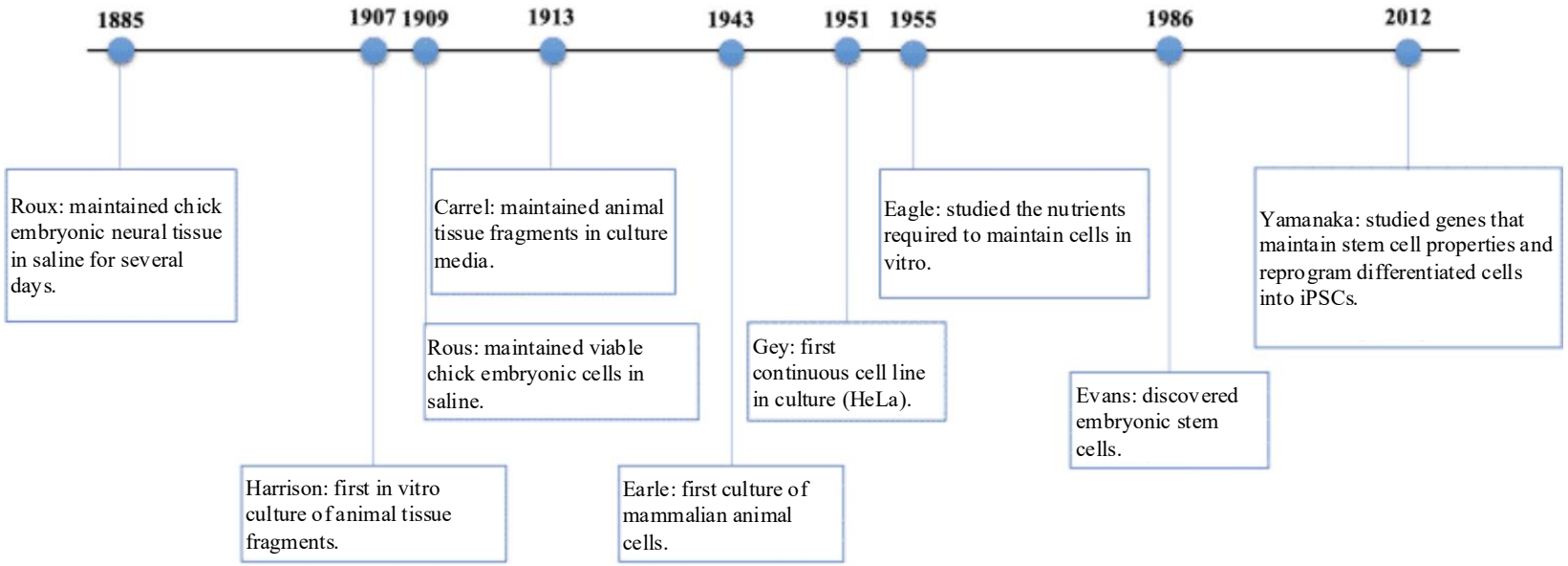
Cell cultures



Cell cultures



Cell cultures

Types of cell culture:

- primary cultures
- cell lines
- stem cells

APPLICATIONS OF CELL CULTURE

Study of cellular activities: transcription, protein synthesis, energy metabolism, drug metabolism, the cell cycle, differentiation, apoptosis.

Study of intracellular dynamics: RNA, hormone receptors, metabolites, calcium, signal transduction pathways, membrane trafficking.

Study of environmental effects: infection, drug action, ligand–receptor interactions, cytotoxicity, mutagenesis, carcinogenesis.

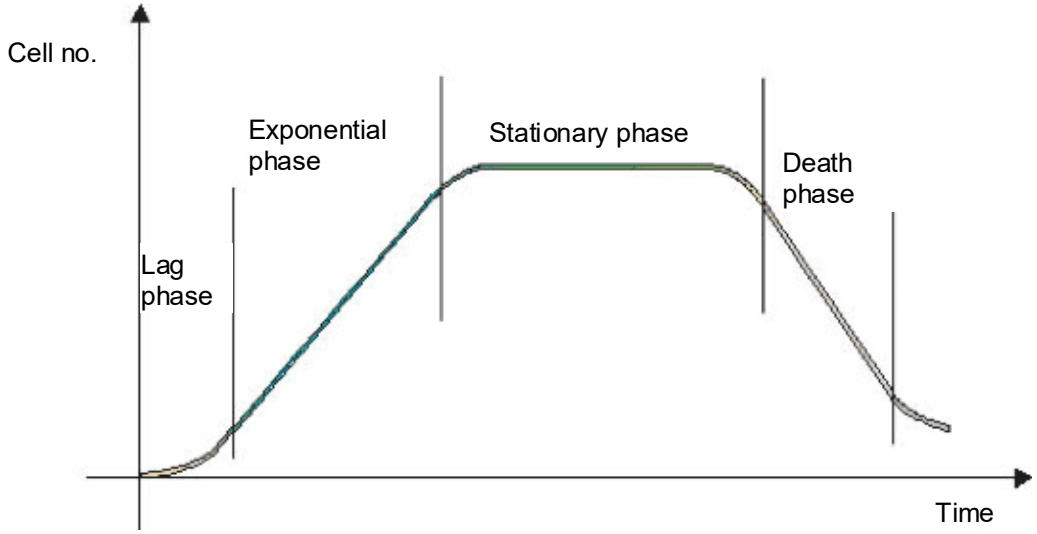
Cell products: secretion of therapeutically important proteins, vaccines, bioreactors, post-translational modifications and product accumulation.

Genetics: genetic analysis, transfection, infection, transformation, immortalization, senescence.

Cell–cell and cell–extracellular environment interactions: morphogenesis, paracrine regulation, cell proliferation, adhesion, motility, invasion and matrix interactions.

Growth curve

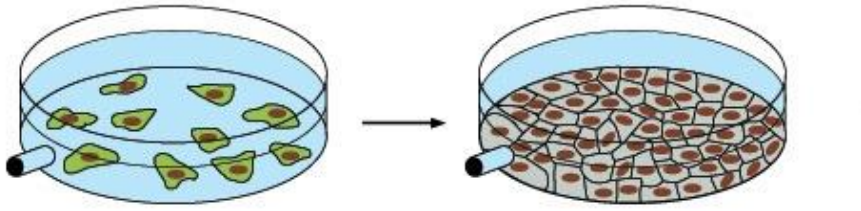
The growth and lifespan of a cell culture are represented by **its growth** curve, which varies with the type of culture.

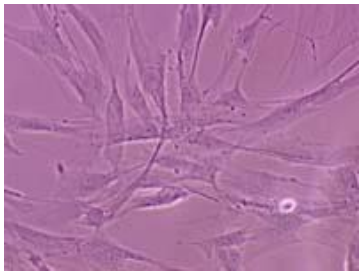


Established cell lines show neither a stationary phase nor a death phase. In these cultures, growth remains exponential because the cells have undergone immortalization.

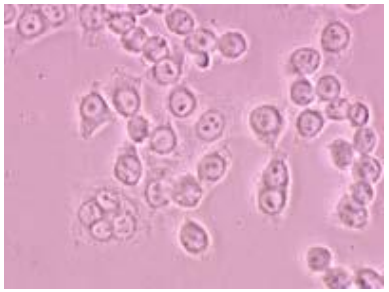
CELL GROWTH

Growth as a monolayer





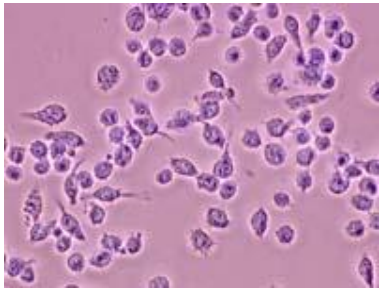
fibroblasts



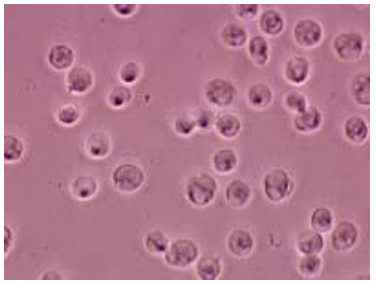
keratinocytes

Growth in suspension





monocytes



lymphocytes

Maintaining a cell culture in vitro

Culture medium: each cell line requires a specific medium. The basis of a culture medium is an isotonic salt solution supplemented with components essential for cell survival, each with a specific function:

•Inorganic salts – control of osmotic pressure and electrolyte balance;

•Amino acids – required for protein synthesis;

•Vitamins and cofactors – essential for regulating multiple cellular functions;

•D-glucose or pyruvate – energy source;

•Phenol red (usually) – pH indicator (yellow at pH 6.5, orange at pH 7.0, red at pH 7.4, violet-red at pH 7.6 and purple at pH 7.8);

•Sodium bicarbonate/HEPES – buffering system.

Antibiotics are added to the culture **medium to** prevent bacterial contamination and, for some cell lines only, growth **factors: G-CSF,** GM-CSF, IL-2, IL-3, IL-6, SCF, EGF, acidic and basic FGF, NGF, etc.

Serum*: fetal bovine serum (FBS) – (Australia, New Zealand, USA, Latin America, etc.)

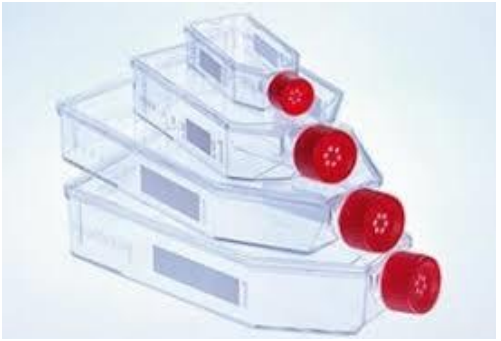
* Serum quality greatly affects cell culture.

Maintaining a cell culture in vitro

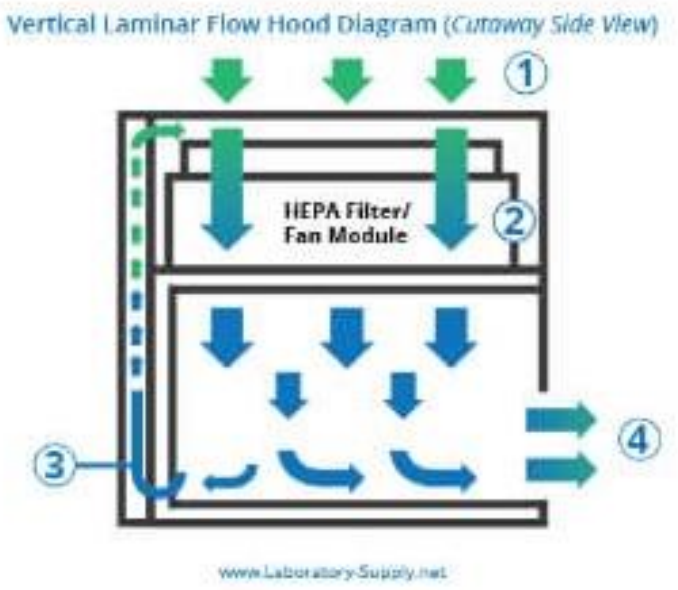
Culture vessels (plastic)







Vertical laminar flow cabinet



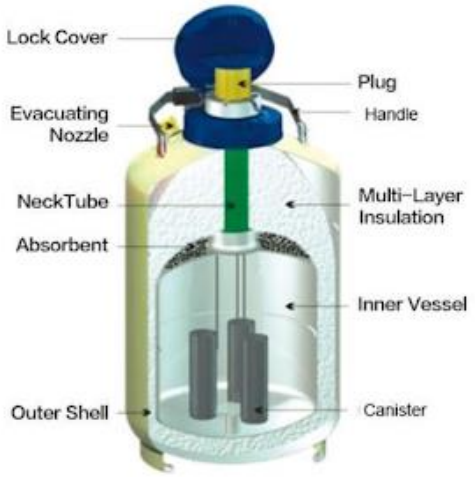


CO2 incubator (5%)
37° C



Maintaining a cell culture in vitro

Cell lines are stored in liquid nitrogen



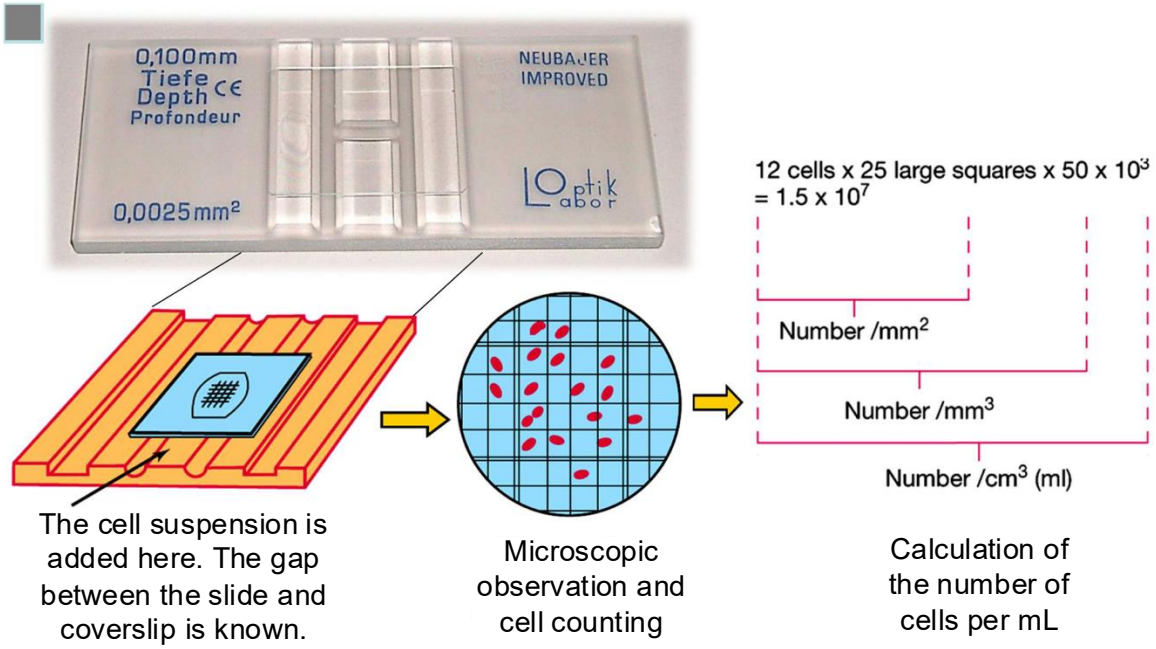
Storage Type





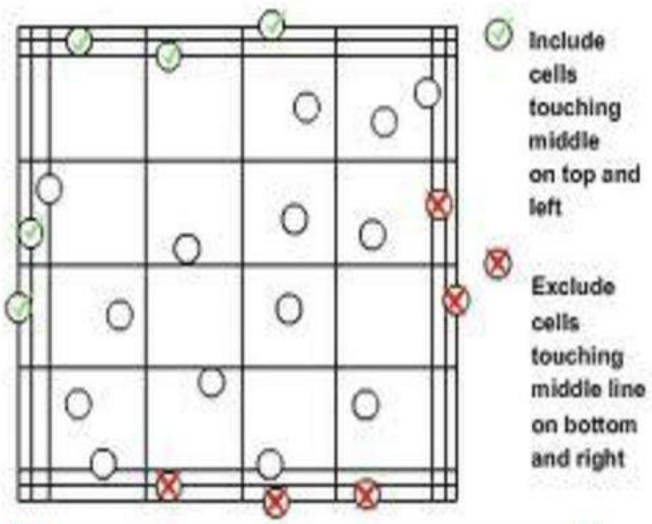


Cell counting with a Bürker chamber



Trypan blue **staining is** commonly used to count viable cells. This method relies on the principle that live cells exclude the dye, whereas dead cells take it up and appear blue under the microscope.

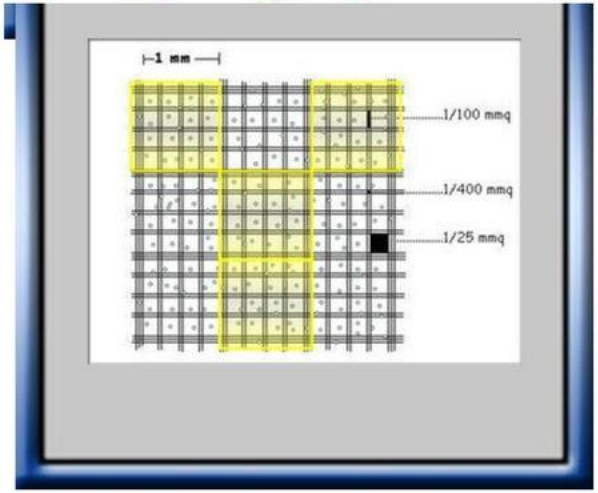
Cell counting with a Bürker chamber

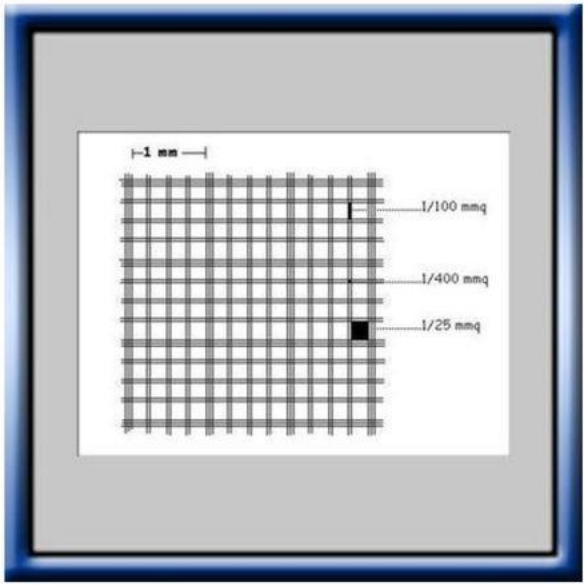


BÜRKER CHAMBER

$$C = n° \times 10 \times 10^4 \times V$$

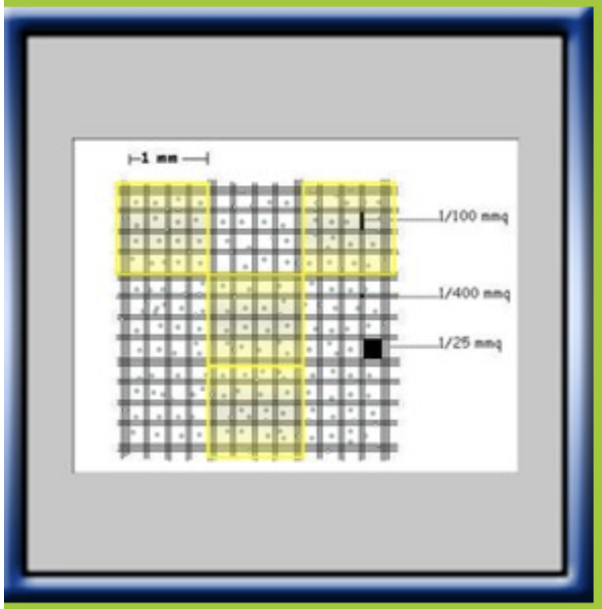
C = cells per mL
n° = (n1+n2+n3+n4)/4
10 = dilution factor
10^4 = conversion factor
V = cell suspension volume





Cell counting with a Bürker chamber

Count cells in 4 squares and calculate the mean to reduce the margin of error.



