Gene Expression in Mammalian Cells and its Applications

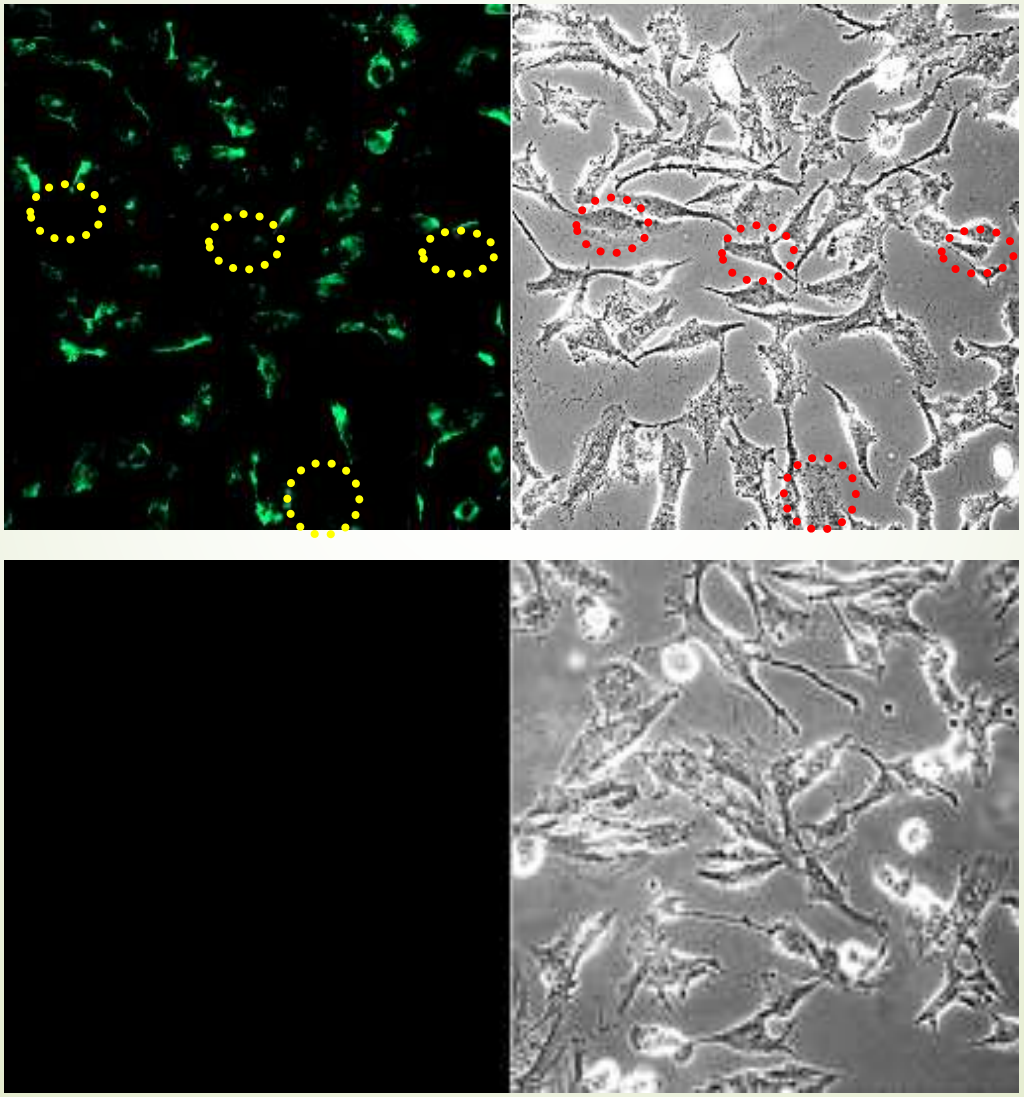
Date: July 21, 2016

Speaker: 轉譯醫學研究中心
方瓊瑤

Gfp reporter plasmids transfect to human neuroblastoma cells

Gfp plasmid

Mock



