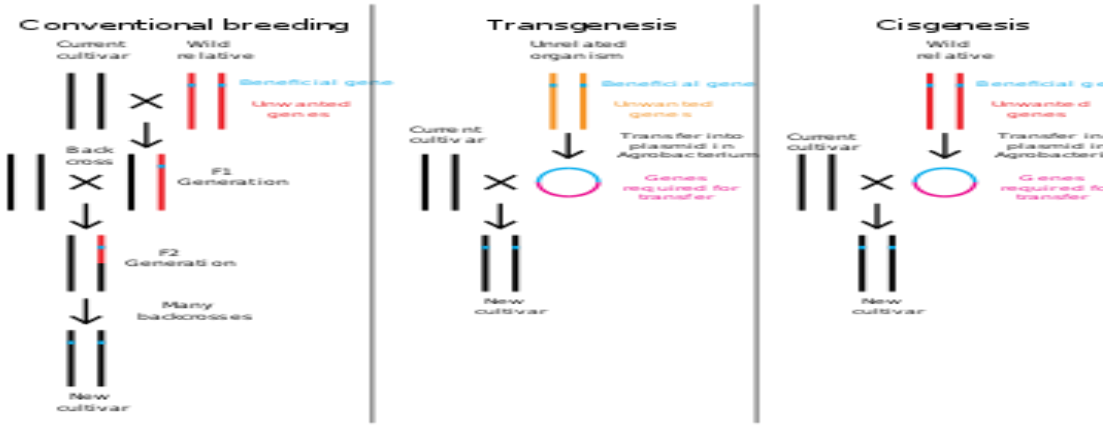
Brief content of lectures of discipline Genetic engineering
Lecture 1 «Introduction to Genetic engineering
L. 1 Genetic engineering
Introduction. Definition.History
Application.
Techniques and methods to study the Genetic engineering
Genetic engineering is a process that alters the genetic structure of an organism by either removing or introducing DNA. Unlike traditional animal and plant breeding, which involves doing multiple crosses and then selecting for the organism with the desired phenotype, genetic engineering takes the gene directly from one organism and inserts it in the other. This is much faster, can be used to insert any genes from any organism (even ones from different domains) and prevents other undesirable genes from also being added.
Genetic engineering could potentially fix severe genetic disorders in humans by replacing the defective gene with a functioning one. It is an important tool in research that allows the function of specific genes to be studied. Drugs, vaccines and other products have been harvested from organisms engineered to produce them. Crops have been developed that aid food security by increasing yield, nutritional value and tolerance to environmental stresses.
The DNA can be introduced directly into the host organism or into a cell that is then fused or hybridised with the host. This relies on recombinant nucleic acid techniques to form new combinations of heritable genetic material followed by the incorporation of that material either indirectly through a vector system or directly through micro-injection, macro-injection or micro-encapsulation.
Genetic engineering does not normally include traditional breeding, in vitro fertilisation, induction of polyploidy, mutagenesis and cell fusion techniques that do not use recombinant nucleic acids or a genetically modified organism in the process. However, some broad definitions of genetic engineering include selective breeding. Cloning and stem cell research, although not considered genetic engineering, are closely related and genetic engineering can be used within them.
Synthetic biology is an emerging discipline that takes genetic engineering a step further by introducing artificially synthesised material into an organism.
Plants, animals or micro organisms that have been changed through genetic engineering are termed genetically modified organisms or GMOs.[1] If genetic material from another species is added to the host, the resulting organism is called **transgenic.** If genetic material from the same species or a species that can naturally breed with the host is used the resulting organism is called **cisgenic**. If genetic engineering is used to remove genetic material from the target organism the resulting organism is termed a **knockout** organism. In Europe genetic modification is synonymous with genetic engineering while within the United States of America and Canada genetic modification can also be used to refer to more conventional breeding methods.



Comparison of conventional plant breeding with **transgenic** and **cisgenic** genetic modification.
History of genetic engineering
Humans have altered the genomes of species for thousands of years through selective breeding, or artificial selection[1] as contrasted with natural selection. More recently, mutation breeding has used exposure to chemicals or radiation to produce a high frequency of random mutations, for selective breeding purposes. Genetic engineering as the direct manipulation of DNA by humans outside breeding and mutations has only existed since the 1970s. The term "genetic engineering" was first coined by J. Williamson in his science fiction novel *Dragon's Island*, published in 1951 – one year before DNA's role in heredity was confirmed by Alfred Hershey and Martha

Chase, and two years before James Watson and Francis Crick showed that the DNA molecule has a double-helix structure – though the general concept of direct genetic manipulation was explored in rudimentary form in Stanley G. Weinbaum's 1936 science fiction story *Proteus Island*.

Lecture 2 Genetic engineering (also genetic modification or genetic manipulation is a set of technologies used to change the genetic makeup of cells»

Genetic engineering, also called **genetic modification** or **genetic manipulation**, is the direct manipulation of an organism's genes using biotechnology. It is a set of technologies used to change the genetic makeup of cells, including the transfer of genes within and across species boundaries to produce improved or novel organisms. New DNA is obtained by either isolating and copying the genetic material of interest using recombinant DNA methods or by artificially synthesising the DNA. A construct is usually created and used to insert this DNA into the host organism. The first recombinant DNA molecule was made by Paul Berg in 1972 by combining DNA from the monkey virus SV40 with the lambda virus. As well as inserting genes, the process can be used to remove, or "knock out", genes. The new DNA can be inserted randomly, or targeted to a specific part of the genome.

An organism that is generated through genetic engineering is considered to be genetically modified (GM) and the resulting entity is a genetically modified organism (GMO). The first GMO was a bacterium generated by Herbert Boyer and Stanley Cohen in 1973. Rudolf Jaenisch created the first GM animal when he inserted foreign DNA into a mouse in 1974. The first company to focus on genetic engineering, Genentech, was founded in 1976 and started the production of human proteins. Genetically engineered human insulin was produced in 1978 and insulin-producing bacteria were commercialised in 1982. Genetically modified food has been sold since 1994, with the release of the Flavr Savr tomato. The Flavr Savr was engineered to have a longer shelf life, but most current GM crops are modified to increase resistance to insects and herbicides. GloFish, the first GMO designed as a pet, was sold in the United States in December 2003. In 2016 salmon modified with a growth hormone were sold.

Genetic engineering has been applied in numerous fields including research, medicine, industrial biotechnology and agriculture. In research GMOs are used to study gene function and expression through loss of function, gain of function, tracking and expression experiments. By knocking out genes responsible for certain conditions it is possible to create animal model organisms of human diseases. As well as producing hormones, vaccines and other drugs genetic engineering has the potential to cure genetic diseases through gene therapy. The same techniques that are used to produce drugs can also have industrial applications such as producing enzymes for laundry detergent, cheeses and other products.

The rise of commercialised genetically modified crops has provided economic benefit to farmers in many different countries, but has also been the source of most of the controversy surrounding the technology. This has been present since its early use; the first field trials were destroyed by anti-GM activists. Although there is a scientific consensus that currently available food derived from GM crops poses no greater risk to human health than conventional food, GM food safety is a leading concern with critics. Gene flow, impact on non-target organisms, control of the food supply and intellectual property rights have also been raised as potential issues. These concerns have led to the development of a regulatory framework, which started in 1975. It has led to an international treaty, the Cartagena Protocol on Biosafety, that was adopted in 2000. Individual countries have developed their own regulatory systems regarding GMOs, with the most marked differences occurring between the US and Europe.

Some genetic engineering techniques are as follows:

1. <u>Artificial selection</u>

A. selective breeding
B. hybridization
C. inbreeding

2. <u>Cloning</u>

3. <u>Gene splicing</u>

4. <u>Gel electrophoresis</u>: analyzing DNA

<u>Artificial selection</u>: breeders choose which organism to mate to produce offspring with desired traits.

