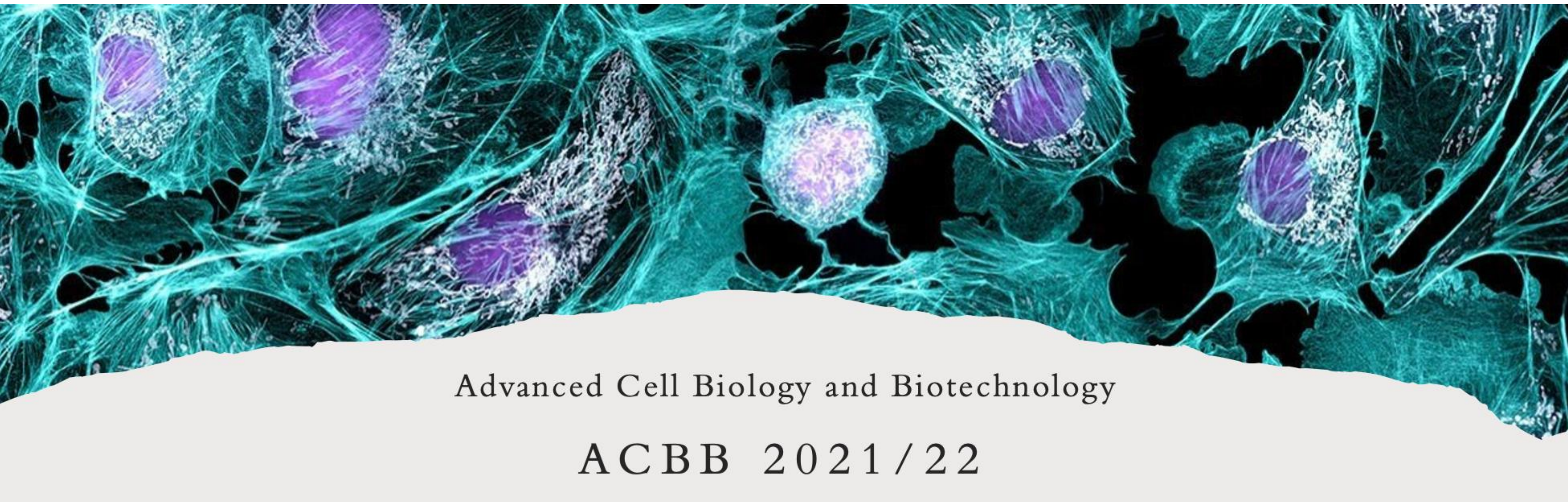
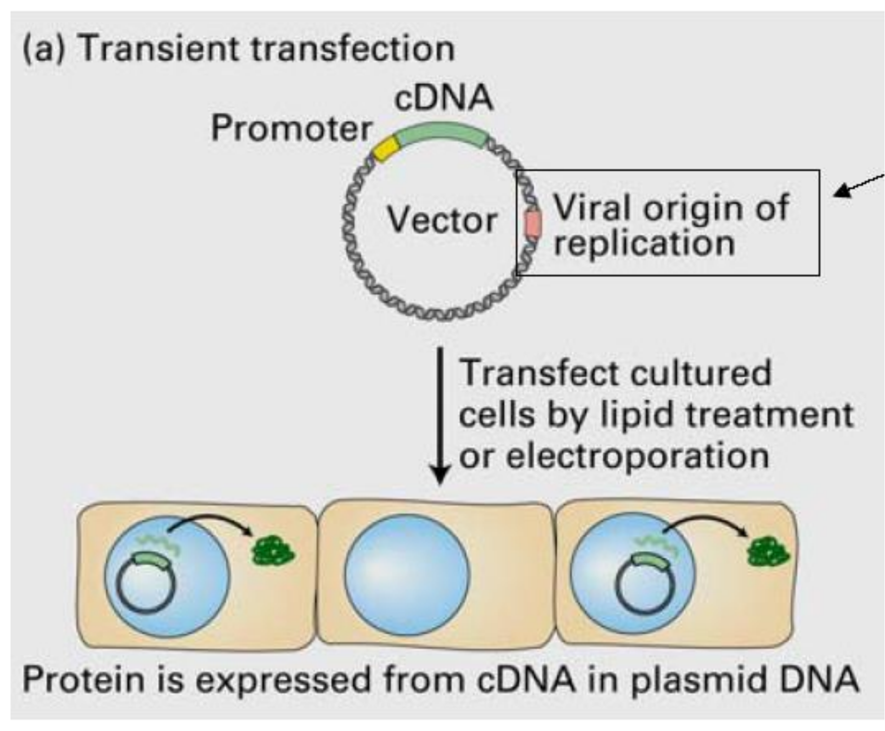
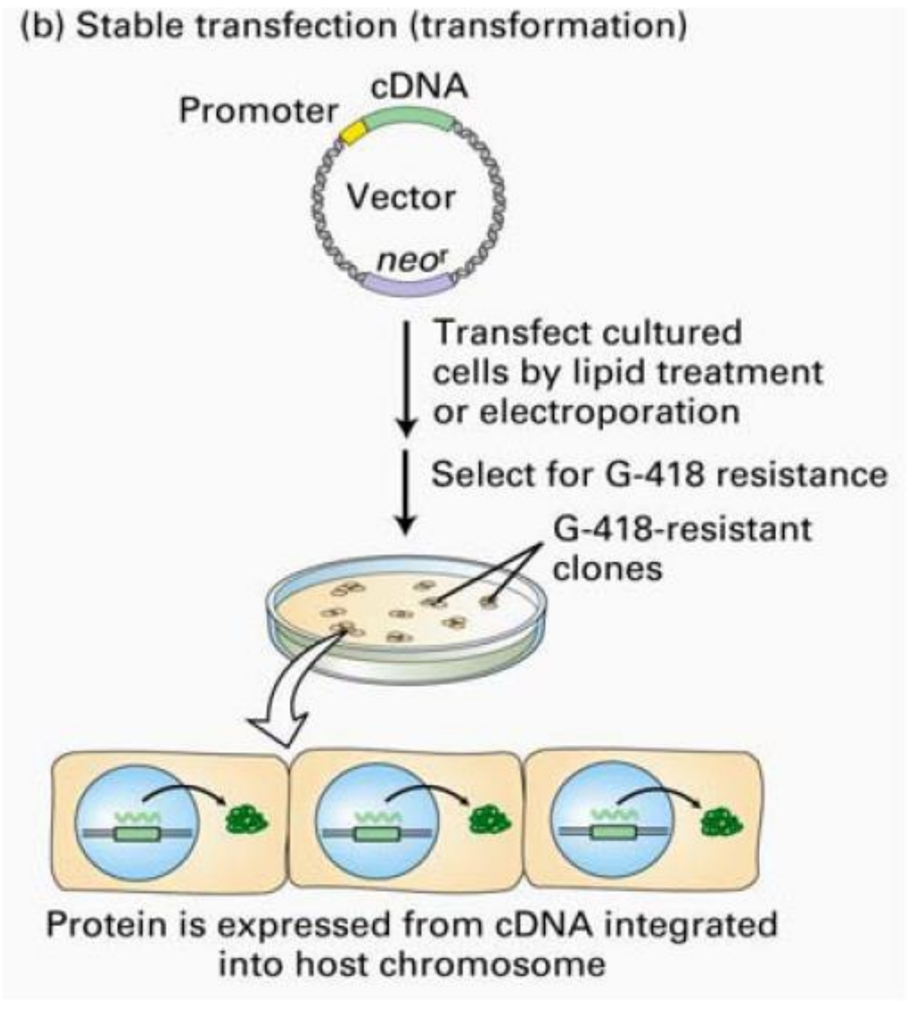


…the lecture is about to begin…

Transient versus Stable Cell Transfection





not interested in biological response → transient transfection of COS cells

Molecular level (protein extract) to study

- protein-protein interaction —> co-IP, FRET
- protein modification (phosphorylation…) —> WB, FRET
- effect of dominant mutations —> WB, co-IP
- effect of dominant-negative mutations —> WB, co-IP

interested in short term biological response → transient transfection of specific cells

interested in long term biological response → stable transfection of specific cells

Cellular level to study

- effect of dominant mutations —> phenotype
- effect of dominant-negative mutations —> phenotype

Gene delivery technologies

CHEMICAL METHODS

use carrier molecules to neutralize or impart a positive charge to the negatively charged nucleic acids:

- Cationic lipid transfection
- Calcium phosphate transfection
- DEAE-dextran transfection
- Delivery by other cationic polymers (e.g., polybrene, PEI, dendrimers)

BIOLOGICAL METHODS

rely on genetically engineered viruses to transfer non-viral genes into cells (also known as transduction):