Cell counting with the Bürker chamber grid:
1) Count cells in the 4 large squares arranged in a Y. Exclude cells within the 3 border lines on two sides only. Example: 84 cells.
2) Calculate the mean:
$84 \div 4 = 21$
3) 21 cells in 0.1 mm^3
4) Multiply by 10^4 to obtain the number of cells per cm^3.
1 cm^3 = 1 mL

21×10^4
cells per mL

Cell counting with an automated counter

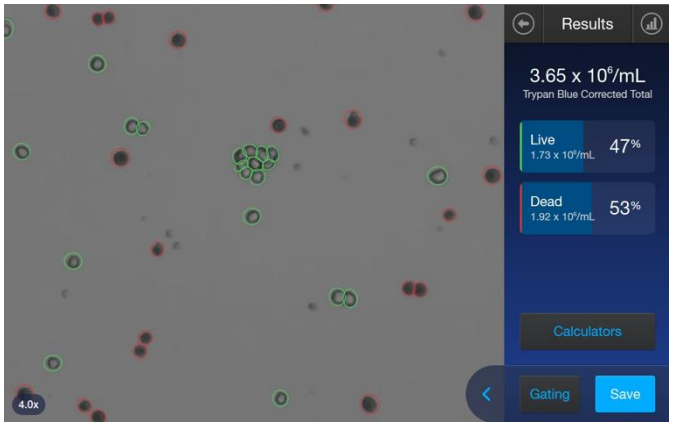












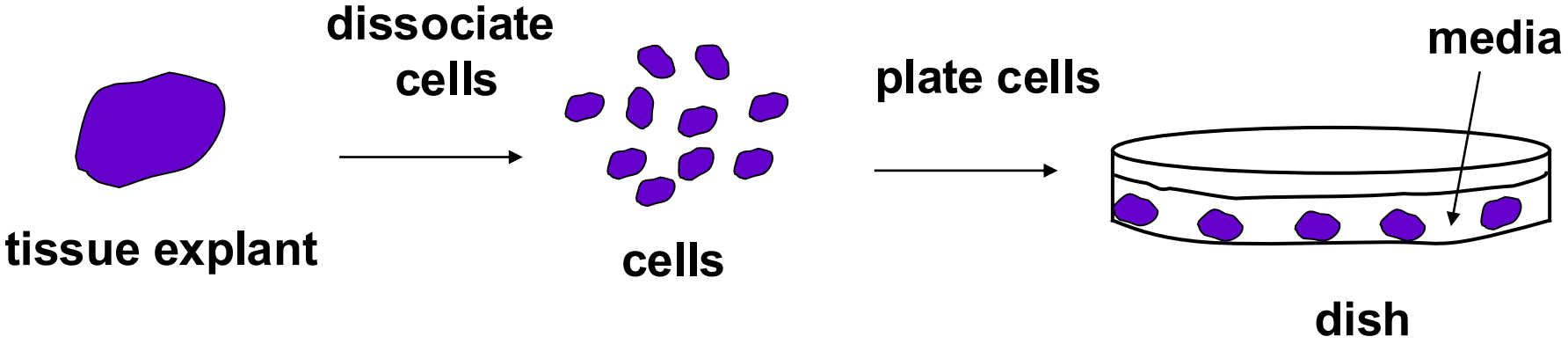
Cell cultures

Types of cell culture:

- primary cultures
- cell lines
- stem cells

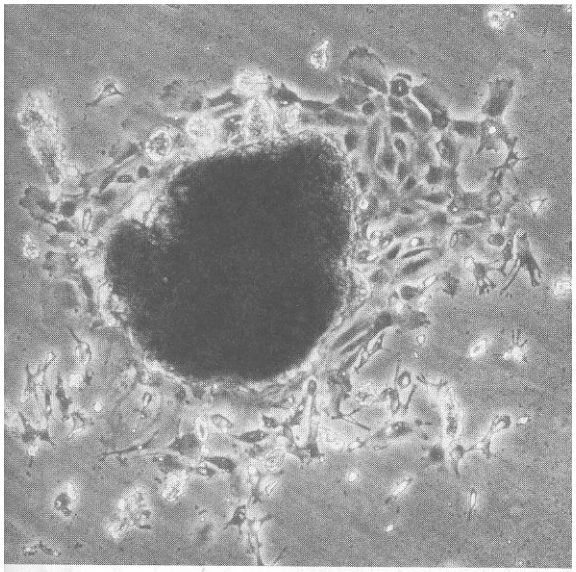
Primary cultures

- **Cells obtained directly from tissues (primary explant)**

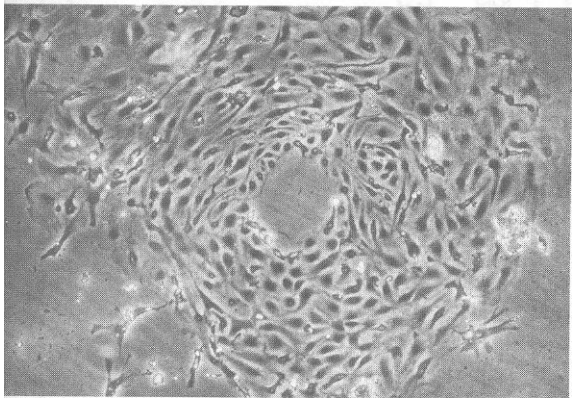


- **Grow and divide in culture dishes**
- **Require specific growth media**
- **Die after 50–100 divisions**

Primary explant culture



3-day culture



7-day culture after removal of the explant

Cell lines

Can originate from several sources:

1. **primary cultures**
2. **transformed cells**
3. **tumor cells**

Cell lines

1) From primary cultures ("normal cells")

- **Rare genetic mutations can produce cells that grow indefinitely (immortalized cells)**

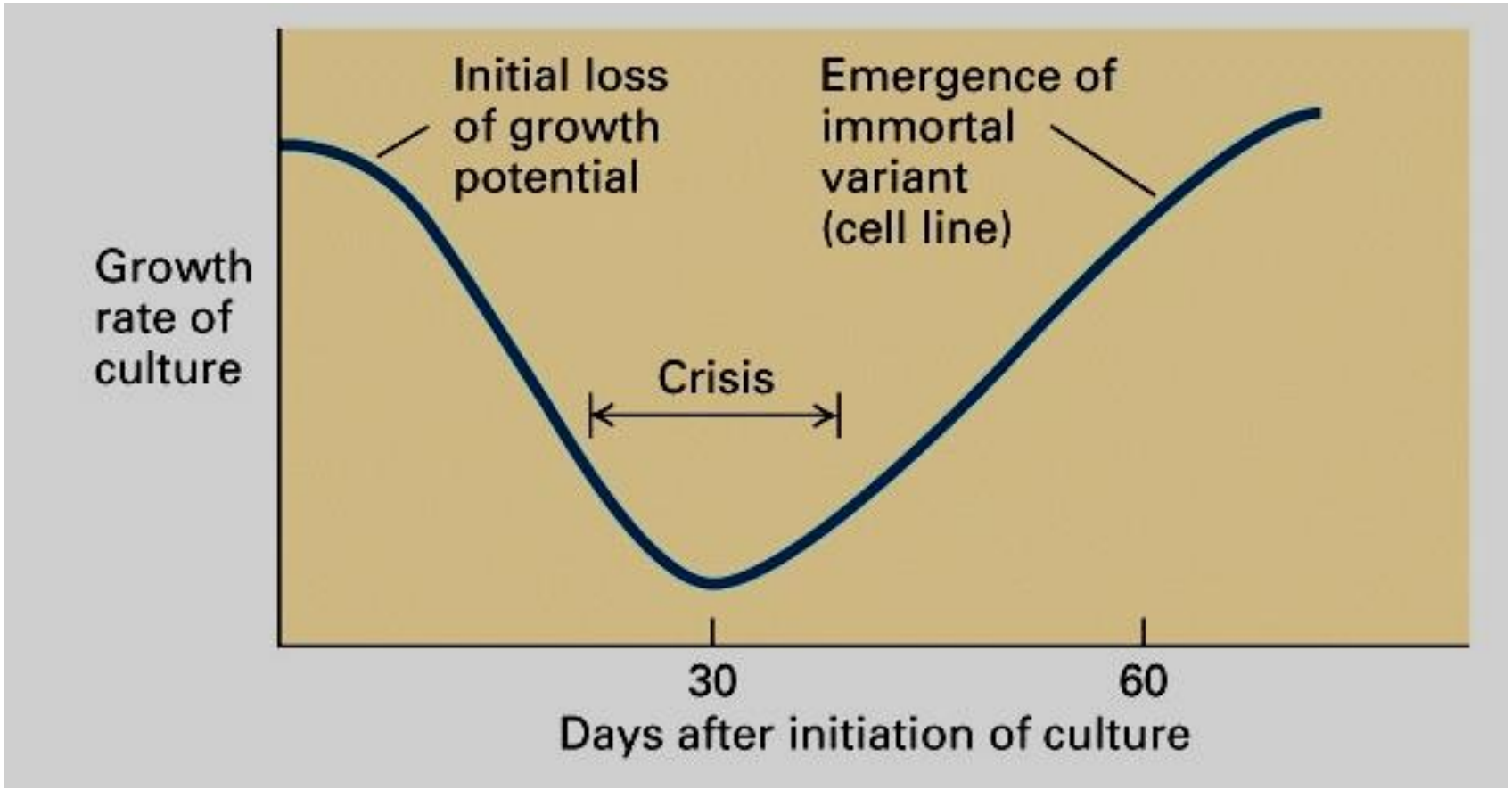
  e.g., BSC-1 cell line (derived from African green monkey kidney)

- **Grow until they contact neighboring cells, then exit the cell cycle (contact inhibition)**

- **Do not form tumors when injected into mice**

Cell lines

Mouse cell line derived from a primary culture



Cell lines

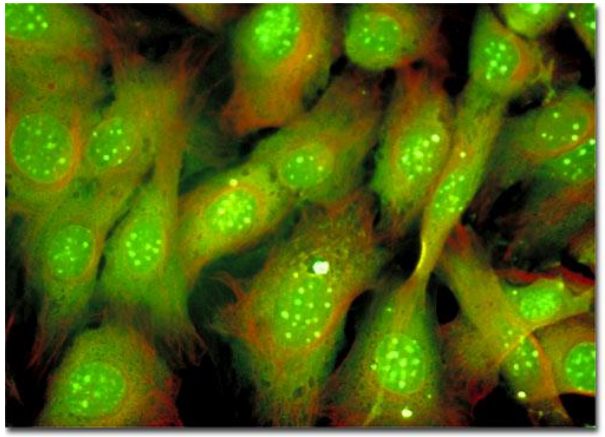
2) From transformed cells

Further genetic changes:
Can be caused by radiation, chemical carcinogens or tumor viruses
Cause morphological changes, loss of growth control and loss of contact inhibition

e.g., 3T3-21F

- **Abnormal karyotypes (Diploidy, heteroploidy and haploidy).**

- **Form tumors when injected into mice**



Cell lines

2) From transformed cells

Immortalized cells

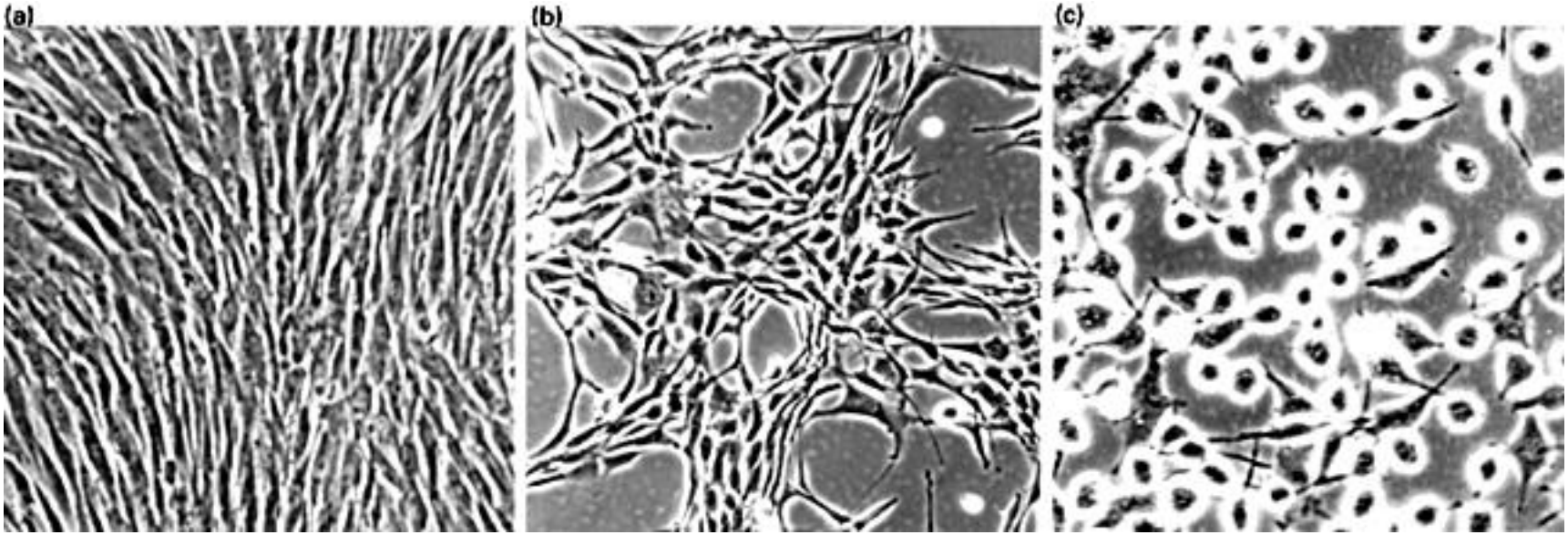
- **Transformation can be induced by:**
  - mutations in p53, Rb or other genes that control the cell cycle
  - telomerase overexpression
  - viral genes: SV40 large T antigen, papillomavirus E6, Epstein–Barr virus
  - chemical mutagens

Do not fully reflect the in vivo situation and are highly genetically unstable!

Cell lines

normal

virus-transformed



contact-inhibited

pile up, rounded

Cell lines

3) From tumor cells

- **Properties similar to those of transformed cells**

e.g., HeLa – cervical carcinoma
(Henrietta Lacks, 1951)



1. Uncontrolled growth
- do not undergo apoptosis
- unlimited replicative potential

2. Ability to invade tissues and metastasize
- lose anchorage dependence for growth
- lose contact inhibition

3. Abnormal karyotypes (diploidy, heteroploidy and haploidy).

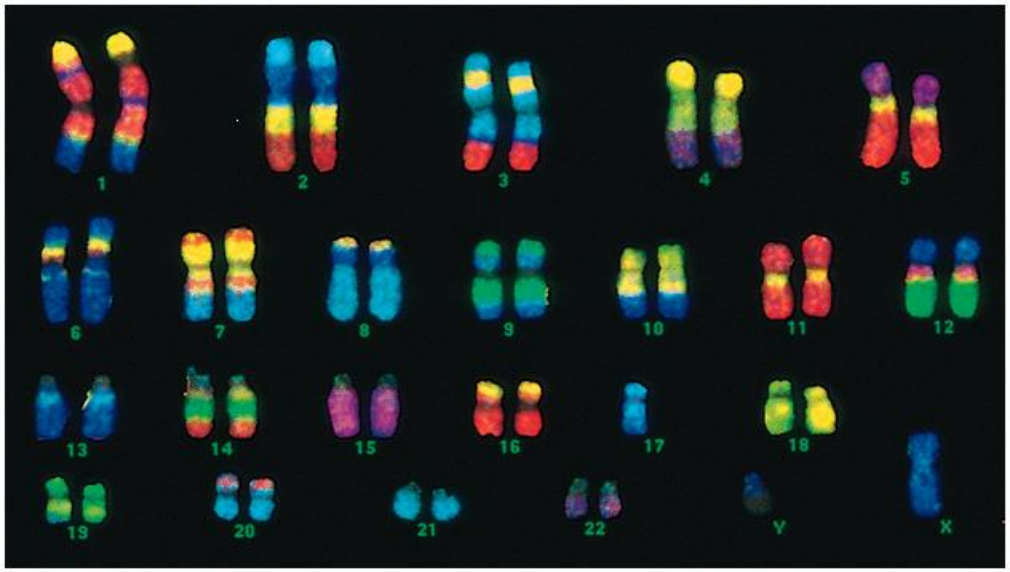


Henrietta Lacks 1920–1951

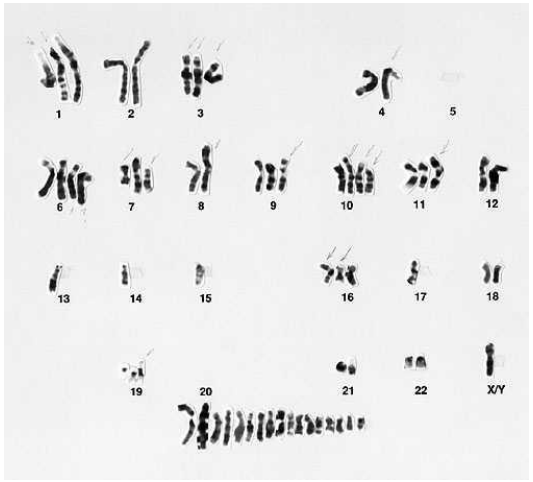


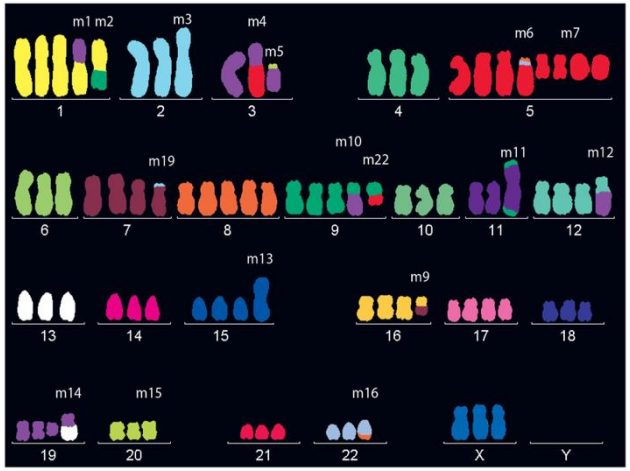
"Henrietta Lacks worked in the tobacco fields of Virginia, as had her enslaved ancestors. When she died of cancer in 1951, doctors took a tissue sample without seeking consent. They soon observed an astonishing phenomenon, never before recorded in medical history: the tumor cells continued to grow outside the body, in the laboratory. Commercialization followed soon afterward, but twenty years would pass before her family discovered a truth as incredible as it was traumatic: Henrietta was 'immortal,' and her cells had given rise to a multibillion-dollar industry."