- They cannot control what genes are passed.
- When they get offspring with the desired traits, the maintain them.

<u>Three types of artificial selection:</u>

A. selective breeding

B. hybridization
C. inbreeding

A. Selective breeding: when animals with desired characteristics are mated to produce offspring with those desired traits.

- Passing of important genes to next generation.
- Example: Champion race horses, cows with tender meat, large juicy oranges on a tree.

C. Inbreeding breeding of organism that genetically similar to maintain desired traits.

- Dogs breeds are kept pure this way.
- Its how a Doberman remains a Doberman.
- It keeps each breed unique from others.
- *Risk: since both have the same genes, the chance that a baby will get a recessive genetic disorder is high.*
- *Risks: blindness, joint deformities.*

Lecture 3 Process of genetic engineering.
Creating a GMO is a multi-step process.
Genetic engineers must first choose what gene they wish to insert into the organism. This is driven by what the aim is for the resultant organism and is built on earlier research. Genetic screens can be carried out to determine potential genes and further tests then used to identify the best candidates. The development of microarrays, transcriptomics and genome sequencing has made it much easier to find suitable genes. The round-up ready gene was discovered after scientists noticed a bacterium thriving in the presence of the herbicide.
Restriction Enzyme A restriction enzyme is an enzyme that cuts double-stranded DNA at a specific recognition nucleotide sequences (A, T, C, G) known as restriction sites.Such enzymes, found in bacteria, are thought to have evolved to provide a defense mechanism against invading viruses.Restriction enzymes are required when removing or replacing genesThey are also very important in the creation of recombinant DNA.
Restriction Enzymes: Genes can be cut at specific DNA sequences by proteins known as Restriction Enzymes. Each recognizes and cuts DNA at a particular sequence (area of Bases). They are INCREDIBLY accurate, they will ONLY cut the area that they recognizeThis amazing ability allows us to cut DNA into fragments so that we can isolate it, separate it, and/or analyze it.
Gene isolation and cloning.
Lecture 4-5. Genetic engineering techniques. Modification of nuclear acids
Recombinant DNA technology-a set of techniques for manipulating DNA, including:
the identification and cloning of genes;
the study of the expression of cloned genes;
and the production of large quantities of gene product.

DNA recombination is sometimes called **gene splicing**, because the genes are "cut & pasted". The new DNA strand created is considered to be artificial, because it would not normally occur in natureBenefit of all life using the exact same DNANot the same thing as "natural" genetic recombinationThis how we engineer drugs to combat bacterial resistance (like MRSA) and how we are able to make non-human organisms make things like human insulin.

Which techniques create recombinant DNA

1. Sexual reproduction: natural
2. selective breeding
3. Hybridization
4. Gene splicing

DNA INSERTION:
During the first 2 steps of genetic engineering, DNA fragments containing the desired gene are obtained and then inserted into DNA that has been removed from the recipient cell (the cell where the DNA is going.). Forming recombinant DNA (New DNA).
To insert the DNA into LIVING cells it is easiest to use bacteria. Bacteria in a solution of salt and the desired DNA will eventually take up the DNA in its own DNA. This is considered DNA insertion. These new bacteria are then cultured (grown) into a large colony. The technical term for a large number of cells grown from a single cells Clone. So this is DNA cloning.
Gel electrophoresis

During gel electrophoresis, DNA is cut with a restriction enzyme into small pieces. Because DNA has a slight negative charge, different charges are placed at either end of a gel containing tray. When the DNA is placed into the tray it will slowly move across the gel (towards the +)Because the pieces are different sizes they move at different speeds (large = slower).
DNA Fingerprinting.
The amazing complexity of the human genome ensures that NO TWO INDIVIDUALS are exactly the same. This biological theory allows for a powerful new tool in criminal investigations. Now, finding bodily fluids and/or skin cells at the scene of a crime or on a victim can link a suspect to a crime with amazing reliability.

Lecture 5. Molecular cloning.
To isolate the candidate gene is the next step in genetic engireering.
Molecular cloning is a set of experimental methods in molecular biology that are used to assemble **recombinant DNA** molecules and to direct their *replication* within **host organisms.**
The use of the word ***cloning*** refers to the fact that the method involves the replication of one molecule to produce a population of cells with identical DNA molecules. Molecular cloning generally uses DNA sequences from two different organisms: the species that is the source of the DNA to be cloned, and the species that will serve as the living host for replication of the recombinant DNA. Molecular cloning methods are central to many contemporary areas of modern biology and medicine.
In a conventional molecular cloning experiment, the DNA to be cloned is obtained from an organism of interest, then treated with enzymes in the test tube to generate smaller DNA fragments. Subsequently, these fragments are then combined with **vector DNA** to generate recombinant DNA molecules. The recombinant DNA is then introduced into a host organism (typically an easy-to-grow, benign, laboratory strain of *E. coli* bacteria). This will generate a population of organisms in which recombinant DNA molecules are replicated along with the host DNA. Because they contain foreign DNA fragments, these are **transgenic** or genetically modified microorganisms (**GMO).** This process takes advantage of the fact that a single bacterial cell can be induced to take up and replicate a single recombinant DNA molecule. This single cell can then be expanded exponentially to generate a large amount of bacteria, each of which contain copies of the original recombinant molecule. Thus, both the resulting bacterial population, and the recombinant DNA molecule, are commonly referred to as "clones". Strictly speaking, *recombinant DNA* refers to DNA molecules, while *molecular cloning* refers to the experimental methods used to assemble them. The idea arose that different DNA sequences could be inserted into a plasmid and that these foreign sequences would be carried into bacteria and digested as part of the plasmid. That is, these plasmids could serve as cloning vectors to carry genes.
Virtually any DNA sequence can be cloned and amplified, but there are some factors that might limit the success of the process. Examples of the DNA sequences that are difficult to clone are inverted repeats, origins of replication, centromeres and telomeres. Another characteristic that limits chances of success is large size of DNA sequence. Inserts larger than 10kbp have very limited success, but bacteriophages such as bacteriophage λ can be modified to successfully insert a sequence up to 40 kbp
The cell containing the gene is opened and the DNA is purified. The gene is separated by using restriction enzymes to cut the DNA into fragments or polymerase chain reaction (PCR) to amplify up the gene segment These segments can then be extracted through gel electrophoresis. If the chosen gene or the donor organism's genome has been well studied it may already be accessible from a genetic library. If the DNA sequence is known, but no copies of the gene are available, it can also be artificially synthesised. Once isolated the gene is ligated into a plasmid that is then inserted into a bacterium. The plasmid is replicated when the bacteria divide, ensuring unlimited copies of the gene are available.
Before the gene is inserted into the target organism it must be combined with other genetic elements. These include a **promoter** and **terminato**r region, which initiate and end **transcription**. A **selectable marker** gene is added, which in most cases confers **antibiotic resistance**, so researchers can easily determine which cells have been successfully transformed. The gene can also be modified at this stage for better expression or effectiveness. These manipulations are carried out using **recombinant DNA** techniques, such as **restriction digests**, ligations and molecular cloning.

Lecture 6. DNA Separation Techniques for different types of DNA. Main principles of electrophoresis for analysis of nucleic acids.

Choice of host organism and cloning vector

Diagram of a commonly used cloning plasmid; **pBR322**. It's a circular piece of DNA 4361 bases long. Two **antibiotic resistance genes** are present, conferring resistance to ***ampicillin*** and ***tetracycline***, and an origin of replication that the host uses to **replicate** the DNA.

Although a very large number of host organisms and molecular cloning vectors are in use, the great majority of molecular cloning experiments begin with a laboratory strain of the bacterium *E. coli* (*Escherichia coli*) and a **plasmid cloning vector**. *E. coli* and plasmid vectors are in common use because they are technically sophisticated, versatile, widely available, and offer rapid growth of recombinant organisms with minimal equipment. If the DNA to be cloned is exceptionally large (hundreds of thousands to millions of base pairs), then a **bacterial artificial chromosome** or **yeast artificial chromosome** vector is often chosen.