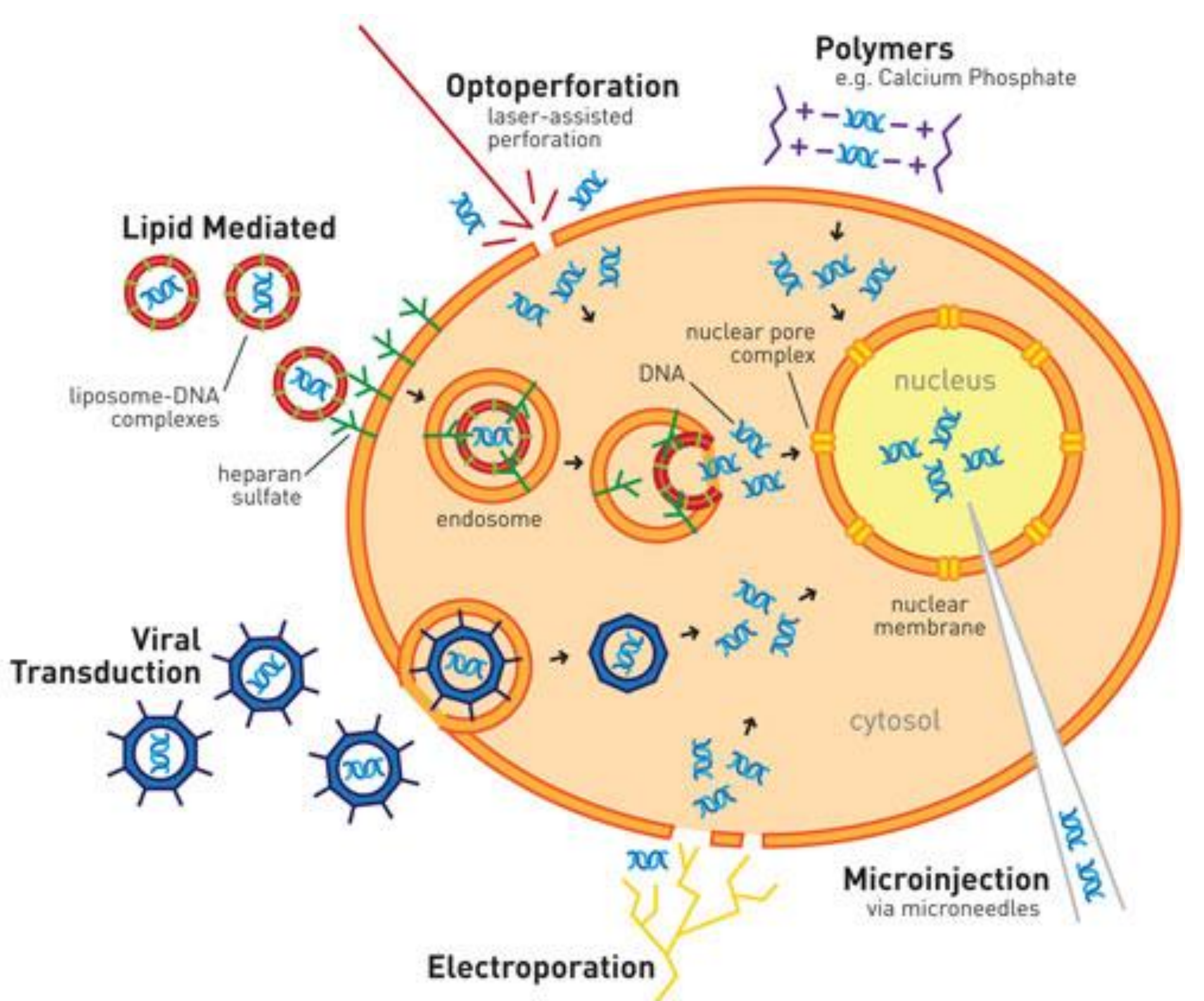
- Viral delivery

PHYSICAL METHODS

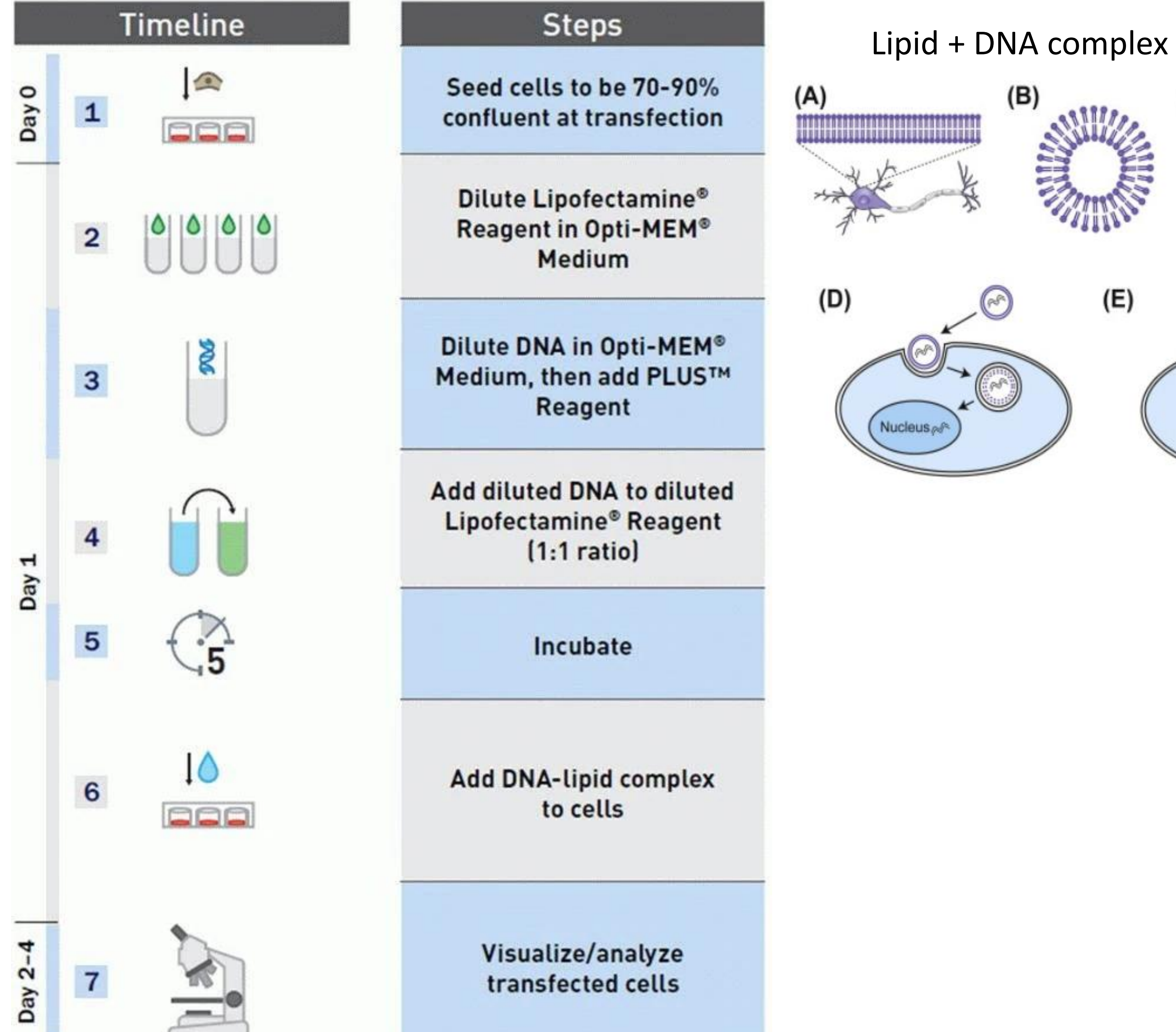
directly deliver nucleic acids into the cytoplasm or the nucleus of the cell and include:

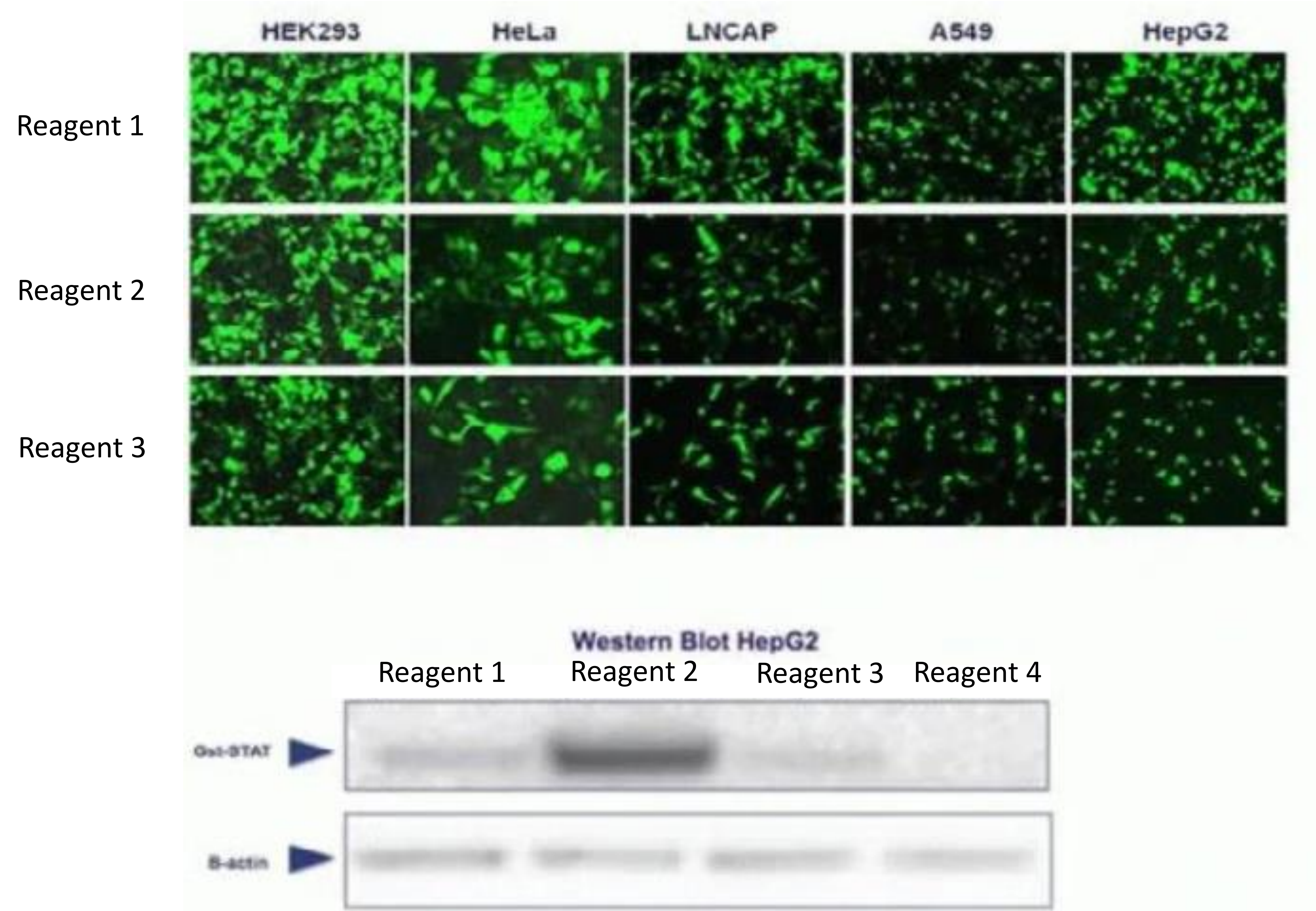
- Electroporation
- Biolistic particle delivery (particle bombardment)
- Direct microinjection
- Laser-mediated transfection (phototransfection)

Gene delivery technologies



Lipid + DNA complex formation




HEK293
HeLa
LNCAP
A549
HepG2
Reagent 1
Reagent 2
Reagent 3
Western Blot HepG2
Reagent 1
Reagent 2
Reagent 3
Reagent 4
B-actin

Transgenic Animals

1- DNA microinjection
- random insertion

2- embryonic stem cell-mediated gene transfer
- random insertion
- homologous recombination (double selection)
- Rosa26 locus

- knock-out animals
- knock-in animals
- conditional knock-out (cre-lox technique, inducible systems)
- siRNA
- CRISPR-CAS9

TRANSGENIC ANIMALS

- What is a transgenic animal?
- Which kind of genes would you like to over-express?
- Which kind of construct do you have to prepare?
- How do you insert the construct into the animal?
- How do you identify the transgenic animals?

What is a transgenic animal?

• a transgenic animal defines an animal which has been genetically modified by the artificial introduction of genetic material in every cell

• the insertion of the foreign DNA (exogenous gene or “transgene”) can
- confer a gain of function (production of a new protein)
- over-expression of an endogenous gene
- expression of a mutated form of an endogenous gene
- expression of a siRNA switching off the expression of a gene
- .....

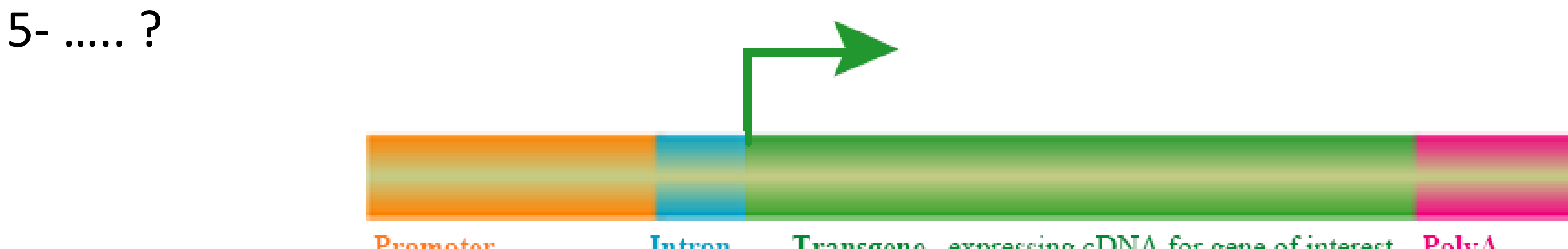
Only for teaching purposes - not for reproduction or sale

- Which genes would you like to over-express?

- It depends from which is your scientific question…

- Which kind of construct do you have to prepare if you want to study:

  1-the over-expression of a protein? (ubiquitous / tissue specific / inducible expression)
  2- the regulation of a promoter?
  3-the down-regulation of a protein?
  4-the localization of a protein?
  5- ….. ?



6-How can you discriminate between endogenous and exogenous proteins?
7-How can you study the expression of the exogenous protein without the expression of the endogenous one?

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GENETIC MODIFICATION of CELLS

- transient vs stable transfection of cells

TRANSGENIC ANIMALS

- Two methods to produce transgenic animals:

    1- DNA microinjection
        - random insertion
    2- embryonic stem cell-mediated gene transfer
        - random insertion
        - homologous recombination (double selection)
        - Rosa26 locus
        - genomic analysis to identify genetically modified animals

- knock-out animals
- knock-in animals

- conditional knock-out (cre-lox technique, inducible systems)
- siRNA

Two methods to produce transgenic mice can be used:

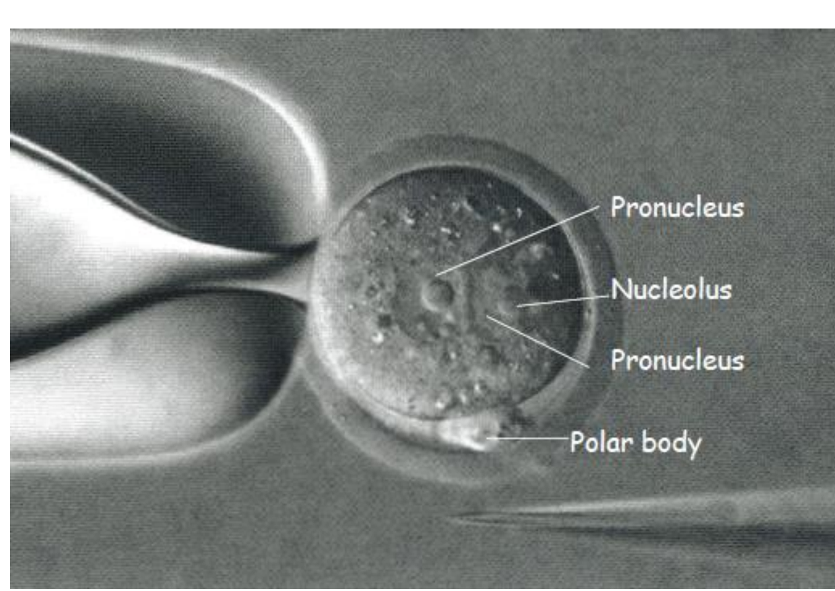
1 - injecting the construct into the pronucleus of a fertilized mouse egg
2 - stably transfecting embryonic stem cells (ES cells) with the construct

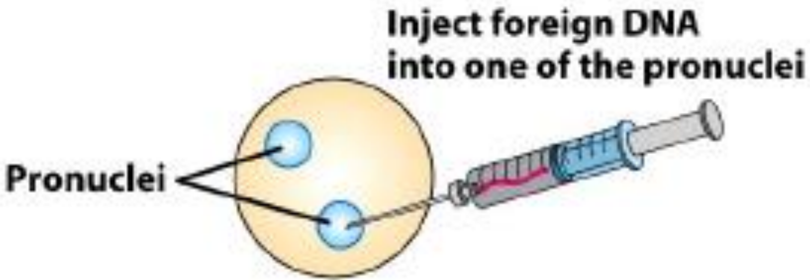
the choice of one method or the other depends on which is the final aim; the first method was used mainly in the past.