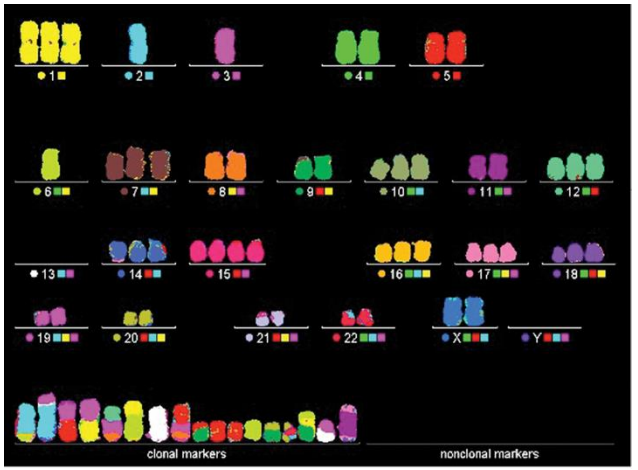
WT



HeLa(s)







HeLa cells in laboratories worldwide have very different karyotypes

Cell lines

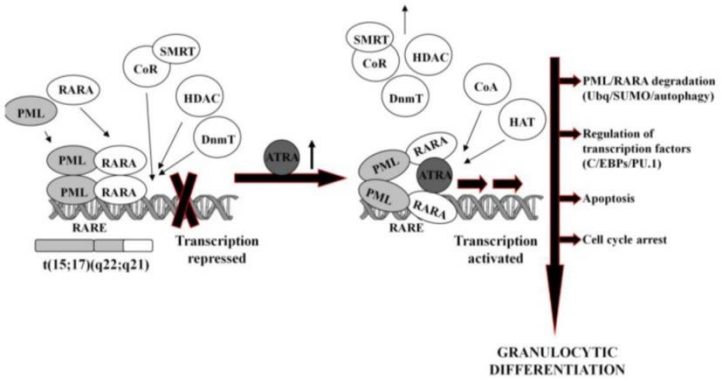
Some cell lines:

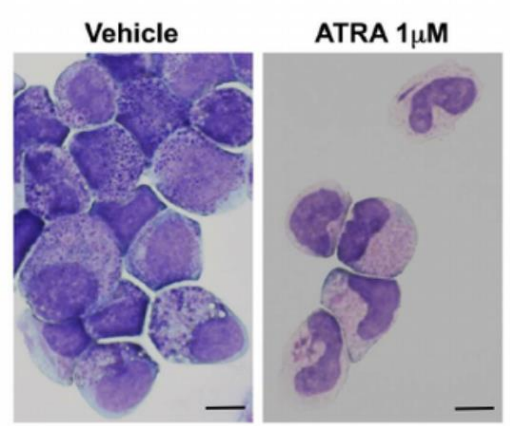
- **retain the properties of the original cells:**
  e.g. liver cell lines
  T-cell lines

- **can differentiate in culture:**
  e.g. epithelial cell lines
  muscle cell lines
  hematopoietic cell lines

Cell lines

NB4 (APL) cell line



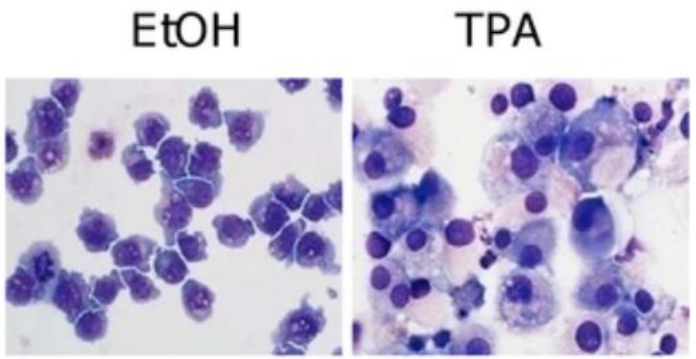


U937(AML) cell line

AML

Phorbol ester (TPA) ↓

Monocytic/Macrophage differentiation



Stem cells

1- Can replicate indefinitely
Stem cells have a high capacity for self-renewal and can therefore undergo many divisions, producing other unspecialized cells like their parent cells.

2- Are unspecialized
Stem cells are undifferentiated and lack specific cellular structures that would enable them to perform specialized functions.

3- Can give rise to specialized cells
Internal or external signals can direct stem cells toward specific differentiation programs controlled by particular genes.

Based on their capacity to generate different cell types, stem cells are classified as:

1- Totipotent: can generate all embryonic and extraembryonic tissues

2- Pluripotent: can generate all derivatives of the three embryonic germ layers

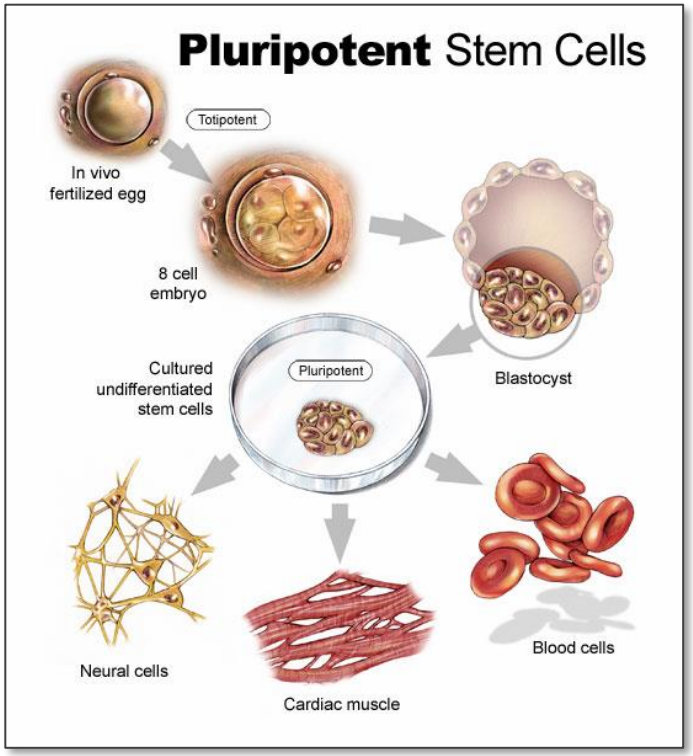
3- Multipotent: form several cell types derived from the same embryonic germ layer

4- Unipotent: self-renewing cells capable of producing a single cell type

Stem cells

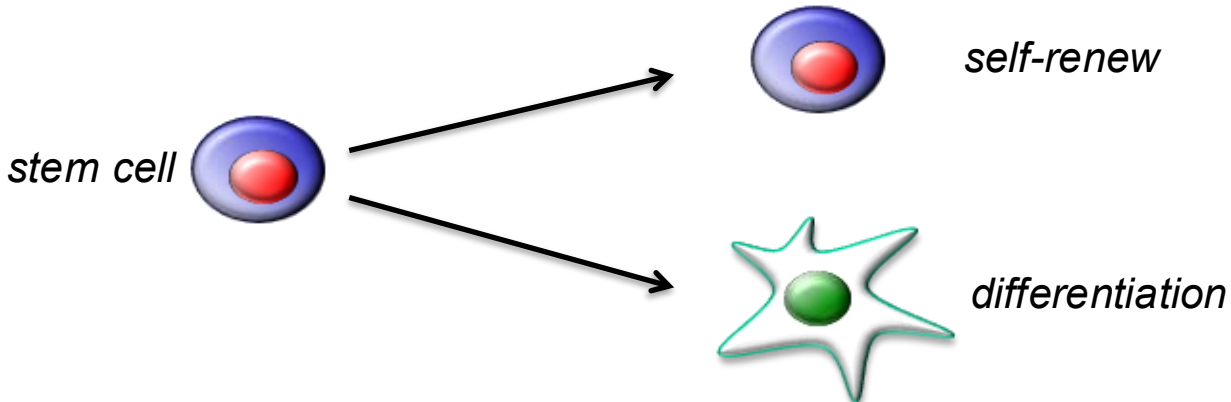
Embryonic stem cells:

- Derived from the embryo at the blastocyst stage (before gastrulation)
- Are pluripotent



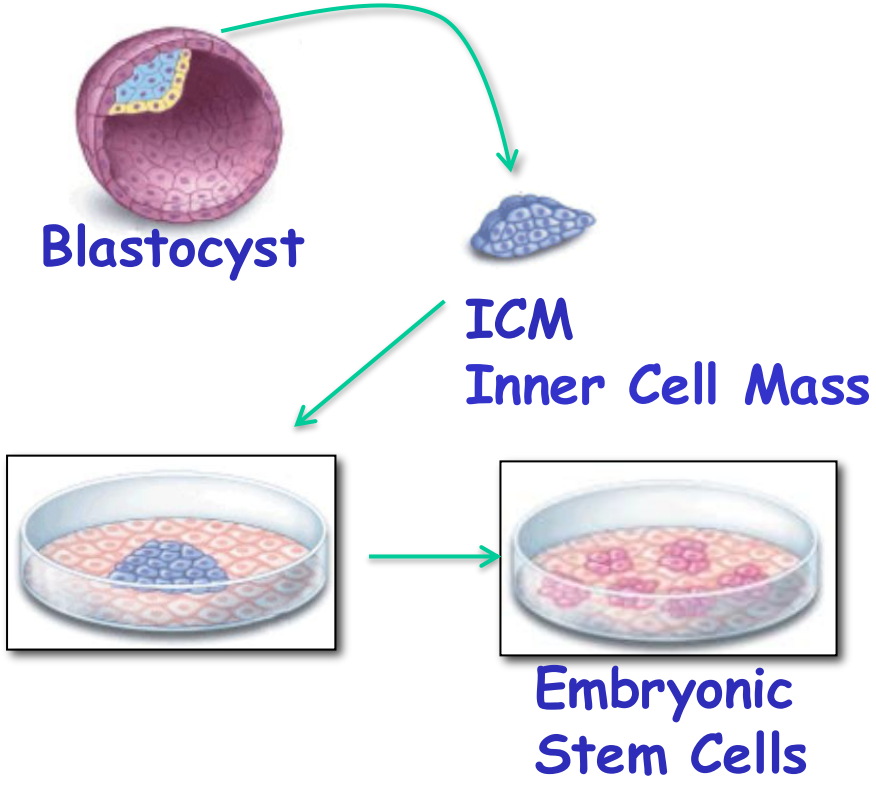
Adult stem cells:

- Present in various tissues throughout the organism's life
- Are multipotent (e.g., HSCs) or unipotent (e.g., spermatogonia)



Embryonic Stem Cells

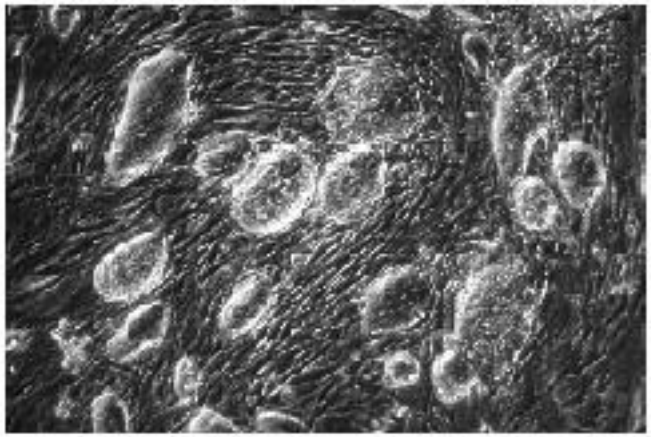
These cells are functionally defined by their ability to remain undifferentiated in culture while retaining the capacity to differentiate into all tissues of the adult organism.

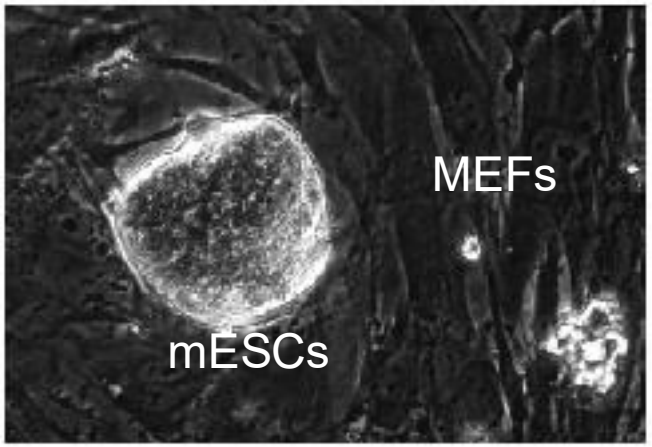


Mouse embryonic stem cells

Mouse embryonic stem cells were first derived in the early 1980s (Evans and Kaufman, Nature 1981; Martin, PNAS 1981).

mESCs grow as colonies ("community effect") and are usually maintained on a feeder cell layer (typically MEFs, mouse embryonic fibroblasts), which provides support and conditions the culture medium.



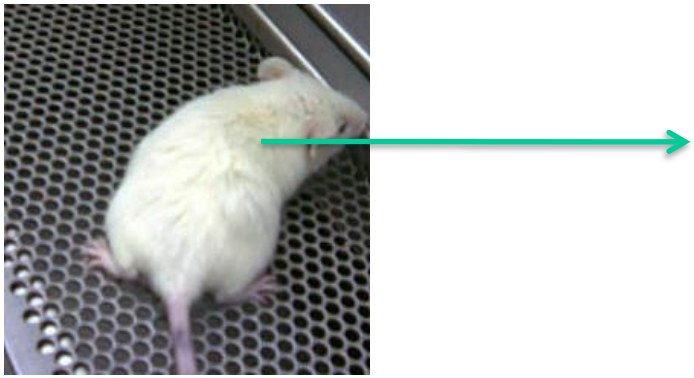


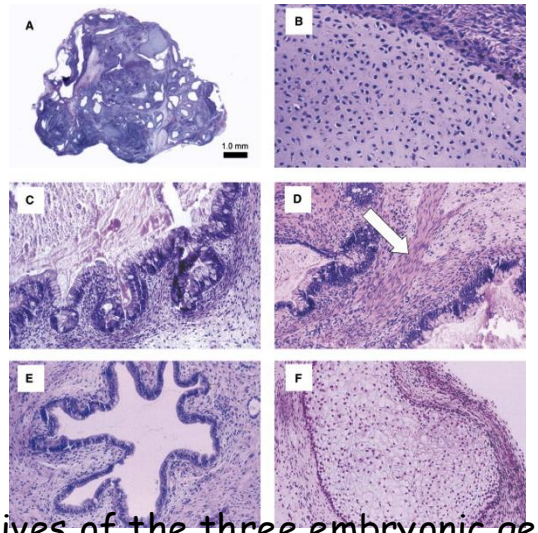
mESC differentiation

Differentiation can be induced in vitro by changing the culture conditions, or in vivo *by* generating **teratomas** or **chimeric mice.**

A teratoma is a tumor whose cells show a high degree of diversity. Derivatives of all three embryonic germ layers can be found within a teratoma.

ES cells injected into an immunodeficient adult mouse form teratomas with varying degrees of differentiation: high-quality ES cells generate highly complex teratomas.





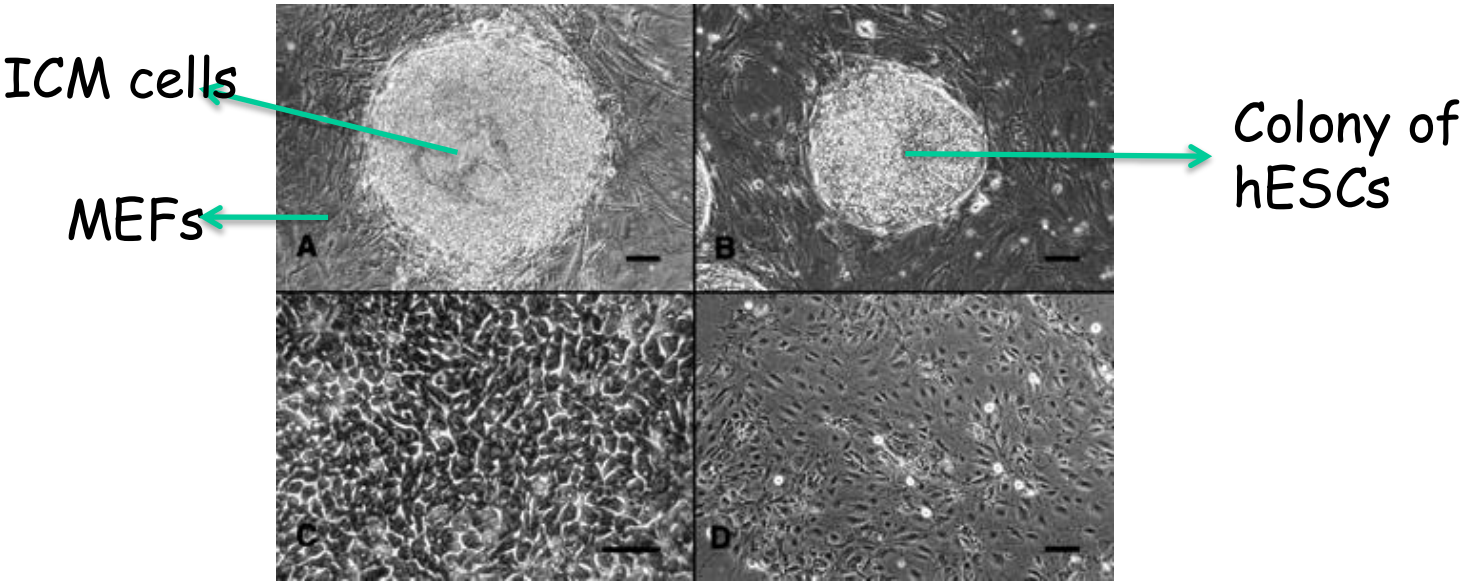
Derivatives of the three embryonic germ layers

Human embryonic stem cells (hESCs)

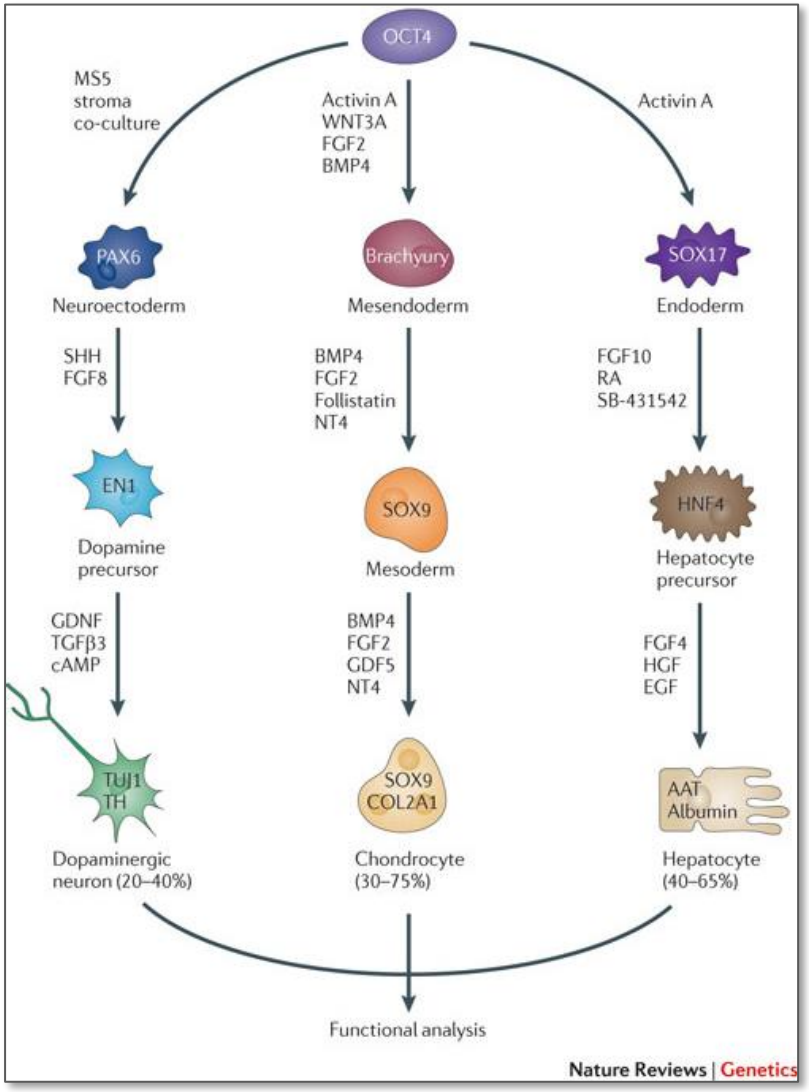
Human embryonic stem cells were first derived in 1998 (Thomson et al., Science 1998).