Specialized applications may call for specialized host-vector systems.

For example, if to harvest a particular protein from the recombinant organism, then an **expression vector** is chosen that contains appropriate signals for transcription and translation in the desired host organism.

Alternatively, if replication of the DNA in different species is desired (for example, transfer of DNA from bacteria to plants), then a multiple host range vector (also termed **shuttle vector**) may be selected. In practice, however, specialized molecular cloning experiments usually begin with cloning into a bacterial plasmid, followed by subcloning into a specialized vector.

Whatever combination of host and vector are used, the vector almost always contains four DNA segments that are critically important to its function and experimental utility:[3]

- DNA *replication origin* is necessary for the vector (and its linked recombinant sequences) to replicate inside the host organism
- one or more unique *restriction endonuclease recognition sites* to serves as sites where foreign DNA may be introduced
- a *selectable genetic marker* gene that can be used to enable the survival of cells that have taken up vector sequences
- a *tag* gene that can be used to screen for cells containing the foreign DNA

Lecture 7. DNA cloning techniques

Cloning: creating an organism that is an exact genetic copy of another

Clone: group of cells or organisms that are genetically identical as a result of asexual reproduction

They will have the same exact DNA as the parent.

How is cloning done?

A single cell is removed from a parent organism.

An entire individual is grown from that cell.

Remember one cell has all the DNA needed to make an entire organism.

Each cell in the body has the same DNA, but cells vary because different genes are turned on in each cell.

How could you clone a human?

Step 1: An egg is removed from a female human

Eggs are haploid: 23 chromosomes.

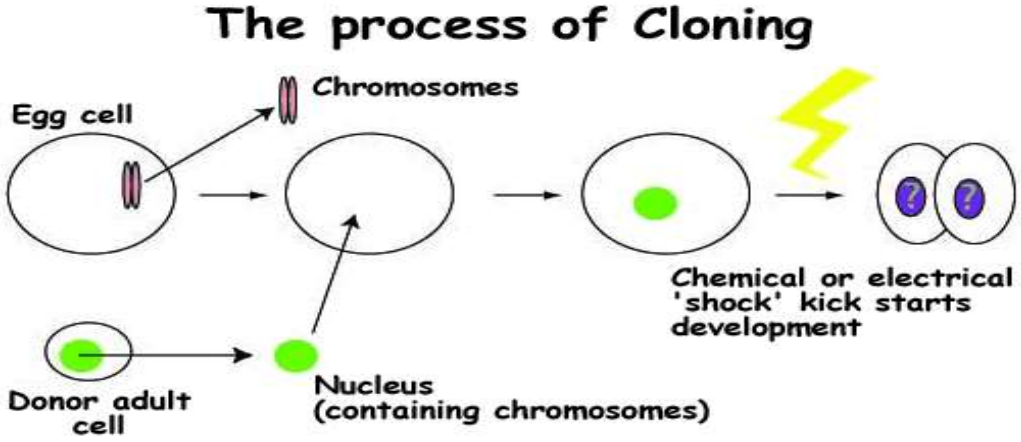
The nucleus of the egg is removed and is thrown away.

Step 2: A body cell is removed from another person.

The nucleus of the body cell is removed
Body cells are diploid: 46 chromosomes.

Step 3:

The nucleus of the diploid body cell is put into the egg.
This egg no longer needs to be fertilized since it has all 46 chromosomes.
Step 4: The egg is then charged with electricity to start mitosis.
Step 5: Its then put into a surrogate mother so it can grow.
Its going to be genetically identical to the parent of the body cell.
But it will be a baby.
Plants and animals can be cloned.



Benefits of cloning:

1. you can make exact copies of organisms with strong traits.
2. Increase food supply
3. Medical purposes: clone organs for transplants.
4. Bring back or Stop species from going extinct.

Risks of cloning:

1. Decreases genetic diversity
2. If one of your clones gets a disease, they all get it: same immune system.
3. Inefficient: high failure rate: 90%+
4. Expensive

Lecture 8. Restriction enzymes to cut the DNA into fragments

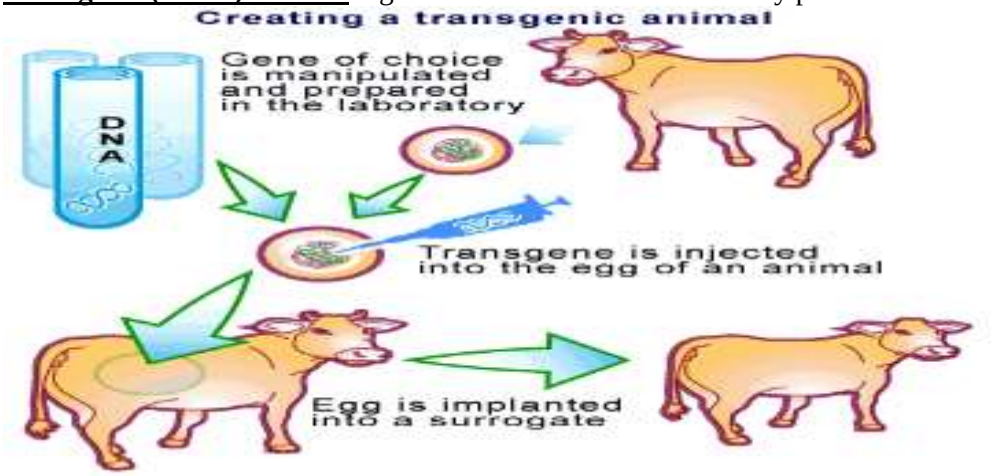
A **restriction enzyme:** enzyme that cuts the DNA at a specific code.
There are thousands of restriction enzymes.
Each cuts DNA at a different sequence.
Some look for GGCC and cut in between the G and C.
Every time GGCC is found in the DNA it is cut by the restriction enzyme

DNA Code:

TTATGGCCATACGGCCTT
AATACCGGTATGCCGGAA
TTATGGCCATACGGCCTT
AATACCGGTATGCCGGAA
TTATGG CCATACGG CCTT
AATACC GGTATGCC GGAA

This DNA segment was cut twice creating three fragments.
Since every one is different, we all have a different amount of times GGCC is found.
My DNA may be cut seven times
Yours may be cut ten times.
This is called **transformation:** when a gene from one organism is transferred to different organism.
The organisms that have DNA transferred to them are called **transgenic organisms**.
trans: means different,
genic: refers to genes

Transgenic (GMO) animals: genes inserted into animals so they produce what humans need.



Virtually any DNA sequence can be cloned and amplified, but there are some factors that might limit the success of the process. Examples of the DNA sequences that are difficult to clone are inverted repeats, origins of replication, centromeres and telomeres. Another characteristic that limits chances of success is large size of DNA sequence. Inserts larger than 10kbp have very limited success, but bacteriophages such as bacteriophage λ can be modified to successfully insert a sequence up to 40 kbp.

- In standard molecular cloning experiments, the cloning of any DNA fragment essentially involves seven steps:
- (1) Choice of host organism and cloning vector,
- (2) Preparation of vector DNA,
- (3) Preparation of DNA to be cloned,
- (4) Creation of recombinant DNA,
- (5) Introduction of recombinant DNA into host organism,
- (6) Selection of organisms containing recombinant DNA,
- (7) Screening for clones with desired DNA inserts and biological properties.

Preparation of vector DNA

The cloning vector is treated with a restriction endonuclease to cleave the DNA at the site where foreign DNA will be inserted. The restriction enzyme is chosen to generate a configuration at the cleavage site that is compatible with the ends of the foreign DNA. Typically, this is done by cleaving the vector DNA and foreign DNA with the same restriction enzyme, for example **EcoRI**.