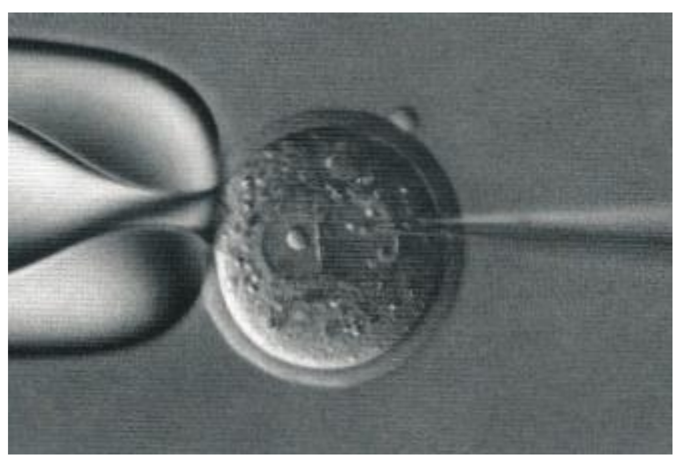
1 - injecting the construct into the pronucleus of a fertilized mouse egg

• the basic technical approach used for the generation of transgenic animals is called "pronuclear injection“:

• harvest freshly fertilized mouse eggs
• inject the male pronucleus with DNA







https://www.youtube.com/watch?v=xHvyICRM6FQ

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1 - injecting the construct into the pronucleus of a fertilized mouse egg

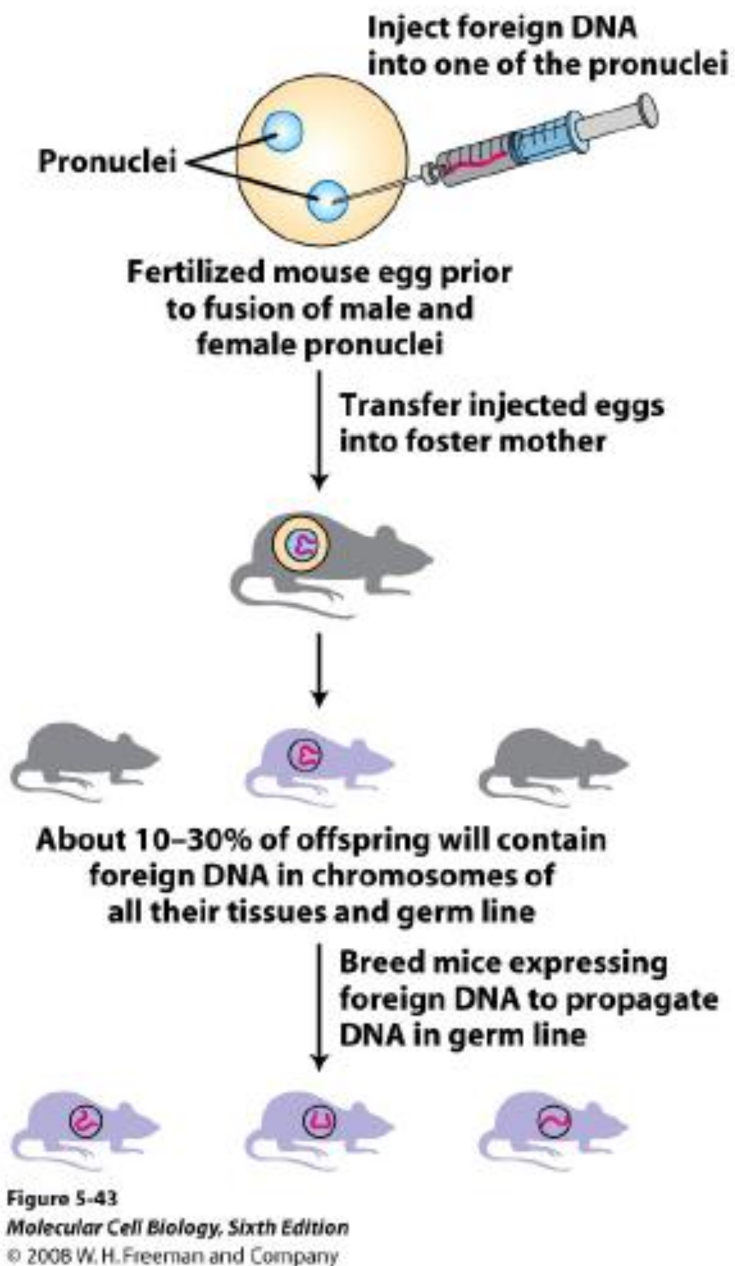

Figure 5-43
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- harvest freshly fertilized mouse eggs
- inject the male pronucleus with DNA
- when the pronuclei have fused to form the diploid zygote nucleus, allow the zygote to divide by mitosis to form a 2-cell embryo
- implant the embryos into the uterus of a **pseudopregnant foster mother**
- some will implant successfully and develop into healthy pups (usually no more than one-third will)

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1 - injecting the construct into the pronucleus of a fertilized mouse egg

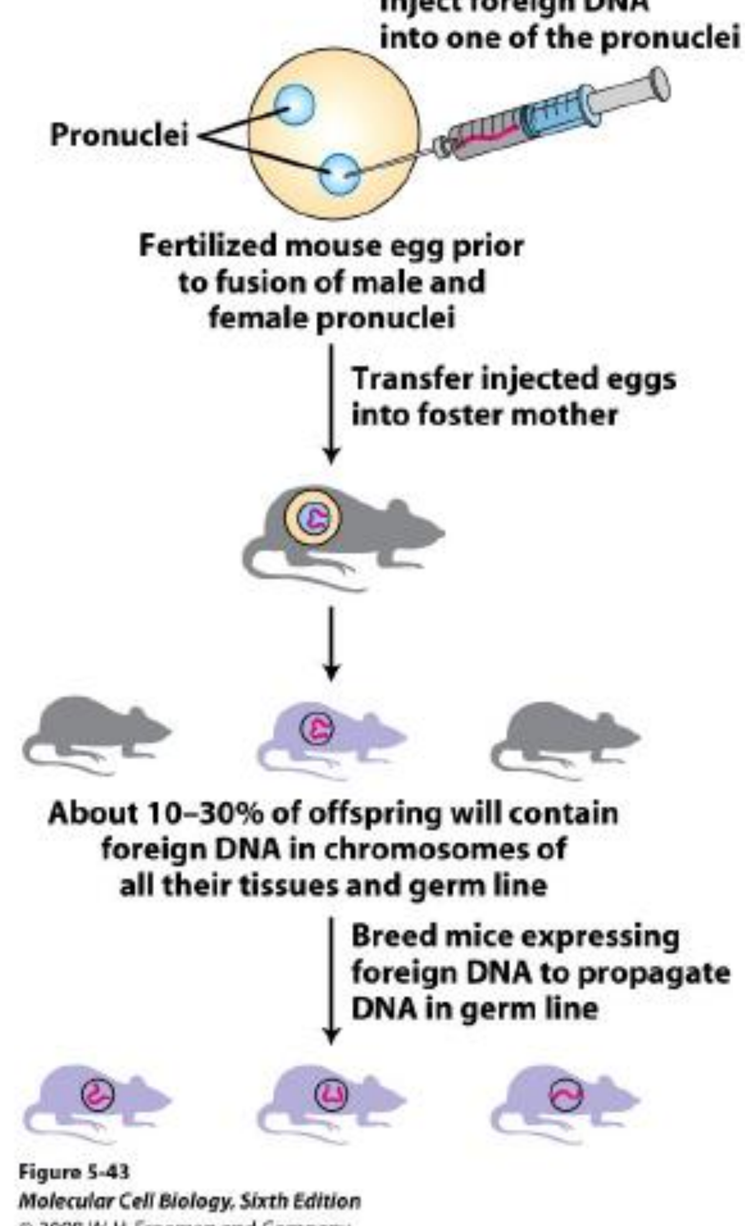

Figure 5-43
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- DNA integrates at **random positions** in the genome

- a loss of function may occur if the transgene interrupts another gene

- which cells or tissues express the exogenous protein?

- the exogenous protein is expressed in a ubiquitous or tissue specific manner according to which promoter is upstream the cDNA

RANDOM VERSUS TARGETED GENE INSERTION

If you do not use a strategy to obtain targeted gene insertion, DNA integrates **RANDOMLY** within the genome

- a loss of function may occur if the transgene interrupts another gene
- no expression may occur if the trangene is inserted in a silenced DNA region
- tissue specific expression may occur if the transgene is inserted downstream a tissue specific promoter
- over-expression may occur if many copies of the construct are inserted in the genome

RANDOM VERSUS TARGETED GENE INSERTION

if you want to obtain:

- selective inactivation of a specific gene (**KNOCK-OUT**)
- replacement of a gene with a mutated/modified copy of the same gene (**KNOCK-IN**)
- proper expression of your transgene (without loss of function of other genes)

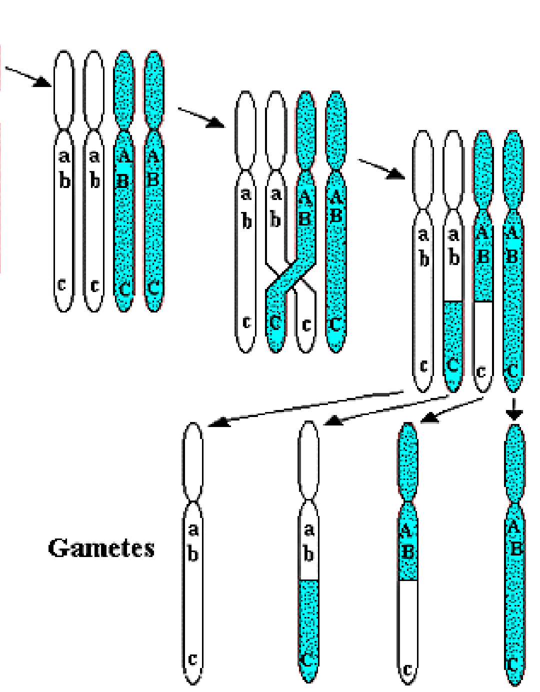
you need **TARGETED GENE EXPRESSION**

you have to:

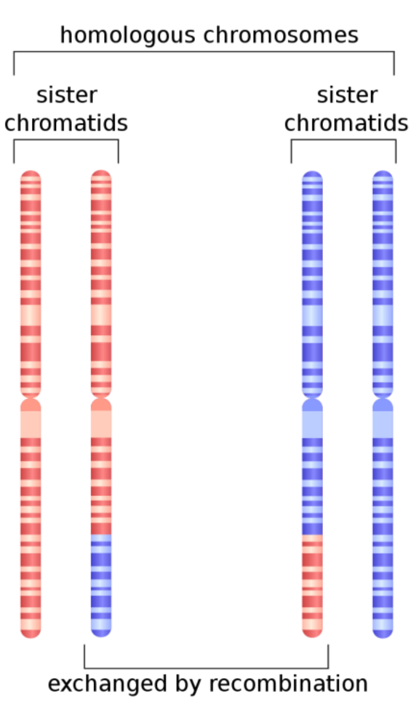
-1- prepare the construct for **HOMOLOGOUS RECOMBINATION**
-2- transfer it into (fertilized egg or embryonic stem cells?)
-3- select cells in which homologous recombination occurred
-4- transfer them into the animal
-5- verify that offspring contains the transgene

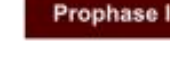
What HOMOLOGOUS RECOMBINATION is?

HOMOLOGOUS RECOMBINATION

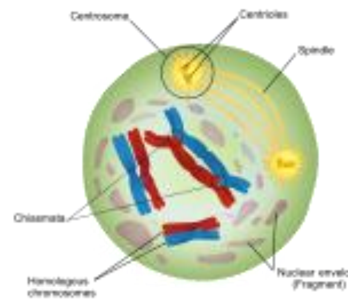
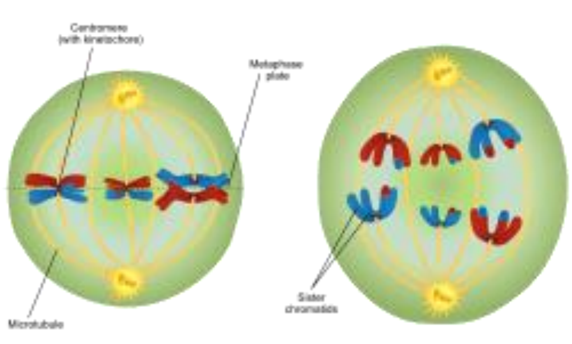
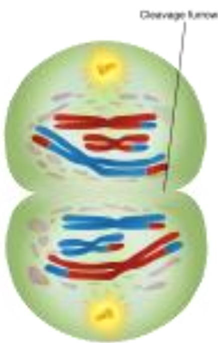


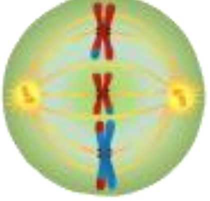
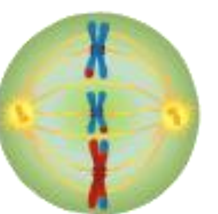
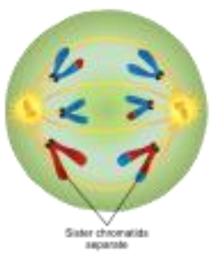
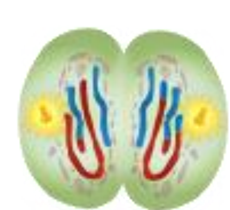
- **Homologous recombination** is a genetic recombination in which nucleotide sequences are exchanged between two similar or identical DNA molecules.
- cells use **homologous recombination** to accurately repair harmful breaks that occur on both strands of DNA.
- **Homologous recombination** produces new combinations of DNA sequences during **meiosis**, the process by which eukaryotes make **gamete cells**, like **sperm** and **egg** cells in animals.





Prophase I | **Metaphase I** | **Anaphase I** | **Telophase I & cytokinesis** | **Prophase II** | **Metaphase II** | **Anaphase II** | **Telophase II & cytokinesis**

The chromosomes condense, and the nuclear envelope breaks down. Crossing-over occurs.

Pairs of homologous chromosomes move to the equator of the cell.

