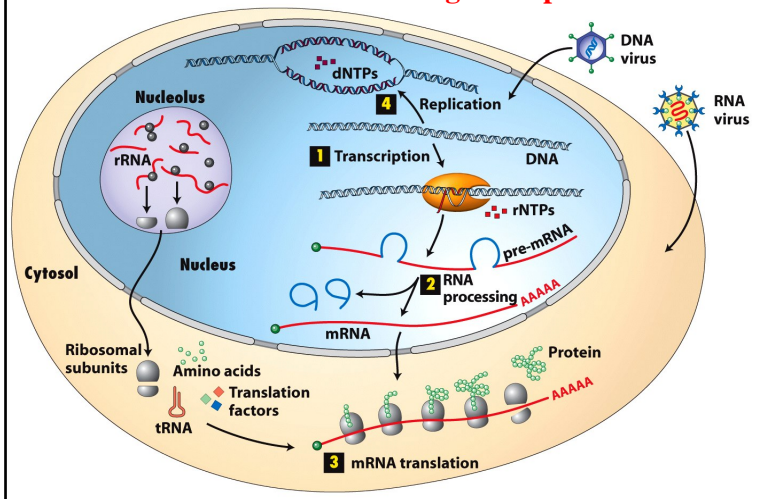
Molecular and Cellular Biology

5a. Basic Molecular Genetic Techniques

Prof. Dr. Klaus Heese

Overview of four basic molecular genetic processes



Practical methods in the laboratory for Protein

isolation, identification and characterization

Molecular and Cell Biology Technologies – Strategy to characterize a gene of interest (goi)

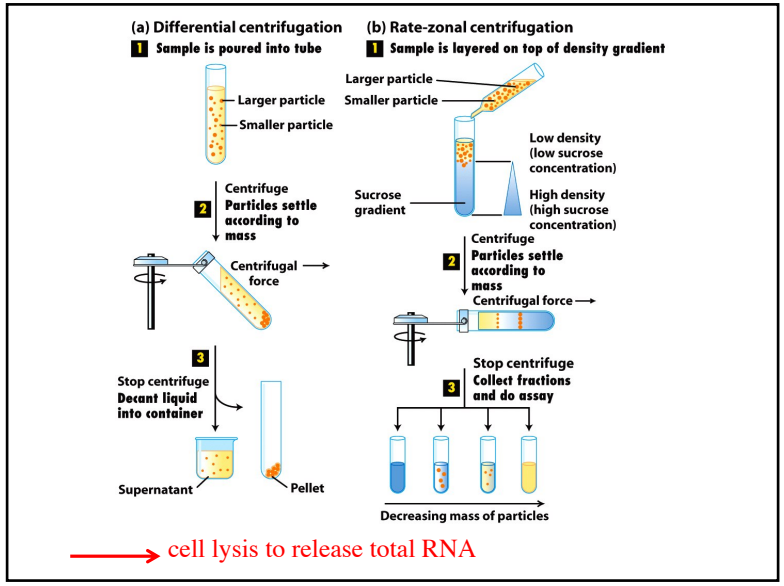
brain **tissue** ---> brain **cells** ---> **t**otal **RNA** ---> **mRNA** --->

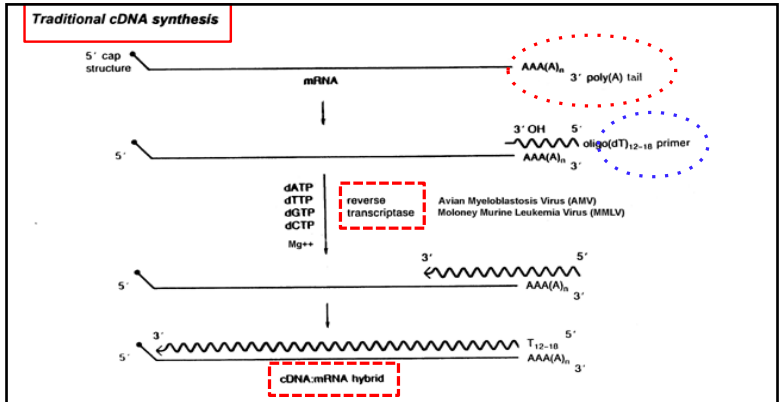
By Reverse Transcriptase (RT) enzyme:
---> **c**omplementary DNA (**c**DNA; single strand) synthesis --->

By polymerase chain reaction (PCR): generation of double strand DNA:
---> dsDNA (goi) (and amplified dsDNA)

cloning: sub-cloning:
---> goi in p_1DNA ---> goi in p_2DNA ---> sequencing
(plasmid DNA)

---> followed by other tests



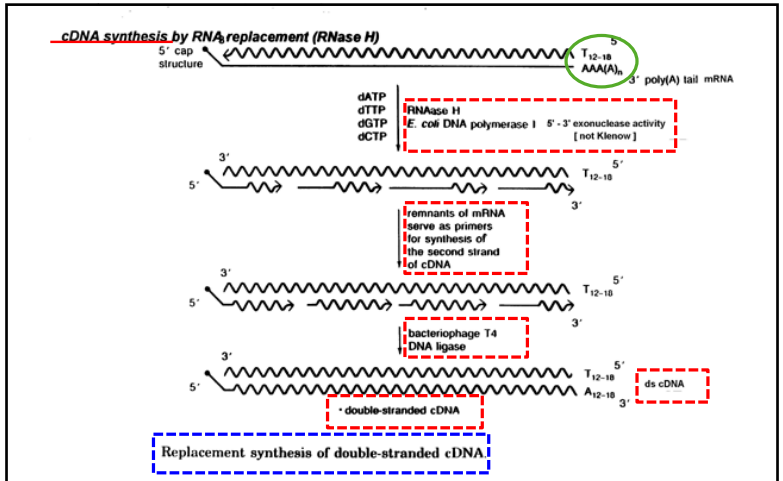


Synthesis of the first strand of cDNA using an oligo(dT) primer and reverse transcriptase.

cDNA is a DNA copy synthesized from mRNA. The enzyme used is reverse transcriptase an RNA-dependent DNA polymerase isolated from a retrovirus (AMV or MMLV). As with other polymerases a short double-stranded sequence is needed at the 3' end of the mRNA which acts as a start point for the polymerase. This is provided by the poly(A) tail found at the 3' end of most eukaryotic mRNAs to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridized (polyT-polyA hybrid). Together with all 4 deoxynucleotide triphosphates, magnesium ions and at neutral pH, the reverse transcriptase synthesises a complementary DNA on the mRNA template. The progress of synthesis can be monitored by incorporation of P-32 radioactive nucleotides.

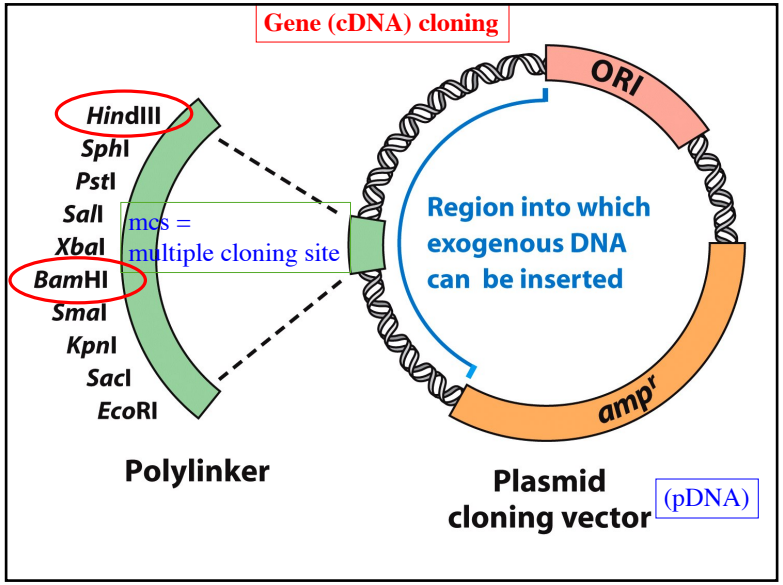
Each mRNA molecule in the mixture with a poly(A) tail can be a template and will produce a cDNA in the form of a single stranded molecule bound to the mRNA (cDNA:mRNA hybrid). The cDNA requires to be converted into a double stranded DNA before it can be manipulated and cloned. This is carried out using another DNA polymerase - DNA Pol I (Klenow fragment). This is the large fragment (75kDa) of DNA polymerase I following its proteolysis with subtilisin; the resulting enzyme has 5'-3' polymerase activity and 3'-5' exonuclease activity but has lost the 5'-3' exonuclease activity associated with the whole enzyme. Commercial sources of the Klenow enzyme use a truncated pol A gene cloned and expressed in E.coli. Klenow polymerase is used to avoid degradation of the newly synthesised cDNAs. To produce the template for the polymerase the mRNA must be removed from the ss cDNA:mRNA hybrid. This is achieved either by boiling or by alkaline treatment. The resulting ss cDNA is used as the template to produce the second DNA strand. As with other polymerases a double stranded primer sequence is needed and this is fortuitously provided during the reverse transcriptase synthesis which produces a short complementary tail at the 5' end of the cDNA. This tail loops back onto the ss cDNA template (the so-called hairpin loop) and provides the primer for the polymerase to start the synthesis of the new DNA strand producing a double stranded cDNA (ds cDNA). A consequence of this method of cDNA synthesis is that the two complementary cDNA strands are covalently joined through the hairpin loop ie. the ds cDNA is essentially a single molecule (shown by electrophoresis on denaturing gels). The hairpin loop is removed by use of a single strand specific nuclease (S1 nuclease from Aspergillus oryzae).

Synthesis of double-stranded cDNA by the self-priming method.



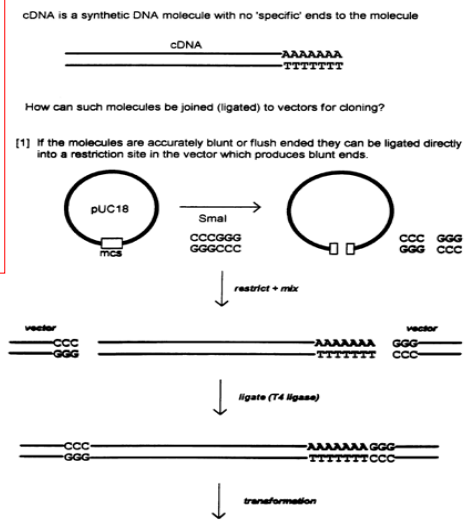
Another strategy for cDNA synthesis employs a ribonuclease (RNase H) which recognises the RNA component of a DNA:RNA hybrid and cleaves the RNA at a number of non-specific sites leaving short oligoribonucleotides attached to the cDNA. These serve as primers for the polymerase to synthesise the second strand cDNA; DNA Pol I (not Klenow) is used since the 5'-3' exonuclease activity is needed to remove RNA in front of the enzyme. The newly synthesised strands of cDNA are joined by ligation using T4 ligase enzyme (+ATP).

Gene (cDNA) cloning

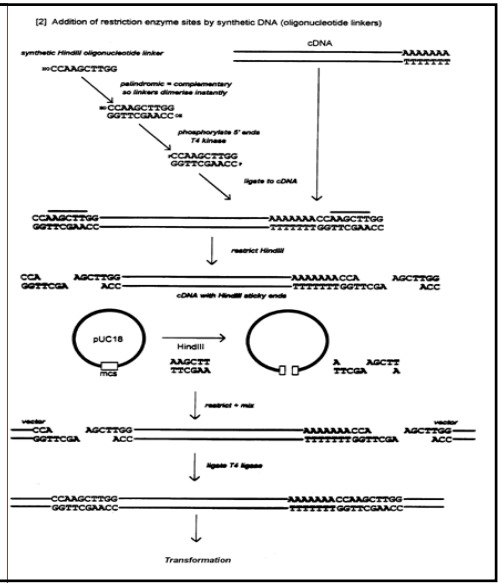


cDNA Library Construction

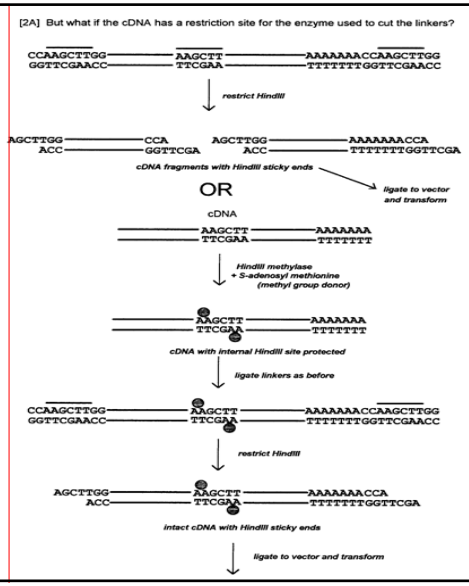
Since the cDNA's resulting from either of the first two methods have no specific ends (ie restriction sites or sticky ends) they sometimes need to be manipulated for efficient joining to a vector DNA molecule. If the cDNA's are blunt-ended they may be cloned directly into a vector which has been cut with any blunt end cutting restriction enzyme such as Sma I. Blunt-end ligations are usually much less efficient than sticky end ligations and so it is usual to provide cDNA's with cohesive ends.



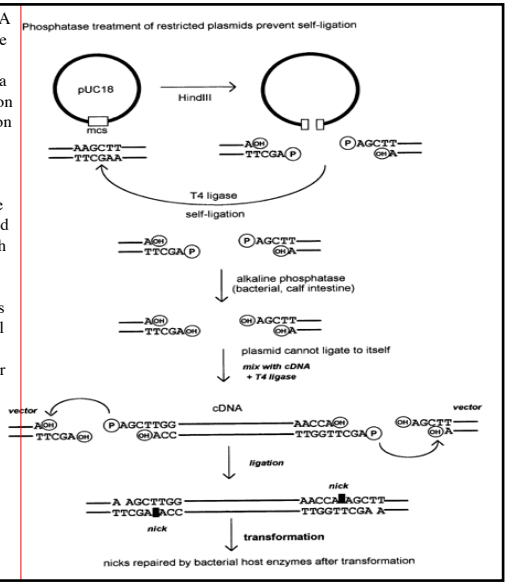
Synthetic cohesive ends can be attached (ligated) to cDNA's with blunt ends. Commonly an oligonucleotide containing a specific restriction site (usually for an enzyme producing a sticky or cohesive end) is made; since most restriction sites are palindromic the synthetic oligonucleotide immediately hybridises to itself (becomes double-stranded). These oligos or 'linkers' are then attached to the ends of the cDNA's using T4 ligase (+ATP); chemically synthesised DNA does not have 5' phosphate groups (5' hydroxyl groups only) essential for this ligation and so oligonucleotides are treated with T4 polynucleotide kinase (+ATP) which phosphorylates the 5' ends. Following ligation to the cDNA's they are then treated with the corresponding restriction enzyme to produce cDNA's with cohesive termini suitable for efficient ligation to a vector with compatible cohesive ends. Ligation of synthetic ends to DNA is efficient because high concentrations of oligonucleotides can be used.



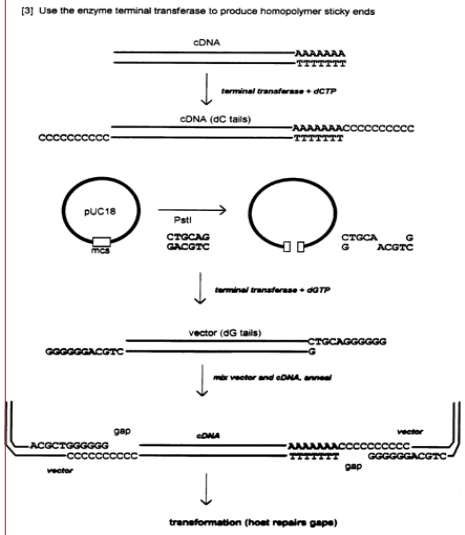
A drawback when using the oligonucleotide linker approach is that any cDNA's containing a restriction site for the same enzyme whose site is incorporated into the oligonucleotide will be cleaved internally when the ends are cleaved, to produce two truncated cDNA molecules. One way to avoid this is to protect any internal restriction sites in the cDNA's by pre-treatment with the methylase enzyme (also known as a methyltransferase) specific for the restriction site being used in the oligos. For example if Hind III oligos are being used the cDNA's can be methylated using Hind III methylase and S-adenosyl methionine (methyl group donor) which protects any internal Hind III sites from subsequent restriction with Hind III. After protection the methylase enzyme is removed and the oligos attached as before.