Most modern vectors contain a variety of convenient cleavage sites that are unique within the vector molecule (so that the vector can only be cleaved at a single site) and are located within a gene (frequently *beta-galactosidase*) whose inactivation can be used to distinguish recombinant from non-recombinant organisms at a later step in the process.

To improve the ratio of recombinant to non-recombinant organisms, the cleaved vector may be treated with an enzyme (***alkaline phosphatase***) that dephosphorylates the vector ends.

Vector molecules with dephosphorylated ends are unable to replicate, and replication can only be restored if foreign DNA is integrated into the cleavage site.

The next step is to isolate the candidate gene. The cell containing the gene is opened and the DNA is purified. The gene is separated by using restriction enzymes to cut the DNA into fragments or polymerase chain reaction (PCR) to amplify up the gene segment These segments can then be extracted through gel electrophoresis. If the chosen gene or the donor organism's genome has been well studied it may already be accessible from a genetic library. If the DNA sequence is known, but no copies of the gene are available, it can also be artificially synthesised. Once isolated the gene is ligated into a plasmid that is then inserted into a bacterium. The plasmid is replicated when the bacteria divide, ensuring unlimited copies of the gene are available.

Before the gene is inserted into the target organism it must be combined with other genetic elements. These include a **promoter** and **terminato**r region, which initiate and end **transcription**. A **selectable marker** gene is added, which in most cases confers **antibiotic resistance**, so researchers can easily determine which cells have been successfully transformed. The gene can also be modified at this stage for better expression or effectiveness.

These manipulations are carried out using **recombinant DNA** techniques, such as **restriction digests**, ligations and molecular cloning.

Lecture 8. Genetic library for genetic engineering

1. A **genomic library** is a collection of the total genomic DNA from a single organism. The DNA is stored in a population of identical vectors, each containing a different **insert** of DNA.
2. In order to construct a genomic library, the organism's DNA is extracted from cells and then digested with a restriction enzyme to cut the DNA into fragments of a specific size.
3. The fragments are then inserted into the vector using **DNA ligase**.
4. Next, the vector DNA can be taken up by a host organism - commonly a population of Escherichia coli or **yeast** - with each cell containing only one vector molecule.
5. Using a host cell to carry the vector allows for easy amplification and retrieval of specific clones from the library for analysis
6. There are several kinds of vectors available with various insert capacities. Generally, libraries made from organisms with larger genomes require vectors featuring larger inserts, thereby fewer vector molecules are needed to make the library. Researchers can choose a vector also considering the ideal insert size to find the desired number of clones necessary for full genome coverage.
7. Genomic libraries are commonly used for sequencing applications. They have played an important role in the whole genome sequencing of several organisms, including the human genome and several model organisms.

History

The first DNA-based genome ever fully sequenced was achieved by two-time Nobel Prize winner, Frederick Sanger, in 1977. Sanger and his team of scientists created a library of the bacteriophage, phi X 174, for use in DNA sequencing. The importance of this success contributed to the ever-increasing demand for sequencing genomes to research **gene therapy**. Teams are now able to catalog polymorphisms in genomes and investigate those candidate genes contributing to maladies such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, and Type 1 diabetes. These are due to the advance of **genome-wide association studies** from the ability to create and sequence genomic libraries. Prior, linkage and candidate-gene studies were some of the only approaches

Genomic library construction

Construction of a genomic library involves creating many recombinant DNA molecules. An organism's genomic DNA is extracted and then digested with a restriction enzyme.

For organisms with very small genomes *(~10 kb)*, the digested fragments can be separated by gel electrophoresis. The separated fragments can then be excised and cloned into the vector separately. However, when a large genome is digested with a restriction enzyme, there are far too many fragments to excise individually. The entire set of fragments must be cloned together with the vector, and separation of clones can occur after. In either case, the fragments are ligated into a vector that has been digested with the same restriction enzyme. The vector containing the inserted fragments of genomic DNA can then be introduced into a host organism.

Below are the steps for creating a genomic library from a large genome.

1. Extract and purify DNA.
2. Digest the DNA with a restriction enzyme. This creates fragments that are similar in size, each containing one or more genes.
3. Insert the fragments of DNA into vectors that were cut with the same restriction enzyme. Use the enzyme DNA ligase to seal the DNA fragments into the vector. This creates a large pool of recombinant molecules.
4. These recombinant molecules are taken up by a host bacterium by **transformation**, creating a DNA library.
5. In order to isolate clones that contain regions of interest from a library, the library must first be **screened**. One method of screening is **hybridization**. Each transformed host cell of a library will contain only one vector with one insert of DNA. The whole library can be plated onto a filter over media. The filter and colonies are prepared for hybridization and then labeled with a probe. The target DNA- insert of interest- can be identified by detection such as autoradiography because of the hybridization with the probe as seen below.
6. Another method of screening is with polymerase chain reaction (PCR). Some libraries are stored as pools of clones and screening by PCR is an efficient way to identify pools containing specific clones.

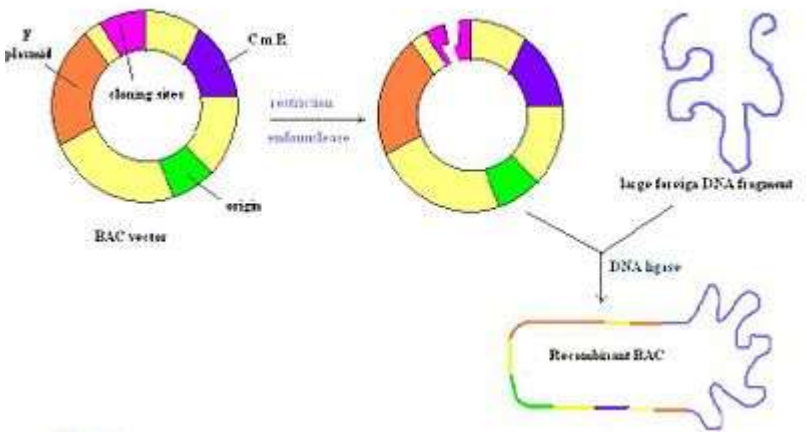
Determining titer of library

After a genomic library is constructed with a viral vector, such as lambda phage, the titer of the library can be determined. Calculating the titer allows researchers to approximate how many infectious viral particles were successfully created in the library. To do this, dilutions of the library are used to transform cultures of E. coli of known concentrations. The cultures are then plated on agar plates and incubated overnight. The number of viral

plaques are counted and can be used to calculate the total number of infectious viral particles in the library. Most viral vectors also carry a marker that allows clones containing an insert to be distinguished from those that do not have an insert. This allows researchers to also determine the percentage of infectious viral particles actually carrying a fragment of the library.
A similar method can be used to titer genomic libraries made with non-viral vectors, such as plasmids and BACs. A test ligation of the library can be used to transform E. coli. The transformation is then spread on agar plates and incubated overnight. The titer of the transformation is determined by counting the number of colonies present on the plates. These vectors generally have a selectable marker allowing the differentiation of clones containing an insert from those that do not. By doing this test, researchers can also determine the efficiency of the ligation and make adjustments as needed to ensure they get the desired number of clones for the library
Screening library.
Lecture 9. Types of vectors. Their characterization and application
Genome size varies among different organisms and the cloning vector must be selected accordingly. For a large genome, a vector with a large capacity should be chosen so that a relatively small number of clones are sufficient for coverage of the entire genome. However, it is often more difficult to characterize an insert contained in a higher capacity vector.
Below is a table of several kinds of vectors commonly used for genomic libraries and the insert size that each generally holds.