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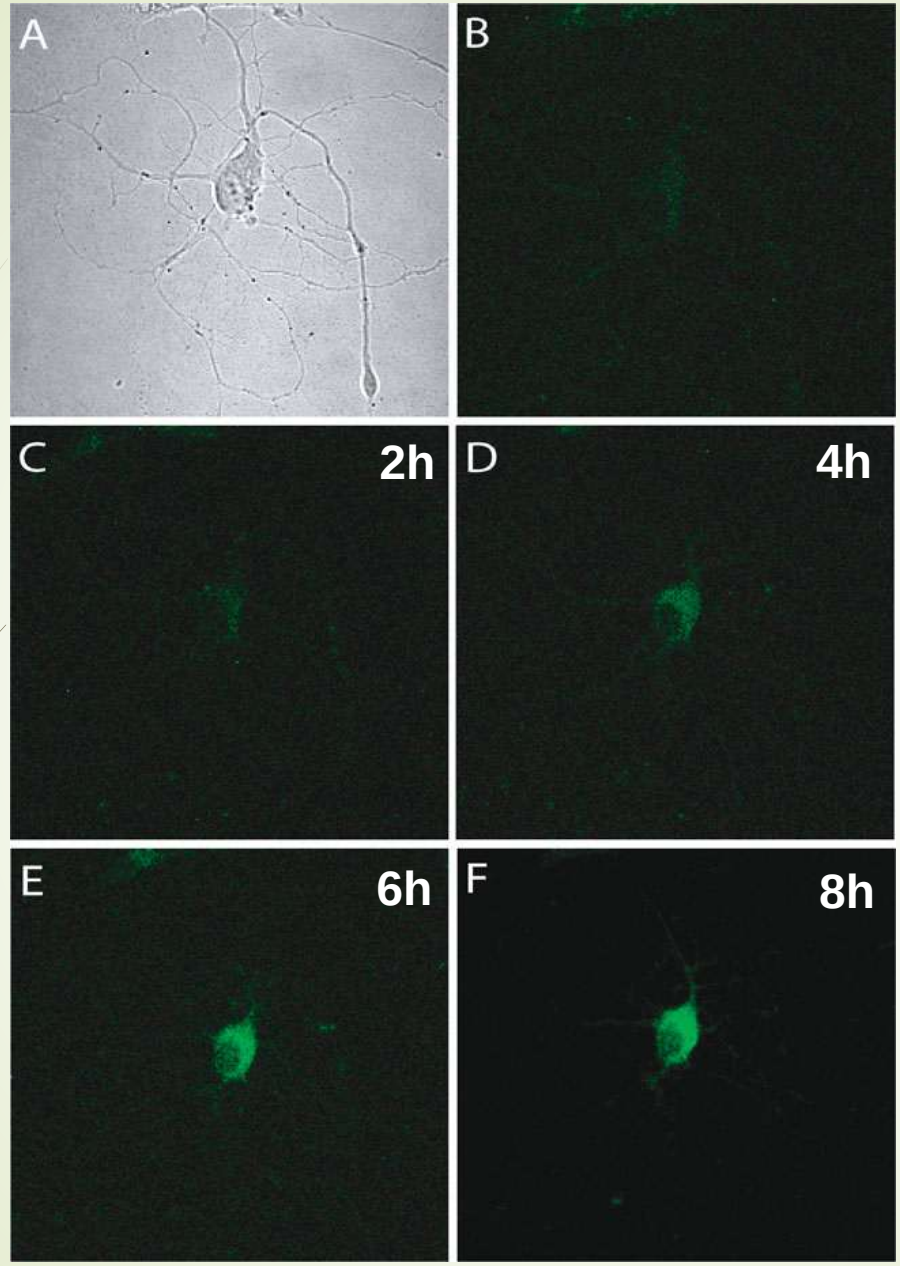


Gria4：L-glutamate-gated ion channels that mediate fast synaptic excitatory **neurotransmission**

Micrographs of the rat hippocampal neuron lipotransfected with in-vitro-transcribed rat Gria4-GFP mRNA