Homologous chromosomes move to the opposite poles of the cell.

Chromosomes gather at the poles of the cells. The cytoplasm divides.

A new spidle forms around the chromosomes.

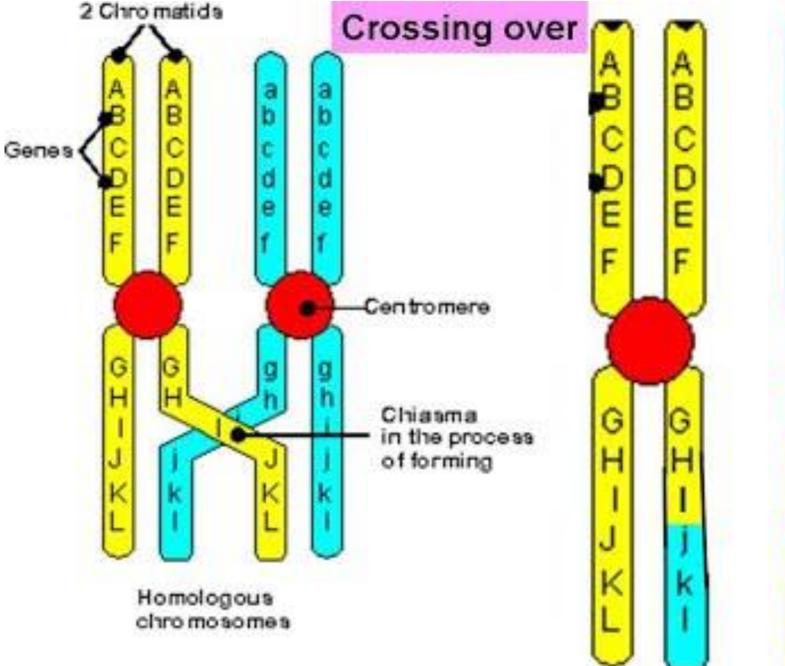
Metaphase II chromosomes line up at the equator.

Centromeres divide. Chromatids move to the opposite poles of the cells.

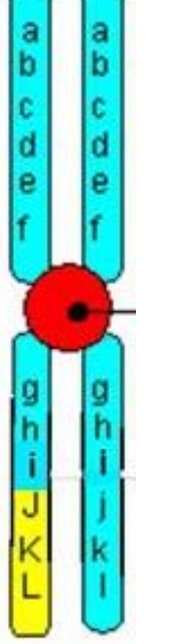
A nuclear envelope forms around each set of chromosomes. The cytoplasm divides.