Vector type	**Insert size (thousands of bases)**
Plasmids	up to 10
Phage lambda (λ)	up to 25
Cosmids	up to 45
Bacteriophage P1	70 to 100
P1 artificial chromosomes (PACs)	130 to 150
Bacterial artificial chromosomes (BACs)	120 to 300
Yeast artificial chromosomes (YACs)	250 to 2000

Plasmids a double stranded circular DNA molecule commonly used for molecular cloning. Plasmids are generally 2 to 4 kilobase-pairs (kb) in length and are capable of carrying inserts up to 15kb. Plasmids contain an origin of replication allowing them to replicate inside a bacterium independently of the host chromosome. Plasmids commonly carry a gene for antibiotic resistance that allows for the selection of bacterial cells containing the plasmid. Many plasmids also carry a reporter gene that allows researchers to distinguish clones containing an insert from those that do not.[3]
Phage lambda (λ) is a double-stranded DNA virus that infects *E. coli*. The λ chromosome is 48.5kb long and can carry inserts up to 25kb. These inserts replace non-essential viral sequences in the λ chromosome, while the genes required for formation of viral particles and infection remain intact. The insert DNA is replicated with the viral DNA; thus, together they are packaged into viral particles. These particles are very efficient at infection and multiplication leading to a higher production of the recombinant λ chromosomes.[3] However, due to the smaller insert size, libraries made with λ phage may require many clones for full genome coverage.[14]
Cosmids vectors are plasmids that contain a small region of bacteriophage λ DNA called *the cos sequence*. This sequence allows the cosmid to be packaged into bacteriophage λ particles. These particles- containing a linearized cosmid- are introduced into the host cell by transduction. Once inside the host, the cosmids circularize with the aid of the host's DNA ligase and then function as plasmids. Cosmids are capable of carrying inserts up to 40kb in size.
Bacteriophage P1 vectors can hold inserts 70 – 100kb in size. They begin as linear DNA molecules packaged into bacteriophage P1 particles. These particles are injected into an E. coli strain expressing Cre recombinase. The linear P1 vector becomes circularized by recombination between two loxP sites in the vector. P1 vectors generally contain a gene for antibiotic resistance and a positive selection marker to distinguish clones containing an insert from those that do not. P1 vectors also contain a P1 plasmid *replicon*, which ensures only one copy of the vector is present in a cell. However, there is a second P1 replicon- called the P1 lytic replicon- that is controlled by an inducible *promoter*. This promoter allows the amplification of more than one copy of the vector per cell prior to DNA extraction.



bac vector

P1 artificial chromosomes (PACs) have features of both P1 vectors and Bacterial Artificial Chromosomes (BACs). Similar to P1 vectors, they contain a plasmid and a lytic replicon as described above. Unlike P1 vectors, they do not need to be packaged into bacteriophage particles for transduction. Instead they are introduced into E. coli as circular DNA molecules through *electroporati*on just as BACs are. Also similar to BACs, these are relatively harder to prepare due to a single origin of replication.

Bacterial artificial chromosomes (BACs) are circular DNA molecules, usually about 7kb in length, that are capable of holding inserts up to 300kb in size. BAC vectors contain a replicon derived from E. coli F factor, which ensures they are maintained at one copy per cell.[4] Once an insert is ligated into a BAC, the BAC is introduced into recombination deficient strains of E. coli by electroporation. Most BAC vectors contain a gene for antibiotic resistance and also a positive selection marker.[2] The figure to the right depicts a BAC vector being cut with a restriction enzyme, followed by the insertion of foreign DNA that is re-annealed by a ligase. Overall, this is a very stable vector, but they may be hard to prepare due to a single origin of replication just like PACs.

Yeast artificial chromosomes.

*Yeast artificial chromosomes (*YACs) are linear DNA molecules containing the necessary features of an authentic yeast chromosome, including telomeres, a centromere, and an origin of replication. Large inserts of DNA can be ligated into the middle of the YAC so that there is an "arm" of the YAC on either side of the insert. The recombinant YAC is introduced into yeast by transformation; selectable markers present in the YAC allow for the identification of successful transformants. YACs can hold inserts up to 2000kb, but most YAC libraries contain inserts 250-400kb in size. Theoretically there is no upper limit on the size of insert a YAC can hold. It is the quality in the preparation of DNA used for inserts that determines the size limit. The most challenging aspect of using YAC is the fact they are prone to rearrangement

Lecture 10. Inserting DNA into the host genome. Gene delivery

A gene gun uses biolistics to insert DNA into plant tissue

There are a number of techniques used to insert genetic material into the host genome. Some bacteria can naturally take up foreign DNA. This ability can be induced in other bacteria via stress (e.g. **thermal** or electric shock), which increases the cell membrane's permeability to DNA; up-taken DNA can either integrate with the genome or exist as **extrachromosomal** DNA. DNA is generally inserted into animal cells using **microinjection**, where it can be injected through the cell's **nuclear envelope** directly into the *nucleus*, or through the use of **viral vectors**.

In plants the DNA is often inserted using ***Agrobacterium*-mediated recombination**, taking advantage of the *Agrobacterium*s T-DNA sequence that allows natural insertion of genetic material into plant cells. Other methods include *biolistics*, where particles of gold or tungsten are coated with DNA and then shot into young plant cells, and electroporation, which involves using an electric shock to make the cell membrane permeable to plasmid DNA. Due to the damage caused to the cells and DNA the transformation efficiency of biolistics and electroporation is lower than agrobacterial transformation and microinjection.

As only a single cell is transformed with genetic material, the organism must be regenerated from that single cell. In plants this is accomplished through the use of tissue culture.

In animals it is necessary to ensure that the inserted DNA is present in the *embryonic stem cells*.

Bacteria consist of a single cell and reproduce clonally so regeneration is not necessary. Selectable markers are used to easily differentiate transformed from untransformed cells. These markers are usually present in the transgenic organism, although a number of strategies have been developed that can remove the selectable marker from the mature transgenic plant

Lecture 11. Confirmation that an organism contains the new gene **.**

Further testing using PCR, Southern hybridization, and DNA sequencing is conducted to confirm that an organism contains the new gene. These tests can also confirm the chromosomal location and copy number of the inserted gene. The presence of the gene does not guarantee it will be **expressed a**t appropriate levels in the target tissue so methods that look for and measure the gene products (RNA and protein) are also used. These include:

northern hybridisation,
quantitative RT-PCR,
Western blot,
immunofluorescence,
ELISA and phenotypic analysis.

The new genetic material can be inserted randomly within the host genome or targeted to a specific location. The technique of **gene targeting uses homologous recombination** to make desired changes to a specific endogenous gene. This tends to occur at a relatively low frequency in plants and animals and generally requires the use of selectable markers. The frequency of gene targeting can be greatly enhanced through *genome editing.*

Genome editing uses artificially engineered nucleases that create specific *double-stranded breaks* at desired locations in the genome, and use the cell's endogenous mechanisms to repair the induced break by the natural processes of homologous recombination and nonhomologous end-joining.

There are four families of engineered nucleases:

meganucleases,
zinc finger nucleases,
transcription activator-like effector nucleases (TALENs),
and the Cas9-guideRNA system (adapted from CRISPR).

TALEN and CRISPR are the two most commonly used and each has its own advantages.

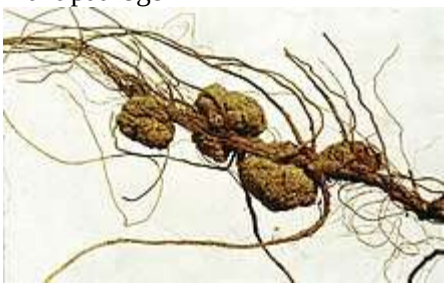
TALENs have greater target specificity, while CRISPR is easier to design and more efficient. In addition to enhancing gene targeting, engineered nucleases can be used to introduce mutations at endogenous genes that generate a *gene knockout*. An intermediate "middle-down" (*средние вниз*) approach in which larger peptide fragments are analyzed may also sometimes be used.

Lecture 12. *Agrobacterium*-mediated recombination

Agrobacterium is a genus of Gram-negative bacteria established by H. J. Conn that uses horizontal gene transfer to cause tumors in plants. *Agrobacterium tumefaciens* is the most commonly studied species in this genus. *Agrobacterium* is well known for its ability to transfer DNA between itself and plants, and for this reason it has become an important tool for genetic engineering.