Ligation will join any compatible DNA ends irrespective of the DNA molecule to which they are attached. In a conventional cloning operation using a plasmid vector with a unique restriction site the majority of the ligation reaction rejoins the restricted plasmid back to itself (self-ligation). This is a consequence of the relatively high concentration of compatible ends - the two compatible sequences at either end of the same molecule! One trick which is often used to prevent an unwanted ligation is to remove the 5' phosphate group from the ends - ligation requires a 5' phosphate group and a 3' hydroxyl group on the ends of the compatible DNA fragments. The enzyme used for this is alkaline phosphatase from bacterial sources or calf intestines. Once treated DNA fragments (eg plasmid) cannot ligate back together. In cDNA cloning this means that ligation can only take place between the plasmid and a cDNA - only a recombinant plasmid is produced (ie one containing an insert).

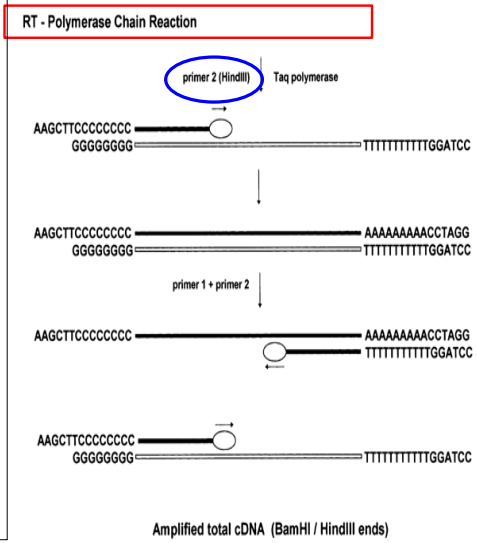


Another technique used to efficiently clone cDNA's is to produce cohesive ends on cDNA's and plasmid exploiting the activity of terminal transferase, a template independent DNA polymerase. This enzyme from calf thymus catalyses the addition of 'homopolymer tails' to the 3' ends of the DNA; in the presence of a single base nucleotide (eg dCTP) the enzyme builds up a string of C's (poly C tail) at both ends of the DNA's. It will use either blunt ended DNA or 3' protruding ends produced by restriction enzymes such as Pst I. Choosing compatible homopolymer tails on cDNA's (eg. poly C) and vector (eg. poly G) allows these ends to anneal (hybridise) together so inserting the fragment into the vector. Using this system there is no requirement to ligate since the inserted fragment is stably maintained until the transformed E.coli host enzymes repair any gaps and covalently seal the plasmid.



The development of the Polymerase Chain Reaction (PCR) using the thermostable DNA polymerase Taq polymerase has seen several applications developed for the production and cloning of cDNA's particularly in situations where only very small quantities of mRNA is available (small amounts of tissue; low abundance of mRNA). The example shown in the figures is only one of many techniques which combine the activities of reverse transcriptase and Taq polymerase in what has become known as RT-PCR. The objective in most of these approaches is to provide sequences at both ends of the cDNA's to which complementary primers can be hybridised to allow amplification of the sequence between. In the strategy shown here, the poly-A tail of the mRNA is used initially (as in the traditional cDNA synthesis) to provide the start point for the reverse transcriptase using oligo-dT as the primer. Note the modified oligo-dT primer contains additionally a restriction site sequence (here e.g. for the Bam HI enzyme). The initial synthesis of cDNA is otherwise exactly the same as in the traditional method. Following the synthesis of the first strand of cDNA and removal of the mRNA (see earlier), the 3' end of the cDNA is homo-polymer tailed using dGTP and terminal transferase - this produces a poly-G tail at the 3' end. The poly-G end can then be used in a similar fashion to the poly A end in the original mRNA.

A second primer - oligo dC can now be used to provide a start point for the DNA polymerase to synthesise the second starnd of cDNA. Note i) that the oligo dC primer also has a restriction site (e.g. Hind III) incorporated at its end and ii) there is now a primer site at both ends of the cDNA (the requirement for PCR amplification). In the presence of the oligo dC primer, dNTP's and Taq polymerase the second strand of cDNA is completed. Note that the synthesis proceeds through the poly-A region and completes the retriction site at the 3' end (Bam HI). Thermal cycling in the presence of both primers (oligo dT and oligo dC) Taq polymerase will amplify the sequences between the two primers in a typical PCR reaction - note that the second restriction site at the 5' end is also completed so that all amplified cDNA's will contain a Hind III site at the 5' end and a Bam HI site at the 3' end. Note also because of these restriction sites that this particular strategy allows directional cloning of cDNA's - useful if an expression library is planned for screening with antibodies. After amplification the final step is the double restriction of the cDNA's with Hind III and Bam HI and ligation into a similarly cut plasmid vector followed by transformation. Note that a plasmid vector cut with two different restriction enzymes will not have compatible ends and cannot therefore be rejoined to itself (self-ligated); only if a fragment with suitably compatible ends (ie a cDNA) is inserted will a (recombinant) plasmid be formed.



RT - Polymerase Chain Reaction

3

Amplified total cDNA (BamHI / HindIII ends)

AAGCTTCCCCCCCC AAAAAAAAACCTAGG
TTTTTTTTTTGGATCC

AAGCTTCCCCCCCC
GGGGGGGG TTTTTTTTTTGGATCC

15-30 cycles

AAGCTTCCCCCCCC AAAAAAAAACCTAGG
TTCGAAGGGGGGGG TTTTTTTTTTGGATCC

AAGCTTCCCCCCCC AAAAAAAAACCTAGG
TTCGAAGGGGGGGG TTTTTTTTTTGGATCC

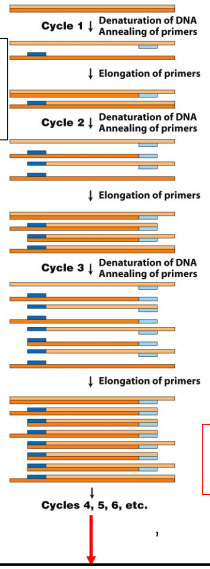
BamHI + HindIII

AGCTTCCCCCCCC AAAAAAAAAC
AGGGGGGGG TTTTTTTTTTGGATC

ligate to BamHI / HindIII cut vector

PCR

polymerase chain reaction



PCR-products with
BamHI and HindIII ends

Gene (cDNA) cloning

Plasmid cloning vector (pDNA)

HindIII
SphI
PstI
SalI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI

mcs =
multiple cloning site

Polylinker

ORI

Region into which
exogenous DNA
can be inserted

ampr

Plasmid
cloning vector

Restriction Enzymes for gene cloning; example for EcorI site:

EcoRI

5′ G A A T T C 3′
3′ C T T A A G 5′

Cleavage

EcoRI

Sticky ends

5′ G A A T T C 3′
3′ C T T A A G 5′

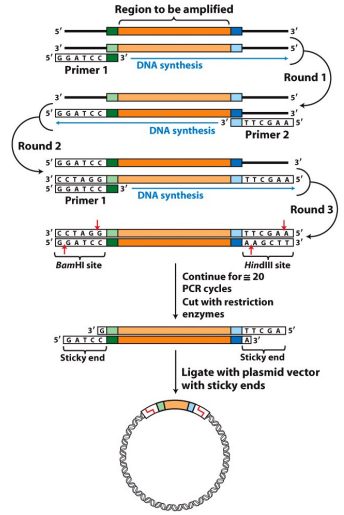
BamHI and HindIII have similar sticky ends

TABLE 5-1 Selected Restriction Enzymes and Their Recognition Sequences

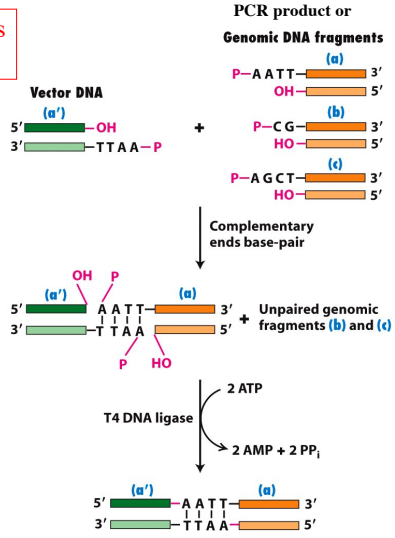
ENZYME	SOURCE MICROORGANISM	RECOGNITION SITE*	ENDS PRODUCED
*Bam*HI	*Bacillus amyloliquefaciens*	-G-G-A-T-C-C- -C-C-T-A-G-G-	Sticky
*Sau*3A	*Staphylococcus aureus*	-G-A-T-C- -C-T-A-G-	Sticky
*Eco*RI	*Escherichia coli*	-G-A-A-T-T-C- -C-T-T-A-A-G	Sticky
*Hin*dIII	*Haemophilus influenzae*	-A-A-G-C-T-T- -T-T-C-G-A-A-	Sticky
*Sma*I	*Serratia marcescens*	-C-C-C-G-G-G- -G-G-G-C-C-C-	Blunt
*Not*I	*Nocardia otitidis-caviarum*	-G-C-G-G-C-C-G-C- -C-G-C-C-G-G-C-G-	Sticky

PCR

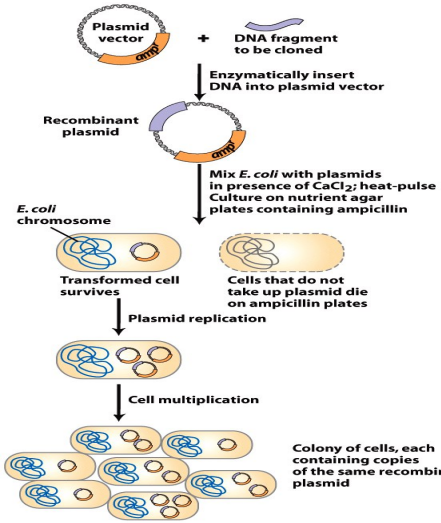
polymerase chain reaction



Cloning of DNA fragments
Into pDNA

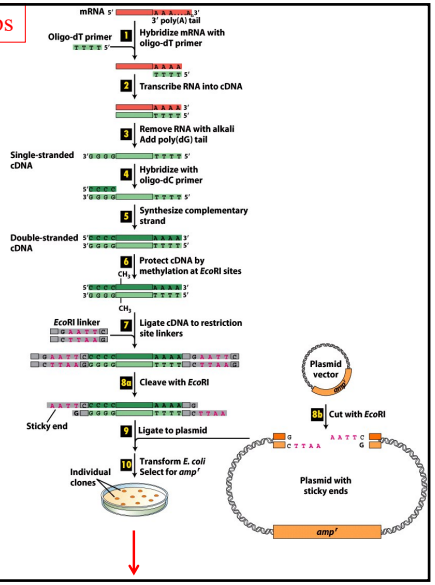


E. coli transformation
with pDNA

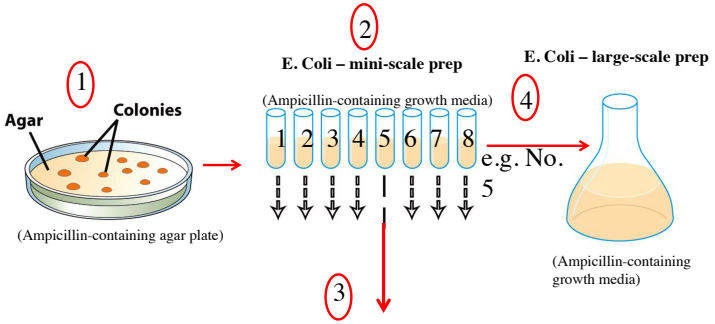


growing (proliferating) bacterial cells (E. coli) used for pDNA amplification

Summary of mRNA cloning steps

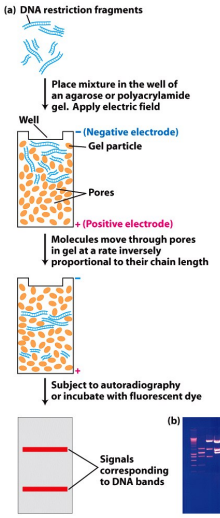


Culturing E. coli cells transformed by plasmid DNA on agar plates, checking for positive clones and amplifying such positive clone



Isolate pDNA and check by PCR and/or restriction enzyme analysis and sequencing for positive clones
--->
e.g. No 5 is positive

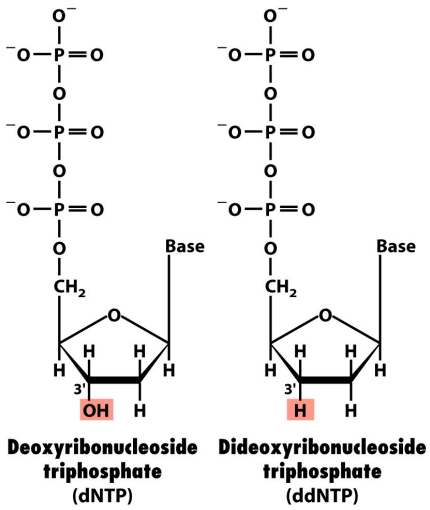
DNA agarose gel to separate (and isolate) DNA fragments



characterization of DNA quality

Sequencing of DNA

characterization of DNA quality by specific gene (pDNA + gene of interest) sequencing.



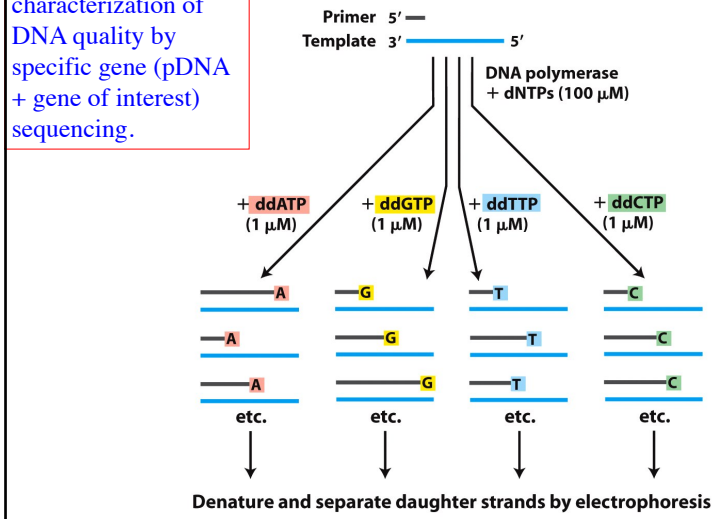
characterization of DNA quality by specific gene (pDNA + gene of interest) sequencing. - **How does DNA sequencing work?**

5′ T A G C T G A C T C 3′
3′ A T C G A C T G A G T C A A G A A C T A T T G G G C T T A A ...

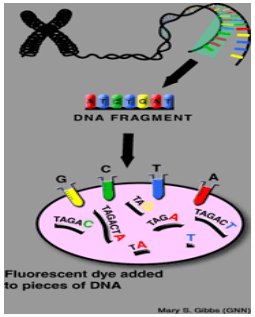
DNA polymerase
+ dATP, dGTP, dCTP, dTTP
+ ddGTP in low concentration

5′ T A G C T G A C T C A G 3′
3′ A T C G A C T G A G T C A A G A A C T A T T G G G C T T A A ...
+
5′ T A G C T G A C T C A G T T C T T G 3′
3′ A T C G A C T G A G T C A A G A A C T A T T G G G C T T A A ...
+
5′ T A G C T G A C T C A G T T C T T G A T A A C C C G 3′
3′ A T C G A C T G A G T C A A G A A C T A T T G G G C T T A A ...

characterization of DNA quality by specific gene (pDNA + gene of interest) sequencing.



Dividing and dying

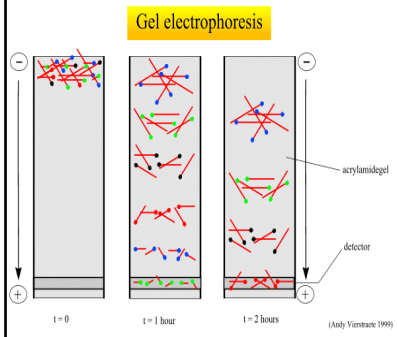


Divided into four batches is added to each batch

One batch of DNA will contain only pieces that end in A,C,T,G

Then a different fluorescent dye is also added to each batch of DNA

How does DNA sequencing work?