Deriving human embryonic stem cells inevitably involves destruction of the embryo.

hESCs can differentiate in *vitro and* generate teratomas in immunodeficient mice. This is the most stringent test for assessing the quality of human ES cells.



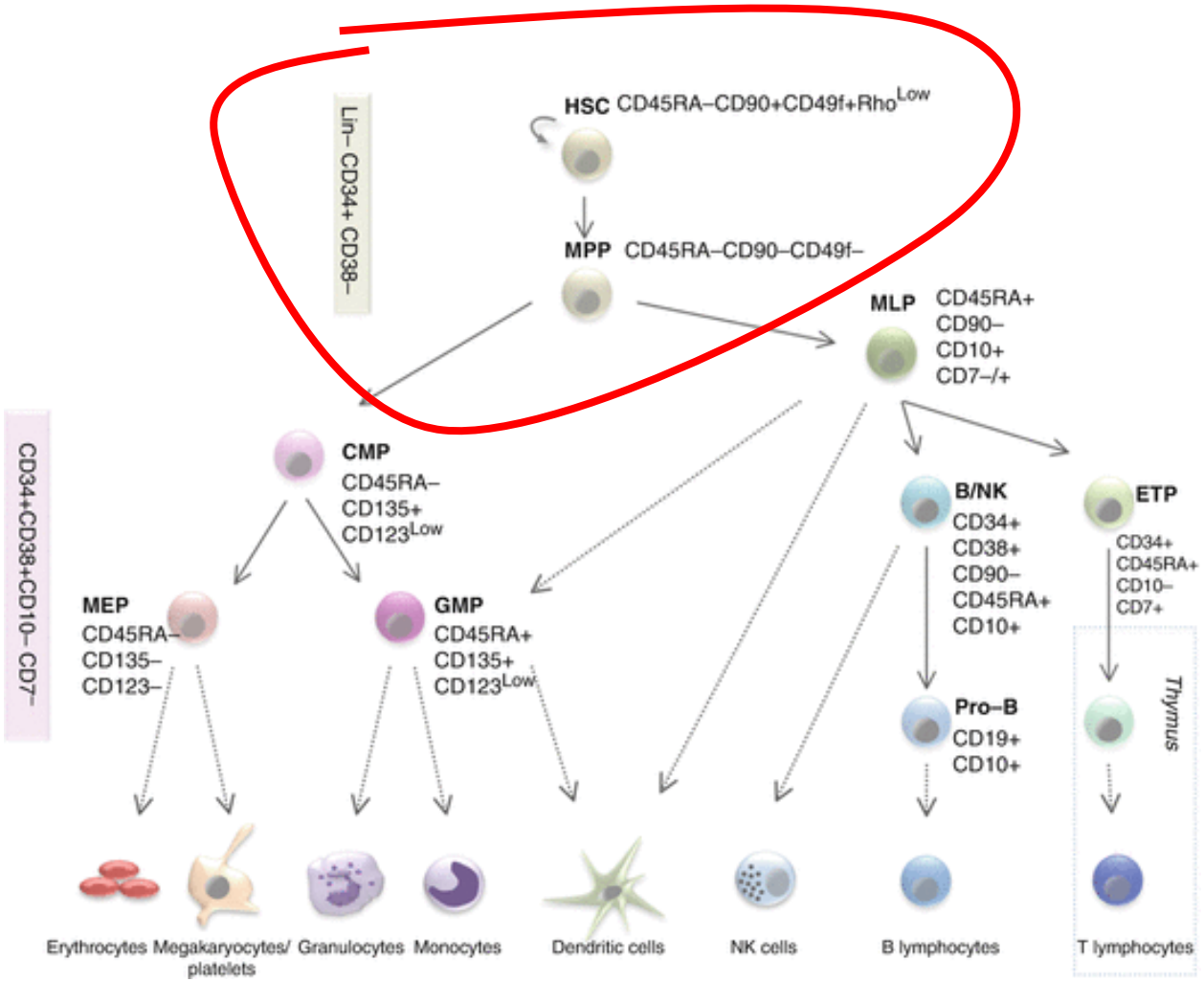
ESC differentiation



(Cohen and Melton, Nat. Rev. Genet. 2011)

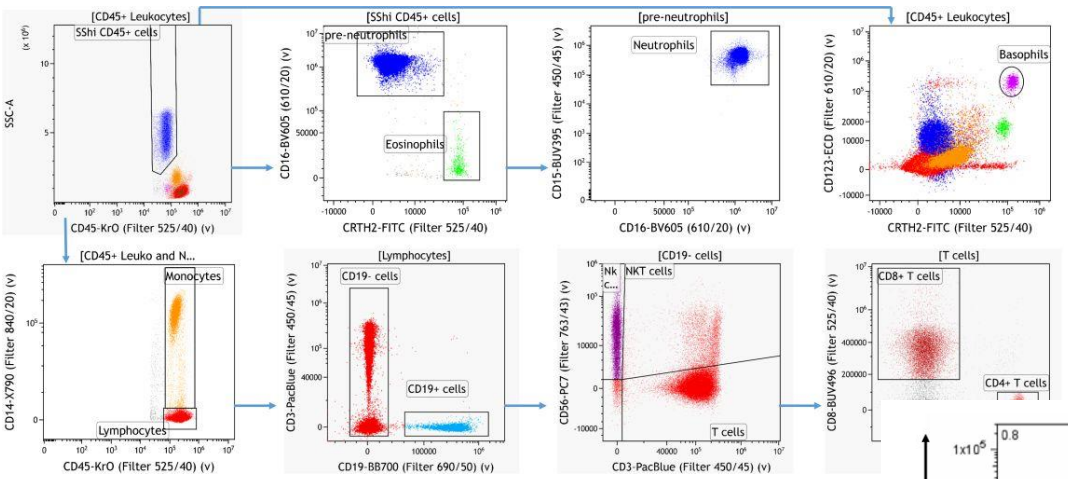
Adult stem cells

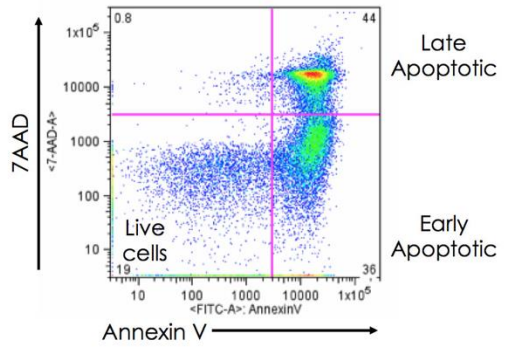
Hematopoietic stem cells

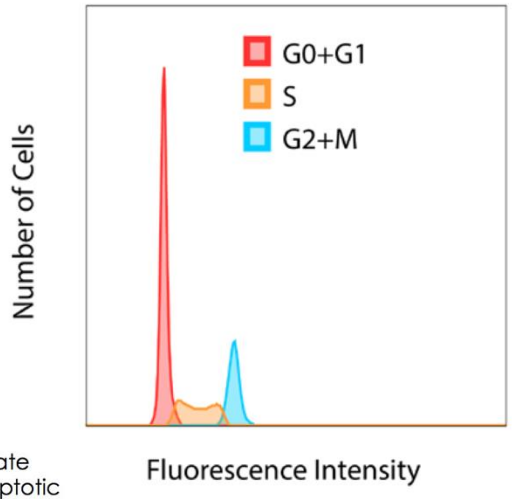


Flow cytometry

Flow cytometry is a laboratory technique used to detect, identify and count specific cells. It can also identify particular intracellular components. This information is obtained using labeling protocols that assess the physical characteristics of cells and the presence or absence of specific markers, or antigens, on their surface or within them. The method enables the study of cells in peripheral blood, bone marrow and body fluids, as well as tumor cells. It can also be used to analyze the cell cycle and cell death.







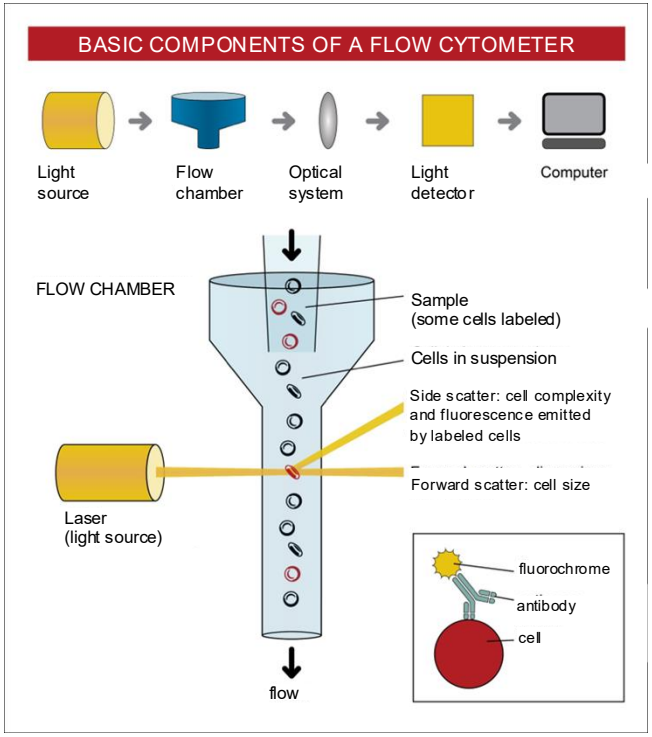
Flow cytometry

The sample is treated with fluorescent antibodies directed against specific intracellular or surface antigens, or with fluorescent intercalating dyes (for cell-cycle and cell-death analysis).

The sample containing the labeled cells is introduced into **a flow cytometer.** Within the instrument, cells enter the flow chamber and pass through a narrow opening, forming a stream in which they are aligned in single file. The cell stream passes through the detection region, where each cell is analyzed at very high speed (hundreds to thousands of cells per second).

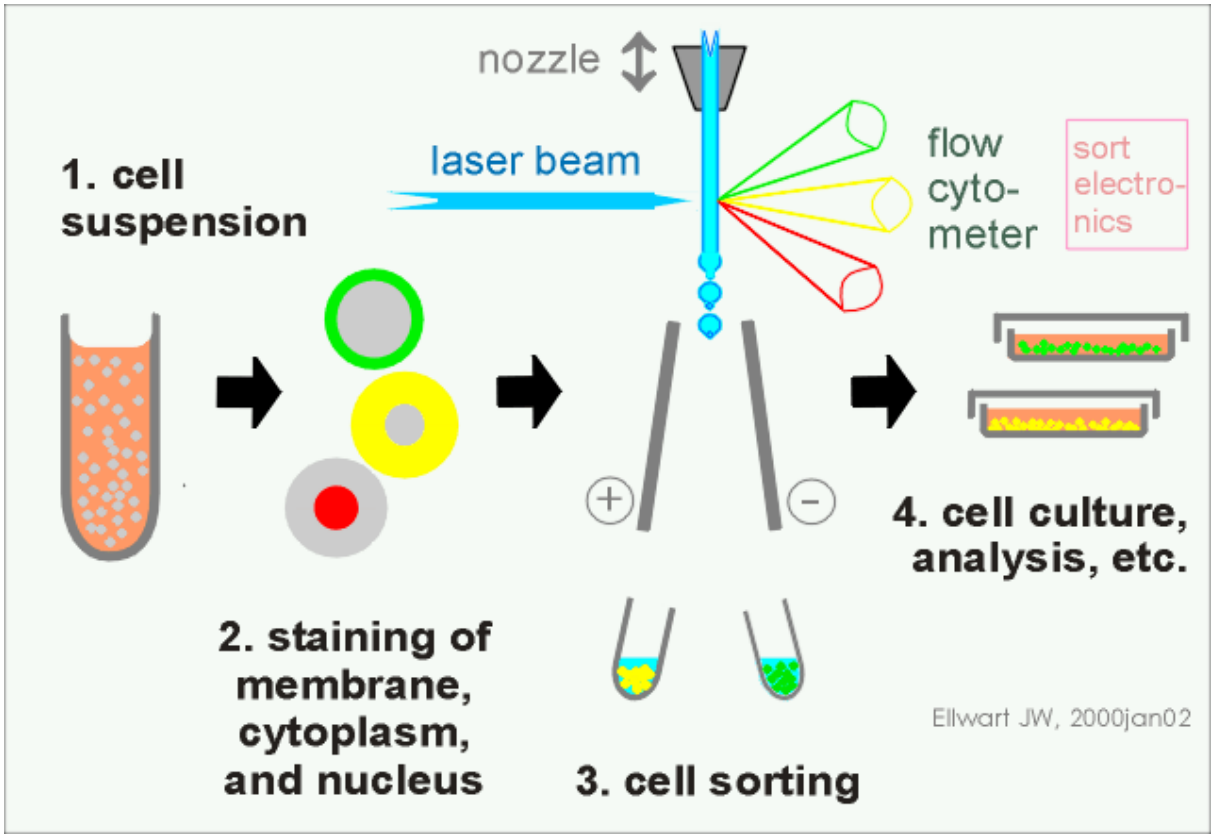
The flow cytometer contains one or more lasers and multiple detectors that identify specific characteristics of each cell. Each laser illuminates the cells in the stream, generating a characteristic light-scattering pattern that depends on the properties of the cell. The measured properties may be physical (cell size and complexity) or depend on the signal emitted by a dye (fluorochrome) excited by the laser. Together, these measurements generate a characteristic profile for each cell in the sample.

The signals recorded by the detectors are amplified by photomultiplier tubes and sent to the computer.



Flow cytometry

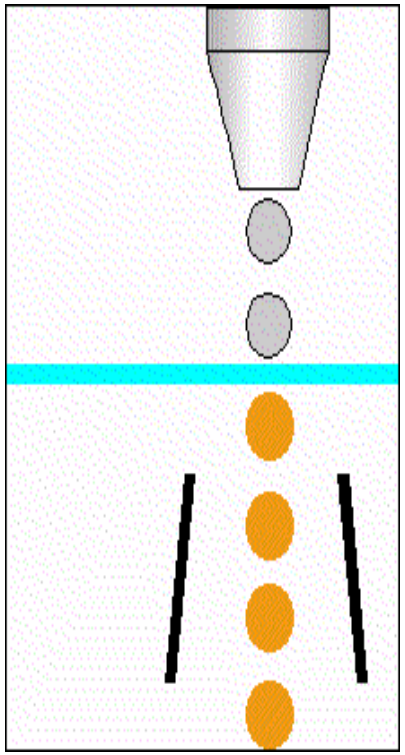
Cells bearing fluorescent markers can also be separated from the remaining cells using a **fluorescence-activated cell sorter.**

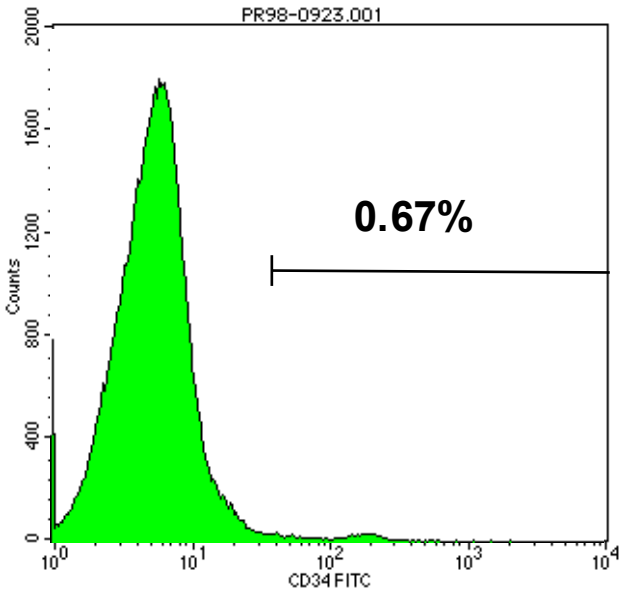




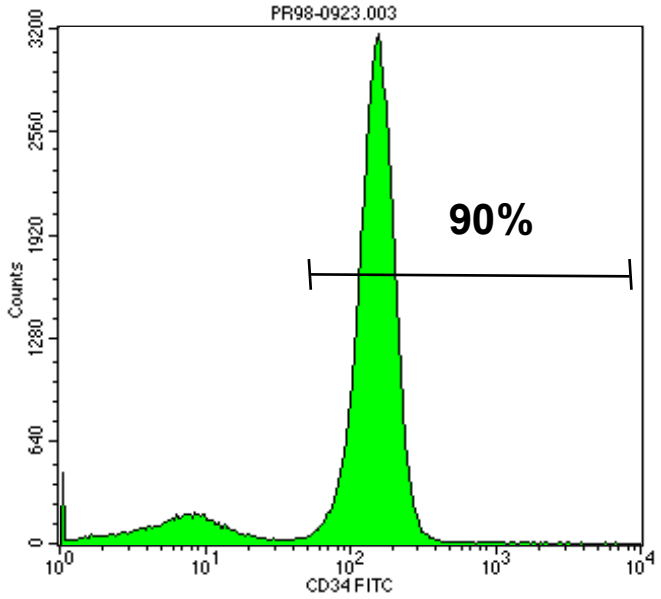
Fluorescence Activated Cell Sorting (FACS)

CD34+ purification:



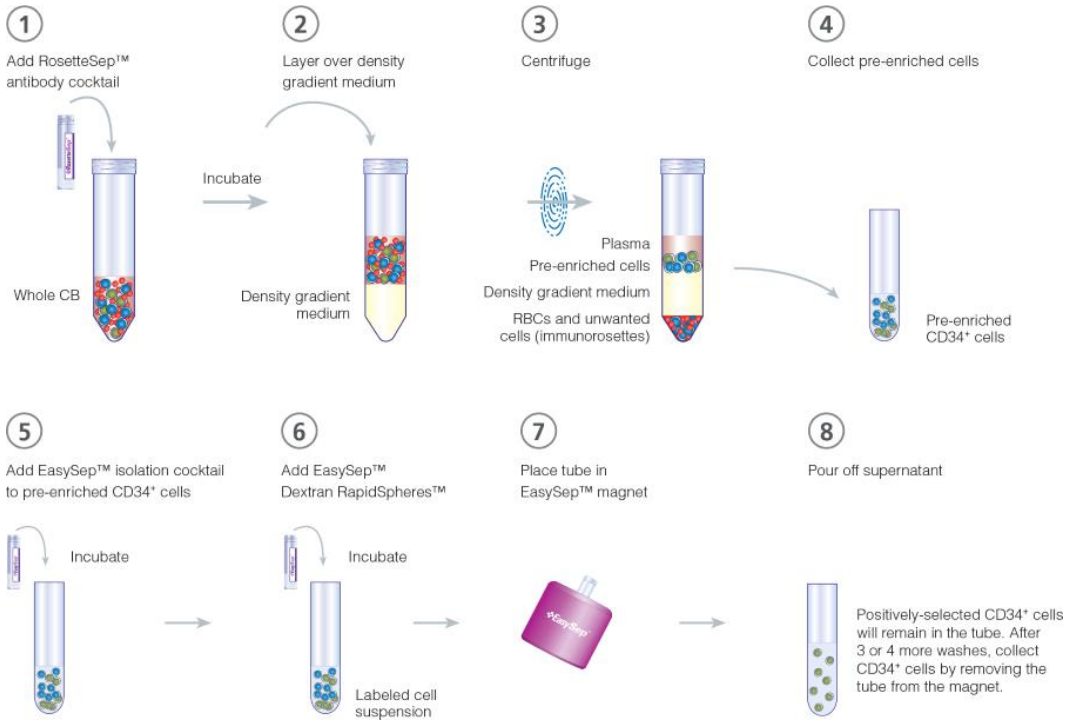


After Sorting



Adult stem cells

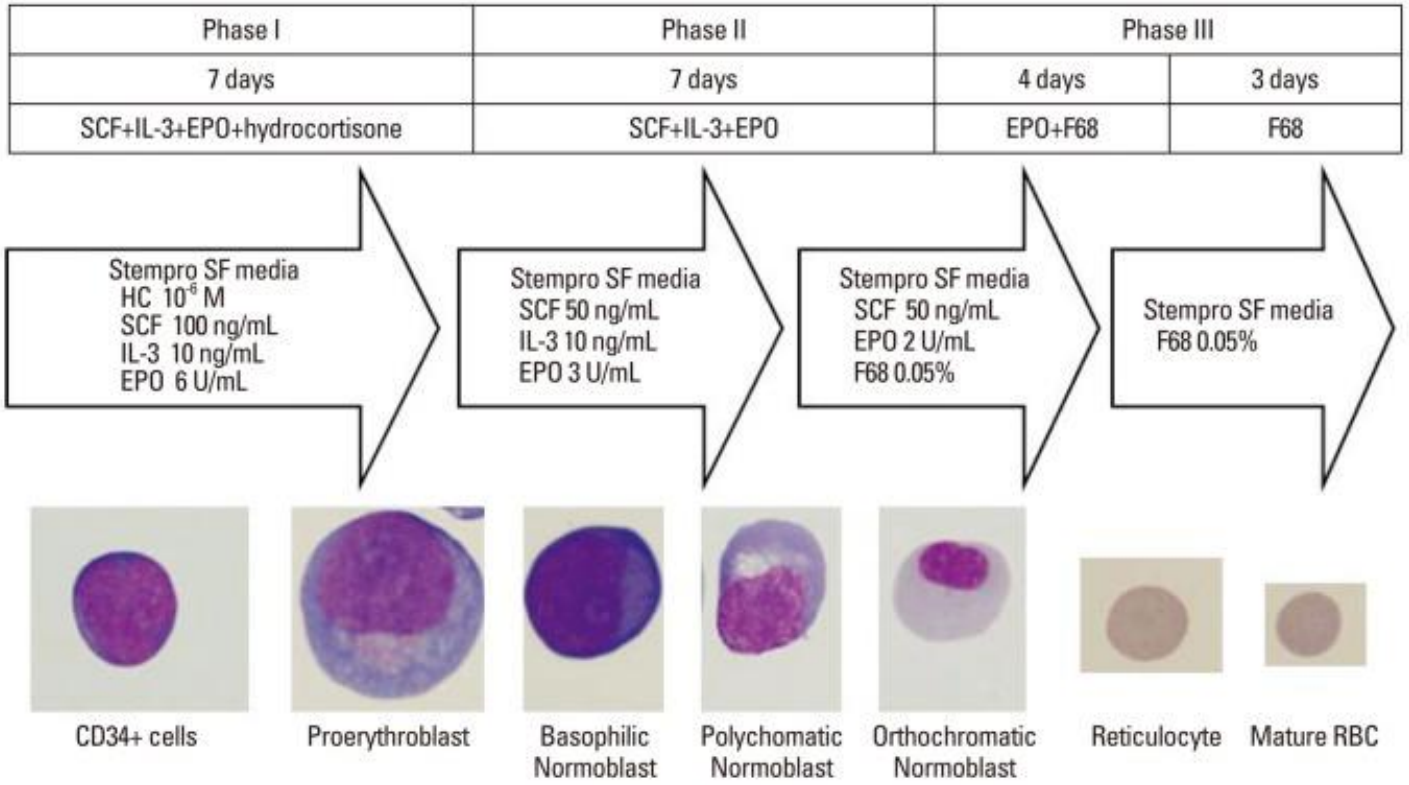
Isolation of CD34+ Cells from Human Cord Blood (CB)



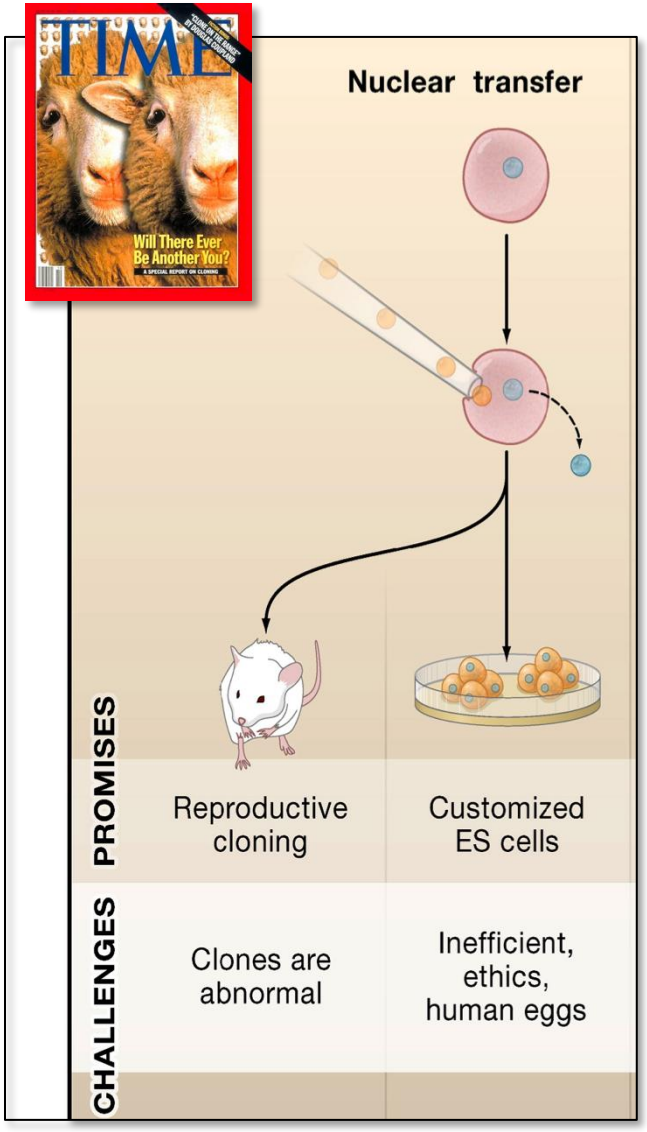
StemCell technology

Adult stem cells

Differentiation of CD34+ HSCs into red blood cells (RBCs)



Somatic Cell Nuclear Transfer (SCNT) and Cell Fusion



These experiments clearly showed that some sort of **Reprogramming Factors** is present in the cytoplasm of the egg and of the embryonic stem cells.

Such factors can “reprogram” a somatic nucleus to an embryonic stem cell-like state.

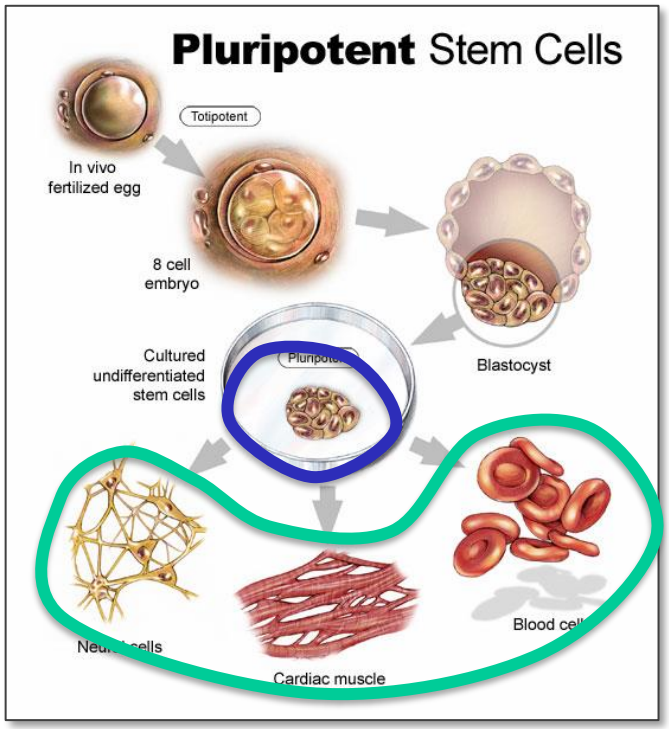
2006

Cell

Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors

Kazutoshi Takahashi[1] and Shinya Yamanaka[1,2,*]



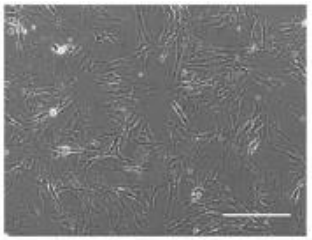


Genes expressed in ESC

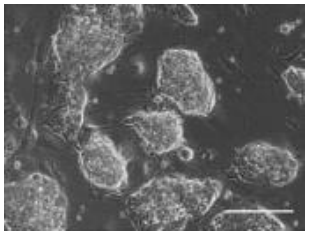
Vs.

Genes expressed in somatic cells

Reprogramming Factors



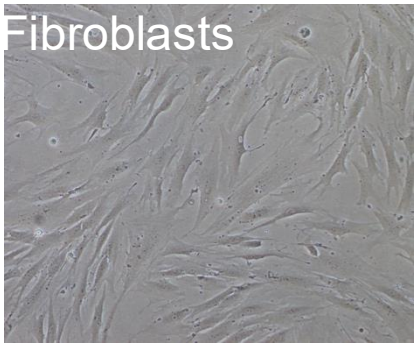
Fibroblasts



ES-like cells: iPS Cells

Reprogramming **is** the process by which a somatic cell **is converted** into a **pluripotent cell.**

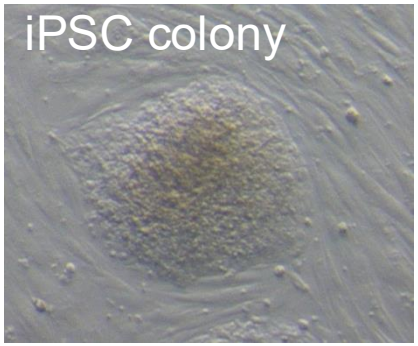
The reprogrammed cell is called an **iPSC** (induced pluripotent stem cell) because it acquires the morphological and functional characteristics **of embryonic stem cells.**



Reprogramming

Yamanaka's reprogramming factors (OSKM):

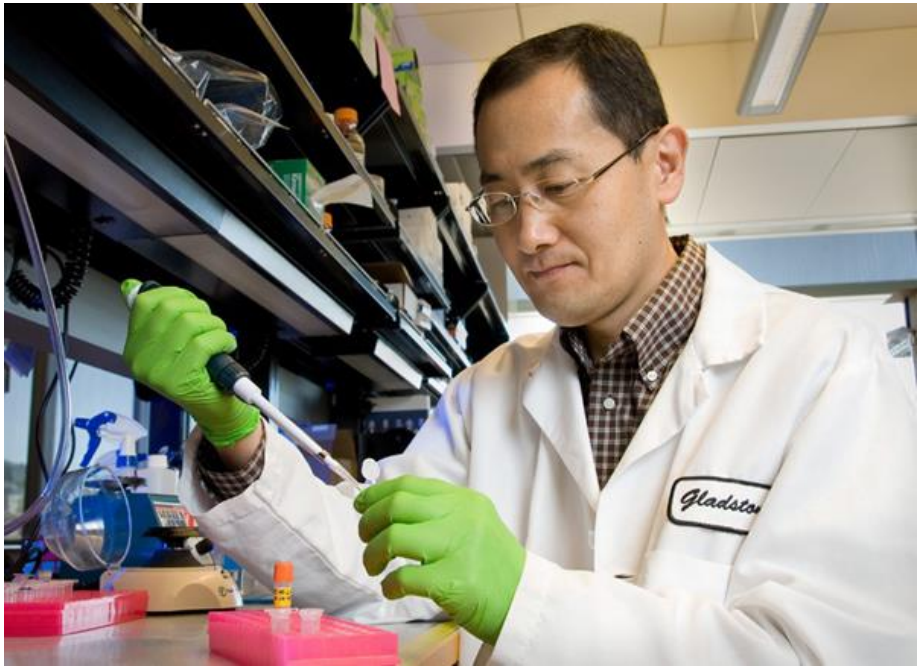
- ✓ **OCT4**
- ✓ **SOX2**
- ✓ **KLF4**
- ✓ **cMyc**



2012 Nobel Prize in Physiology or Medicine

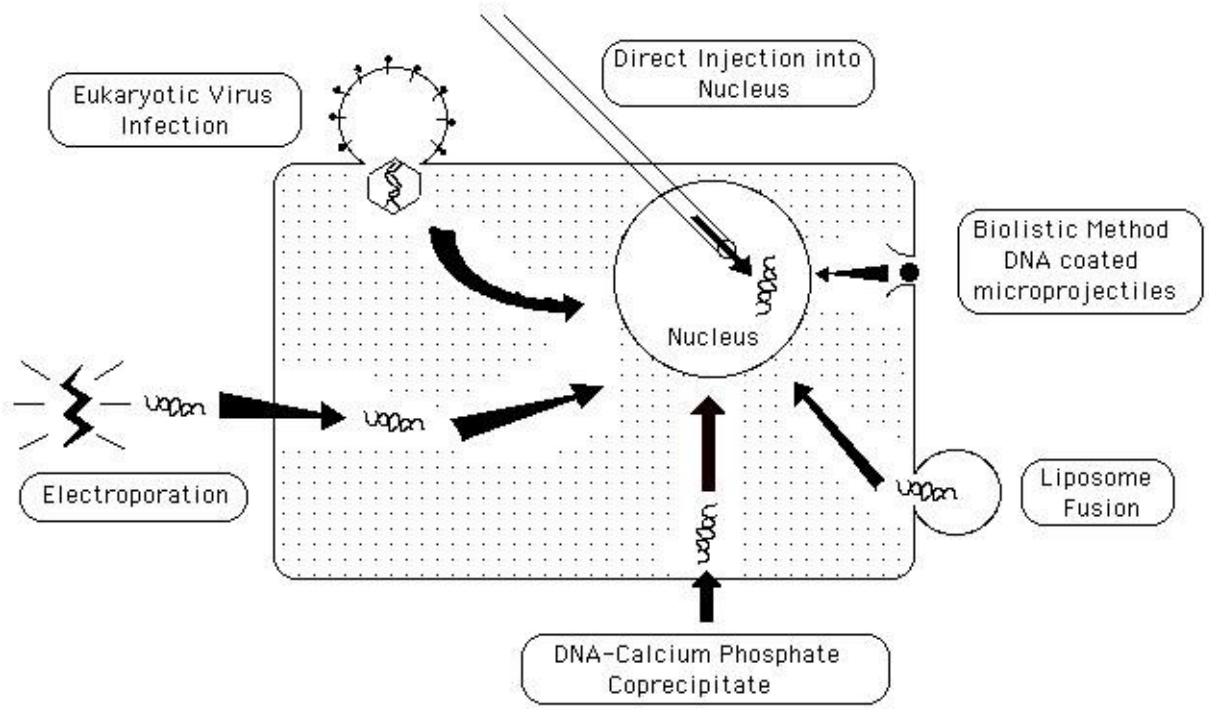


John Gurdon



Shinya Yamanaka

Gene transfer into animal cells



Introducing DNA into mammalian cells

1- Direct transfer:
- microinjection
- bombardment with DNA-coated metal particles

2- Transfection:
- DEAE-dextran
- Calcium chloride
- Electroporation
- Liposomes

3- Transduction:
- viruses

TRANSFECTION

- **DEAE-dextran:** an inert carbohydrate polymer (dextran) linked to a positively charged chemical group (DEAE, diethylaminoethyl)

- **Calcium phosphate precipitation: uses** a phosphate buffer and calcium chloride

- **Electroporation**

- **Lipofection:** uses liposomes

Co-precipitation methods

- **DEAE-dextran**
- **Calcium phosphate**

DEAE-dextran and calcium phosphate form insoluble complexes or precipitates with DNA, which cultured cells take up by endocytosis.

- Work well only with adherent cells
- Require large amounts of DNA (10–40 µg)
- Can be used to generate stable clones (efficiency <1%)
- Used for viral packaging

Electroporation

Cells are placed in a DNA-containing solution and exposed to a brief electrical pulse that creates transient pores in their membranes.



- Works well only with cells grown in suspension
- Works very well with linear constructs and oligonucleotides
- Requires large amounts of DNA (10–40 μg)
- Special kits are available for difficult-to-transfect cells (AMAXA)

Electroporation

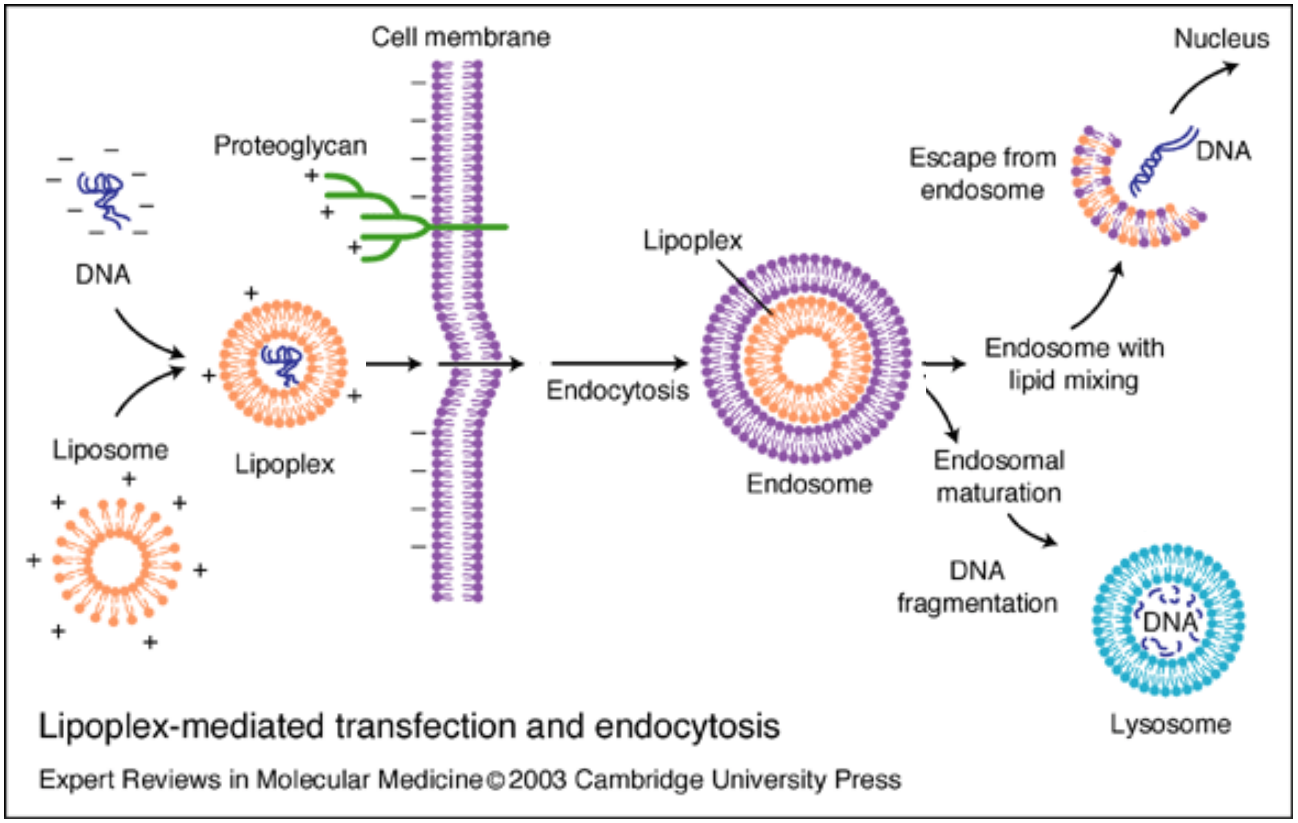


Lipofection

DNA is incubated with cationic liposomes that bind to the negatively charged surface of cultured cells. DNA enters through membrane fusion or endocytosis. Many commercial products are available (and expensive).