The genus *Agrobacterium* is quite heterogeneous. Recent taxonomic studies have reclassified all of the *Agrobacterium* species into new genera, such as *Ahrensia*, *Pseudorhodobacter*, *Ruegeria*, and *Stappia*, but most species have been controversially reclassified as *Rhizobium* species

Plant pathogen



The large growths on these roots are galls induced by *Agrobacterium* sp.

Agrobacterium tumefaciens causes crown-gall disease in plants. The disease is characterised by a *tumour*-like growth or gall on the infected plant, often at the junction between the root and the shoot. Tumors are incited by the conjugative transfer of a DNA segment (T-DNA) from the bacterial tumour-inducing (Ti) plasmid. The closely related species, *Agrobacterium rhizogenes*, induces root tumors, and carries the distinct Ri (root-inducing) plasmid. Although the taxonomy of *Agrobacterium* is currently under revision it can be generalised that 3

biovars exist within the genus, *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, and *Agrobacterium vitis*. Strains within *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are known to be able to harbour either a Ti or Ri-plasmid, whilst strains of *Agrobacterium vitis*, generally restricted to grapevines, can harbour a Ti-plasmid. Non-*Agrobacterium* strains have been isolated from environmental samples which harbour a Ri-plasmid whilst laboratory studies have shown that non-*Agrobacterium* strains can also harbour a Ti-plasmid. Some environmental strains of *Agrobacterium* possess neither a Ti nor Ri-plasmid. These strains are avirulent.

The large growths on these roots are *galls* induced by *Agrobacterium* sp

The plasmid T-DNA is integrated semi-randomly into the genome of the host cell,[7] and the tumor morphology genes on the T-DNA are expressed, causing the formation of a gall. The T-DNA carries genes for the biosynthetic enzymes for the production of unusual amino acids, typically octopine or nopaline. It also carries genes for the biosynthesis of the plant hormones, auxin and cytokinins, and for the biosynthesis of *opines*, providing a carbon and nitrogen source for the bacteria that most other micro-organisms can't use, giving *Agrobacterium* a selective advantage.

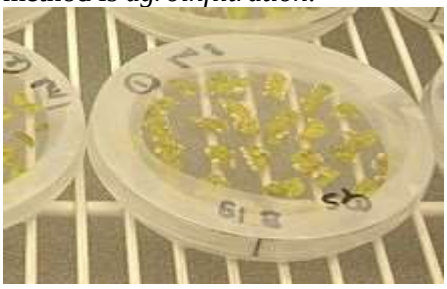
By altering the hormone balance in the plant cell, the division of those cells cannot be controlled by the plant, and tumors form. The ratio of auxin to cytokinin produced by the tumor genes determines the morphology of the tumor (root-like, disorganized or shoot-like).

Uses in biotechnology

Horizontal gene transfer

The ability of *Agrobacterium* to transfer genes to plants and fungi is used in genetic engineering for plant improvement. A modified Ti or Ri plasmid can be used. The plasmid is 'disarmed' by deletion of the tumor inducing genes; the only essential parts of the T-DNA are its two small (25 base pair) border repeats, at least one of which is needed for plant transformation.

The genes to be introduced into the plant are cloned into a *plant transformation vector* that contains the T-DNA region of the disarmed *plasmid*, together with a selectable marker (such as *antibiotic resistance*) to enable selection for plants that have been successfully transformed. Plants are grown on media containing antibiotic following transformation, and those that do not have the T-DNA integrated into their genome will die. An alternative method is *agroinfiltration*.



Plant (*S. chacoense*) transformed using *Agrobacterium*. Transformed cells start forming calluses on the side of the leaf pieces

Transformation with *Agrobacterium* can be achieved in multiple ways.

Protoplasts or alternatively leaf-discs can be incubated with the *Agrobacterium* and whole plants regenerated using *plant tissue culture*. In agroinfiltration the *Agrobacterium* may be injected directly into the leaf tissue of a plant. This method transforms only cells in immediate contact with the bacteria, and results in transient expression of plasmid DNA.

Agroinfiltration is commonly used to transform tobacco (*Nicotiana*). A common transformation protocol for *Arabidopsis* is the *floral dip method*: *inflorescence* are dipped in a suspension of *Agrobacterium*, and the bacterium transforms the *germline cells* that make the female **gametes**.

The seeds can then be screened for antibiotic resistance (or another marker of interest), and plants that have not integrated the plasmid DNA will die when exposed to the correct condition of antibiotic.[1]

Agrobacterium does not infect all plant species, but there are several other effective techniques for plant transformation including the gene gun.

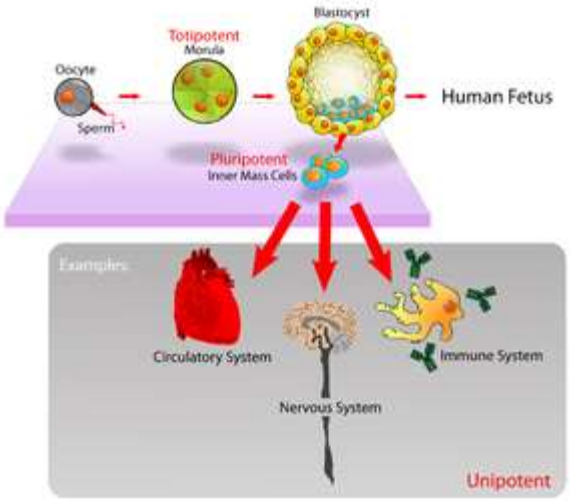
Agrobacterium is listed as being the vector of genetic material that was transferred to these USA GMOs:

- Soybean
- Cotton
- Corn
- Sugar Beet
- Alfalfa
- Wheat
- Rapeseed Oil (Canola)
- Creeping bentgrass (for animal feed)
- Rice (Golden Rice)

The *transformation* of fungi using *Agrobacterium* is used primarily for research purposes and follows similar approaches as for plant transformation. The Ti plasmid system is modified to include DNA elements to select for transformed fungal strains, after co-incubation of *Agrobacterium* strains carrying these plasmids with fungal species.

Lecture 13. Embryonic stem cell in genetic engineering

Embryonic stem cells (ESCs), derived from the blastocyst stage of early mammalian embryos, are distinguished by their ability to differentiate into any embryonic cell type and by their ability to self-renew. It is these traits that makes them valuable in the scientific and medical fields. ESCs have a normal karyotype, maintain high telomerase activity, and exhibit remarkable long-term proliferative potential.



Pluripotent. Embryonic stem cells are able to develop into any type of cell, excepting those of the placenta. Only embryonic stem cells of the morula are totipotent: able to develop into any type of cell, including those of the placenta.

Embryonic stem cells of the inner cell mass are pluripotent, meaning they are able to differentiate to generate primitive ectoderm, which ultimately differentiates during gastrulation into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. These germ layers generate each of the more than 220 cell types in the adult human body. When provided with the appropriate signals, ESCs initially form precursor cells that in subsequently differentiate into the desired cell types. Pluripotency distinguishes embryonic stem cells from adult stem cells, which are multipotent and can only produce a limited number of cell types.

Self-Renewal

Under defined conditions, embryonic stem cells are capable of self-renewing indefinitely in an undifferentiated state. Self-renewal conditions must prevent the cells from clumping and maintain an environment that supports an unspecialized state. Typically this is done in the lab with media containing serum and leukemia inhibitory factor or serum-free media supplements with two inhibitory drugs ("2i"), the MEK inhibitor PD03259010 and GSK-3 inhibitor CHIR99021.