In electrophoresis, DNA is placed at one end of a gel

Cause the DNA molecules to move through the gel

DNA molecules become separated into different bands according to their size.

DNA sequencing process

① A mixture of DNA go into sequencing machine.

② Move past a laser that causes the dye molecules to fluoresce.

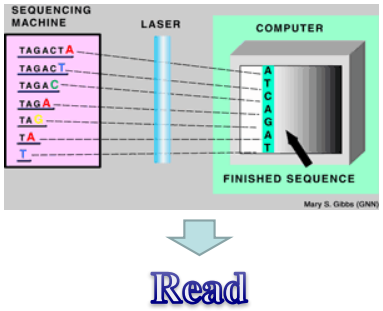
③ A detector reads the color of the fluorescence.

1: blue-T, blue-TAGACT

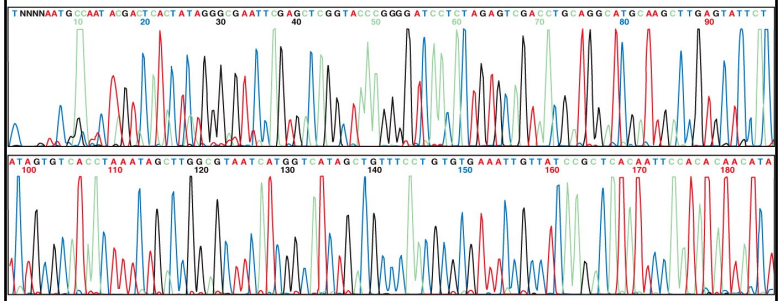
2: red-TA, red-TAGA

3: yellow-TAG

4: green-TAGAC



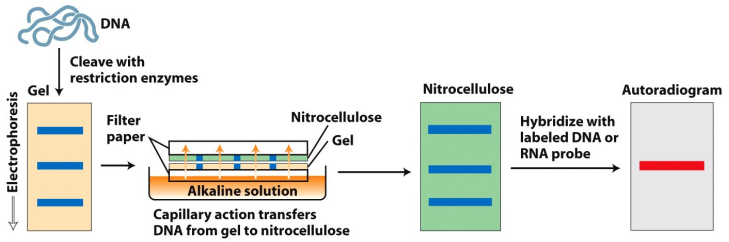
characterization of DNA quality by specific gene (pDNA + gene of interest) sequencing.



Separation method is similar to SDS-PAGE (see slides further below)

Qualitative and quantitative DNA (Southern-) and RNA (Northern-) analysis by Southern blot and Northern blot.

Southern blot (Northern blots (RNA) similar, but more difficult due to RNA degradation)

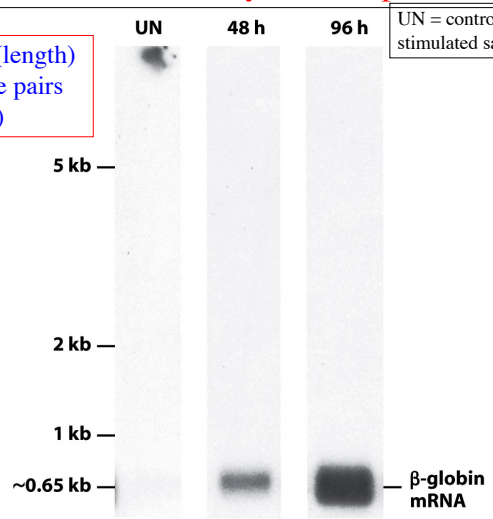


use isotope labeled probes or digoxigenin-dUTP-labeled probes. DIG-labeled hybrids are detected with an anti-DIG-alkaline phosphatase conjugate and the substrates NBT (nitroblue tetrazolium salt) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt), which give a light-blue precipitate. Substrates can be also fluorescent and detected with photo-films.

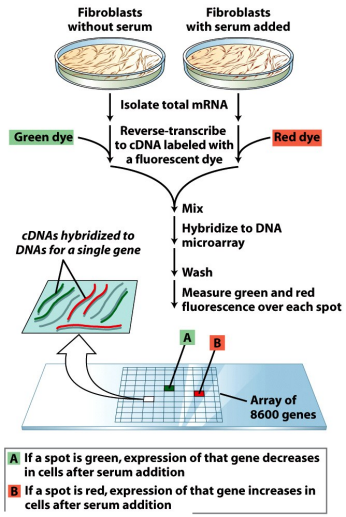
Northern blots (RNA) analysis example

RNA size (length) in kilo base pairs (kbp or kb)

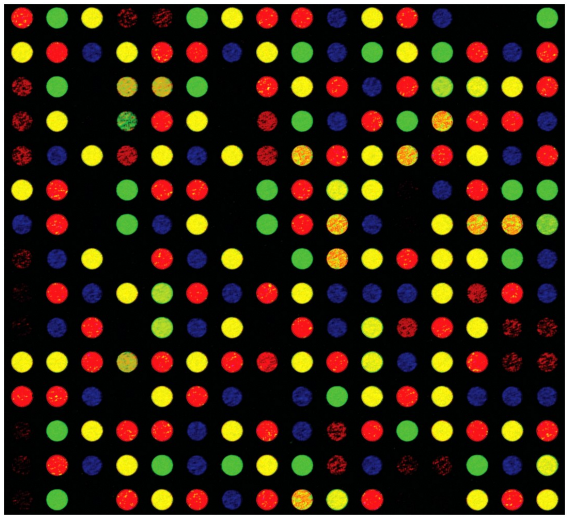
UN = control or un-stimulated sample)

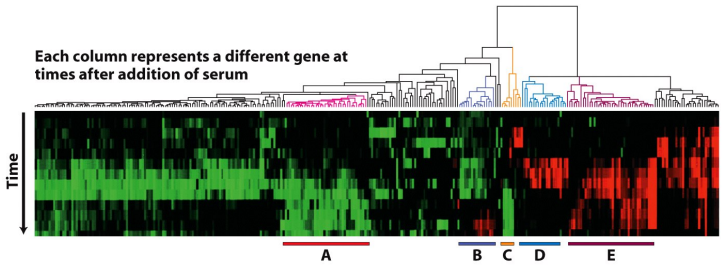


Comparative Gene (mRNA) Expression Analysis



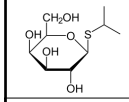
Comparative Gene (mRNA) Expression Analysis





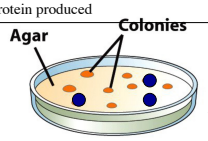
Expression of proteins in E.coli (bacteria, prokaryotic cell)

IPTG (isopropylthio-β-galactoside)

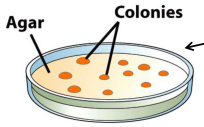


blue: LacZ protein produced that acts on Xgal to turn it blue

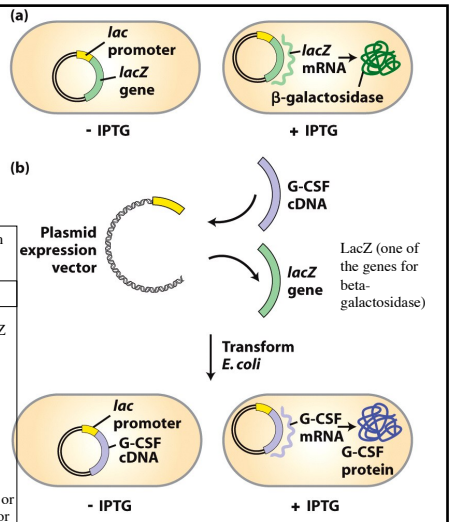
white: cloning into mcs successful and no LacZ protein produced

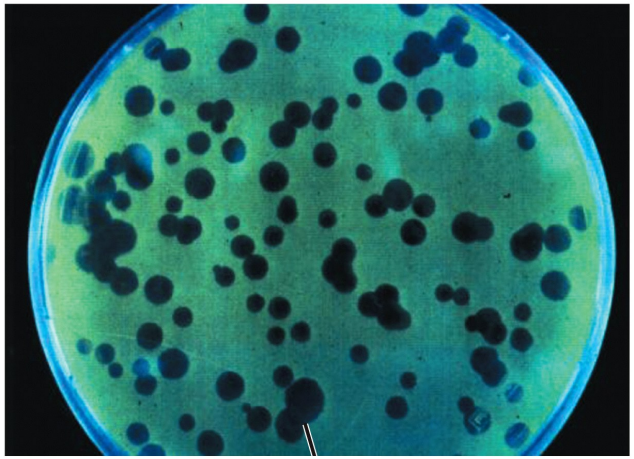


+ X-gal Substrate; if lacZ gene has been kept and mcs inside the LacZ

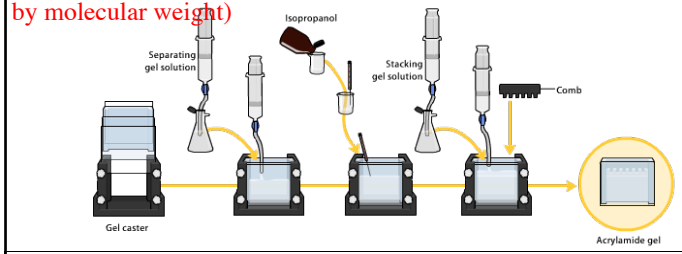


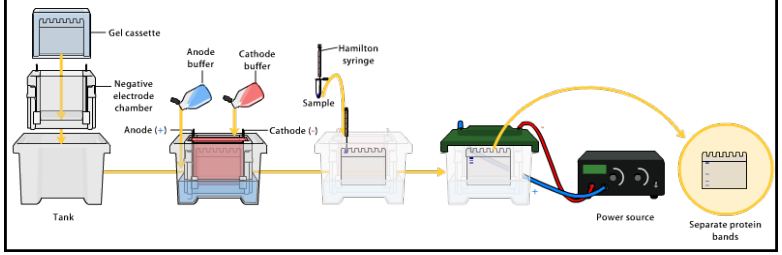
if no LacZ gene or if a normal vector has been used to transform eukaryotic cells





SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, describes a technique widely used in biochemistry for protein separation by molecular weight)



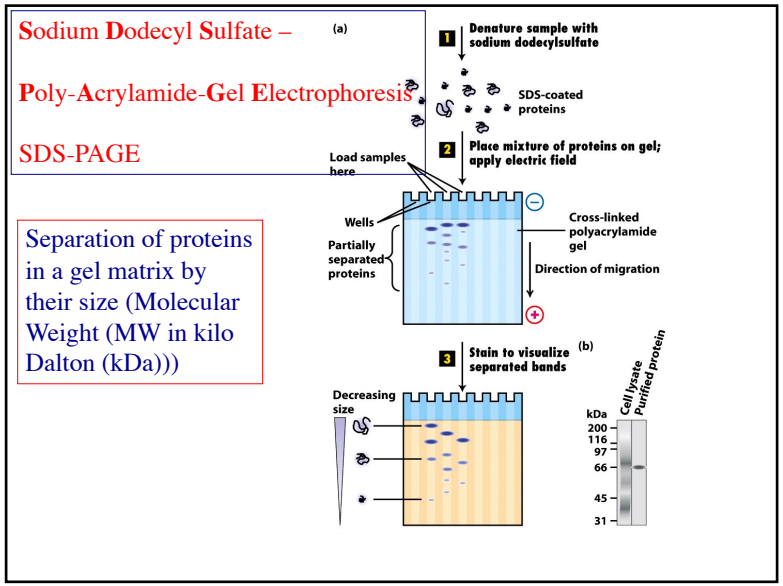


Sodium **D**odecyl **S**ulfate –

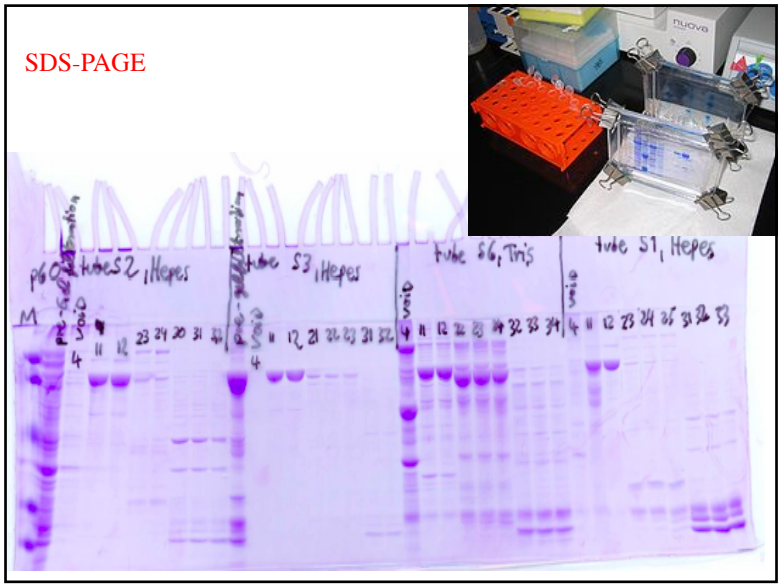
Poly-**A**crylamide-**G**el **E**lectrophoresis

SDS-PAGE

Separation of proteins in a gel matrix by their size (Molecular Weight (MW in kilo Dalton (kDa)))



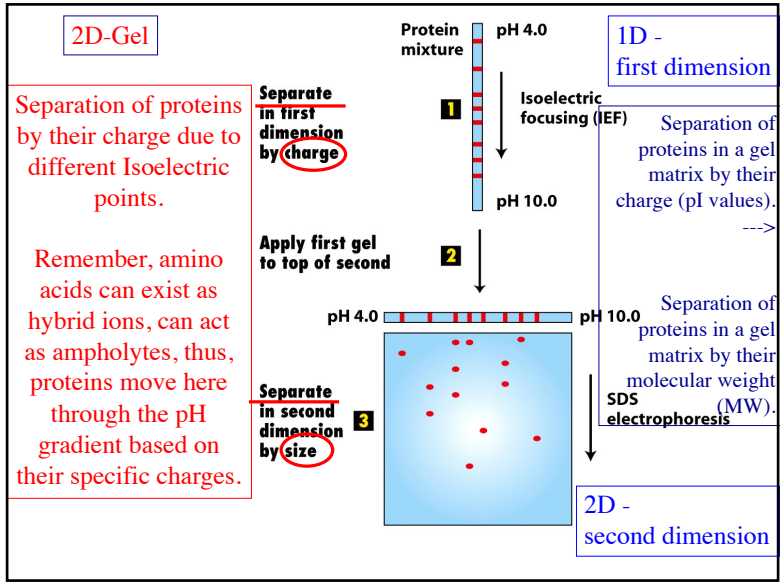
SDS-PAGE



2D-Gel

Separation of proteins by their charge due to different Isoelectric points.

Remember, amino acids can exist as hybrid ions, can act as ampholytes, thus, proteins move here through the pH gradient based on their specific charges.



1D - first dimension

Separation of proteins in a gel matrix by their charge (pI values). --->

Separation of proteins in a gel matrix by their molecular weight (MW).