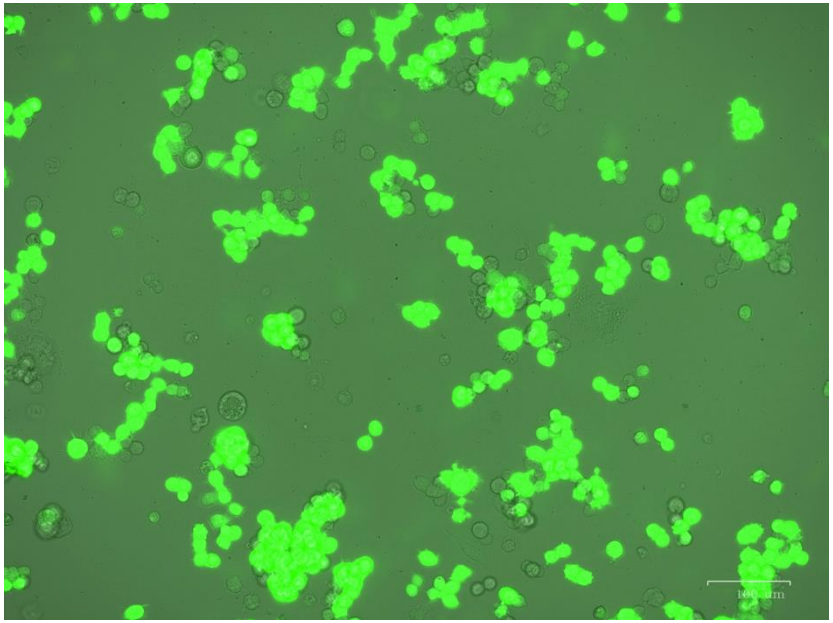
- Simple, efficient and reproducible
- High transfection efficiency (even >80%)
- Does not require large amounts of DNA (1–10 μg)
- Also works very well with oligonucleotides (both DNA and RNA)

Lipofection

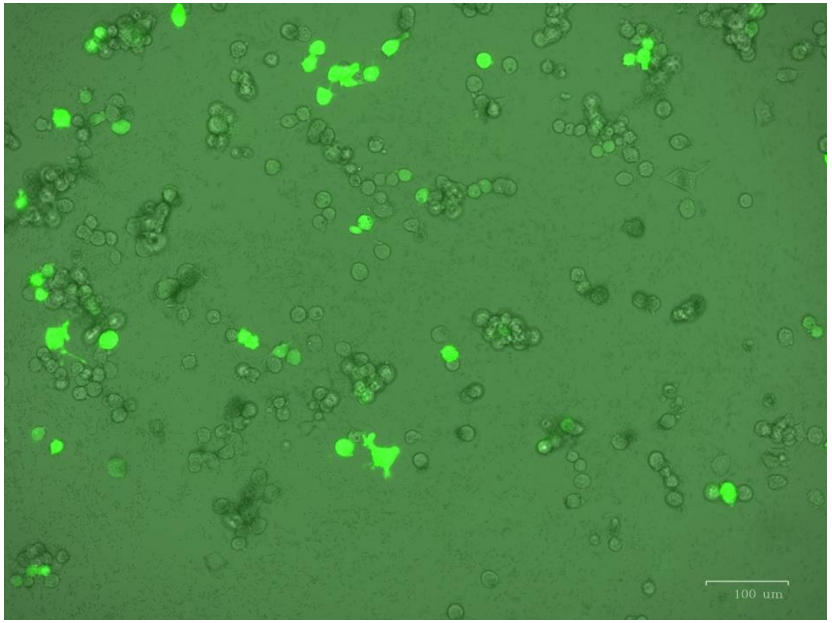


N2A, 24h

Lipofectamine 2000

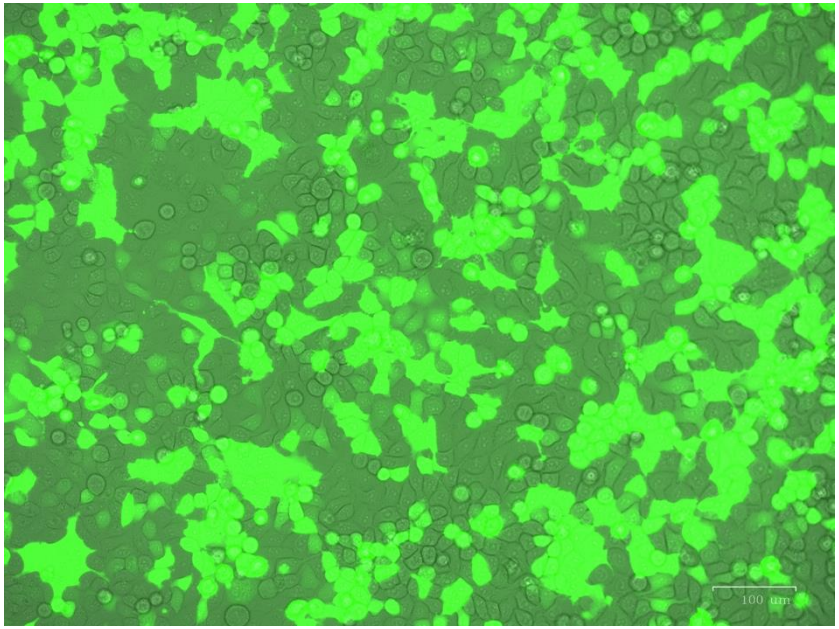


Calcium phosphate

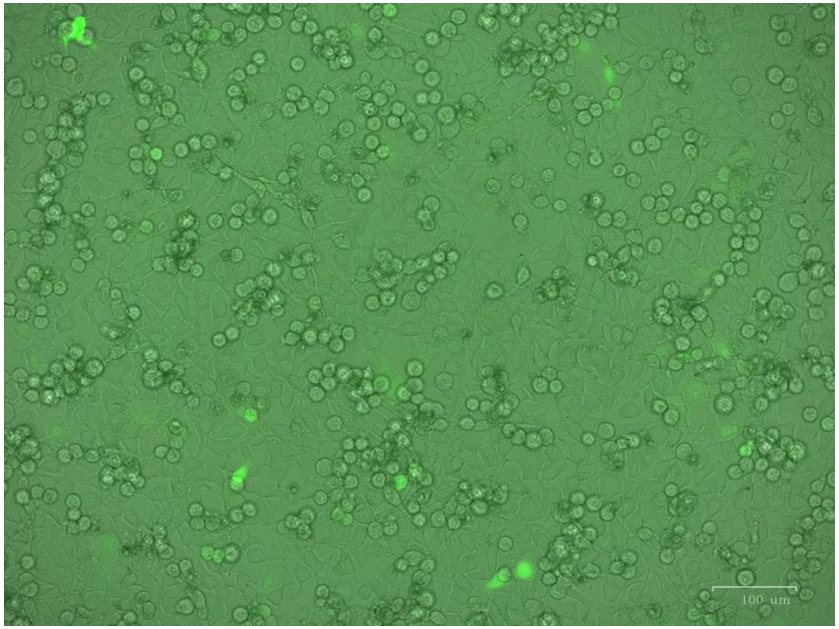


HeLa, 24h

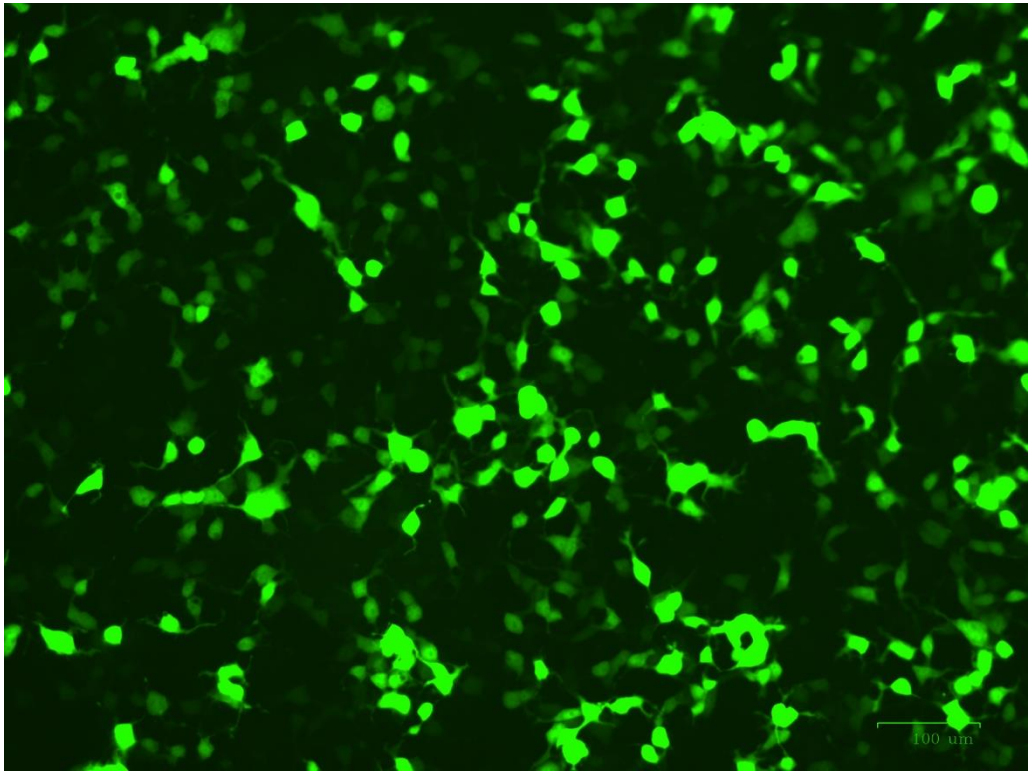
Lipofectamine 2000

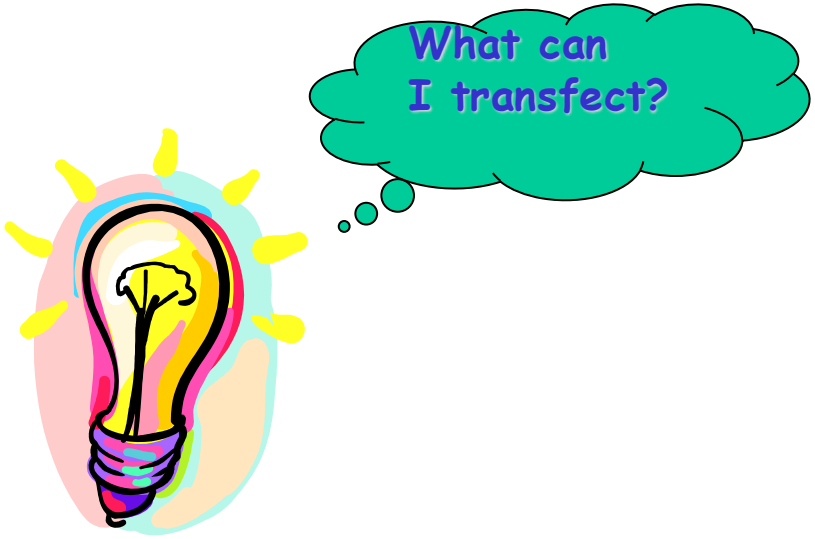


Calcium phosphate



293T cells transfected using calcium phosphate





Nucleic acids:

single- or double-stranded
circular or linear

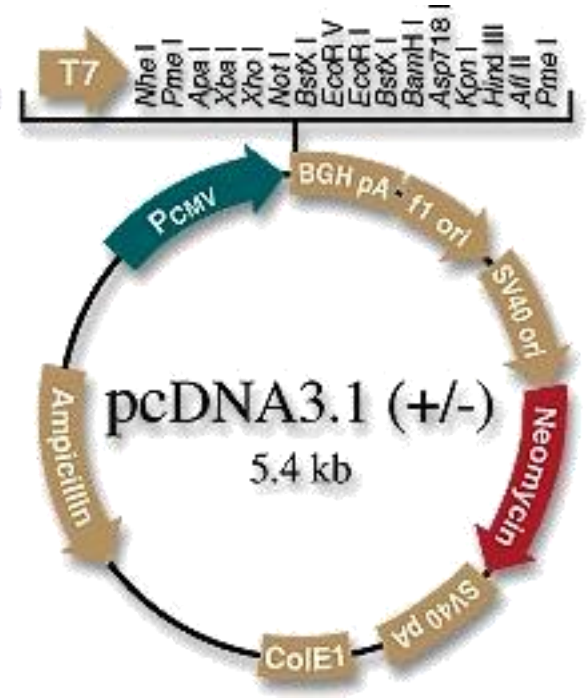
Note

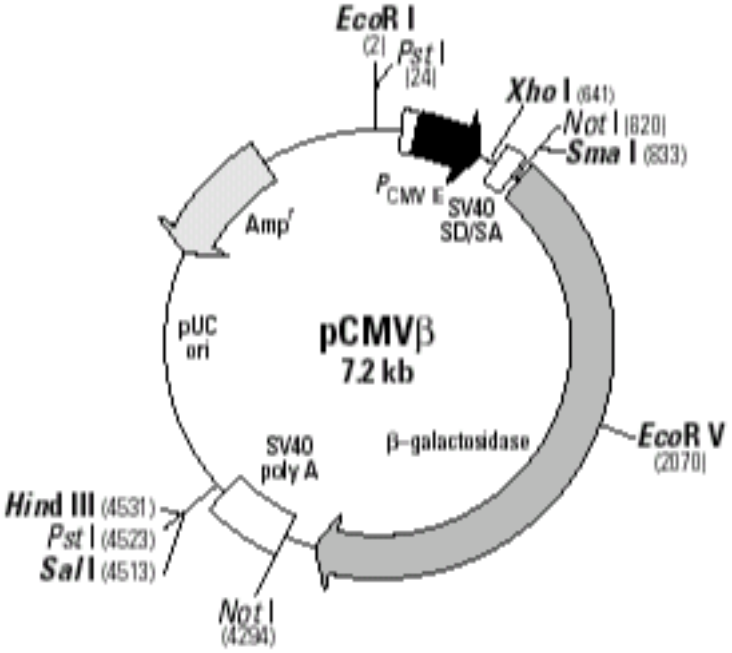
Gene transfer techniques are never 100% efficient. A second gene that produces an identifiable phenotype (a reporter or marker gene) must therefore be introduced.

Expression vectors for mammalian cells

Components:

- origin of replication and bacterial selection marker
- gene of interest cloned into the MCS
- a promoter and terminator functional in mammalian cells
- a reporter or marker gene





Reporter and marker genes

(GFP, LacZ, drug resistance, etc.)

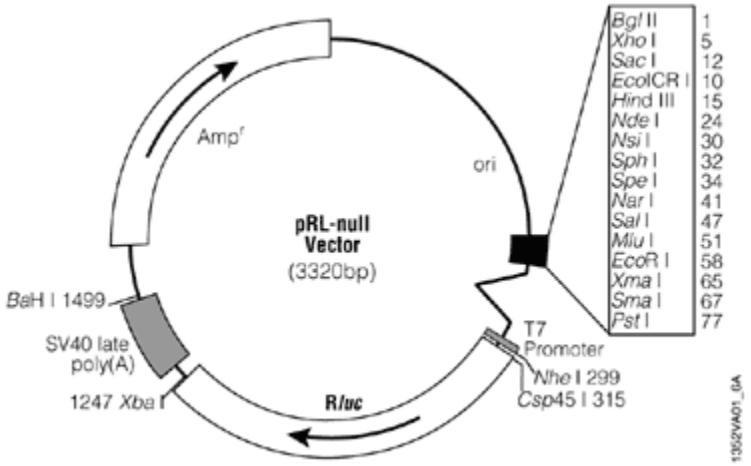
•Ideally, these should be included in the same construct as the gene of interest (not always possible).

•A reporter can also be co-transfected on a separate construct, added at a lower molar concentration than the gene of interest. Statistically, cells that receive the gene of interest will also receive the reporter.

Reporter genes

Also used in promoter activity assays. The promoter under investigation is cloned upstream of a reporter gene encoding a protein that can be readily measured.

Luciferase (luc): a protein from the firefly Photinus pyralis that catalyzes luciferin oxidation in the presence of ATP, producing AMP, carbon dioxide and light. Light emission is measured with a luminometer.





GENE INTEGRATION

Transfection methods usually produce transient expression of the transgene (>100 copies per cell). Because it is not integrated into the genome, it is lost during subsequent cell divisions.

Stable expression can be obtained by:

- **Use selectable markers to select cells** in which random integration has occurred after transfection (a rare event). Selectable markers encode proteins that confer resistance to specific antibiotics (neomycin, puromycin, etc.) on the cells expressing them.