MEYOSIS I

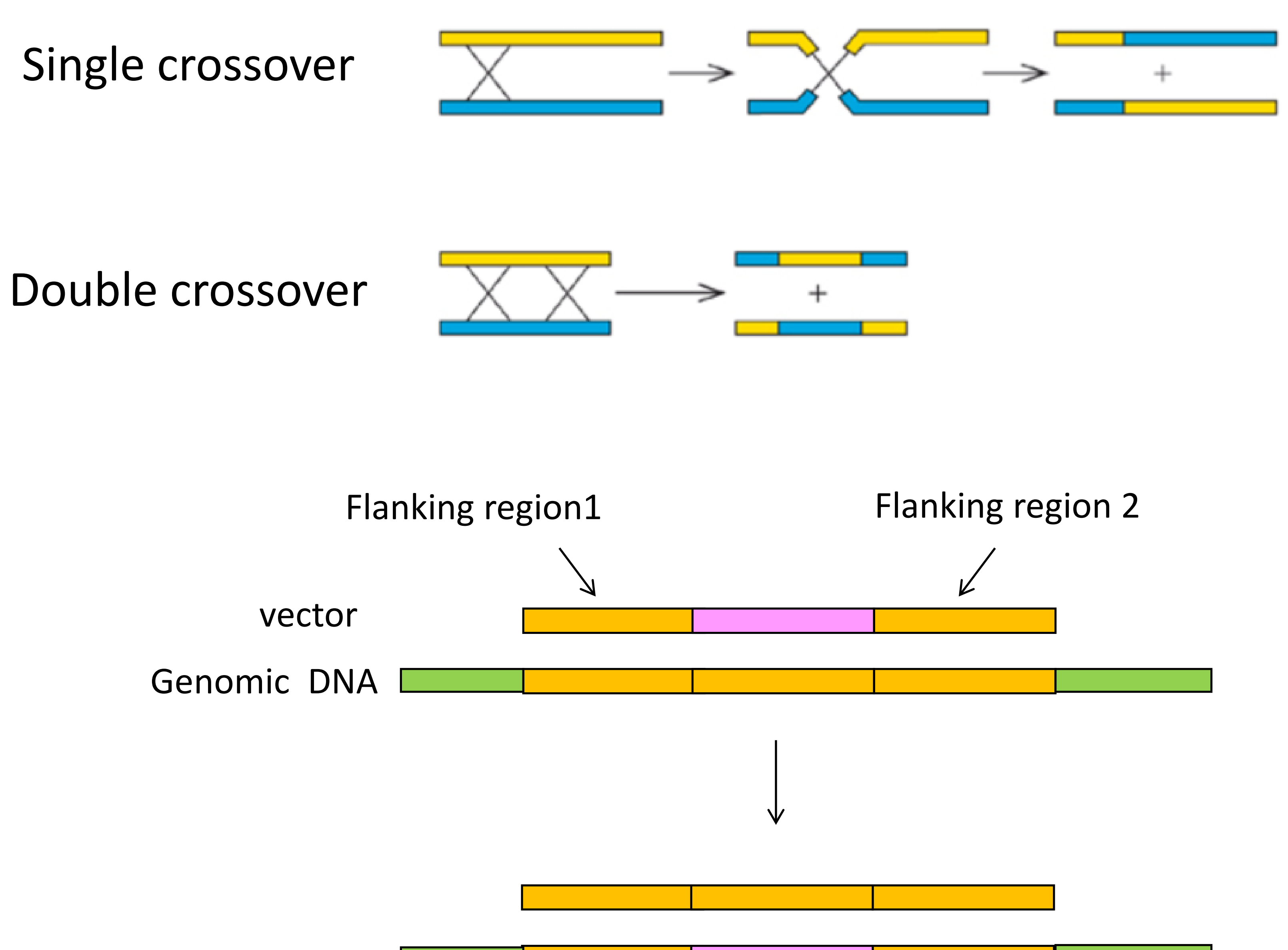
MEYOSIS II

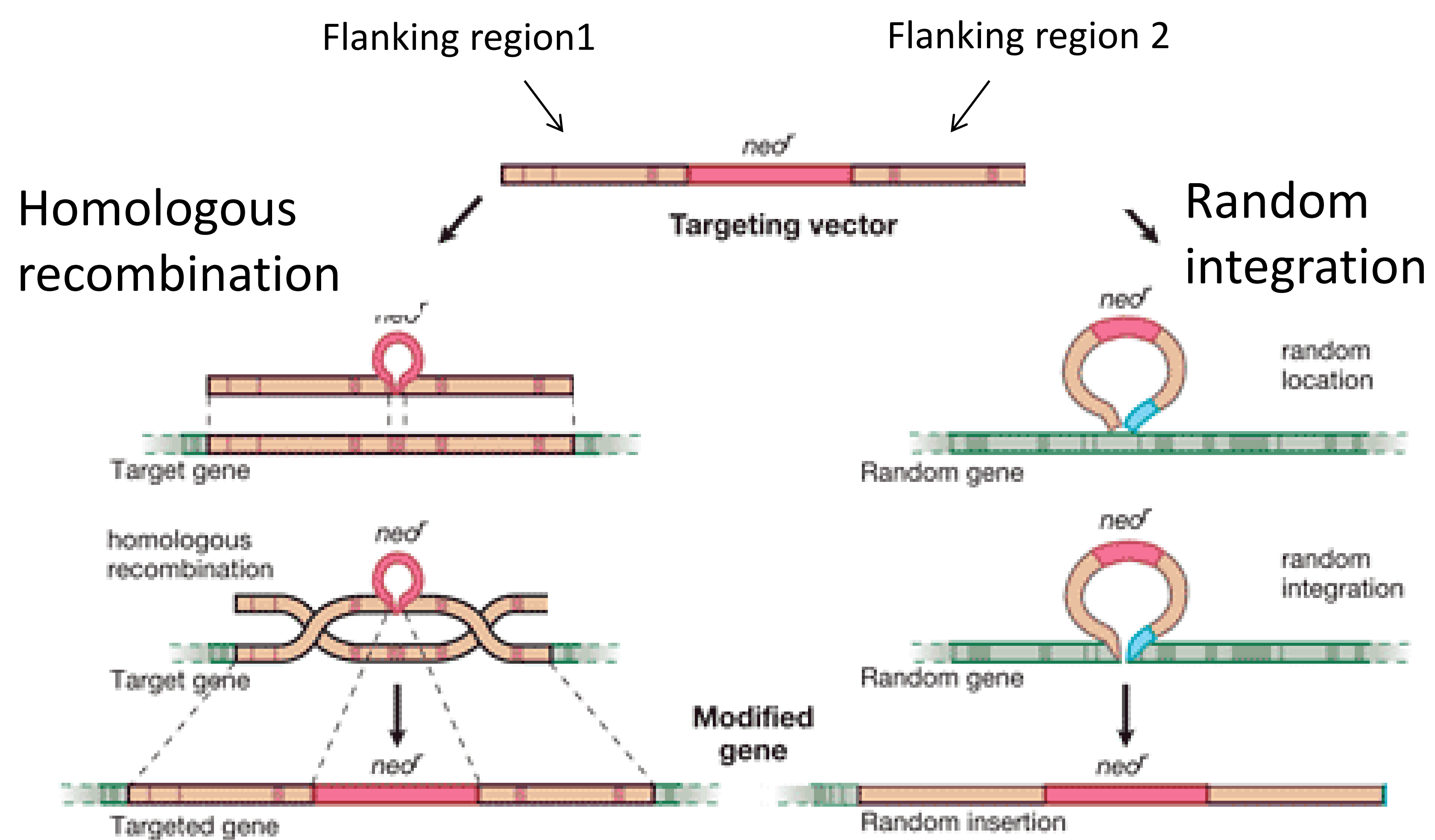


Crossing over



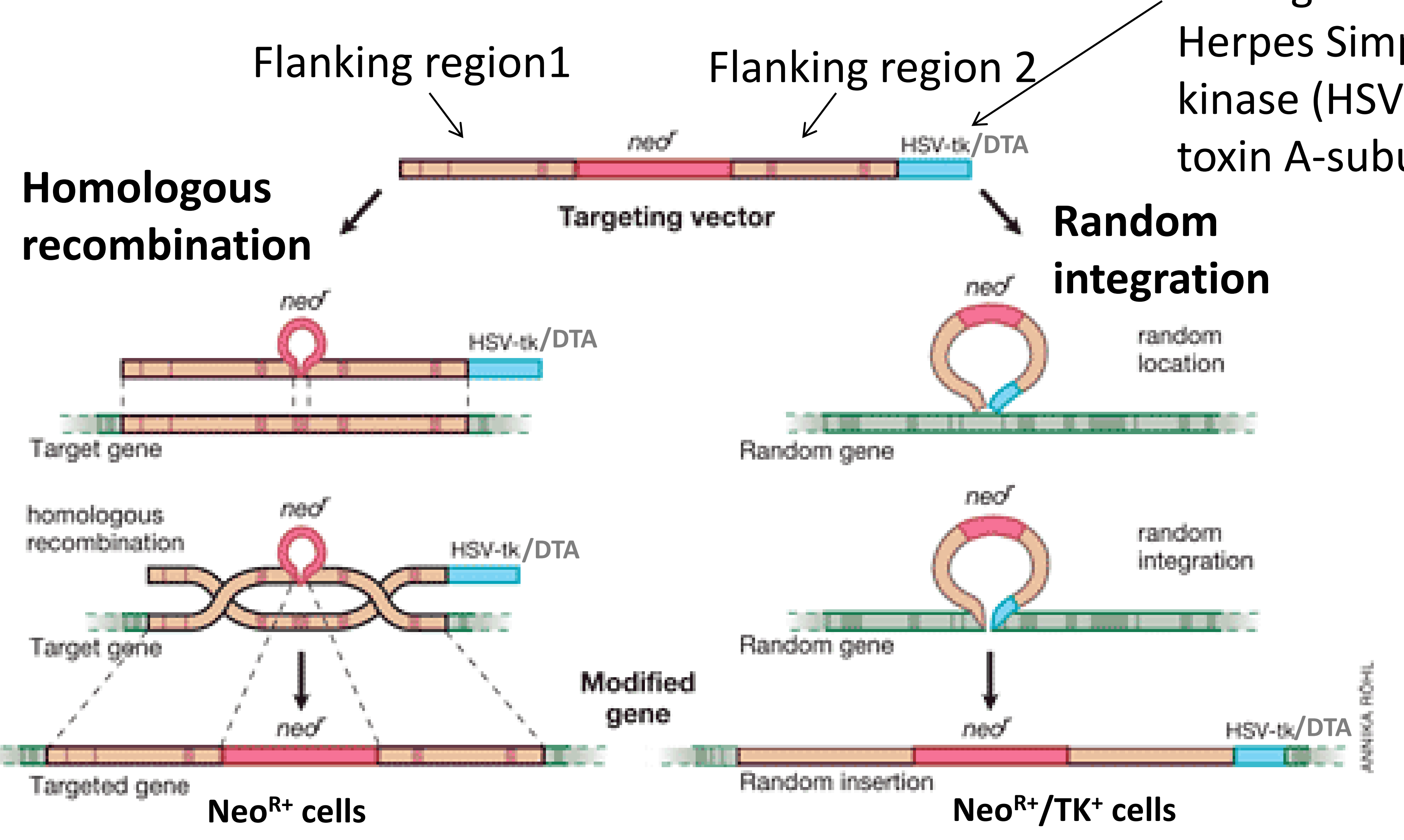
HOMOLOGOUS RECOMBINATION



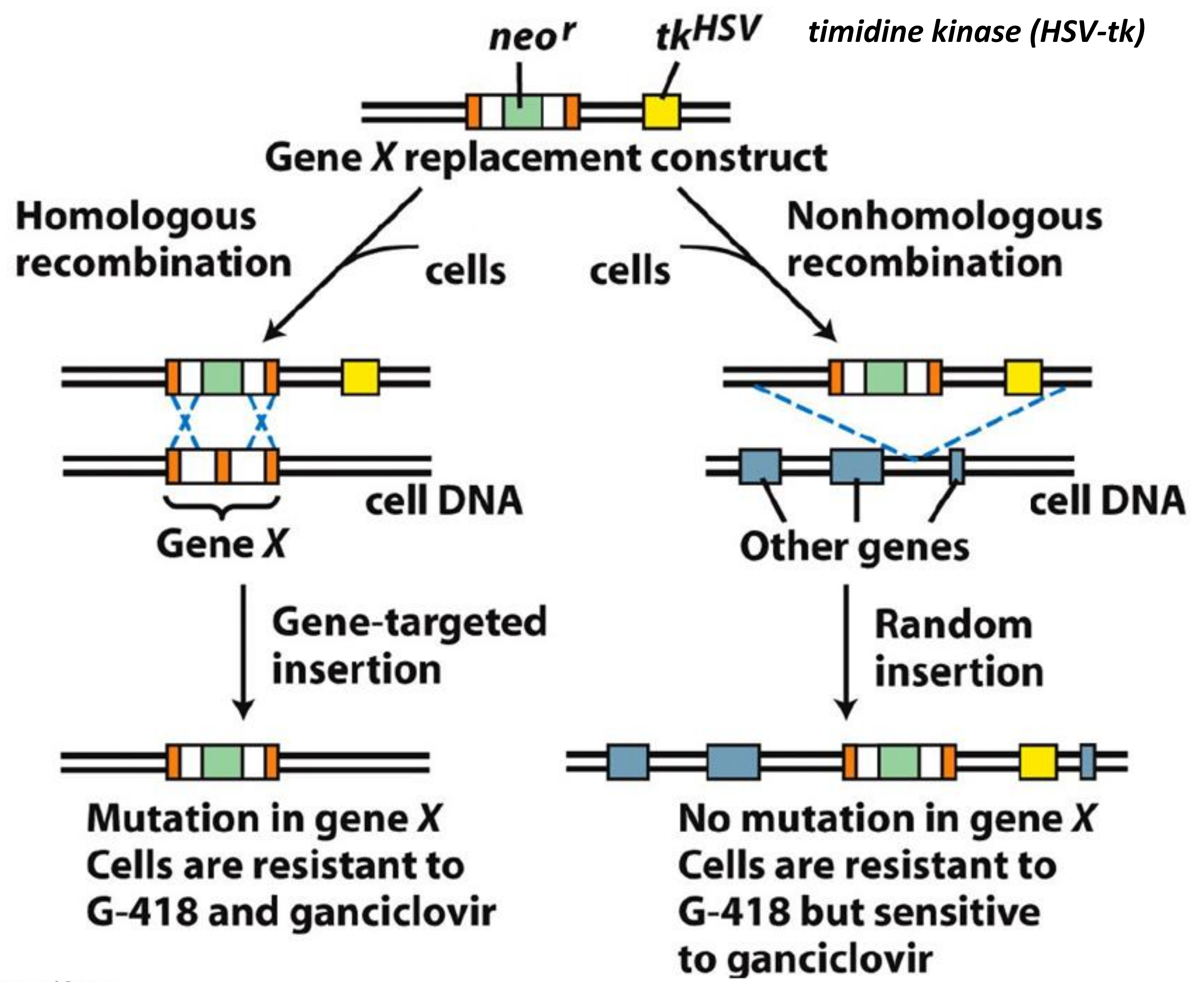


- Which strategy can you follow to obtain **homologous recombination** and distinguish it from **random integration**?

Adding to the construct the Herpes Simplex Virus Timidine kinase (HSV-tk) or Diphtheria toxin A-subunit (DTA)



HSV-tk will convert gancyclovir into a toxic drug and kills HSV-tk+ cells



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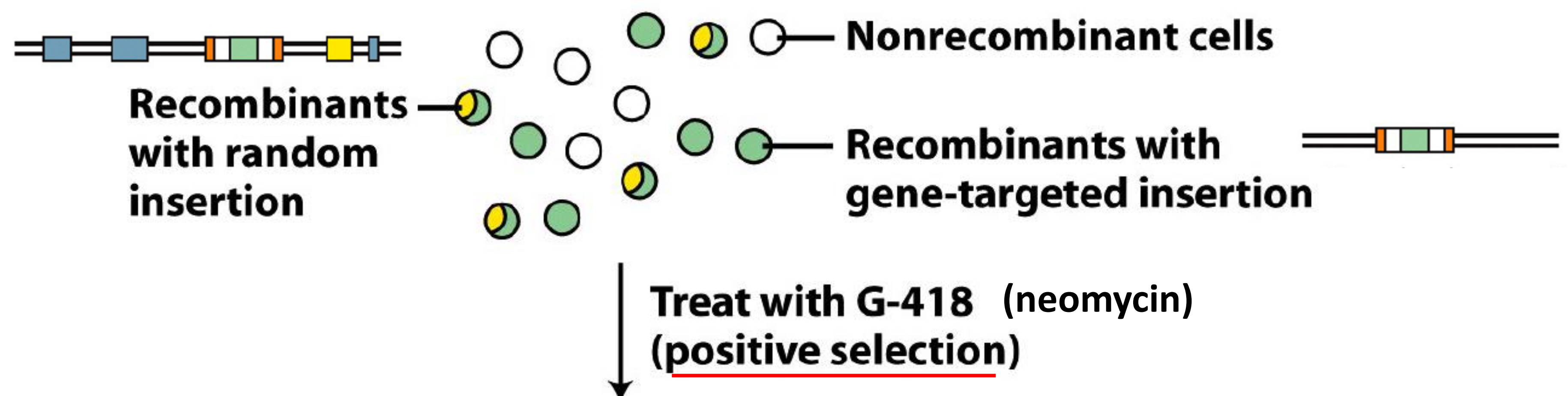
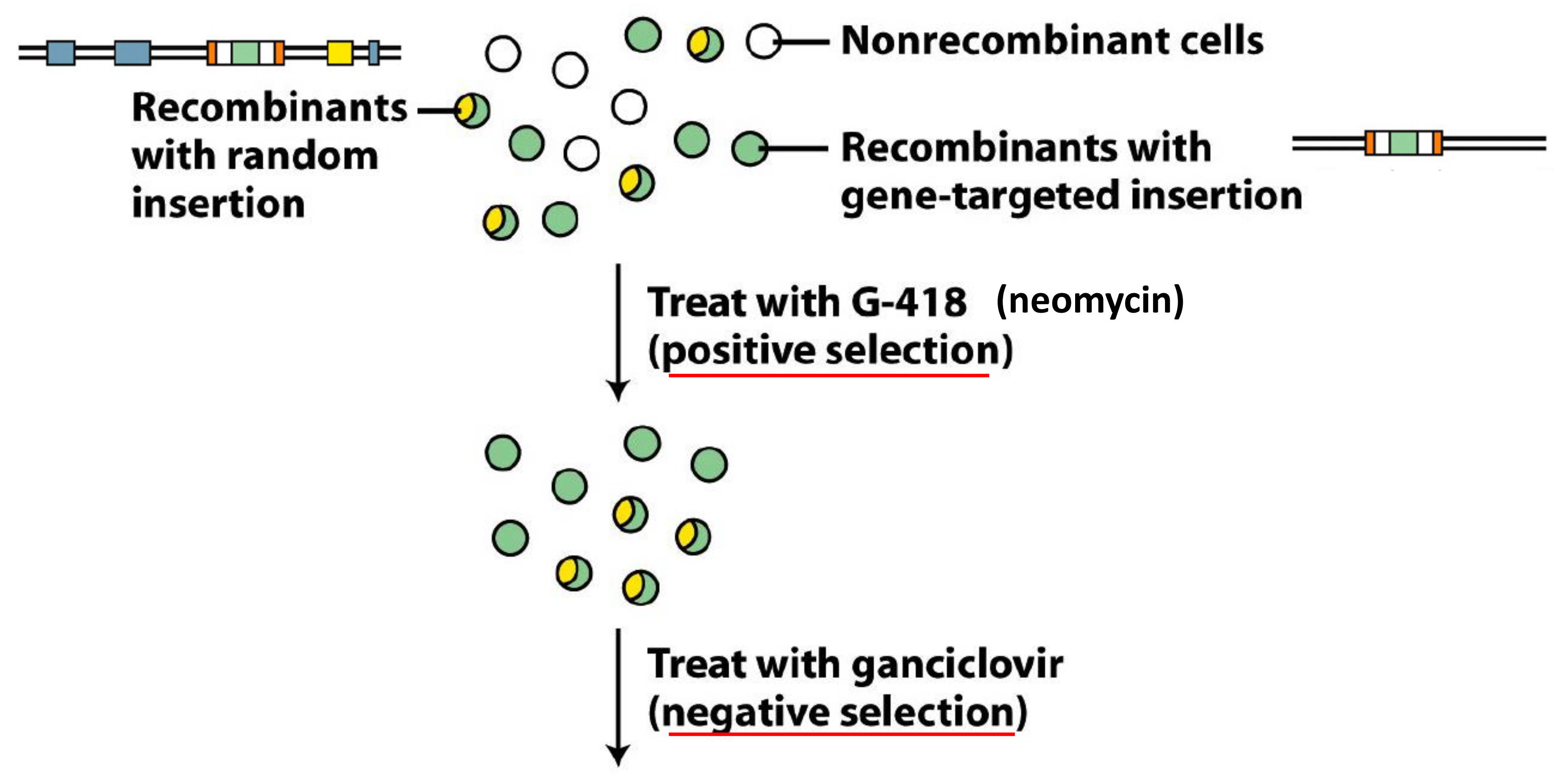
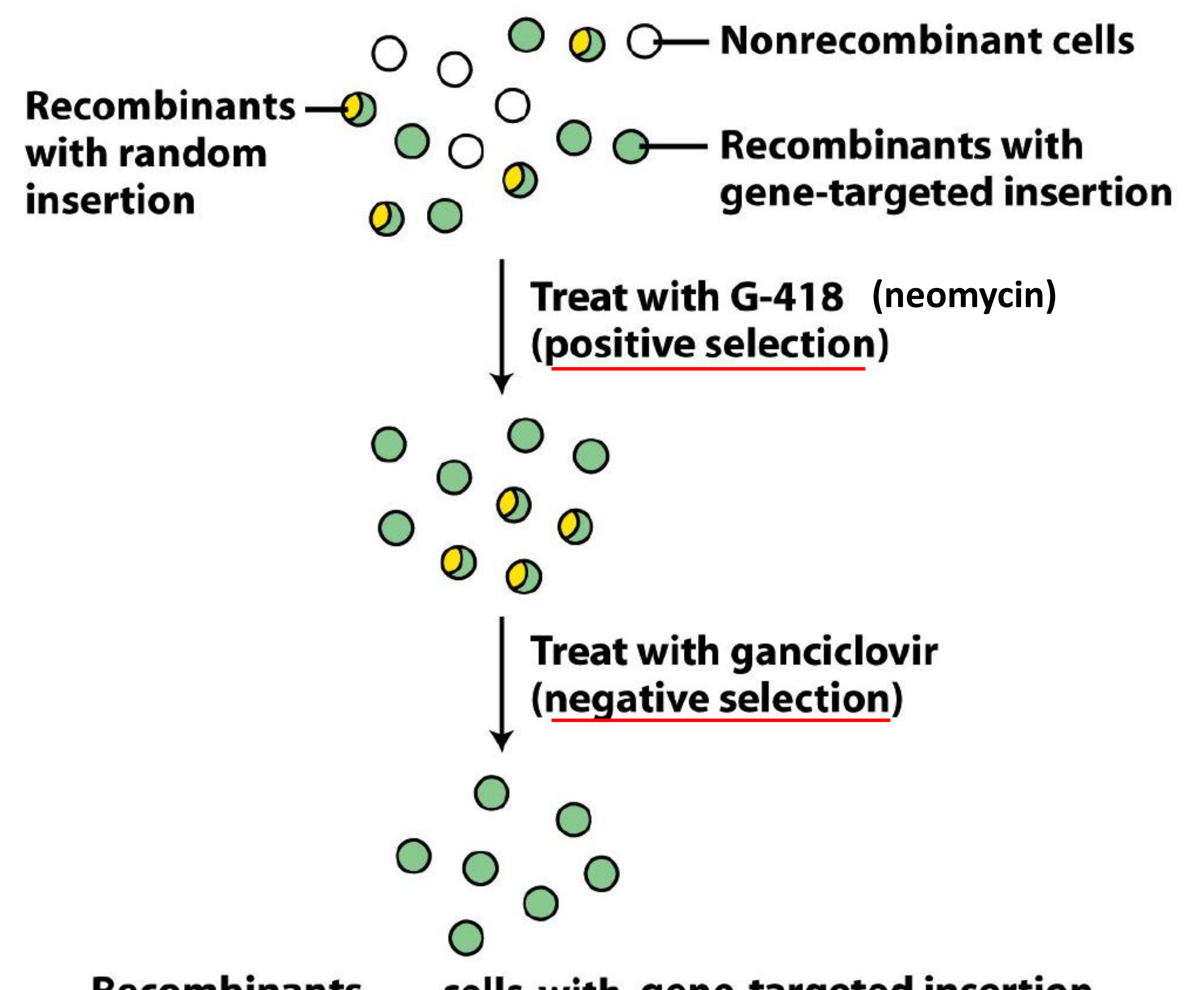