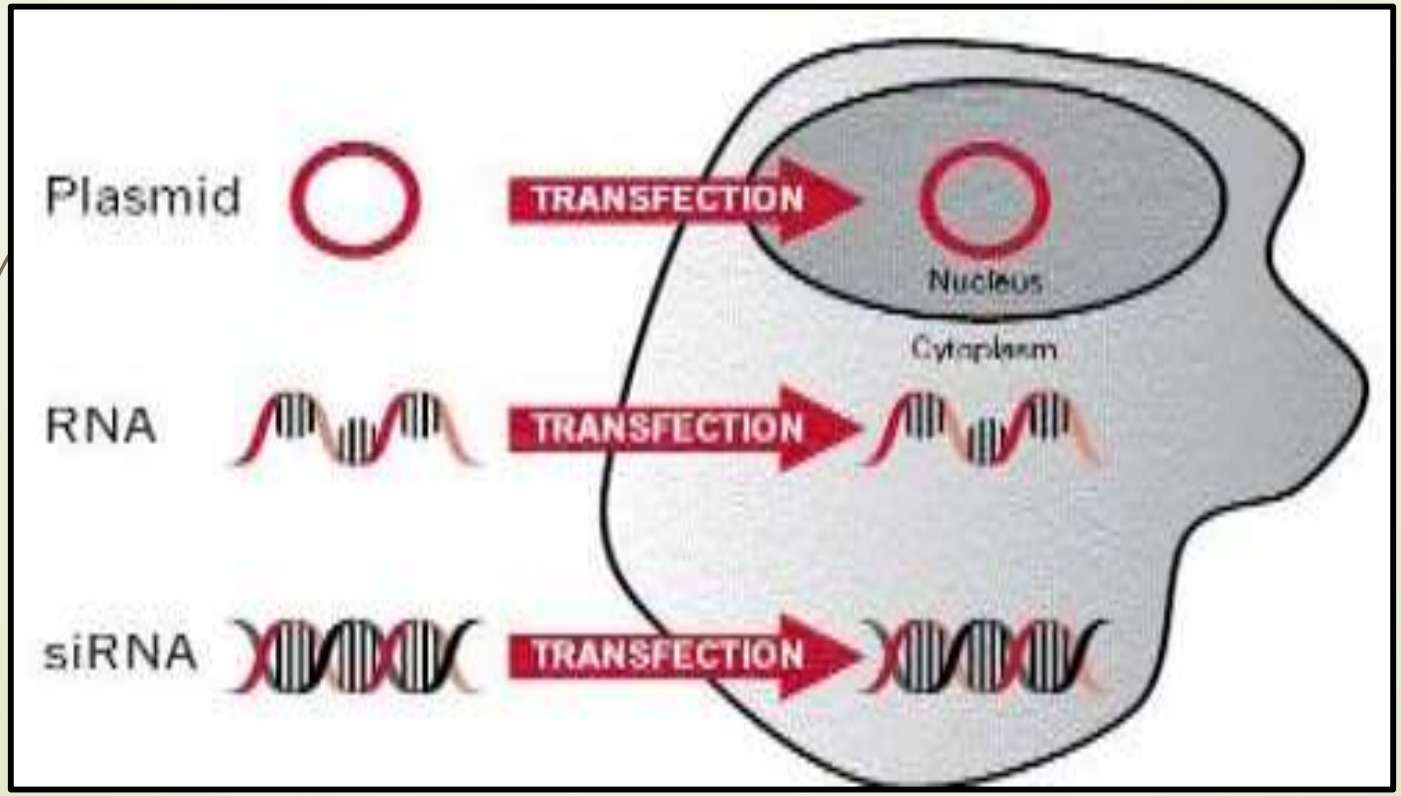
Anal Bioanal Chem (2010) 397:3173–3178

Presentation Overview

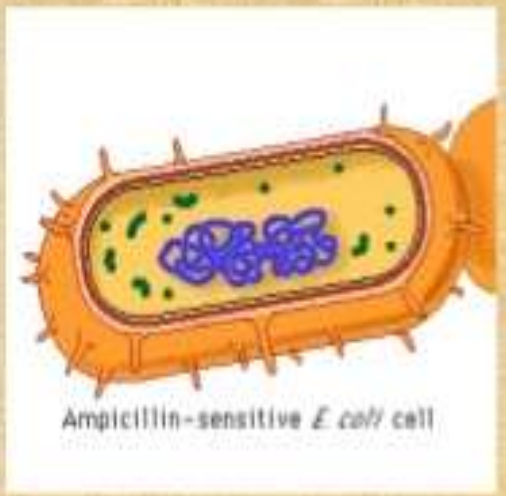
- Terminology
- Factors Affecting Transfection
- Transfection Workflow
- Common Transfection Methods

Transfection

- transporting DNA, RNA or macromolecules into an **eukaryotic cells**



Transfection vs. Transformation

Cloning (uptake of genetic material)		Cancer
Transformation	**Transfection**	**Transformation**
In unicellular organisms like prokaryotes (bacteria) or unicellular eukaryotes (amoeba)	In Metazoan Eukaryotic Cells	Advancement of a metazoan eukaryotic cell from being non-cancerous to cancerous.
 Ampicillin-sensitive *E. coli* cell		