2D - second dimension

2D Gel analysis after

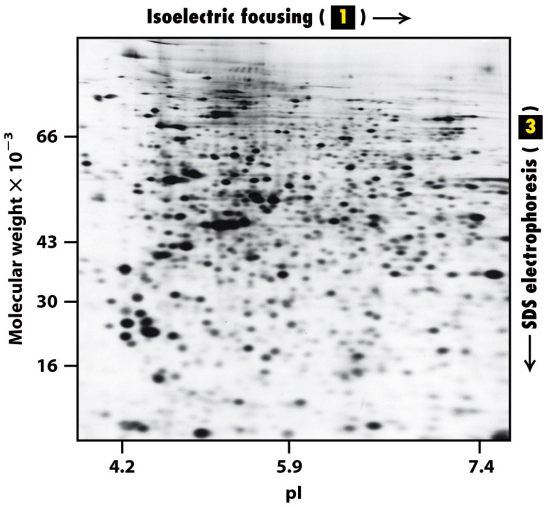
a) Coloring of proteins

Or

b) Radioisotope labelling

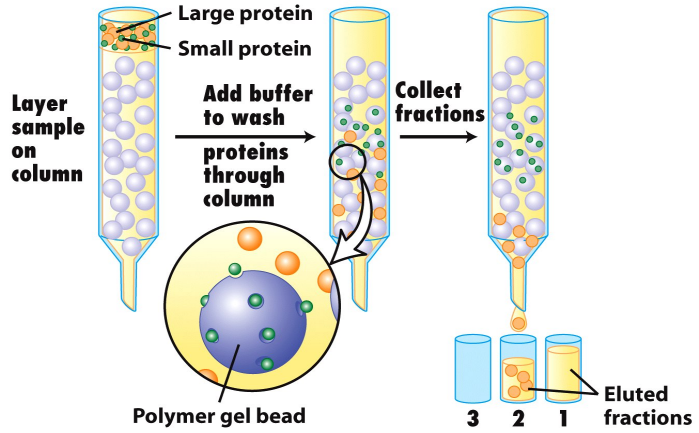
Or

c) DIG labelling



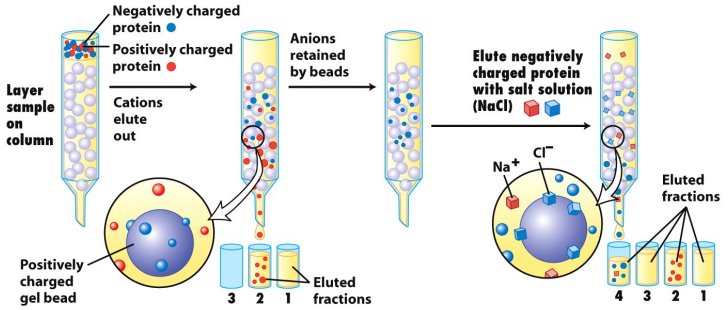
Separation of Proteins by their Size

Gel filtration chromatography

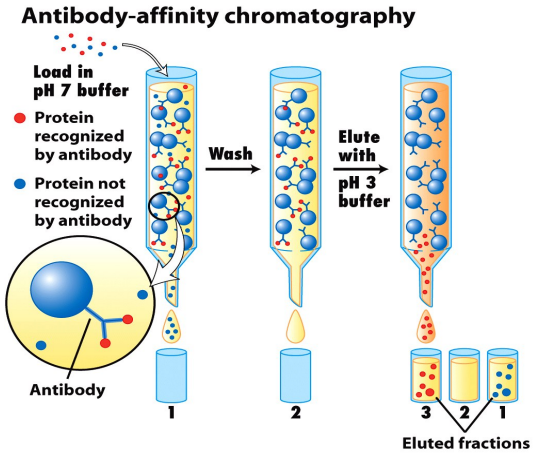


Separation of Proteins by their Charge

Ion-exchange chromatography

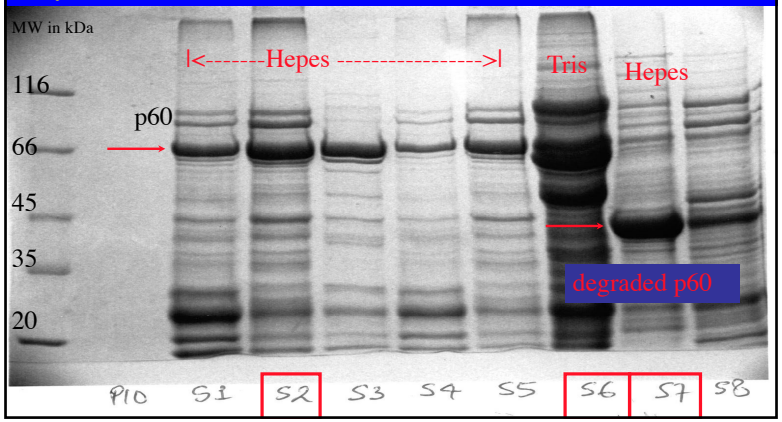


Separation of Molecules by their Specific Interaction – Immune-Affinity: Antibody-Antigen-Interaction

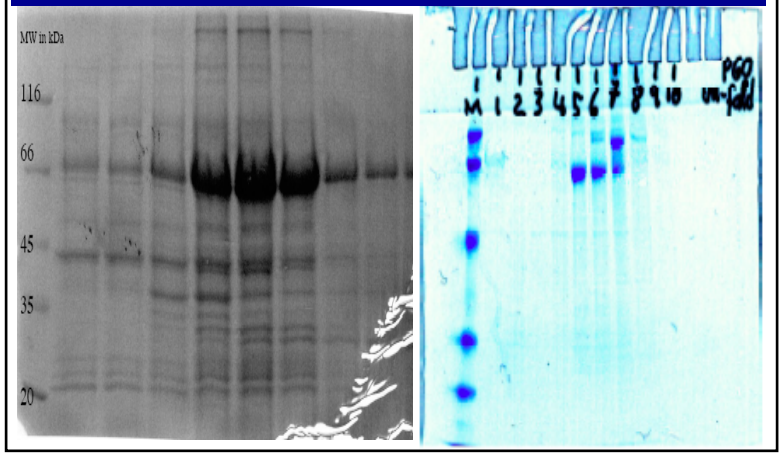


Purification of 6xHis-tag-p60TRP protein from Ecoli by NiNTA-columns

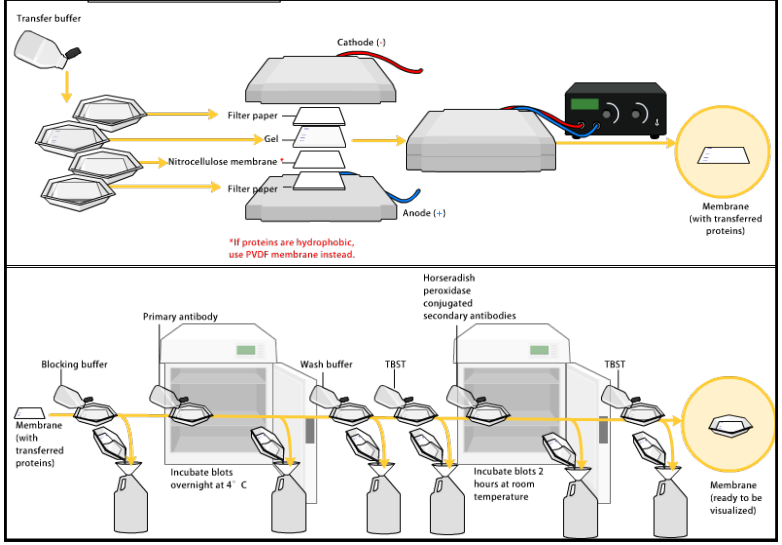
p60TRP from E.coli supernatant after lysis in HEPES-buffer / 500 mM NaCl : total cell lysate loaded on SDS-gel matrix for SDS-PAGE analysis



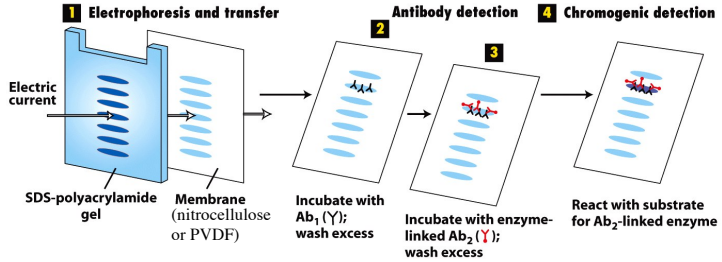
Purification of p60TRP-6xHistidin-tag protein from E.coli by Ni-NTA-columns. Total cell lysates (HEPES buffer) were purified using the Ni-NTA-columns and different 1.5ml fractions collected and analyzed by SDS-PAGE.

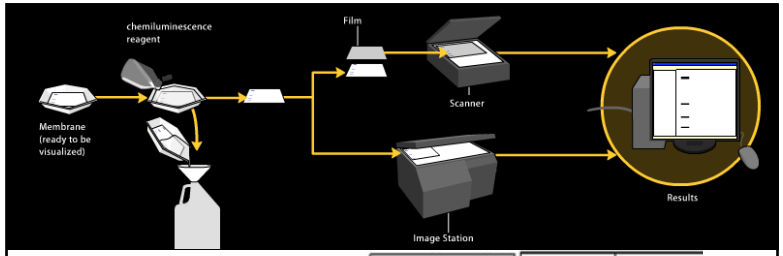


Western Blotting



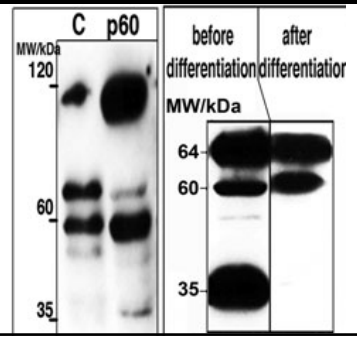
Western Blotting





Western Blotting

Chemiluminescent detection



Molecular and Cell Biology Technologies –
Strategy to characterize a gene of interest (goi)

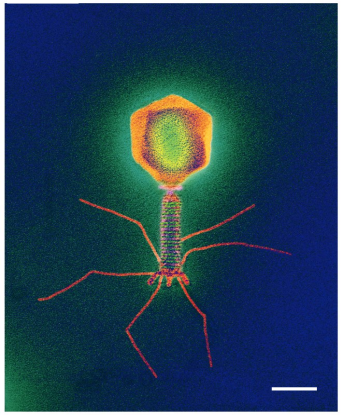
brain **tissue** ---> brain **cells** ---> **t**otal **RNA** ---> **m**RNA ---> **c**DNA ---> **ds**DNA (goi) ---> goi in p_1DNA ---> goi in p_2DNA --->
RT --- PCR
cloning
sub-cloning
sequencing
---> check tissue/organ-specific expression of goi by Northern-/Southern blot or cDNA library screening
cell lysis
expression of goi in E.coli ---> check by SDS-PAGE / Western blot
---> purification by Affinity-/Ion-Exchange-Chromatography
---> (if possible: crystallization ---> X-ray analysis ---> 3D-structure
---> drug development
---> (if not possible: expression of goi in eukaryotic cell system:
---> transfection using electroporation system or lipofectamine solution or e.g. lentivirus system
---> visualizing by confocal microscopy

---> (if not possible: expression of goi in eukaryotic cell system: add signal peptide to goi to express extracellular
---> transfection using electroporation system or lipofectamine solution or e.g. lentivirus system

---> check goi in cell culture supernatant (native folding?!?)
---> check by SDS-PAGE / Western blot
---> purification by Affinity-/Ion-Exchange-Chromatography
---> (if possible: crystallization ---> X-ray analysis ---> 3D-structure
---> drug development

Application of Virology in Modern Molecular & Cellular Biotechnology

A bacteriophage is a virus that infects only bacteria

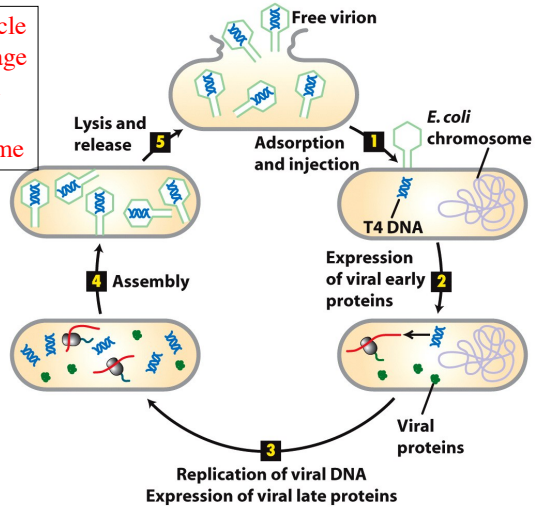


Bacteriophage T4

a non-enveloped DNA virus, mainly/only lytic pathway

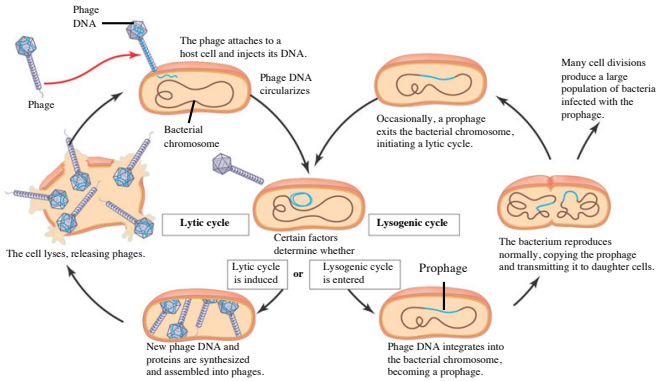
scale-bar = 50 nm

Lytic Replication cycle of E.coli bacteriophage T4, a non-enveloped virus with a double-stranded DNA genome

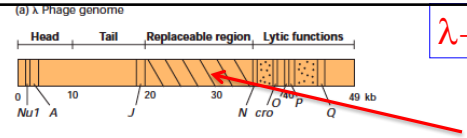


λ-phage

The lytic and lysogenic cycles of phage λ, a temperate phage

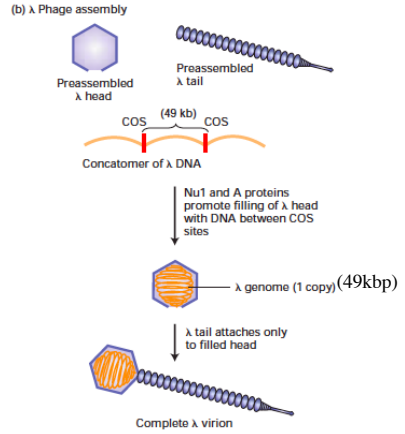


λ-phage



(25kbp/λ-genome)

cDNA library

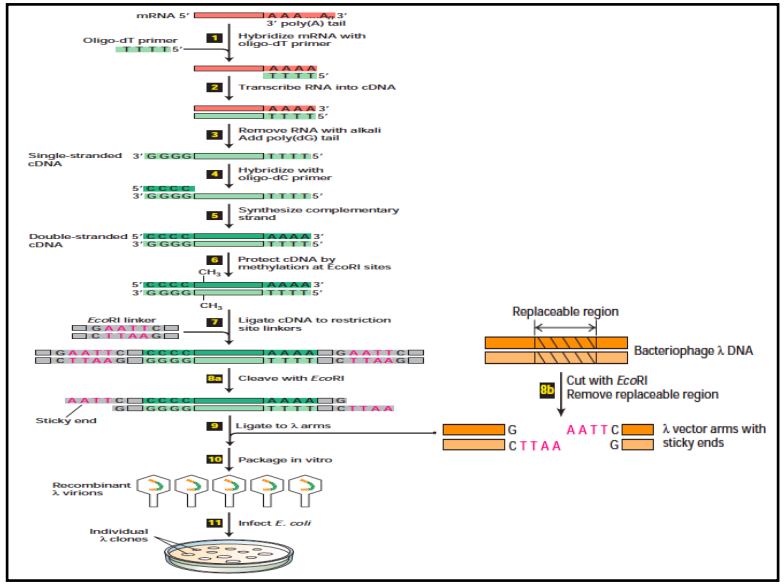


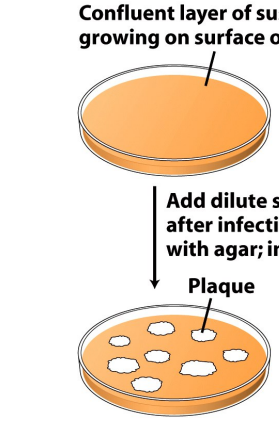
total mRNA

total RNA

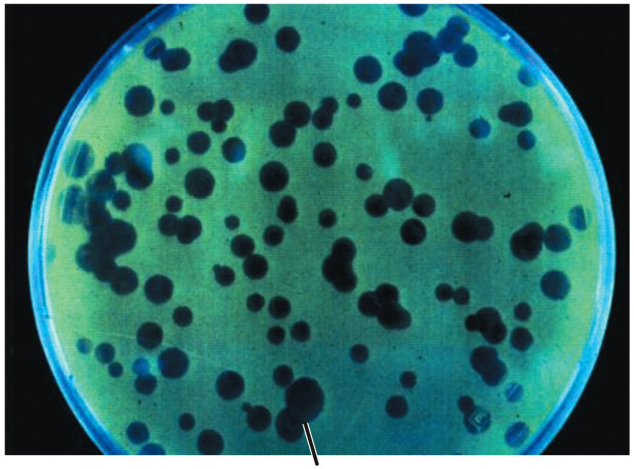
cell

tissue

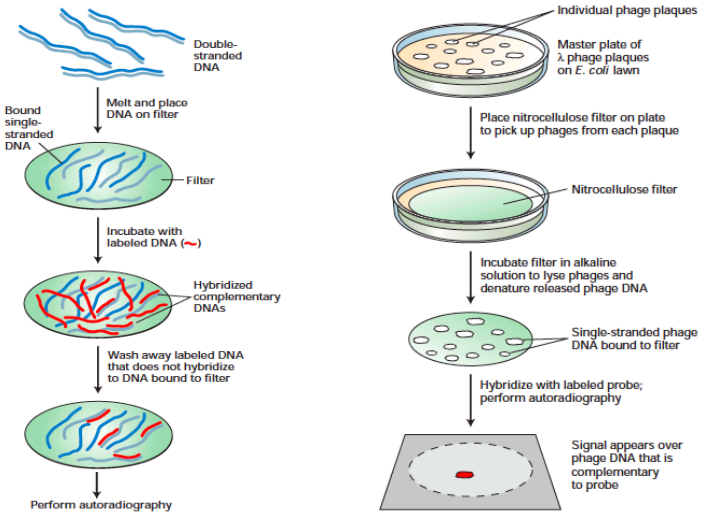




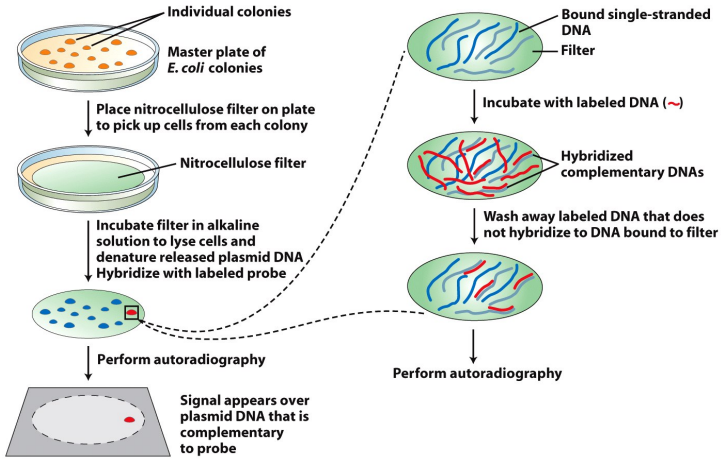
Each plaque represents cell lysis initiated by one viral particle (agar restricts movement so that virus can infect only contiguous cells)