Nonrecombinant cells
Recombinants with random insertion
Recombinants with gene-targeted insertion
Treat with G-418
(neomycin)
(positive selection)

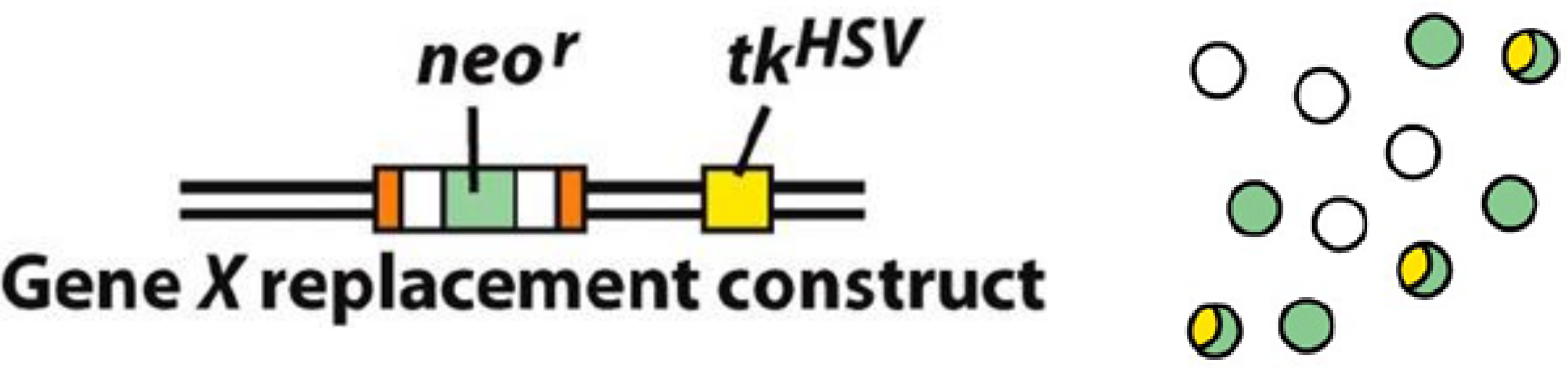


Nonrecombinant cells
Recombinants with random insertion
Recombinants with gene-targeted insertion
Treat with G-418
(neomycin)
(positive selection)
Treat with ganciclovir
(negative selection)

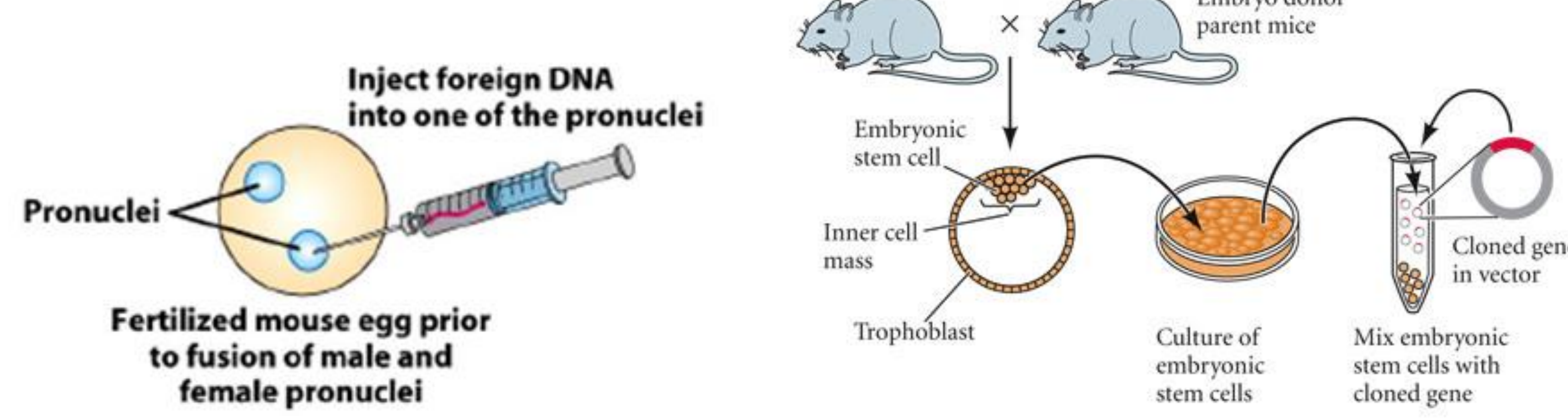


Nonrecombinant cells
Recombinants with random insertion
Recombinants with gene-targeted insertion
Treat with G-418
(neomycin)
(positive selection)
Treat with ganciclovir
(negative selection)
Recombinants
cells with
gene-targeted insertion

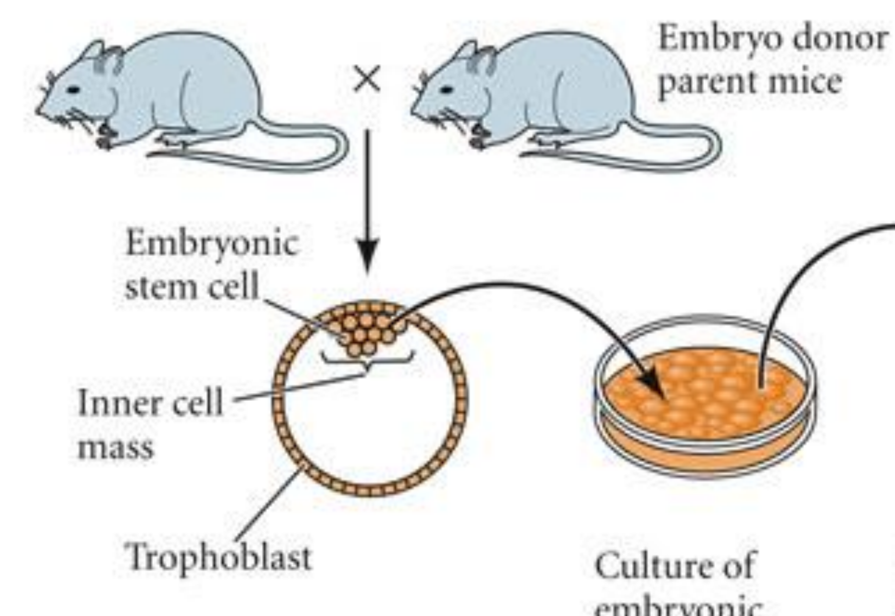
In which cells do we have to introduce this construct to obtain homologous recombination?



DNA microinjection in **fertilized eggs** or transfection of **embryonic stem cells**?



Embryonic stem (ES) cells

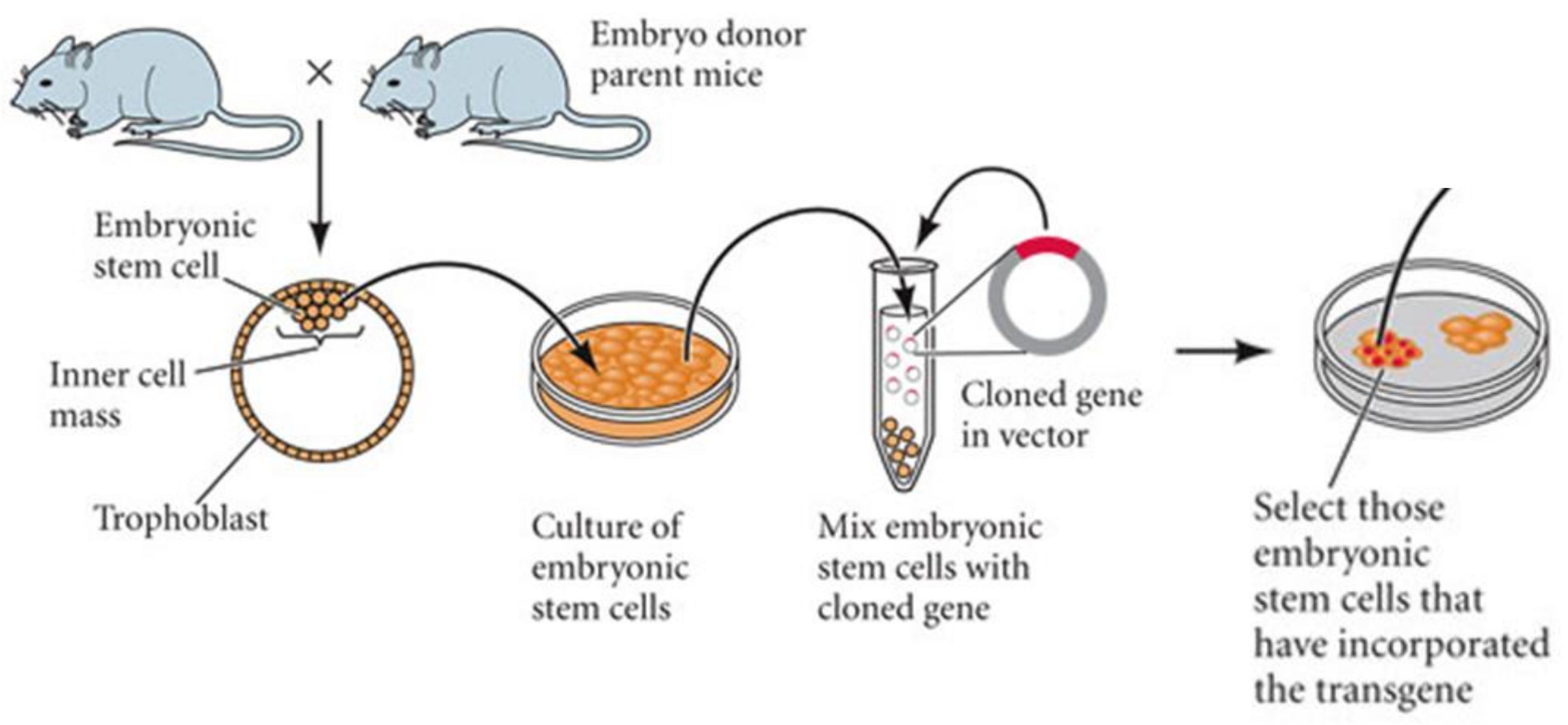


• During development, there is a stage (the **blastocyst**) when only two cell types are present:
1-the **outer trophoblast cells**, which will form the fetal portion of the placenta
2-the **inner cell mass**, whose cells will give rise to the embryo itself.

• if a cell of the **inner cell mass** from one mouse is transferred into the blastocyst of a second mouse, donor cells can contribute to every organ of the host embryo.

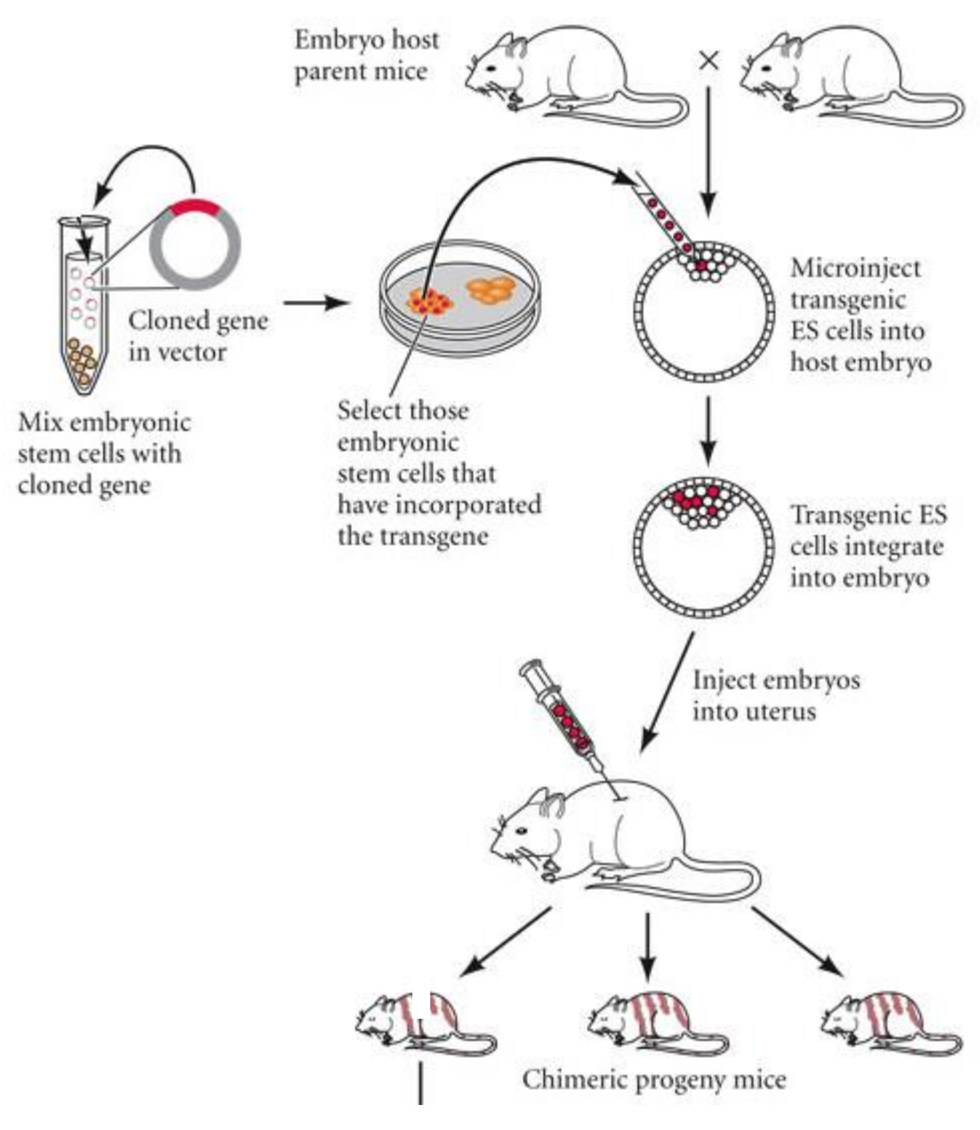
• Inner cell mass blastomeres can be isolated from an embryo and be grown in *in vitro* culture. These cells are called **embryonic stem cells** (**ES cells**). They retain their full potential to produce all the cells of the mature animal, including its gametes.