Growth

ESCs divide very frequently due to a shortened G1 phase in their cell cycle. Rapid cell division allows the cells to quickly grow in number, but not size, which is important for early embryo development. In ESCs, cyclin A and cyclin E proteins involved in the G1/S transition are always expressed at high levels. Cyclin-dependent

kinases such as CDK2 that promote cell cycle progression are overactive, in part due to downregulation of their inhibitors. Retinoblastoma proteins that inhibit the transcription factor E2F until the cell is ready to enter S phase are hyperphosphorylated and inactivated in ESCs, leading to continual expression of proliferation genes. These changes result in accelerated cycles of cell division. Although the shortened G1 phase has been linked to maintenance of pluripotency, ESCs grown in serum-free 2i conditions do express hypo-phosphorylated active Retinoblastoma proteins and have an elongated G1 phase. Despite this difference in the cell cycle when compared to ESCs grown in media containing serum these cells have similar pluripotent characteristics. Pluripotency factors Oct4 and Nanog play a role in transcriptionally regulating the ESC cell cycle

Uses

Due to their plasticity and potentially unlimited capacity for self-renewal, embryonic stem cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. Pluripotent stem cells have shown promise in treating a number of varying conditions, including but not limited to: spinal cord injuries, age related macular degeneration, diabetes, neurodegenerative disorders (such as Parkinson's disease), AIDS, etc. In addition to their potential in regenerative medicine, embryonic stem cells provide a possible alternative source of tissue/organs which serves as a possible solution to the donor shortage dilemma. There are some ethical controversies surrounding this though.

Ethical debate section below). Aside from these uses, ESCs can also be used for research on early human development, certain genetic disease, and *in vitro* toxicology testing.

Lecture 14. Biolistics

Electroporation, or **electropermeabilization**, is a microbiology technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing chemicals, drugs, or DNA to be introduced into the cell (also called ***electrotransfer***). In microbiology, the process of electroporation is often used to transform bacteria, yeast, or plant protoplasts by introducing new coding DNA. If bacteria and plasmids are mixed together, the plasmids can be transferred into the bacteria after electroporation, though depending on what is being transferred cell-penetrating peptides or *CellSqueeze* could also be used. Electroporation works by passing thousands of volts across a distance of one to two millimeters of suspended cells in an electroporation cuvette (1.0 – 1.5 kV, 250 – 750 V/cm). Afterwards, the cells have to be handled carefully until they have had a chance to divide, producing new cells that contain reproduced plasmids. This process is approximately ten times more effective than chemical transformation.

Electroporation is also highly efficient for the introduction of foreign genes into tissue culture cells, especially *mammalian* cells. For example, it is used in the process of producing *knockout mice*, as well as in tumor treatment, gene therapy, and cell-based therapy. The process of introducing foreign DNA into eukaryotic cells is known as transfection.

Electroporation is highly effective for transfecting cells in suspension using electroporation cuvettes. Electroporation has proven efficient for use on tissues in vivo, for in utero applications as well as in ovo transfection. Adherent cells can also be *transfected* using electroporation, providing researchers with an alternative to trypsinizing their cells prior to transfection. One downside to electroporation, however, is that after the process the gene expression of over 7,000 genes can be affected.[6] This can cause problems in studies where gene expression has to be controlled to ensure accurate and precise results.

Although bulk electroporation has many benefits over physical delivery methods such as microinjections and *gene guns*, it still has limitations including low cell viability. Miniaturization of electroporation has been studied leading to *microelectroporation* and *nanotransfection o*f tissue utilizing electroporation based techniques via nanochannels to minimally invasively deliver cargo to the cells.

Cell fusion is of interest not only as an essential process in cell biology, but also as a useful method in biotechnology and medicine. Artificially induced fusion can be used to investigate and treat different diseases, like diabetes, regenerate axons of the central nerve system, and produce cells with desired properties, such as in cell vaccines for cancer immunotherapy. However, the first and most known application of cell fusion is production of monoclonal antibodies in hybridoma technology, where hybrid cell lines (hybridomas) are formed by fusing specific antibody-producing B lymphocytes with a myeloma (B lymphocyte cancer) cell line.

Laboratory practice

Cuvettes for in-vitro electroporation. These are plastic with aluminium electrodes and a blue lid. They hold a maximum of 400 μl.

Electroporation is performed with ***electroporators***, purpose-built appliances which create an electrostatic field in a cell solution. The cell *suspension* is *pipetted* into a glass or plastic cuvette which has two aluminium electrodes on its sides.

For bacterial electroporation, typically a suspension of around 50 microliters is used. Prior to electroporation, this suspension of bacteria is mixed with the plasmid to be transformed. The mixture is pipetted into the cuvette, the voltage and capacitance are set, and the cuvette is inserted into the electroporator. The process requires direct contact between the electrodes and the suspension. Immediately after electroporation, one milliliter of liquid medium is added to the bacteria (in the cuvette or in an Eppendorf tube), and the tube is incubated at the bacteria's optimal temperature for an hour or more to allow recovery of the cells and expression of the plasmid, followed by bacterial culture on agar plates.

The success of the electroporation depends greatly on the purity of the plasmid solution, especially on its salt content. Solutions with high salt concentrations might cause an electrical discharge (known *as arcing*), which often reduces the viability of the bacteria. For a further detailed investigation of the process, more attention should be paid to the output impedance of the porator device and the *input impedance* of the cells suspension (e.g. *salt* content).

Since the cell membrane is not able to pass current (except in ion channels), it acts as an electrical capacitor. Subjecting membranes to a high-voltage electric field results in their temporary breakdown, resulting in pores that are large enough to allow macromolecules (such as DNA) to enter or leave the cell.

Additionally, electroporation can be used to increase permeability of cells during in Utero injections and surgeries. Particularly, the electroporation allows for a more efficient transfection of DNA, RNA, shRNA, and all nucleic acids into the cells of mice and rats. The success of in vivo electroporation depends greatly on voltage, repetition, pulses, and duration. Developing central nervous systems are most effective for in vivo electroporation due to the visibility of ventricles for injections of nucleic acids, as well as the increased permeability of dividing cells. Electroporation of injected in *utero embryos* is performed through the uterus wall, often with forceps-type electrodes to limit damage to the embryo.

In vitro and animal studies.

In vivo gene electrotransfer was first described in 1991 and today there are many preclinical studies of gene electrotransfer. The method is used to deliver large variety of therapeutic genes for potential treatment of several diseases, such as: disorders in immune system, tumors, metabolic disorders, monogenetic diseases, cardiovascular diseases, analgesia…

With regards to irreversible electroporation, the first successful treatment of malignant cutaneous tumors implanted in mice was completed in 2007 by a group of scientists who achieved complete tumor ablation in 12 out of 13 mice. They accomplished this by sending 80 pulses of 100 microseconds at 0.3 Hz with an electrical field magnitude of 2500 V/cm to treat the cutaneous tumors.

The first group to look at electroporation for medical applications was led by Lluis M Mir at the Institute Gustave Roussy. In this case, they looked at the use of reversible electroporation in conjunction with impermeable macromolecules. The first research looking at how nanosecond pulses might be used on human cells was conducted by researchers at Eastern Virginia Medical School and Old Dominion University, and published in 2003.

Lecture 15. Medical applications of electroporation. Application of genetic engineering in research, industrial, agriculture *Medical applications*.

Irreversible electroporation

The first medical application of electroporation was used for introducing poorly permeant anticancer drugs into tumor nodules. Soon also gene electrotransfer became of special interest because of its low cost, easiness of realization and safety. Namely, viral vectors can have serious limitations in terms of immunogenicity and pathogenicity when used for DNA transfer.

A higher voltage of electroporation was found in pigs to irreversibly destroy target cells within a narrow range while leaving neighboring cells unaffected, and thus represents a promising new treatment for cancer, heart disease and other disease states that require removal of tissue. Irreversible electroporation (IRE) has since proven effective in treating human cancer, with surgeons at Johns Hopkins and other institutions now using the technology to treat *pancreatic cancer* previously thought to be unresectable.