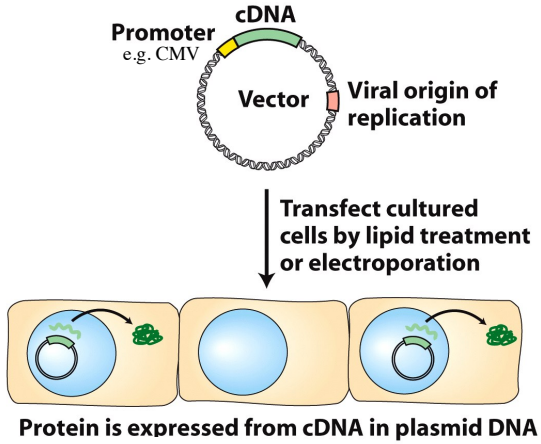
Plaque



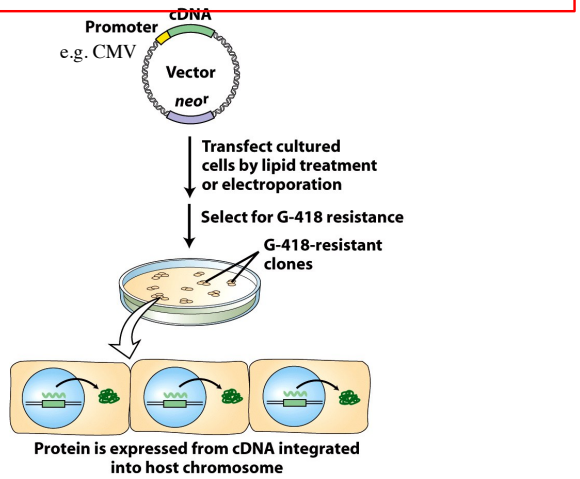
Screening colonies with isotope labeled probes (e.g. a cDNA library cloned into λ DNA)

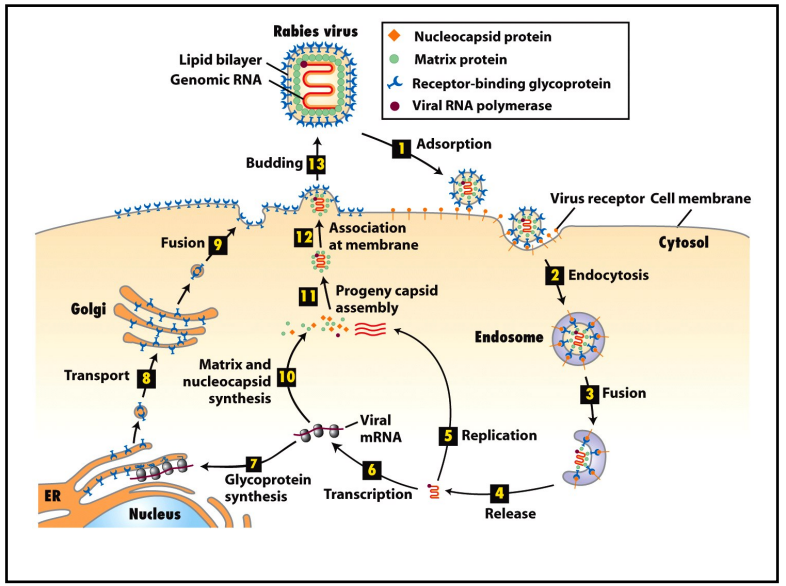


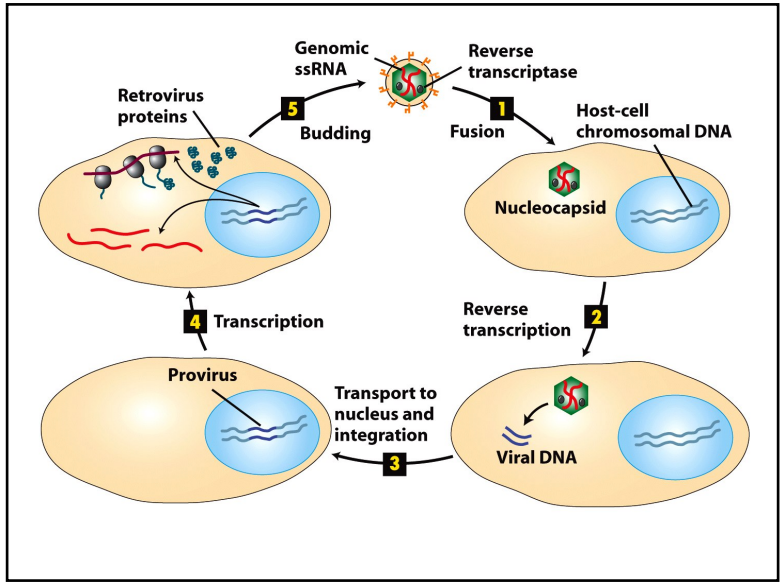
Transient transfection of eukaryotic cells



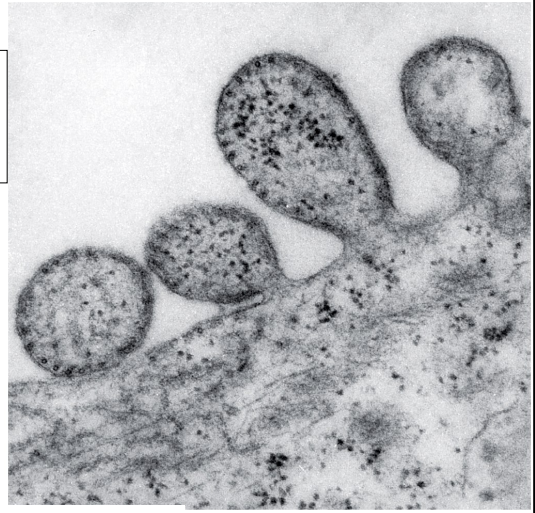
Stable transfection (transformation) of eukaryotic cells

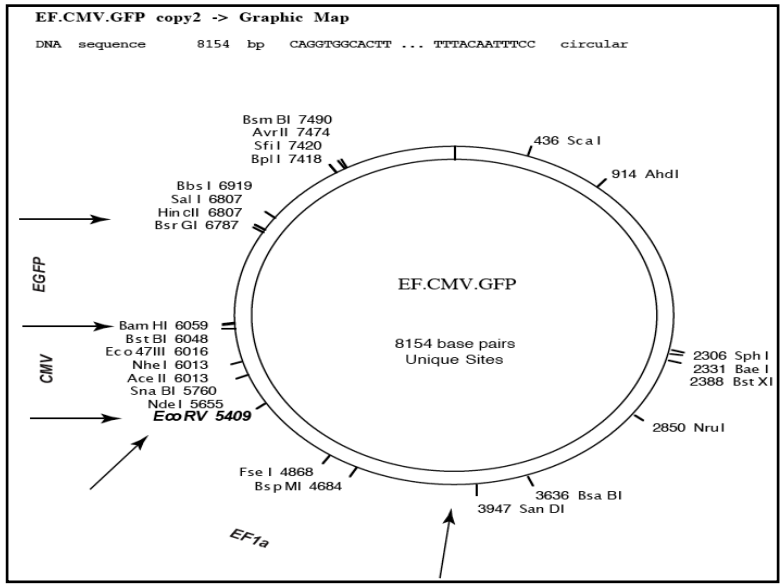


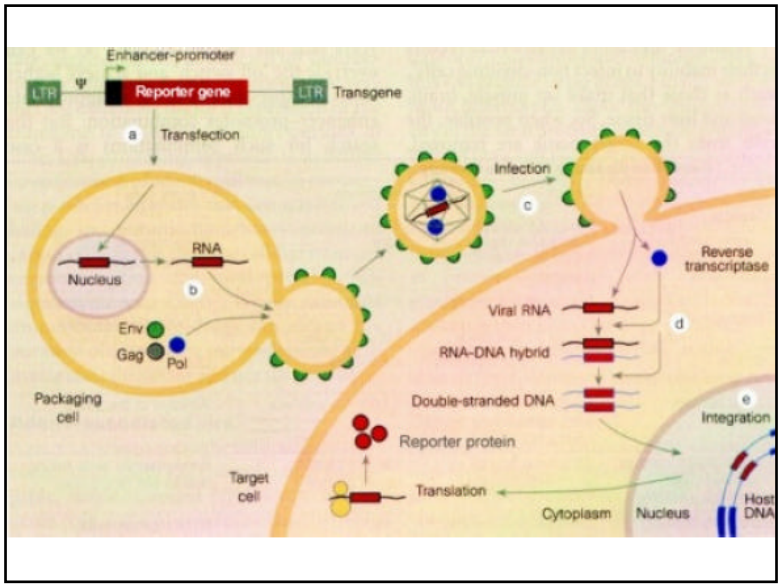




Progeny virions of enveloped viruses are released by budding from infected cells.

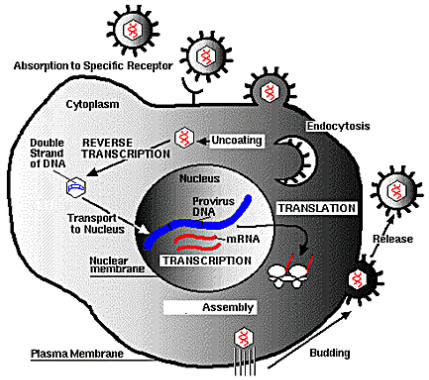




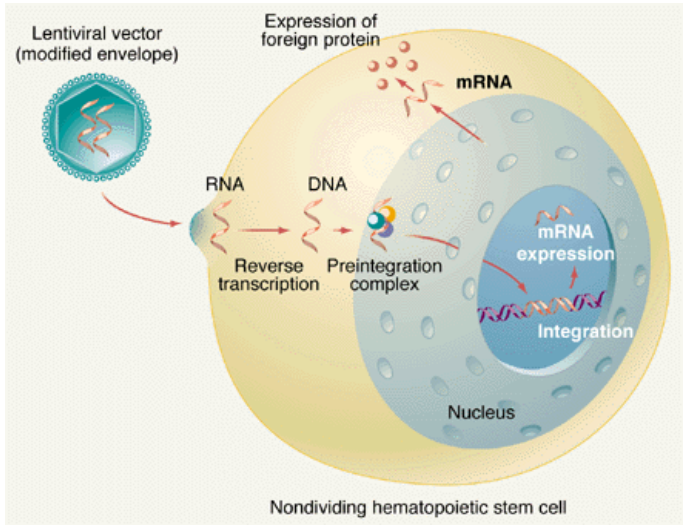


Lentiviruses

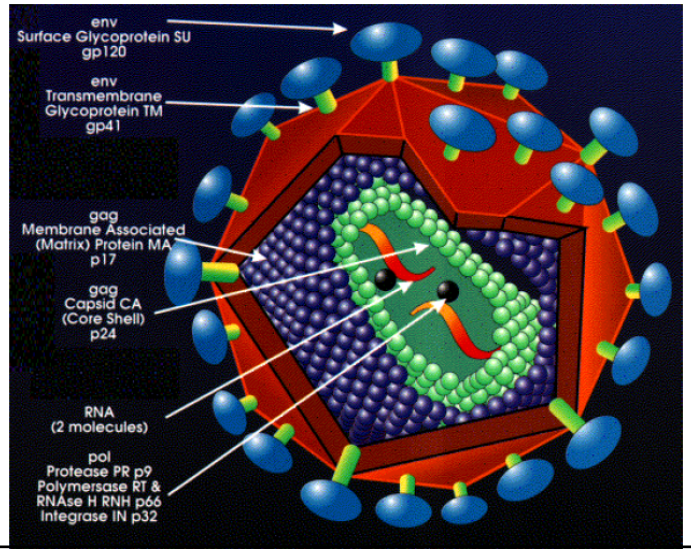
- Retrovirus Family
- Single Strand (+) RNA virus
- Contains 2 copies
- Can infect non-dividing cells
- Contains regulatory proteins
- Latent or active viral states
- Spread by blood and other bodily fluids
- Long lived virus leading to reservoir for persistent infection
- Virus type present in many vertebrate species



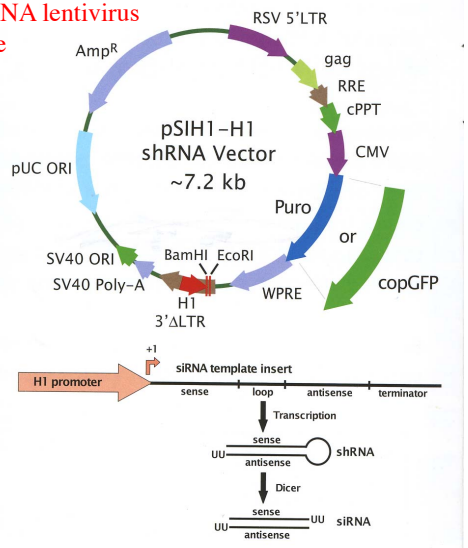
Retrovirus replication



Structure of HIV

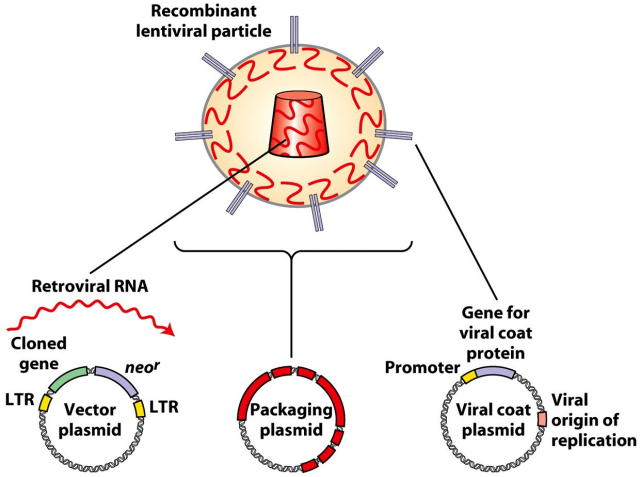


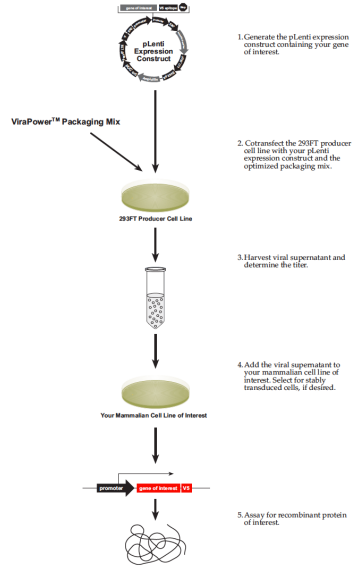
A general siRNA lentivirus pDNA sample



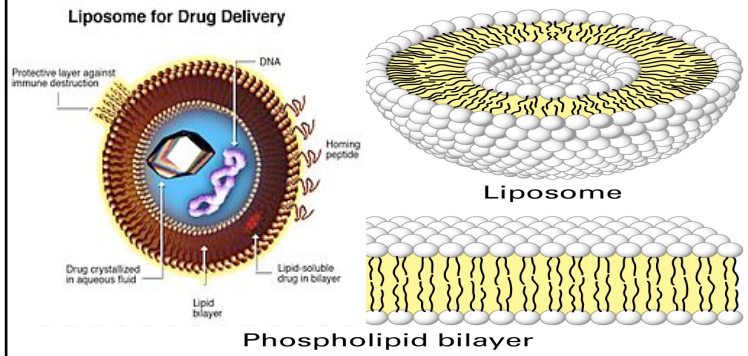
GFP = Green Fluorescent Protein

Generation of a Lentivirus for Target-Cell Transduction





Cross-sectional views of the three structures formed by phospholipids in aqueous solutions



The white spheres depict the hydrophilic heads of the phospholipids, and the squiggly black lines (in the yellow regions) represent the hydrophobic tails. Shown are a spherical micelle with a hydrophobic interior composed entirely of fatty acyl chains; a spherical liposome, which has two phospholipid layers and an aqueous center; and a two-molecule-thick sheet of phospholipids, or bilayer, the basic structural unit of bio-membranes.