Factors Affecting Transfection

Cell Health

- Cells should be grown in appropriate medium with all necessary factors
- Cultures must be free of contamination
- Fresh medium must be used if it contains chemically unstable components, such as thiamine
- Cells should be incubated at 37°C with CO_2 supplied at the correct percentage (5–10%) and 100% relative humidity
- Cells should be maintained in log phase growth

Factors Affecting Transfection

Cell Culture

Confluency and Growth Phase

- Cells should be transfected at 40–80% confluency (cell type dependent)
  - Too few cells cause cell cultures to grow poorly without cell-to-cell contact
  - Too many cells result in contact inhibition, making cells resistant to uptake of DNA and other macromolecules
- Actively dividing cells take up DNA better than quiescent cells (breakdown and perforation of the nuclear membrane during mitosis enable nuclear delivery)

Number of Passages for Primary Cells

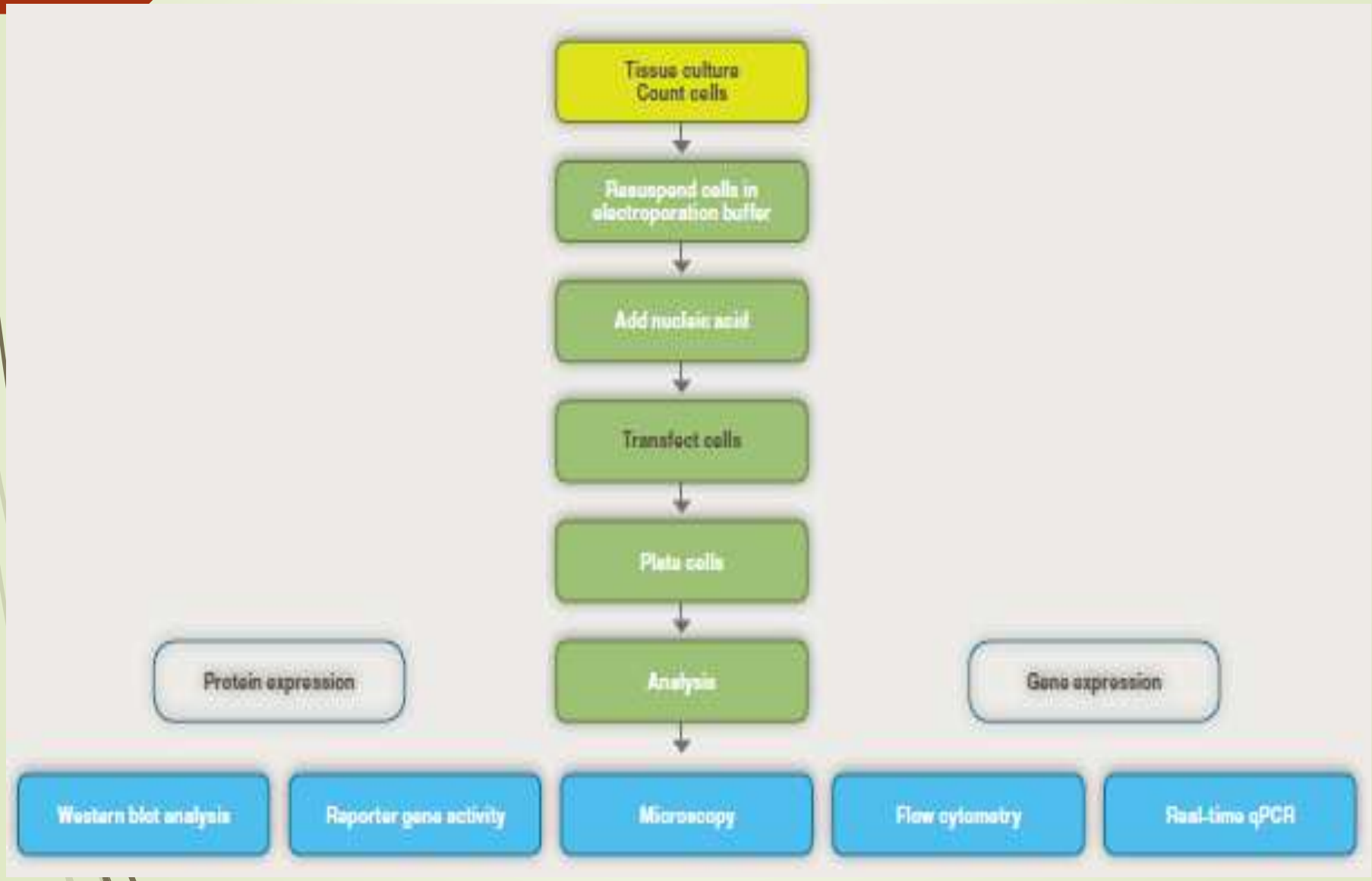
- The number of passages should be low (<50)
- The number of passages for cells used in a variety of experiments should be consistent
- Cell characteristics can change over time with immortalized cell lines and cells may not respond to the same transfection conditions
- Cells may not respond to the same transfection conditions after repeated passages