• it is not necessary to obtain ES cells from the inner cell mass every time, because **ES cell lines are available**.



ES cells are almost **totipotent**, since each of them can contribute to all tissues, except the trophoblast, if injected into a host embryo.

2- EMBRYONIC STEM CELL-MEDIATED GENE TRANSFER



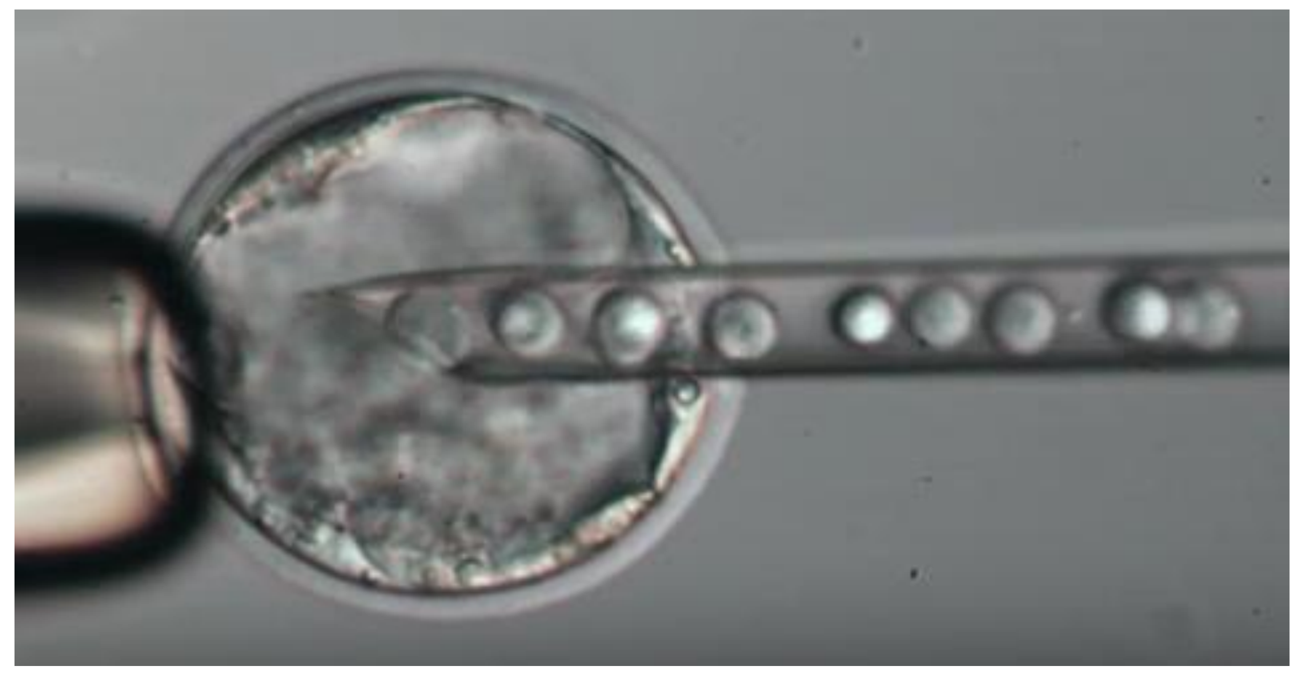
- stably transfect ES cells with the construct (using positive and negative selection as previously discussed: G418 and gancyclovir).

- microinject stably transfected ES cell into another host embryo; ES will integrate into the host.

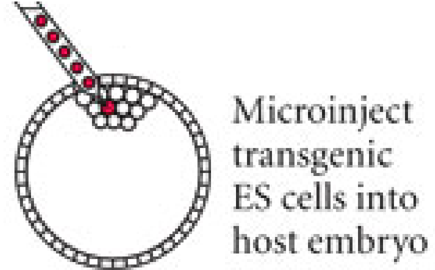
- implant the embryos into the uterus of a **pseudopregnant** foster mother.

The result is a **chimeric mouse**: some of the chimera cells will be derived from the host inner cell mass, some will be derived from the donor stably transfected embryonic stem cells.

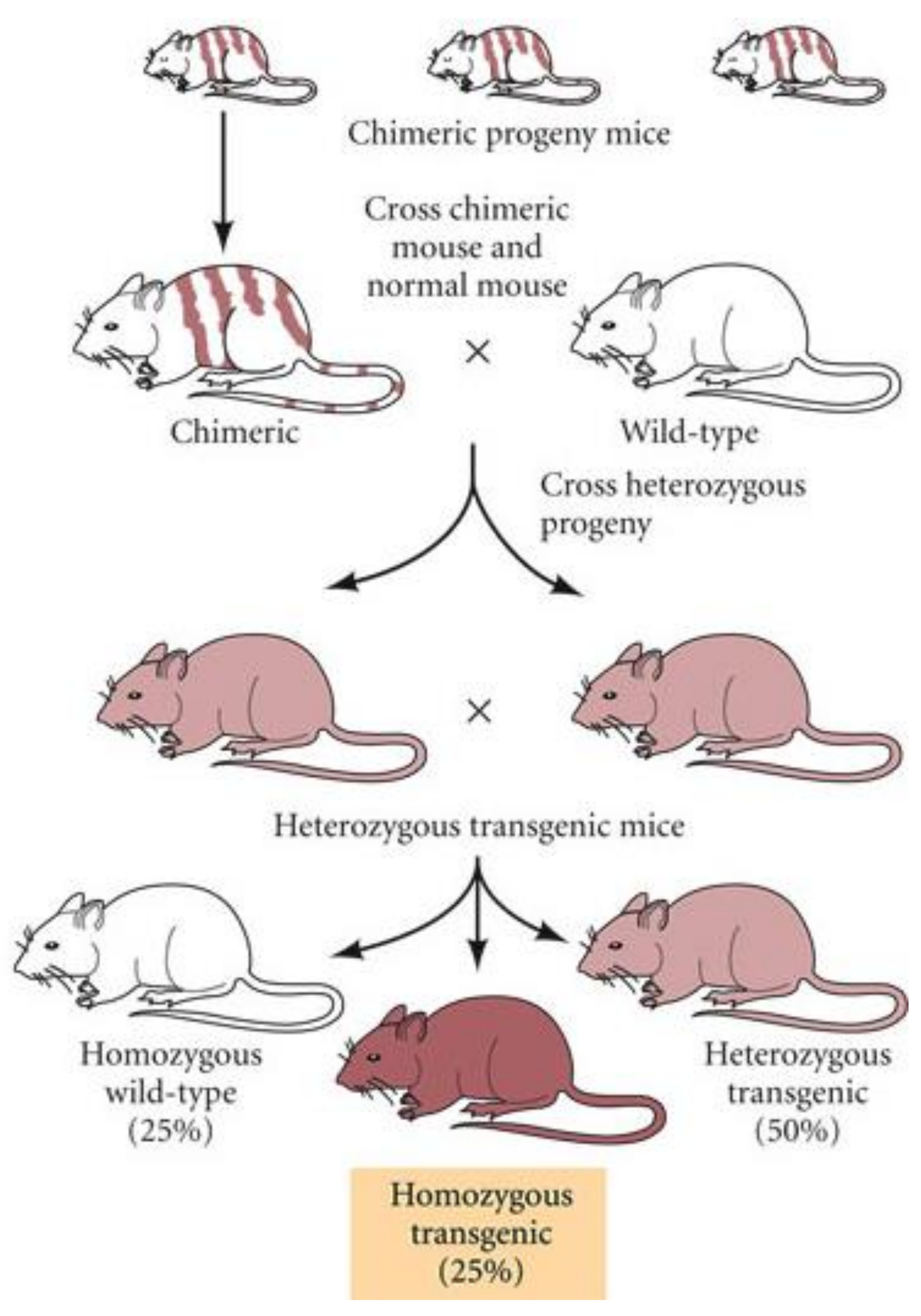
2- EMBRYONIC STEM CELL-MEDIATED GENE TRANSFER



https://www.youtube.com/watch?v=YFm0c4EEWQg



2- EMBRYONIC STEM CELL-MEDIATED GENE TRANSFER



- If the treated cells become part of the germ line of the mouse, some of its gametes will be derived from the donor cell.
- If the chimeric mouse is mated with a wild-type mouse, some of its progeny will carry one copy of the inserted gene.
- When these heterozygous transgenic mice are mated to one another, about 25% of the resulting offspring will be homozygous for the inserted gene in every cell of their bodies (:the inserted gene will be present in both chromosomes).

Mario Capecchi, Martin Evans and Oliver Smithies were awarded the **2007 Nobel Prize for Physiology or Medicine** in recognition of their discovery of how **homologous recombination** can be used to introduce **genetic modifications** in mice through embryonic stem cells.

Mario Capecchi

Martin Evans

Oliver Smithies

NEO^R

Neomycin resistance gene encodes an **aminoglycoside 3‘-phosphotransferase**.

G418, also known as G418 sulfate and Geneticin, is an aminoglycoside antibiotic similar in structure to gentamicin B1, produced by *Micromonospora rhodorangea*.

G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells.

Selection in **mammalian cells** is usually achieved in three to seven days with concentrations ranging from 400 to 1000 µg/ml.
Cells that are dividing are affected sooner than those that are not.

NEO^R cassette contains promoter sequences, a cDNA coding for aminoglycoside 3‘-phosphotransferase, followed by a polyadenilation site.

1 - injecting the construct into the pronucleus of a fertilized mouse egg

2- embryonic stem cell-mediated gene transfer

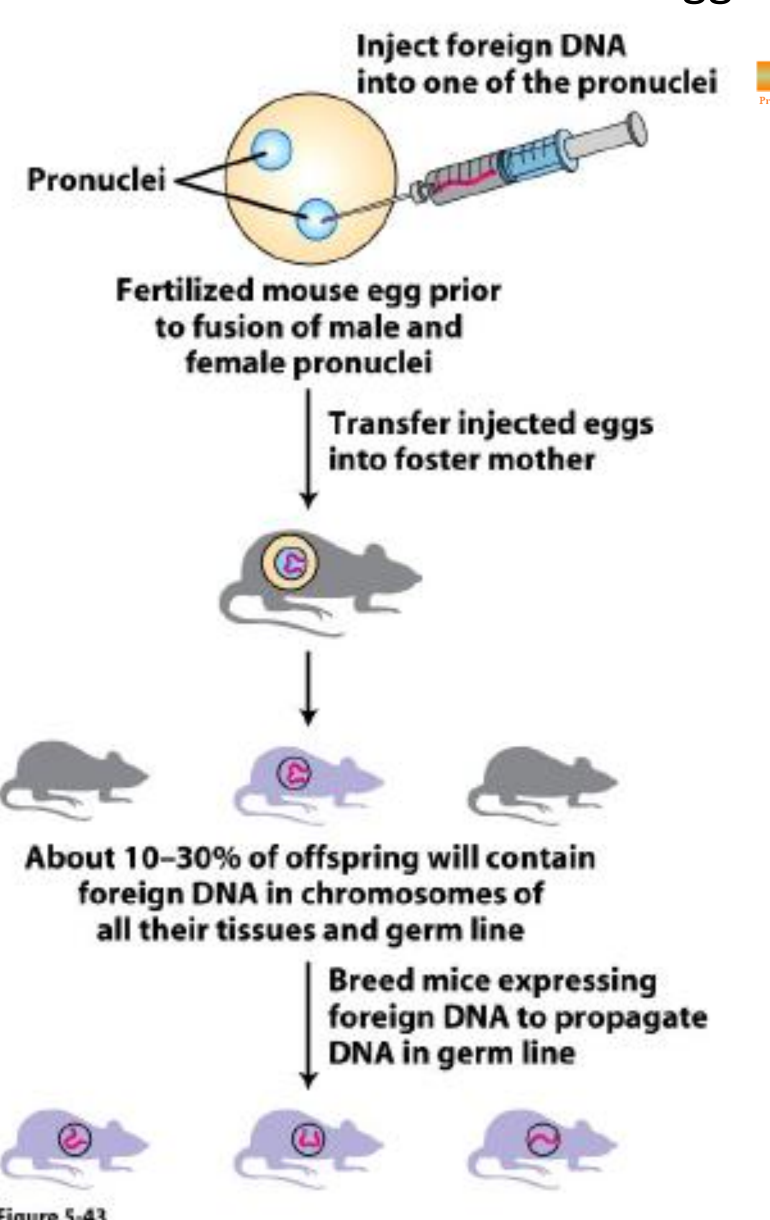


Figure 5-43
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Promoter | Intron | **Transgene** - expressing cDNA for gene of interest | PolyA