Practical Applications in Modern

Protein Cellular Biology

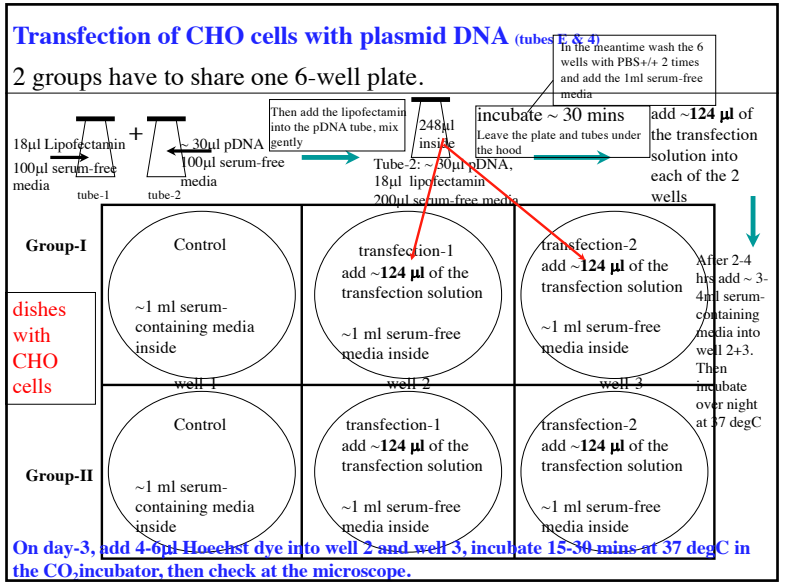
Intracellular Protein

Visualization / Localization

with a Gene-**GFP**

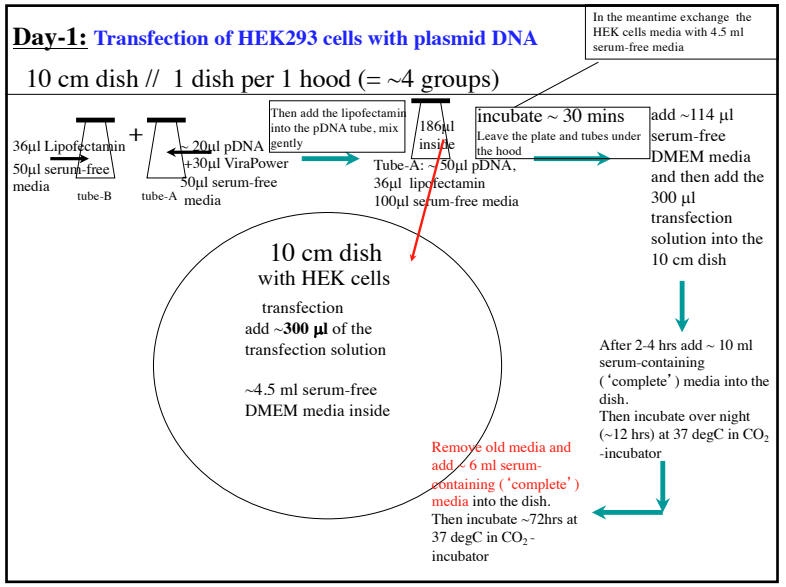
Cell Transfection with a Gene-**GFP**

(pDNA) using a Lipid System



Cell Transfection with a Gene-**GFP**

(pDNA) using a Lenti-Virus System



Day-2: **Transfection of PC12 cells using lenti-virus system**
Add the 2ml lenti-virus solution directly into the well-3.

2 groups have to share one 6-well plate.

	well-1	well-2	well-3
Group-I	Control 3 ml 'complete' media inside	Control 3 ml 'complete' media inside	+ add **2ml** of the lentivirus solution 1 ml 'complete' media inside
Group-II	Control 3 ml 'complete' media inside	Control 3 ml 'complete' media inside	+ add **2ml** of the lentivirus solution 1 ml 'complete' media inside

dishes with PC12 cells

---> incubate ~ 1-2 days, exchange with fresh 3ml 'complete' media after ~ 1-2 days into all wells

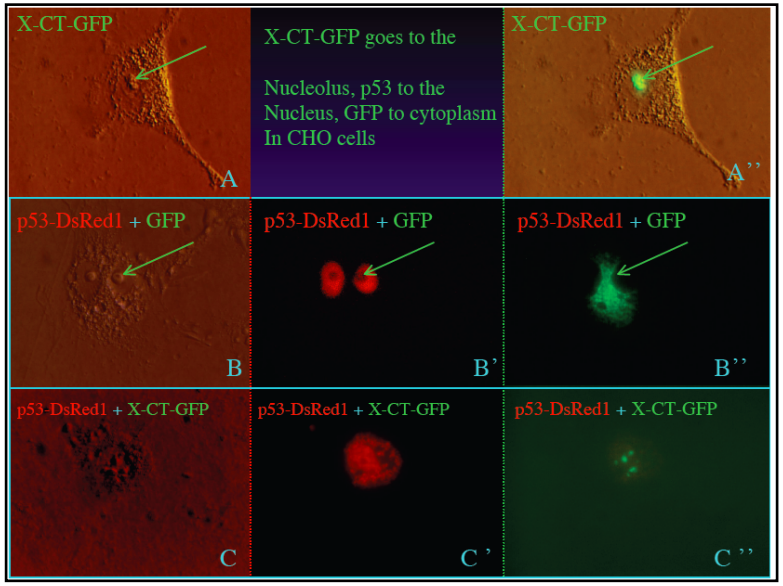
Day-3: **Stimulation of PC12 cells with NGF (stock-solution: 10µg/ 100µl or 100ng/1µl; 100ng/ml final concentration) Adding 3µl = 300ng of the stock-vial into 3 ml media gives you 300ng/3ml = 100ng/ml]. Add the 3µl directly into the dish.**

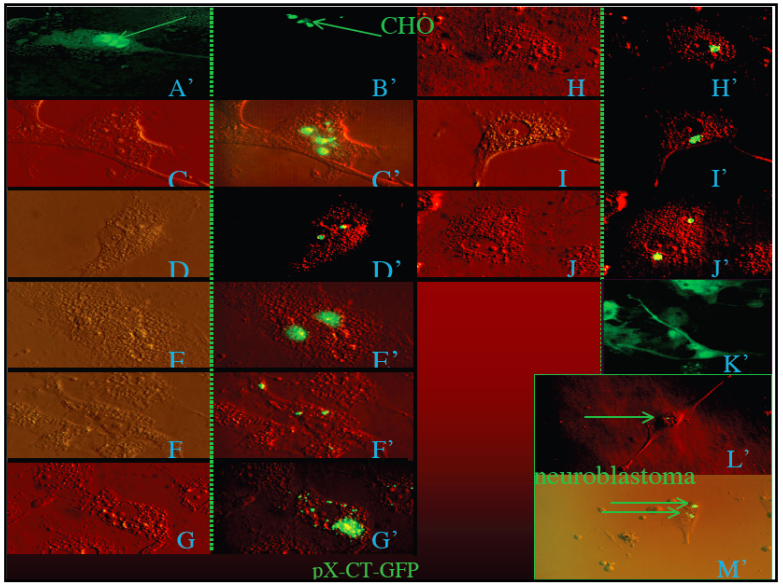
2 groups have to share one 6-well plate. First get fresh media in all 3 wells

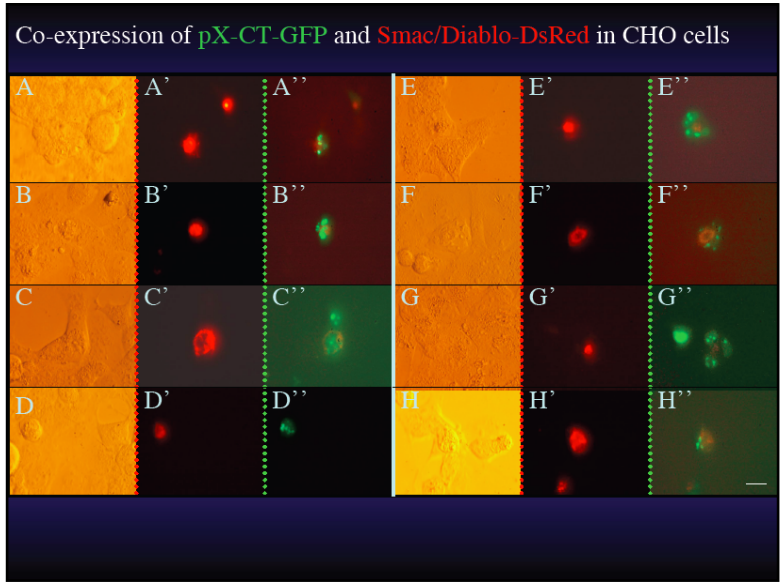
	well-1	well-2	well-3
Group-I	Control 3 ml complete media inside	+ NGF add **3 µl** of the stock solution 3 ml complete media inside	+ NGF add **3 µl** of the stock solution (transfected cells) 3 ml complete media inside
Group-II	Control 3 ml complete media inside	+ NGF add **3 µl** of the stock solution 3 ml complete media inside	+ NGF add **3 µl** of the stock solution (transfected cells) 3 ml complete media inside

dishes with PC12 cells

On day-6 to -14 (check with supervisor), add 4-6µl Hoechst dye into well-3, incubate 15-30 mins at 37 degC in the CO_2incubator, then check at the microscope.



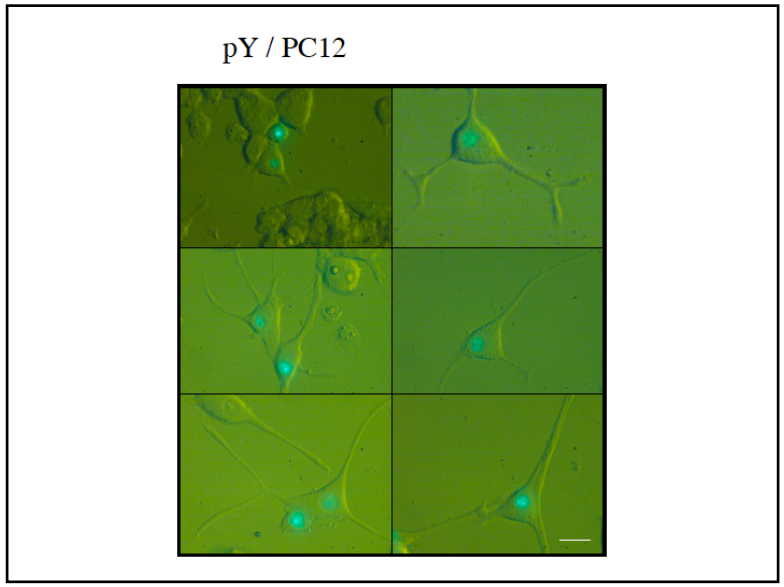


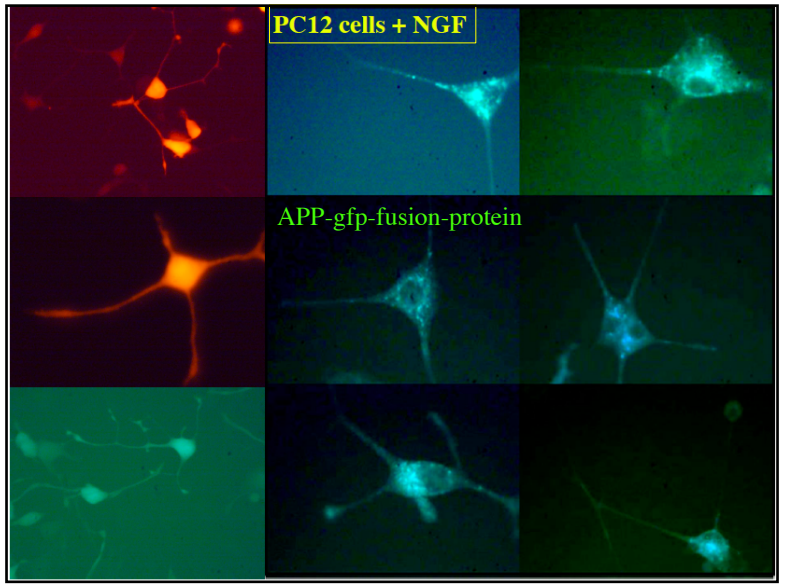


Stimulation of PC12 cells with NGF (stock-solution: 10µg/ 100µl or 100ng/1µl; 100ng/ml final concentration) Adding 3µl = 300ng of the stock-vial into 3 ml media gives you 300ng/3ml = 100ng/ml]. Add the 3µl directly into the dish.

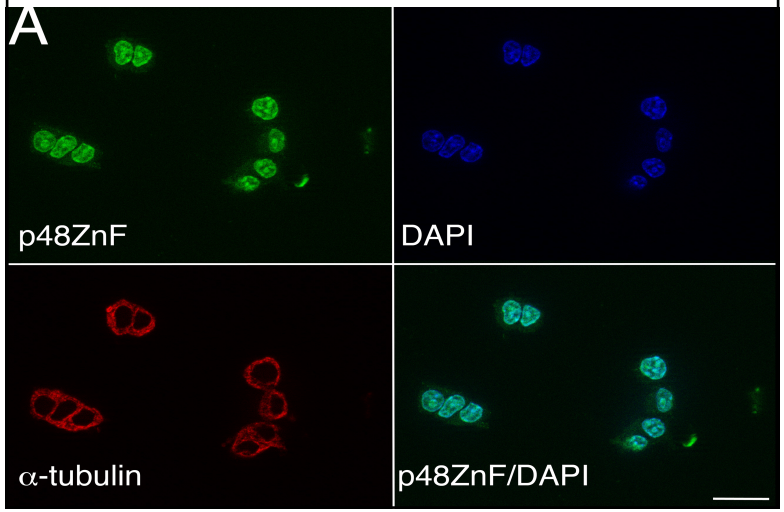
2 groups have to share one 6-well plate.