- **Using viral vectors**

Selectable markers for mammalian cells

Positive selection:

- neomycin phosphotransferase (neo)
- hygromycin phosphotransferase (hyg)
- puromycin resistance (pur)

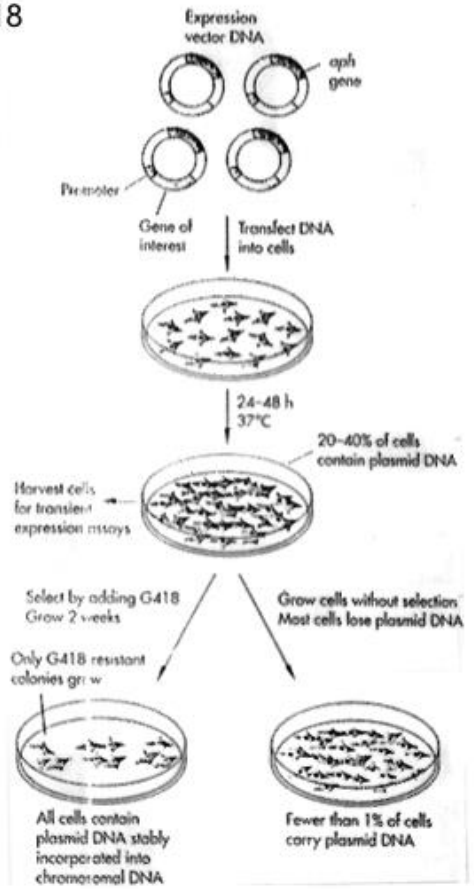
Negative selection:

- thymidine kinase (tk)
- cytosine deaminase (cd)
- hypoxanthine phosphoribosyltransferase (hprt)

Random integration and positive selection

The neomycin (neo) resistance selection procedure (G418 selection) allows for **isolation of stable transfectants**

Drug-selection using G418



Viral vectors

Baculovirus

Retrovirus

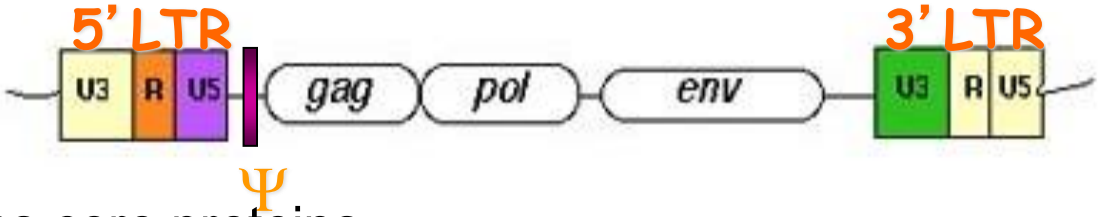
Lentivirus

Adenovirus

Adeno-associated viruses

RETROVIRUS

The retroviruses initially used for gene transfer were derived from mice and were ecotropic (infecting only mice and rats). Spontaneous mutants capable of infecting other mammals, including primates, were selected from these retroviruses (amphotropic viruses). Example: Moloney murine leukemia virus (Mo-MLV).



gag- encodes core proteins

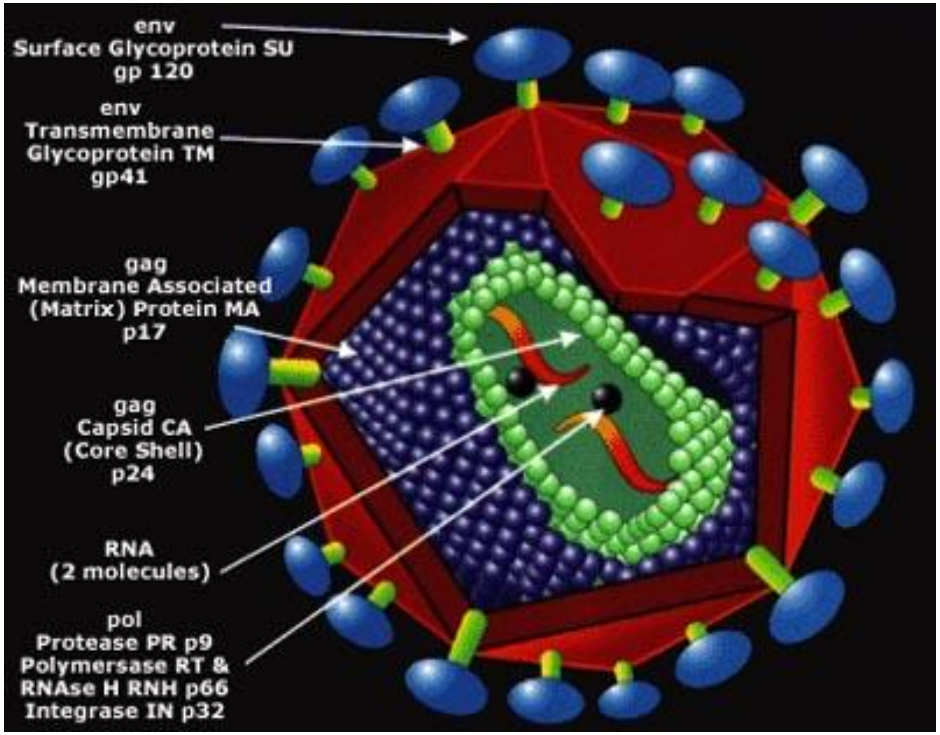
pol- encodes multifunctional proteins (RT, DNA polymerase, protease and integrase)

env- encodes viral envelope proteins (changes in these proteins alter tropism)

LTR- Long terminal repeat: divided into U3, R and U5; contains control elements for transcription initiation and termination, reverse transcription and integration

¬ - sequence required for packaging RNA into the virion

Structure of a retroviral particle:

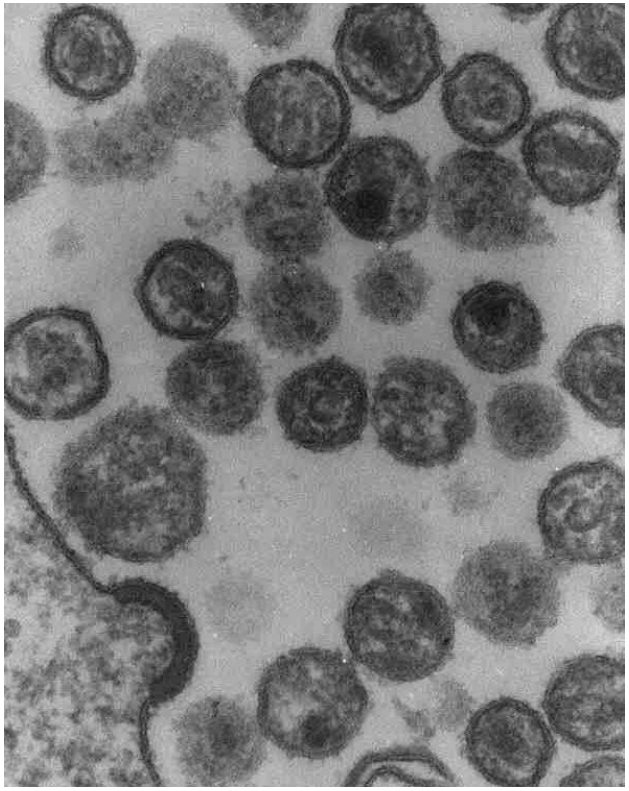


Retroviral gene transfer

•Can accommodate long sequences, including genes as well as cDNA (approximately 7.5 kb)

•Allow stable expression through integration into the genome

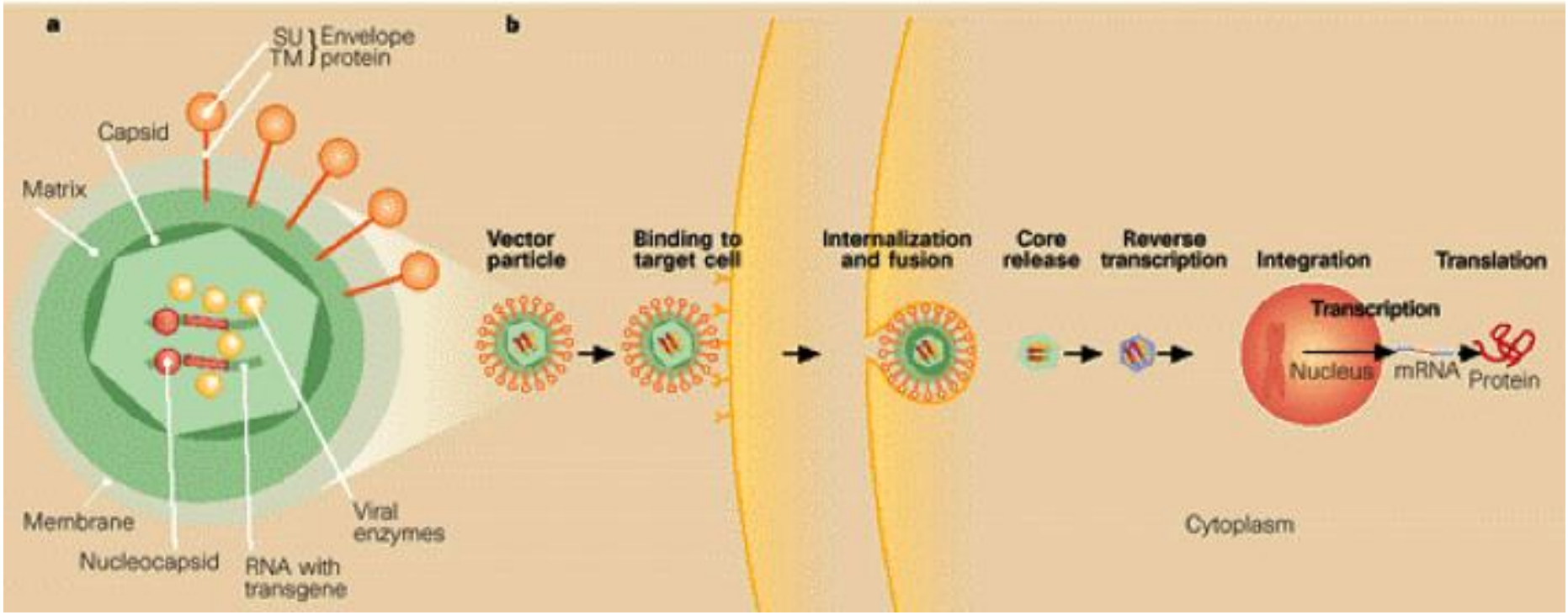
•Infect only dividing cells (the viral genome enters the nucleus only when the nuclear envelope breaks down during mitosis)



•Have low immunogenicity because viral proteins are absent

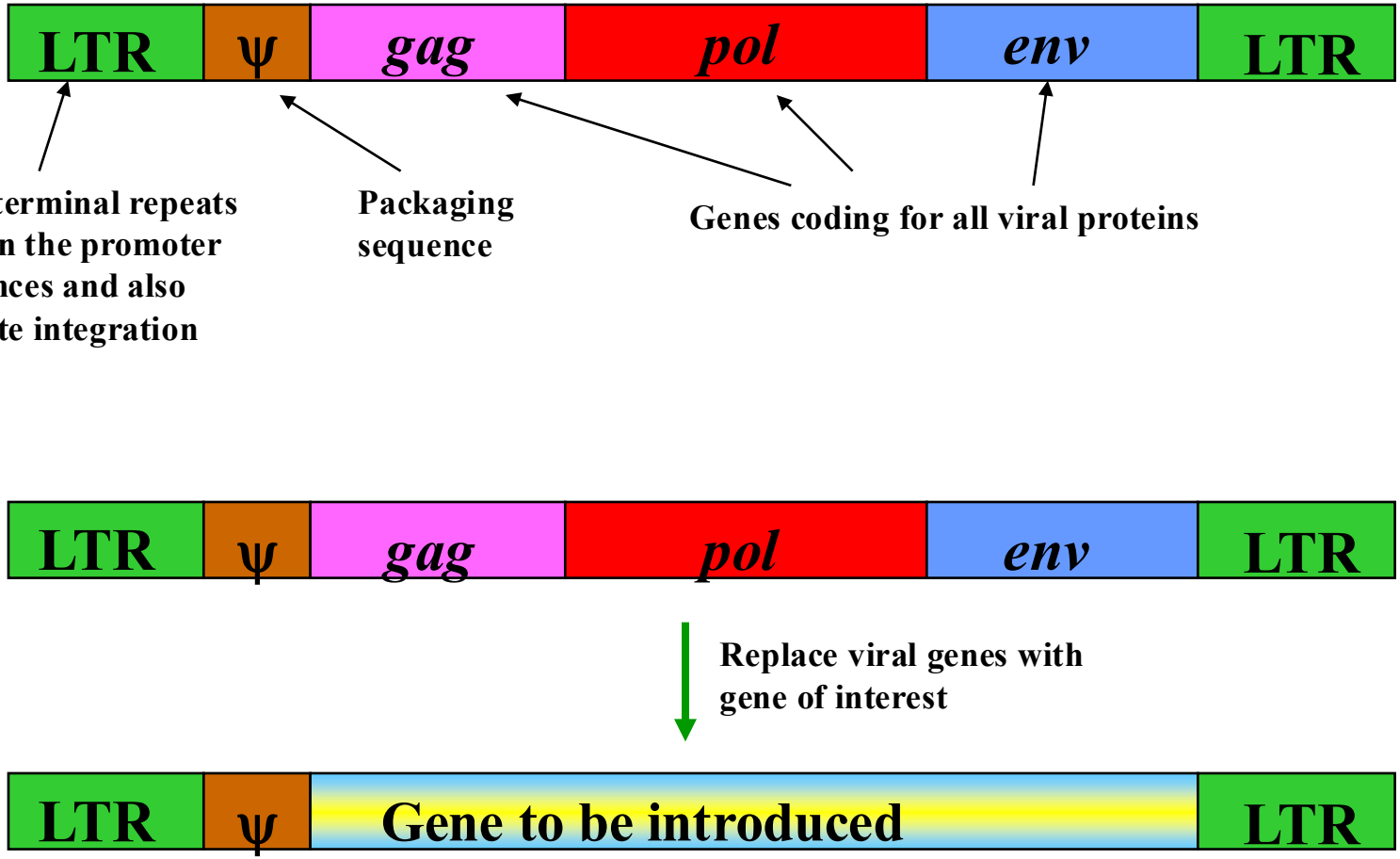
•Developed for use in gene therapy as well

Life cycle



Note: Infection and integration do not require expression of retroviral genes. Removing viral sequences can produce defective retroviruses (capable of infecting cells but unable to replicate).

Retroviral genome



Recombinant (defective) retroviral DNA (a.k.a. retroviral vector DNA)

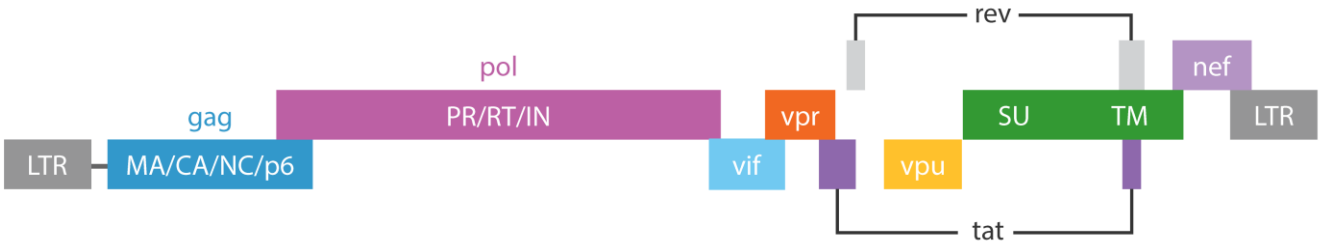
Lentiviral vectors

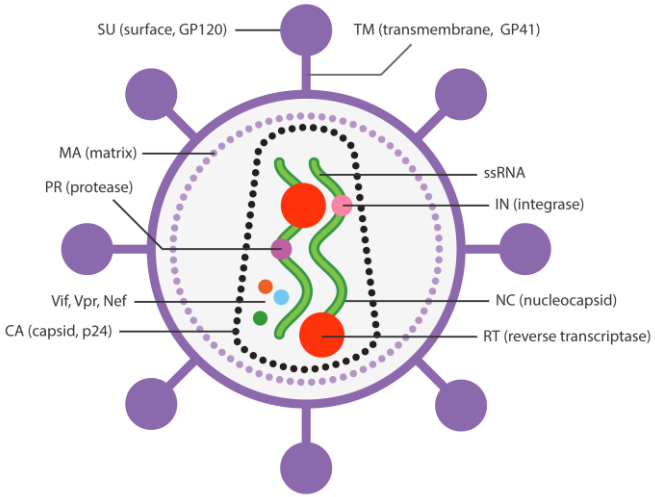
Lentiviruses are retroviruses that can infect both dividing and non-dividing cells (the pre-integration complex can cross an intact nuclear envelope).

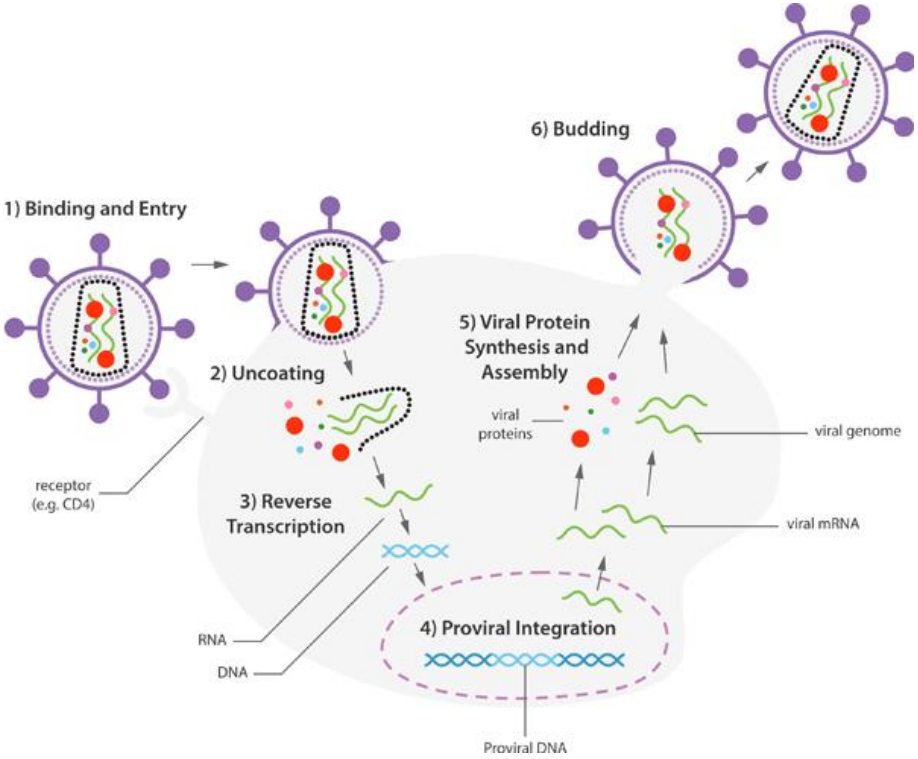
Can infect quiescent and differentiated cells: neurons, macrophages, hematopoietic stem cells, muscle cells and liver cells

Example of a lentivirus: HIV-1

Lentivirus

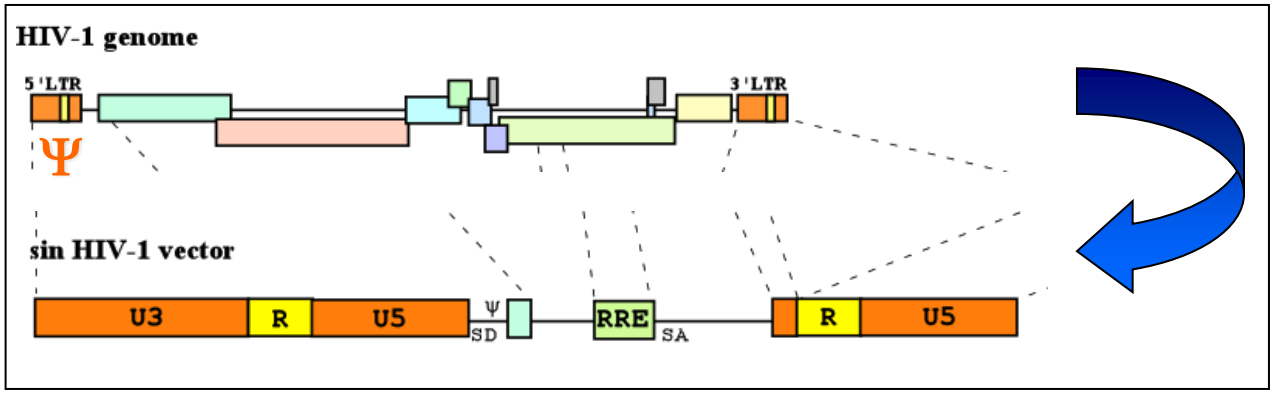

rev
pol
nef
gag
PR/RT/IN
vpr
SU
TM
LTR
LTR
MA/CA/NC/p6
vif
vpu
tat


SU (surface, GP120)
TM (transmembrane, GP41)
MA (matrix)
ssRNA
PR (protease)
IN (integrase)
Vif, Vpr, Nef
NC (nucleocapsid)
CA (capsid, p24)
RT (reverse transcriptase)