Embryo host parent mice

Mix embryonic stem cells with cloned gene

Cloned gene in vector

Select those embryonic stem cells that have incorporated the transgene

Microinject transgenic ES cells into host embryo

Transgenic ES cells integrate into embryo

Inject embryos into uterus

Chimeric progeny mice

GENETIC MODIFICATION of CELLS

- transient vs stable transfection of cells

TRANSGENIC ANIMALS

- Two methods to produce transgenic animals:

1- DNA microinjection
  - random insertion

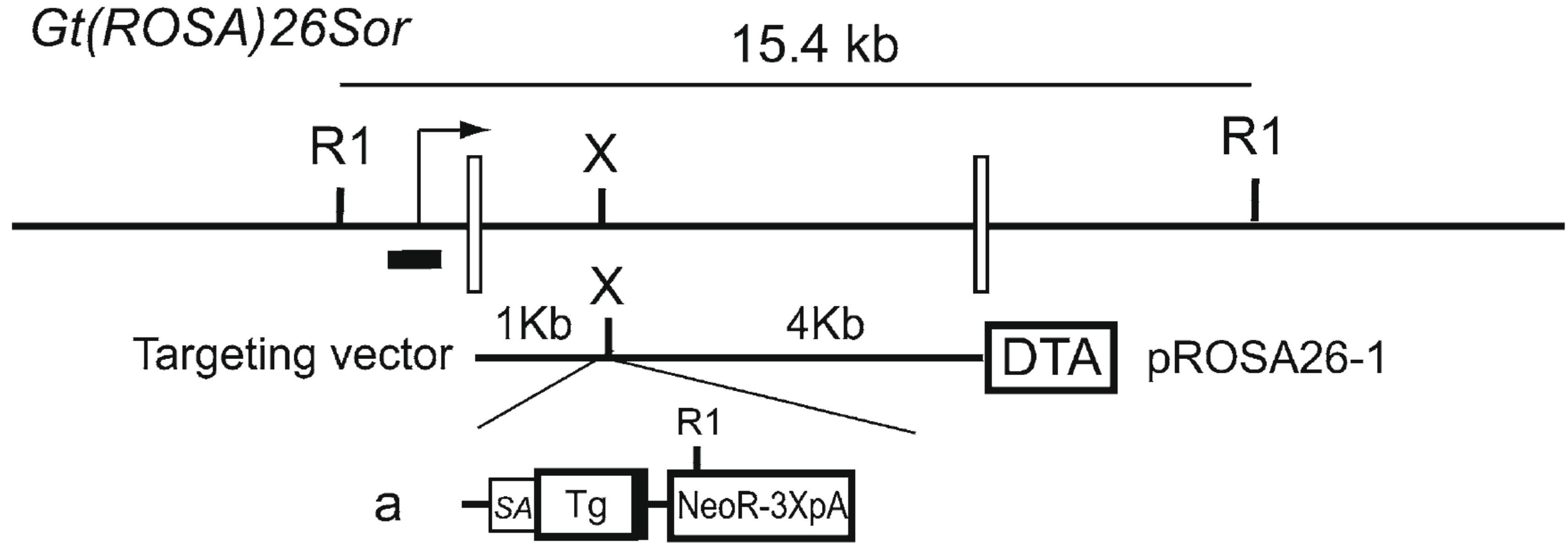
2- embryonic stem cell-mediated gene transfer
  - random insertion
  - homologous recombination (double selection)
  - Rosa26 locus
  - genomic analysis to identify genetically modified animals

- knock-out animals
- knock-in animals

- conditional knock-out (cre-lox technique, inducible systems)
- siRNA

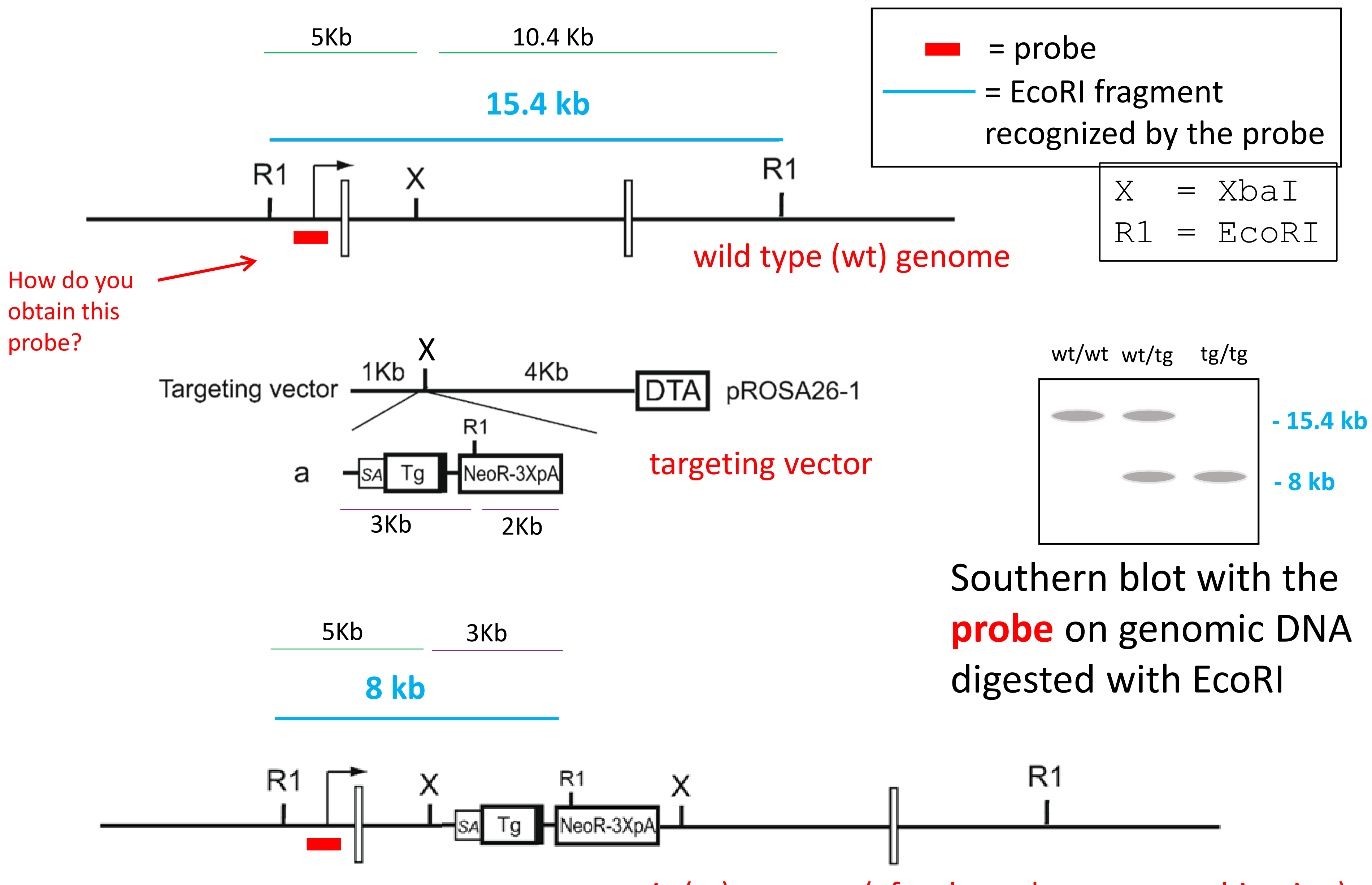
The ROSA26 locus

Targeting strategy to insert single-copy transgenes (Tg) into the ROSA26 locus.



• pROSA26-1 is an example of targeting vector used to insert transgenes into intron 1 of the ROSA-26 gene
• the basic vector contains 5 kb of ROSA26 genomic sequences to drive homologous recombination and the gene for the **Diphtheria toxin A-subunit (DTA)** as negative selection marker
• the transgene (Tg), in most cases preceded by a splice acceptor (SA) site, is cloned into a unique Xba1 (X) site

Genomic analysis to identify genetically modified animals



Nucleic acids are readily labeled with tags that facilitate detection or purification. A variety of enzymatic or chemical methods are available to generate nucleic acids labeled with radioactive phosphates, fluorophores, or nucleotides modified with biotin or digoxygenin for example.

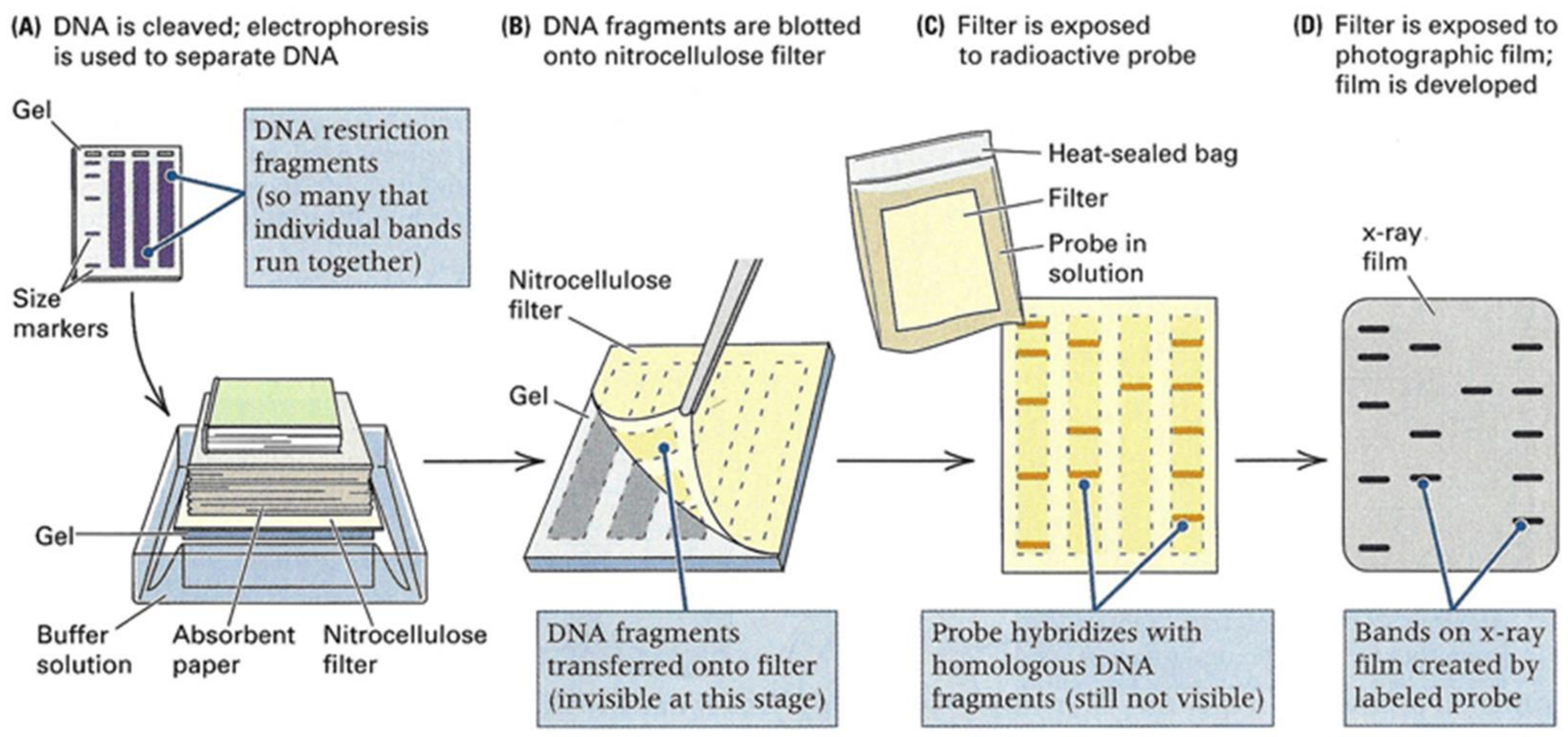
Nucleic acids may be labeled at their 5´ end, their 3´ end, or throughout the molecule depending on the application.

For hybridization reactions (Southern or northern blotting) it is usually advantageous to generate high specific activity probes with label distributed throughout the nucleic acid, through techniques such as nick translation, random priming, by PCR or in vitro transcription using labeled dNTPs or NTPs.

For applications involving protein interactions, such as gel-shift assays or pull-downs, it is generally beneficial to generate end-labeled probes to prevent steric interference of the interaction. This can be achieved with end-labeling protocols or with PCR using primers bearing the required modification.

Application	Reaction	Recommended Enzyme
DNA 5´ End Labeling	5´ 5´	T4 Polynucleotide Kinase
Labeling by PCR		*Taq* DNA Polymerase
DNA 3´ End Labeling	3´	Terminal™ Transferase
	3´	Klenow Fragment (3´ → 5´ exo-)
Single Nucleotide Terminator Labeling		Therminator DNA Polymerase
Random Priming	Random primers	Klenow Fragment (3´ → 5´ exo-)
Nick Translation	Nick	DNA Polymerase I (*E. coli*)

Southern blot



Can PCR be used to identify transgenic animals?