Group-I dishes with PC12 cells	Control 3 ml complete media inside	+ NGF add **3 µl** of the stock solution 3 ml complete media inside	+ NGF add **3 µl** of the stock solution 3 ml complete media inside
Group-II	Control 3 ml complete media inside	+ NGF add **3 µl** of the stock solution 3 ml complete media inside	+ NGF add **3 µl** of the stock solution 3 ml complete media inside

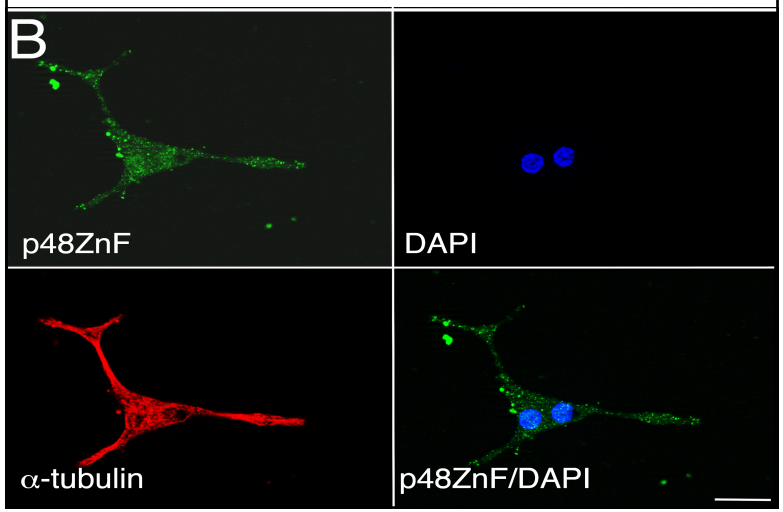




Living proliferating cells: p48ZnF in the nucleus



Living differentiated neuronal cells: p48ZnF in the cytoplasm

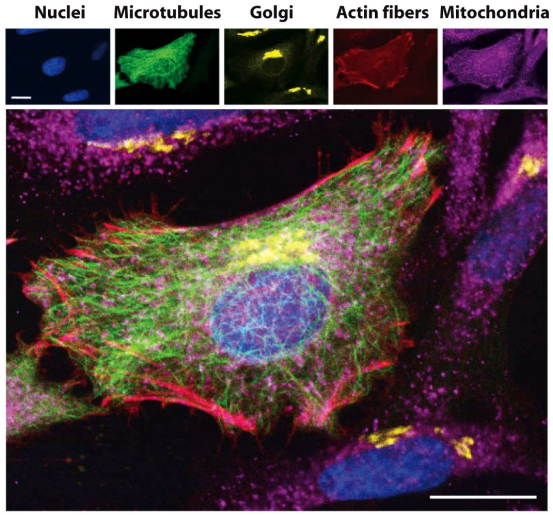


Molecular and Cellular Biology

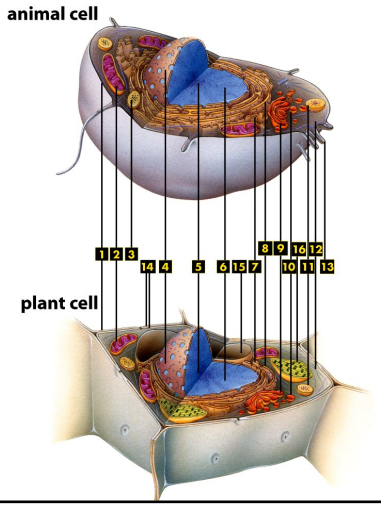
5b. Visualizing, Fractionating, and Isolation of Cells

Prof. Dr. Klaus Heese

The Cell and its Major Systems & Compartments

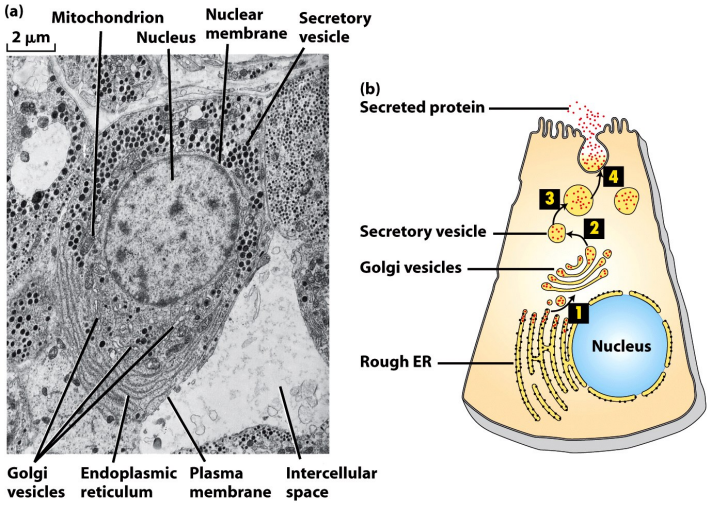


The Cell and its Major Systems & Compartments



1 Plasma membrane
2 Mitochondria
3 Lysosomes
4 Nuclear envelope
5 Nucleolus
6 Nucleus
7 Smooth endoplasmic reticulum (SER)
8 Rough endoplasmic reticulum (ER)
9 Golgi complex
10 Secretory vesicles
11 Peroxisomes
12 Cytoskeletal fibers
13 Microvilli
14 Cell wall
15 Vacuole
16 Chloroplast

The Cell and its Major Systems & Compartments



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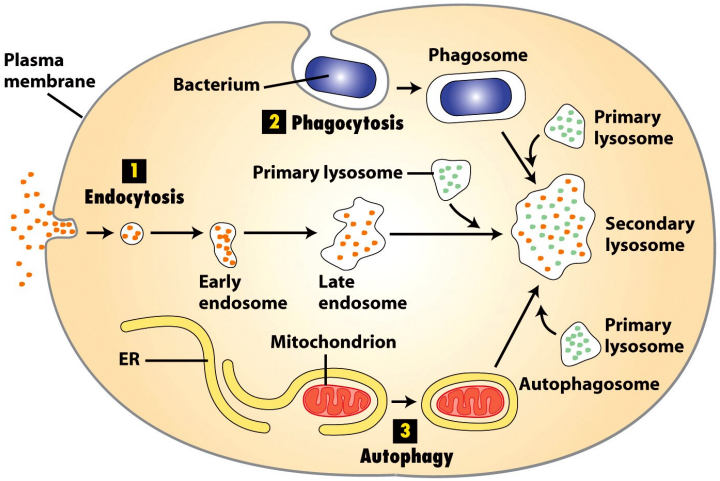
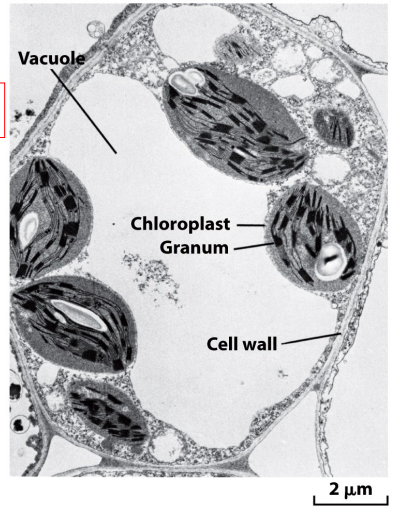
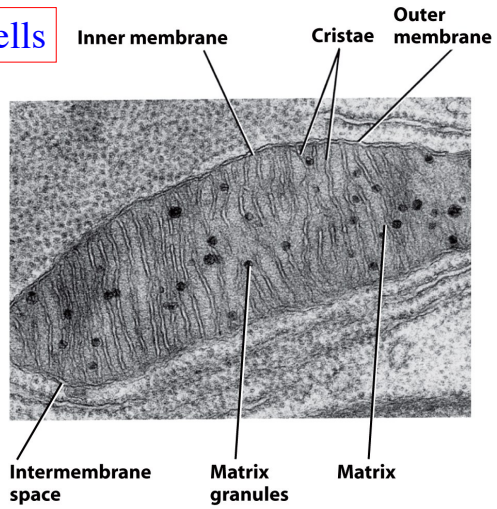


The Cell and its Major Systems & Compartments

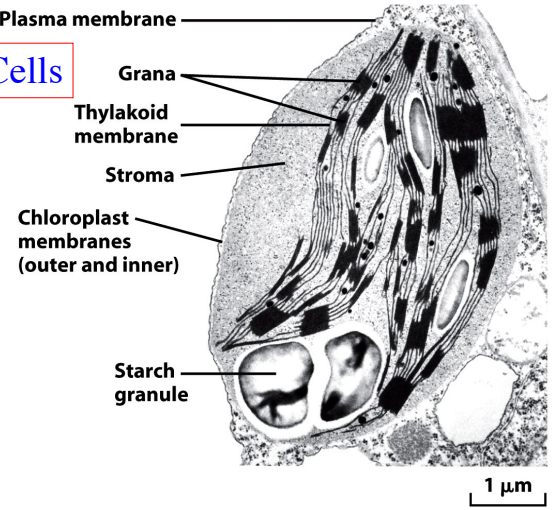
Plant Cells



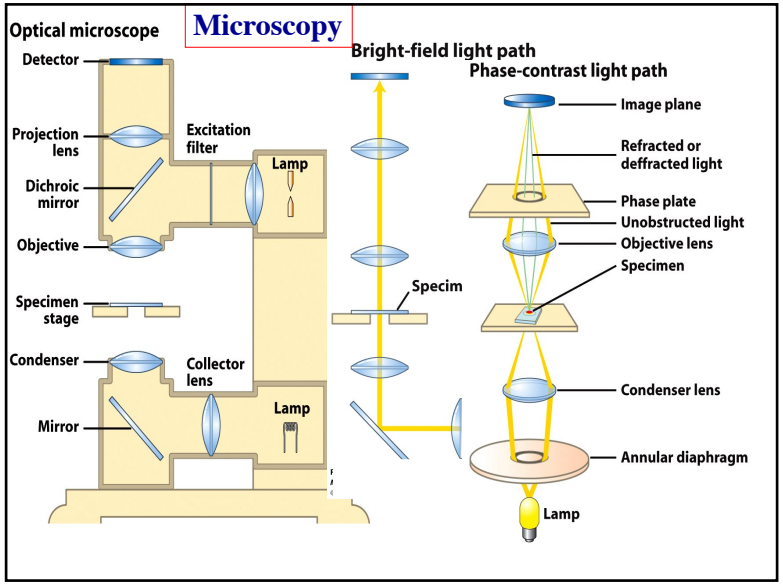
Plant Cells



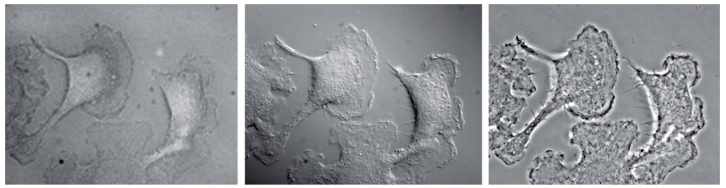
Plant Cells



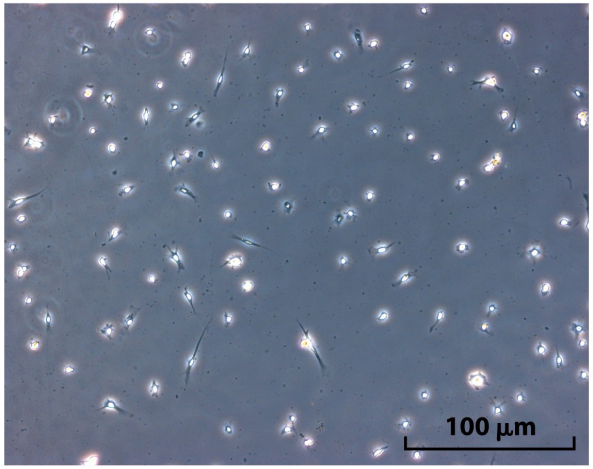
Microscopy



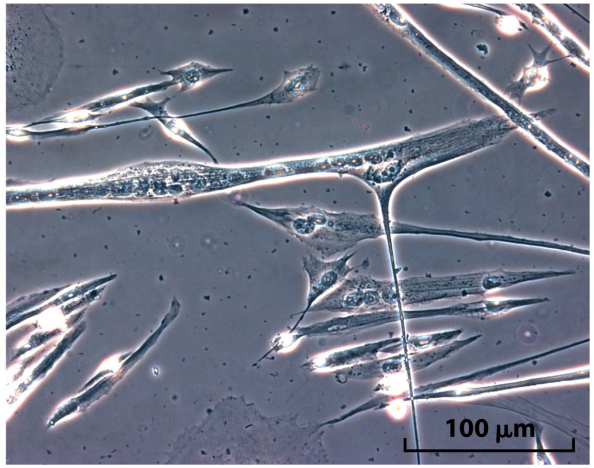
Microscopy



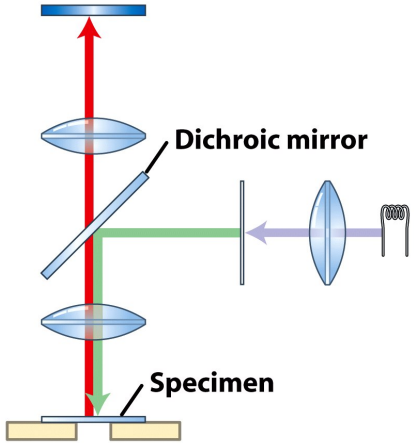
Undifferentiated cells

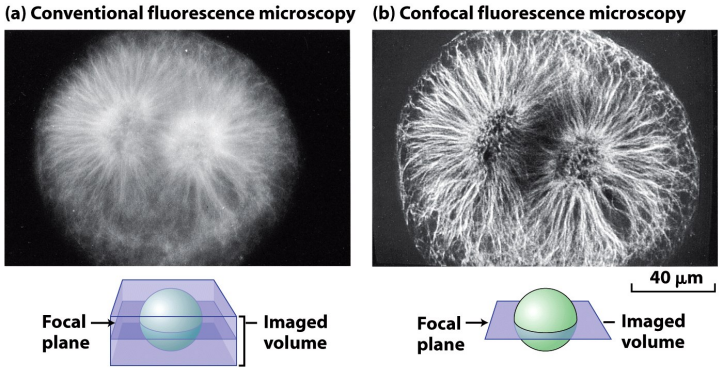


Differentiated cells



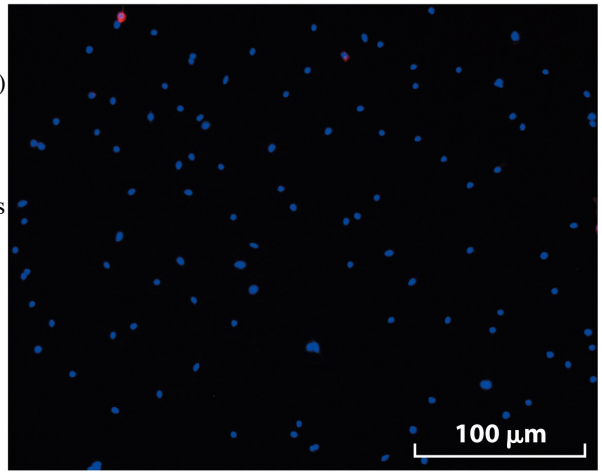
Epifluorescence light path

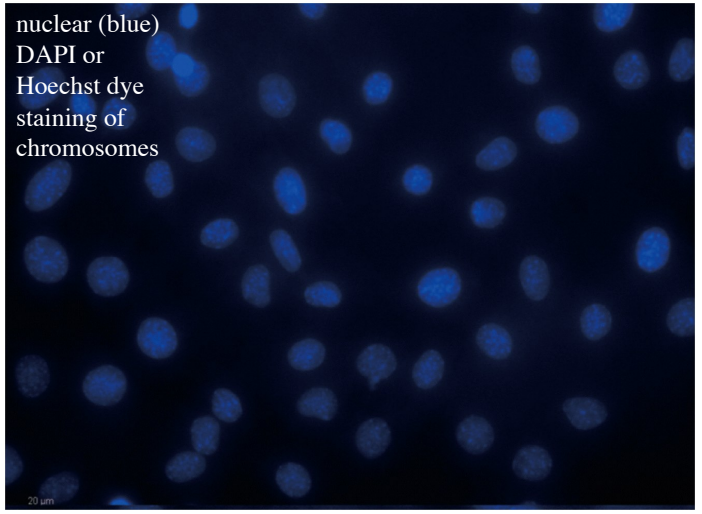




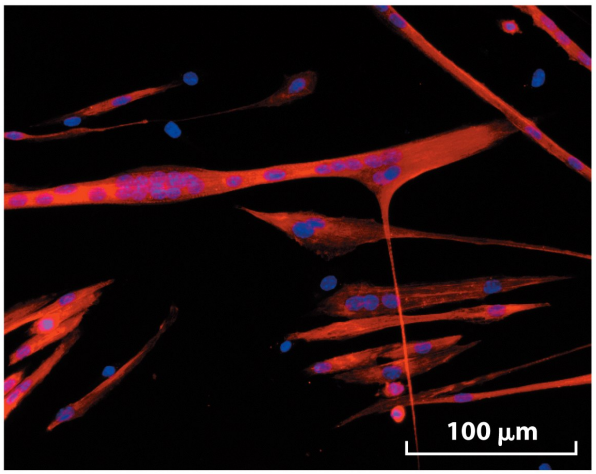
Undifferentiated cells

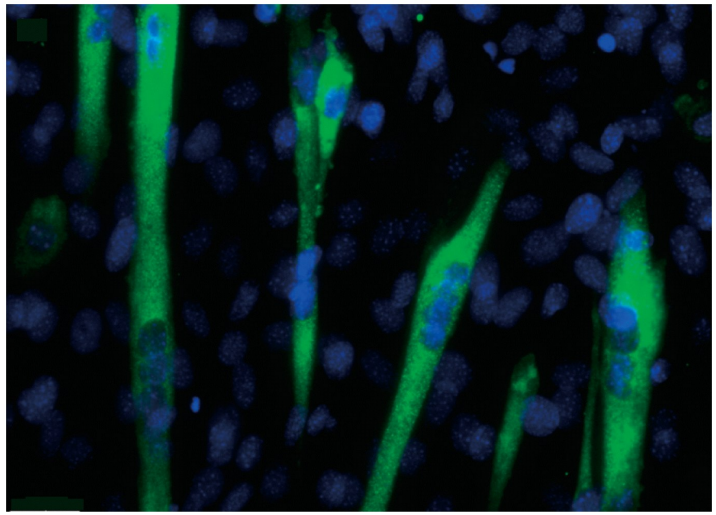
nuclear (blue) DAPI or Hoechst dye staining of chromosomes