6) Budding
1) Binding and Entry
5) Viral Protein Synthesis and Assembly
2) Uncoating
viral proteins
viral genome
receptor (e.g. CD4)
3) Reverse Transcription
viral mRNA
RNA
DNA
4) Proviral Integration
Proviral DNA

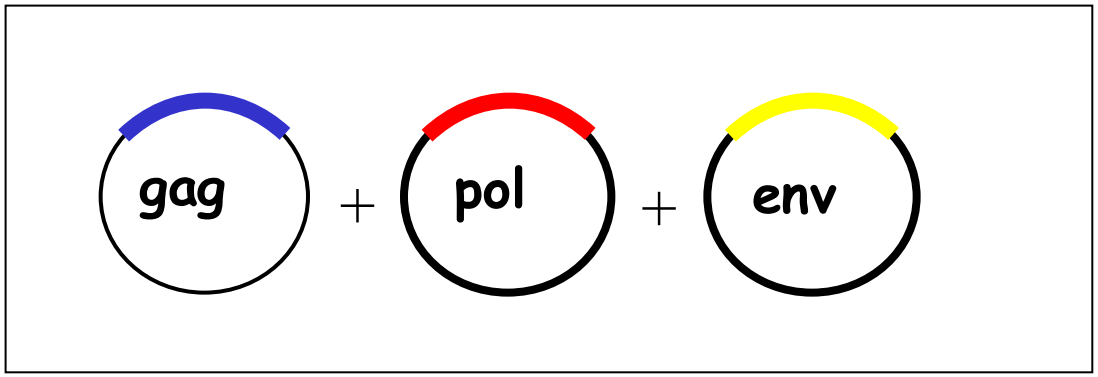
LENTIVIRUS (HIV)

Vector:



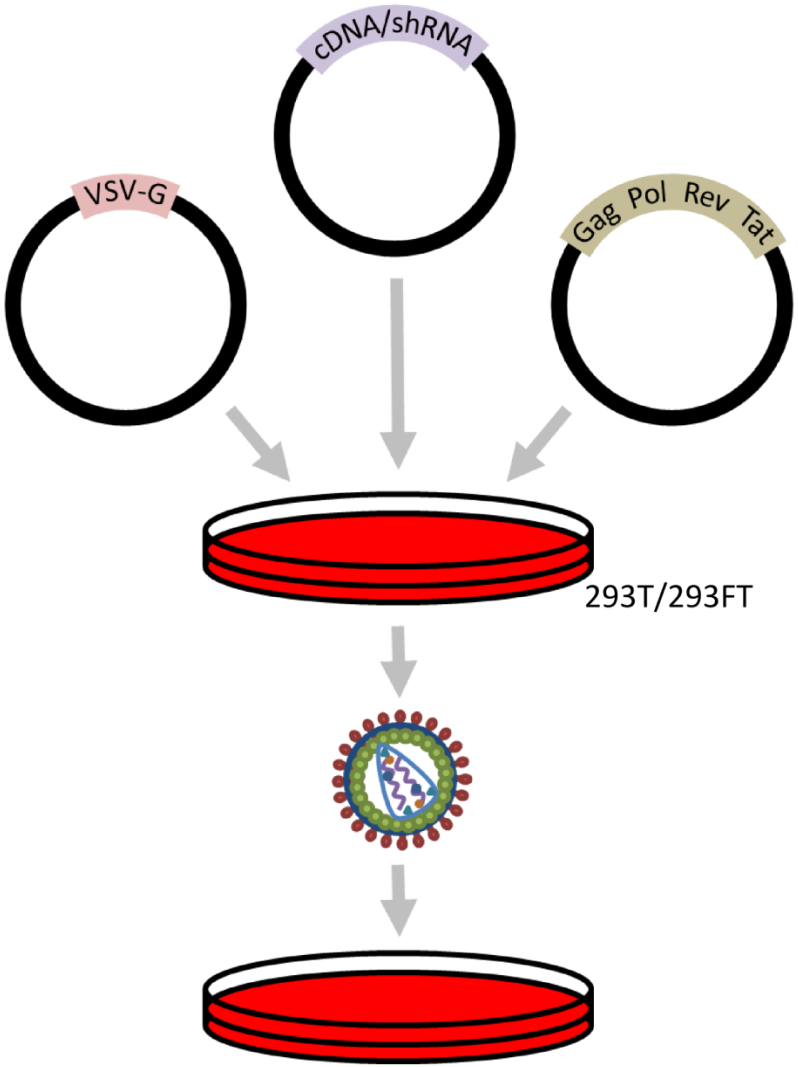
Modify the genome by removing viral genes

Packaging plasmids:



Proteins required for packaging are supplied on plasmids

Production of viral particles



1) Transfect packaging cells

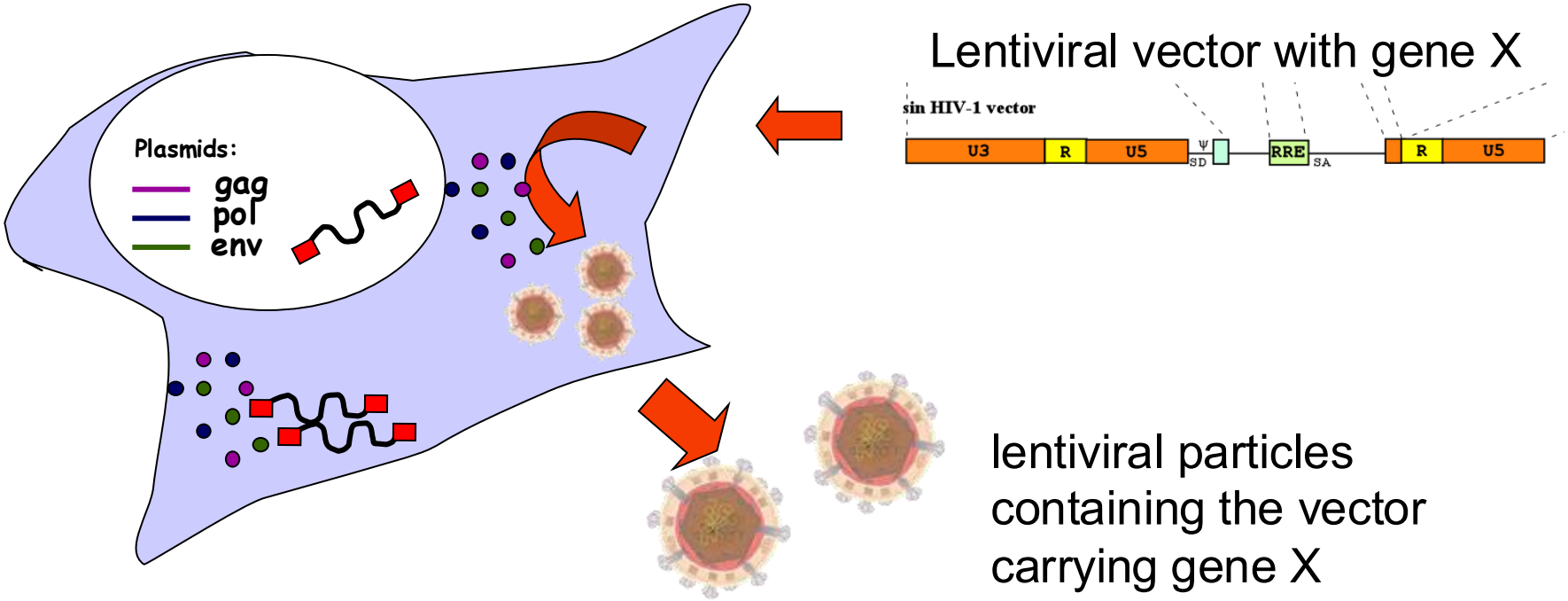
2) Collect virus particles

3) Transduce target cells

Production of viral particles

•Packaging cells (mouse 3T3 fibroblasts) are transformed with the packaging plasmids (gag, pol, env).

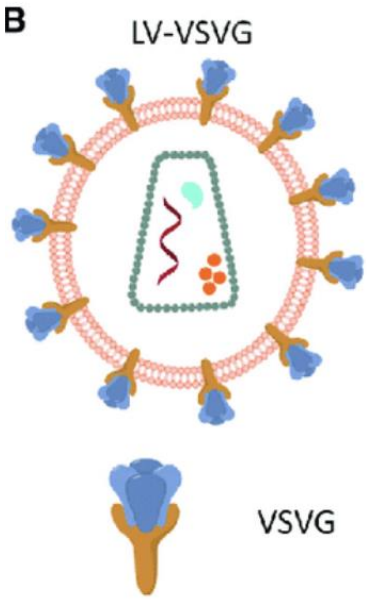
•The lentiviral vector is then transfected into the same cells. RNA transcribed from the LTRs encounters all the proteins it requires, supplied by the helper system, and is selectively packaged together with reverse transcriptase (RT) into viral particles.



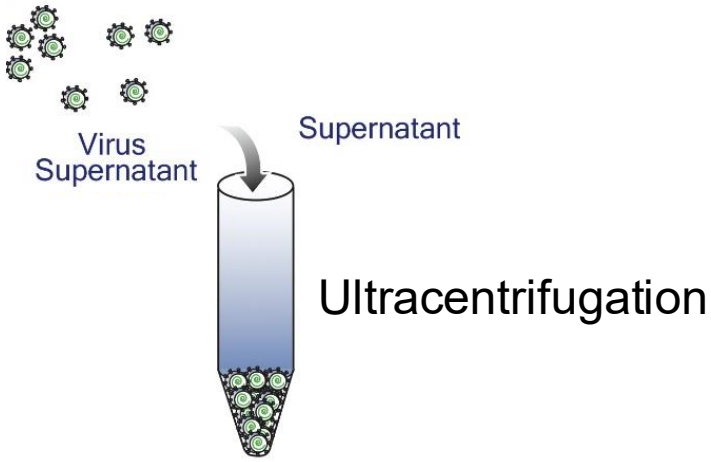
Production of viral particles

Vesicular **stomatitis virus glycoprotein G (VSV-G) is** used as the envelope protein during packaging because of its broad tropism.

VSV-G binds members of the LDL receptor family and mediates membrane fusion, enabling these viruses to infect a wide range of cell types from many host species, including rodent and human cells.



Purification of viral particles



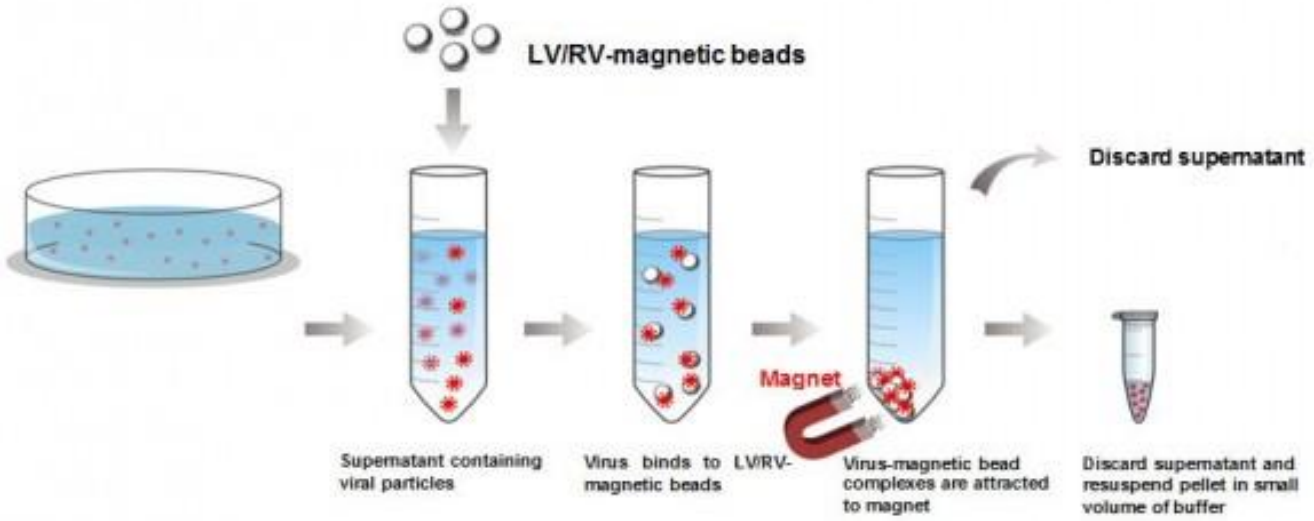
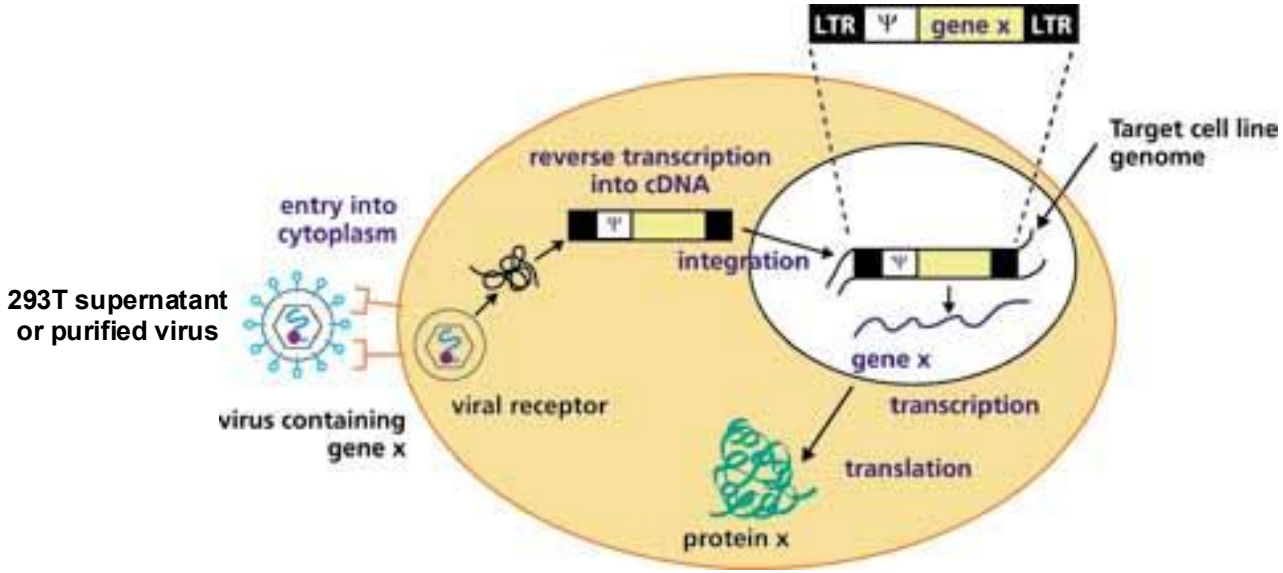


Fig.1. Protocol to capture and concentrate virus using Lentivirus (LV) / Retrovirus (RV) magnetic beads.

Infection of cells with retroviral particles

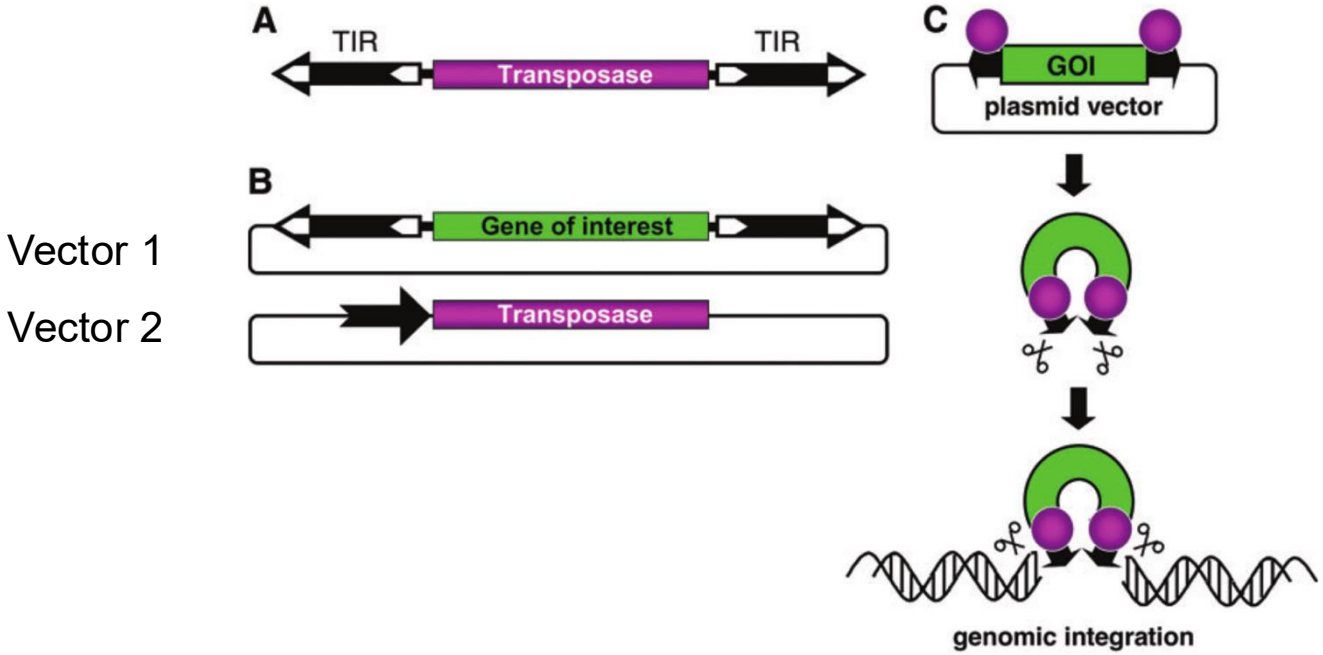


Viral RT reverse-transcribes the RNA into DNA, which integrates into the genome. Only gene X is transcribed, while all viral genes are absent.

Lentiviral vectors allow stable transgene expression

Transposon vectors

(A) Autonomous transposable elements consist of terminal inverted repeats (TIRs; black arrows) flanking the transposase gene. (B) Transposition vector system. One component contains the DNA of interest between the TIRs in a plasmid vector. The other is a transposase expression plasmid, in which the black arrow indicates the promoter driving transposase expression. (C) The transposon carrying the DNA of interest is excised from the donor plasmid and integrated into a chromosomal site by the transposase.

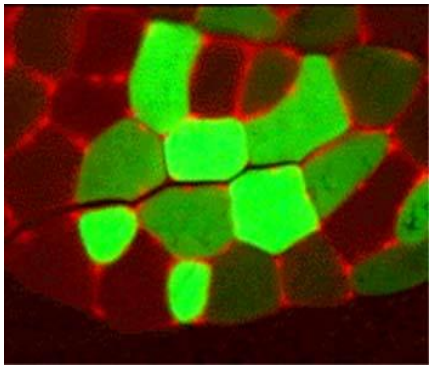


The cells must be transfectable!

How can I select cells that received the transgene?

- Include the GFP gene in the lentiviral vector/transposon





→ FACS/Sorting

- Include a positive selection gene (e.g., puromycin resistance) in the lentiviral vector/transposon → 3–5 days selection → Cells with an integrated gene