Also first phase I clinical trial of gene electrotransfer in patients with metastatic melanoma was reported. Electroporation mediated delivery of a plasmid coding gene for interleukin-12 (pIL-12) was performed and safety, tolerability and therapeutic effect were monitored. Study concluded, that gene electrotransfer with pIL-12 is safe and well tolerated. In addition partial or complete response was observed also in distant non treated metastases, suggesting the systemic treatment effect. Based on these results they are already planning to move to Phase II clinical study. There are currently several ongoing clinical studies of gene electrotransfer, where safety, tolerability and effectiveness of immunization with DNA vaccine, which is administered by the electric pulses is monitored.

Although the method is not systemic, but strictly local one, it is still the most efficient non-viral strategy for gene delivery.

N-TIRE.

A recent technique called non-thermal irreversible electroporation (N-TIRE) has proven successful in treating many different types of tumors and other unwanted tissue. This procedure is done using small electrodes (about 1mm in diameter), placed either inside or surrounding the target tissue to apply short, repetitive bursts of electricity at a predetermined voltage and frequency. These bursts of electricity increase the resting transmembrane potential (TMP), so that nanopores form in the plasma membrane. When the electricity applied to the tissue is above the electric field threshold of the target tissue, the cells become permanently permeable from the formation of nanopores. As a result, the cells are unable to repair the damage and die due to a loss of homeostasis. N-TIRE is unique to other tumor ablation techniques in that it does not create thermal damage to the tissue around it.

Reversible electroporation.

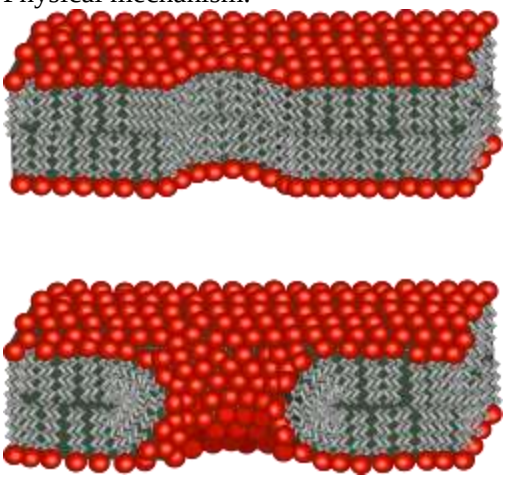
Contrastingly, reversible electroporation occurs when the electricity applied with the electrodes is below the electric field threshold of the target tissue. Because the electricity applied is below the cells' threshold, it allows the cells to repair their phospholipid bilayer and continue on with their normal cell functions. Reversible electroporation is typically done with treatments that involve getting a drug or gene (or other molecule that is not normally permeable to the cell membrane) into the cell. Not all tissue has the same electric field threshold; therefore careful calculations need to be made prior to a treatment to ensure safety and efficacy.

One major advantage of using N-TIRE is that, when done correctly according to careful calculations, it only affects the target tissue. Proteins, the extracellular matrix, and critical structures such as blood vessels and nerves are all unaffected and left healthy by this treatment. This allows for a quicker recovery, and facilitates a more rapid replacement of dead tumor cells with healthy cells.

Before doing the procedure, scientists must carefully calculate exactly what needs to be done, and treat each patient on an individual case-by-case basis. To do this, imaging technology such as CT scans and MRI's are commonly used to create a 3D image of the tumor. From this information, they can approximate the volume of the tumor and decide on the best course of action including the insertion site of electrodes, the angle they are inserted in, the voltage needed, and more, using software technology. Often, a CT machine will be used to help with the placement of electrodes during the procedure, particularly when the electrodes are being used to treat tumors in the brain.

The entire procedure is very quick, typically taking about five minutes. The success rate of these procedures is high and is very promising for future treatment in humans. One disadvantage to using N-TIRE is that the electricity delivered from the electrodes can stimulate muscle cells to contract, which could have lethal consequences depending on the situation. Therefore, a paralytic agent must be used when performing the procedure. The paralytic agents that have been used in such research are successful; however, there is always some risk, albeit slight, when using anesthetics.

H-FIRE.
A more recent technique has been developed called high-frequency irreversible electroporation (H-FIRE). This technique uses electrodes to apply bipolar bursts of electricity at a high frequency, as opposed to unipolar bursts of electricity at a low frequency. This type of procedure has the same tumor ablation success as N-TIRE. However, it has one distinct advantage, H-FIRE does not cause muscle contraction in the patient and therefore there is no need for a paralytic agent.
Drug and gene delivery.
Electroporation can also be used to help deliver drugs or genes into the cell by applying short and intense electric pulses that transiently permeabilize cell membrane, thus allowing transport of molecules otherwise not transported through a cellular membrane. This procedure is referred to as electrochemotherapy when the molecules to be transported are chemotherapeutic agents or gene electrotransfer when the molecule to be transported is DNA. Scientists from Karolinska Institutet and the University of Oxford use electroporation of exosomes to deliver siRNAs, antisense oligonucleotides, chemotherapeutic agents and proteins specifically to neurons after inject them systemically (in blood). Because these exosomes are able to cross the blood brain barrier, this protocol could solve the problem of poor delivery of medications to the central nervous system, and potentially treat Alzheimer's, Parkinson's Disease and brain cancer, among other conditions.
Bacterial transformation is generally the easiest way to make large amounts of a particular protein needed for biotechnology purposes or in medicine. Since gene electrotransfer is very simple, rapid and highly effective technique it first became very convenient replacement for other transformation procedures.
Physical mechanism.



Schematic cross-section showing the theoretical arrangement of lipids in a hydrophobic pore (top) and a hydrophilic pore (bottom).
Lipid bilayer mechanics
Electroporation allows cellular introduction of large highly charged molecules such as DNA which would never passively diffuse across the hydrophobic bilayer core. This phenomenon indicates that the mechanism is the creation of nm-scale water-filled holes in the membrane. Although electroporation and dielectric breakdown both result from application of an electric field, the mechanisms involved are fundamentally different. In dielectric breakdown the barrier material is ionized, creating a conductive pathway. The material alteration is thus chemical in nature. In contrast, during electroporation the lipid molecules are not chemically altered but simply shift position, opening up a pore which acts as the conductive pathway through the bilayer as it is filled with water.
Electroporation is a dynamic phenomenon that depends on the local transmembrane voltage at each point on the cell membrane. It is generally accepted that for a given pulse duration and shape, a specific transmembrane voltage threshold exists for the manifestation of the electroporation phenomenon (from 0.5 V to 1 V). This leads to the definition of an electric field magnitude threshold for electroporation (E_{th}). That is, only the cells within areas where $E \geqq E_{th}$ are electroporated. If a second threshold (E_{ir}) is reached or surpassed, electroporation will compromise the viability of the cells, *i.e.*, irreversible electroporation (IRE).
Electroporation is a multi-step process with several distinct phases. First, a short electrical pulse must be applied. Typical parameters would be 300–400 mV for < 1 ms across the membrane (note- the voltages used in cell experiments are typically much larger because they are being applied across large distances to the bulk solution so the resulting field across the actual membrane is only a small fraction of the applied bias). Upon application of this potential the membrane charges like a capacitor through the migration of ions from the surrounding solution. Once the critical field is achieved there is a rapid localized rearrangement in lipid morphology. The resulting

structure is believed to be a "pre-pore" since it is not electrically conductive but leads rapidly to the creation of a conductive pore. Evidence for the existence of such pre-pores comes mostly from the "flickering" of pores, which suggests a transition between conductive and insulating states. It has been suggested that these pre-pores are small (~3 Å) hydrophobic defects. If this theory is correct, then the transition to a conductive state could be explained by a rearrangement at the pore edge, in which the lipid heads fold over to create a hydrophilic interface. Finally, these conductive pores can either heal, resealing the bilayer or expand, eventually rupturing it. The resultant fate depends on whether the critical defect size was exceeded which in turn depends on the applied field, local mechanical stress and bilayer edge energy.

Electrotransfer