after restriction enzyme digestion.

2.2. YAC vectors

A yeast artificial chromosome cloning vector consists of two copies of a yeast telomeric sequence (telomeres are the sequences at the ends of chromosomes), a yeast centromere, a yeast ars (an autonomously replicating sequence where replication DNA begins) and appropriate selectable markers.

2.3. Cloning and transformation

Donor DNA, prepared as above, is mixed with the dephosphorylated YAC vector DNA and ligated.

The recipient yeast strain, AB 1380 (auxotrophic for uracil and tryptophan), is grown in rich media, and the polysaccharide cell wall is partially broken down by lyticase in order to produce yeast spheroplasts.

The spheroplasts are transformed with the ligated DNA. The cell walls are regenerated in the agar, and the YAC clones start multiplying to form colonies.

2.4. Screening

Most of the YAC colonies grow within the agar, and not on the surface. They need to be picked individually, either manually or by robot. YAC library screening is based on either hybridization or polymerase chain reaction (PCR) methodology.

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