Differentiated cells





Microscopy

a) Immunecytochemistry using fluorescence-labeled (**red** / **green**) antibodies directed specifically to proteins of the cell

a) Genes expressing fusion proteins with e.g. **green fluorescence proteins (GFP)**

The Cell and its Major Systems & Compartments

Microscopy

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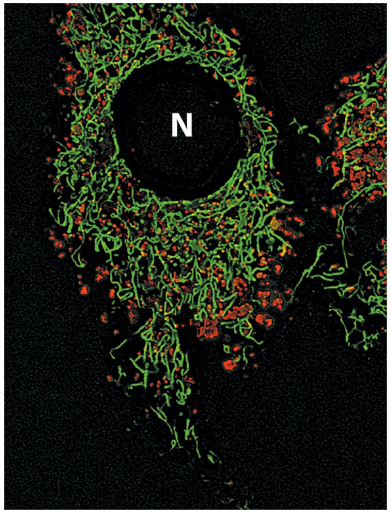

Microscopy

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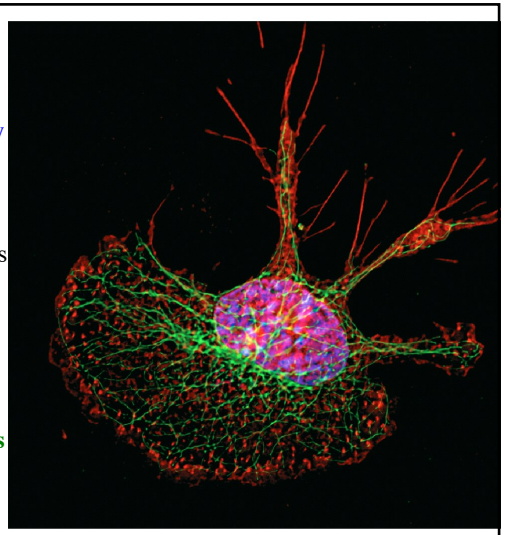
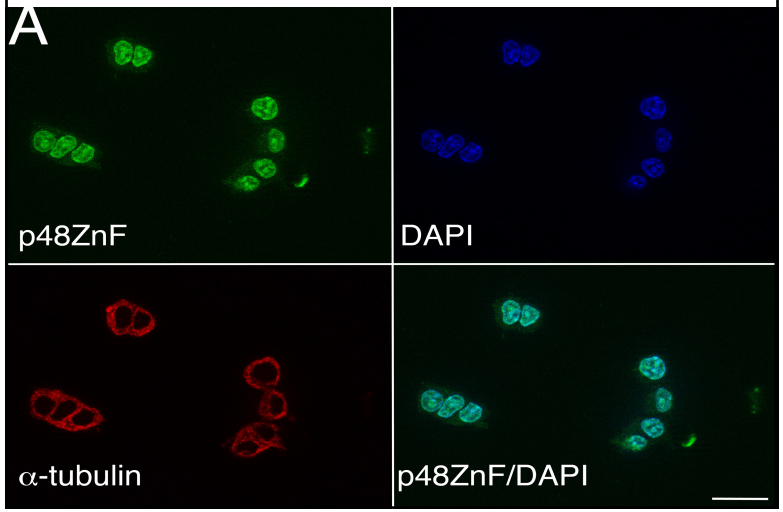
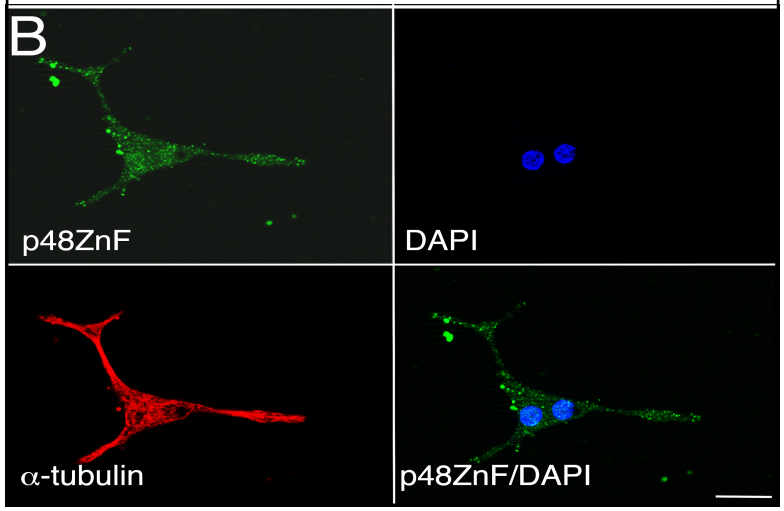


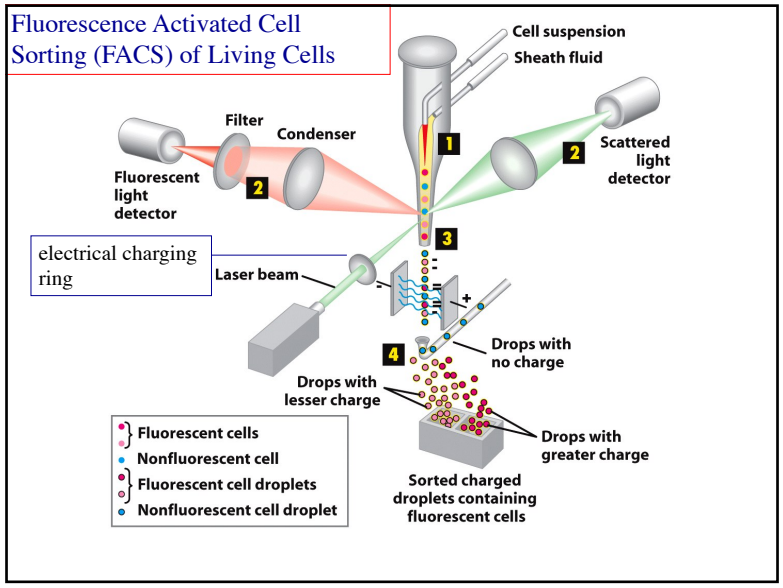
Living proliferating cells: p48ZnF in the nucleus

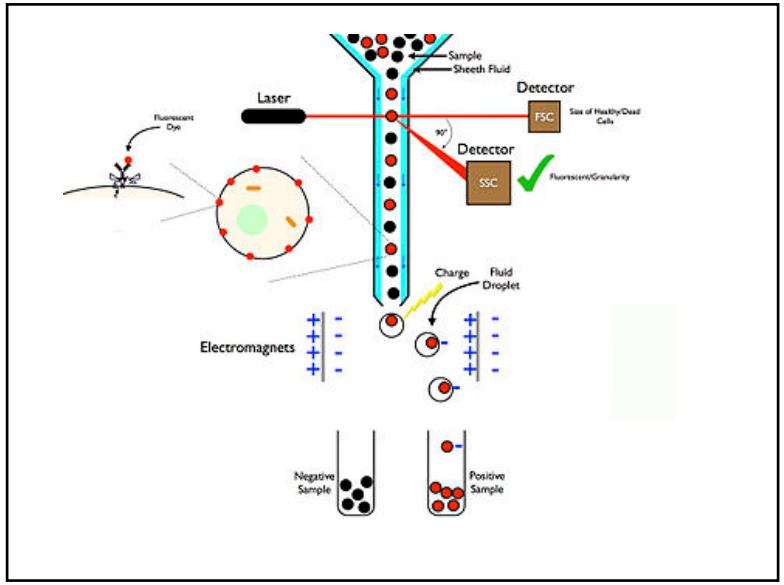


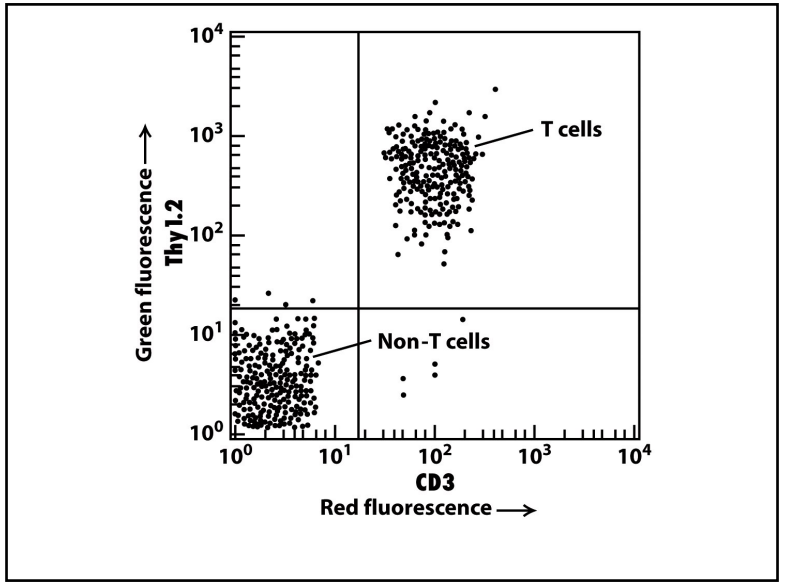
Living differentiated neuronal cells: p48ZnF in the cytoplasm



Fluorescence Activated Cell Sorting (FACS) of Living Cells

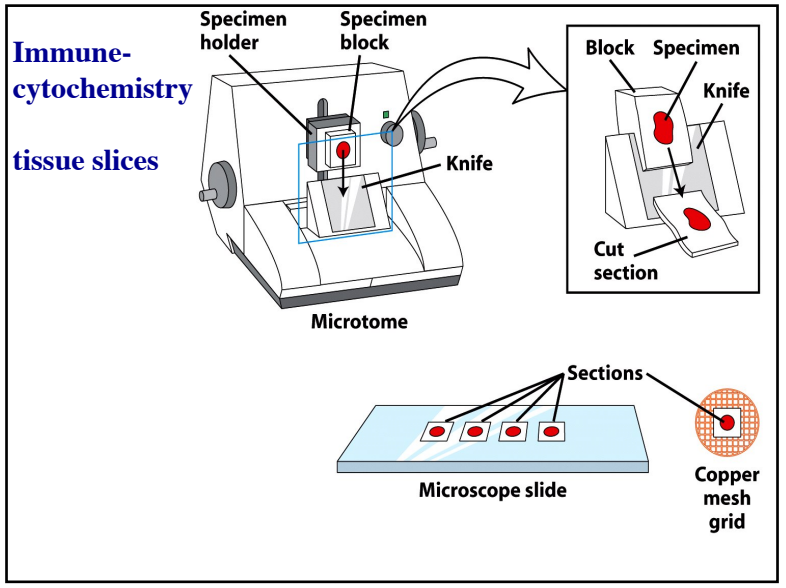


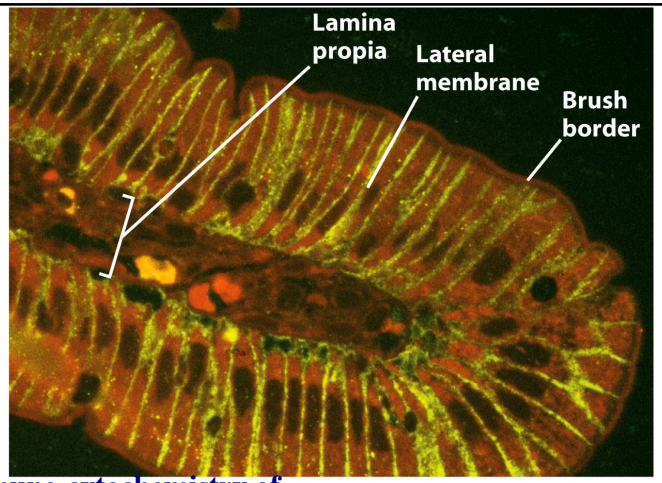




Immune-cytochemistry

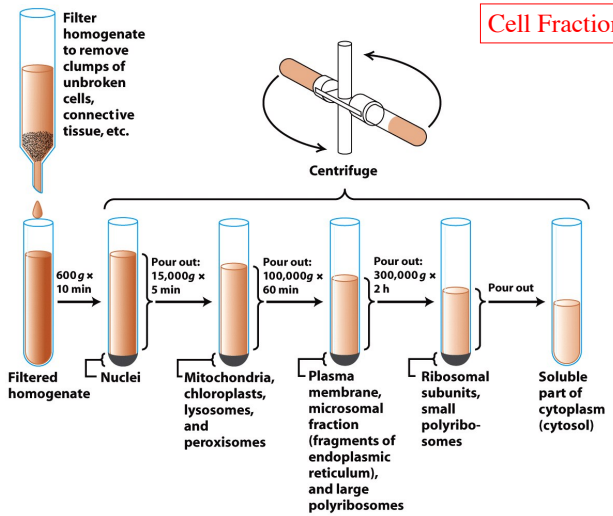
tissue slices



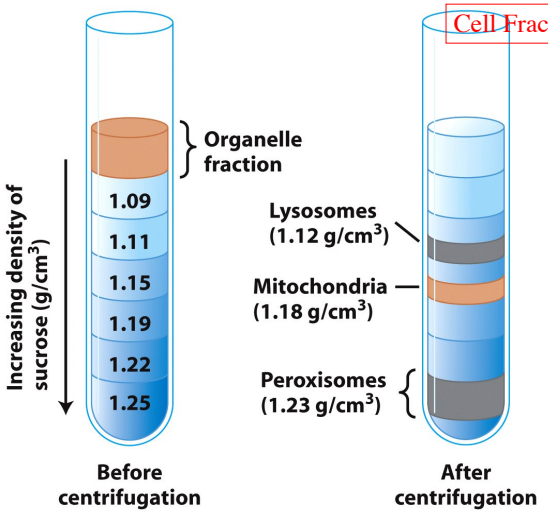


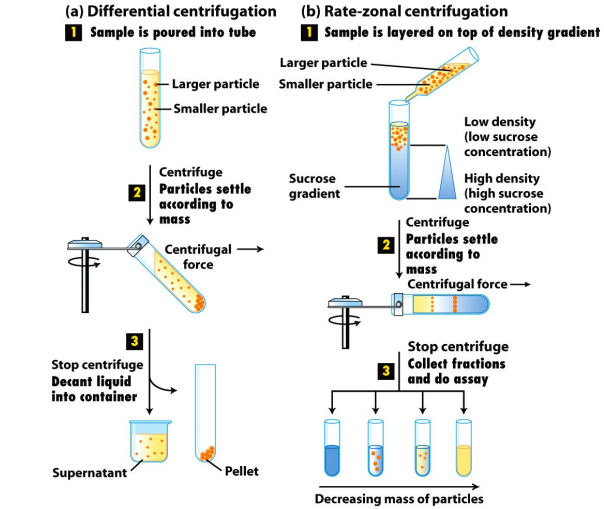
Immune-cytochemistry of tissue slices

Cell Fractionation



Cell Fractionation





→ cell lysis to release proteins → SDS-PAGE