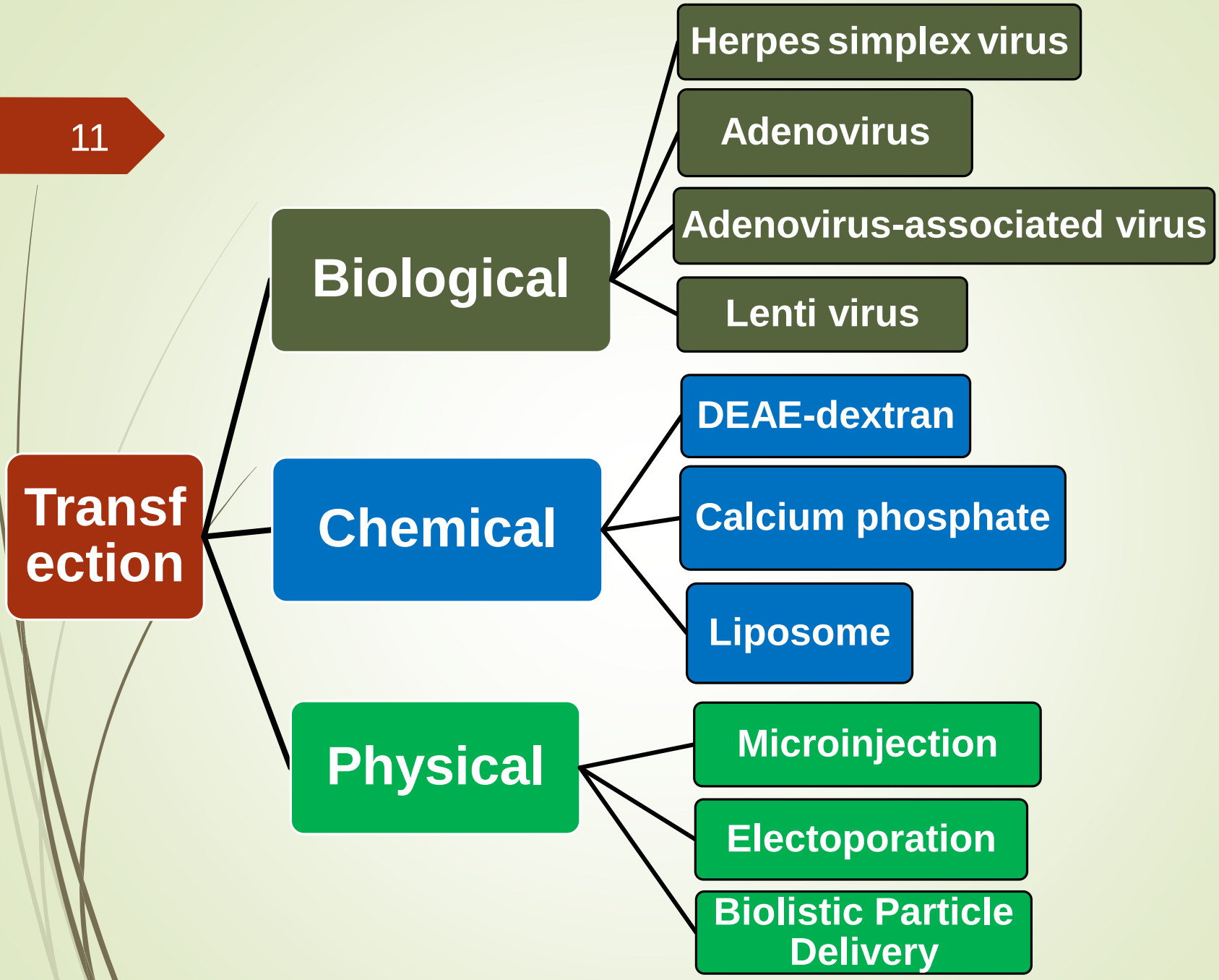
Factors Affecting Transfection

DNA Quality and Quantity

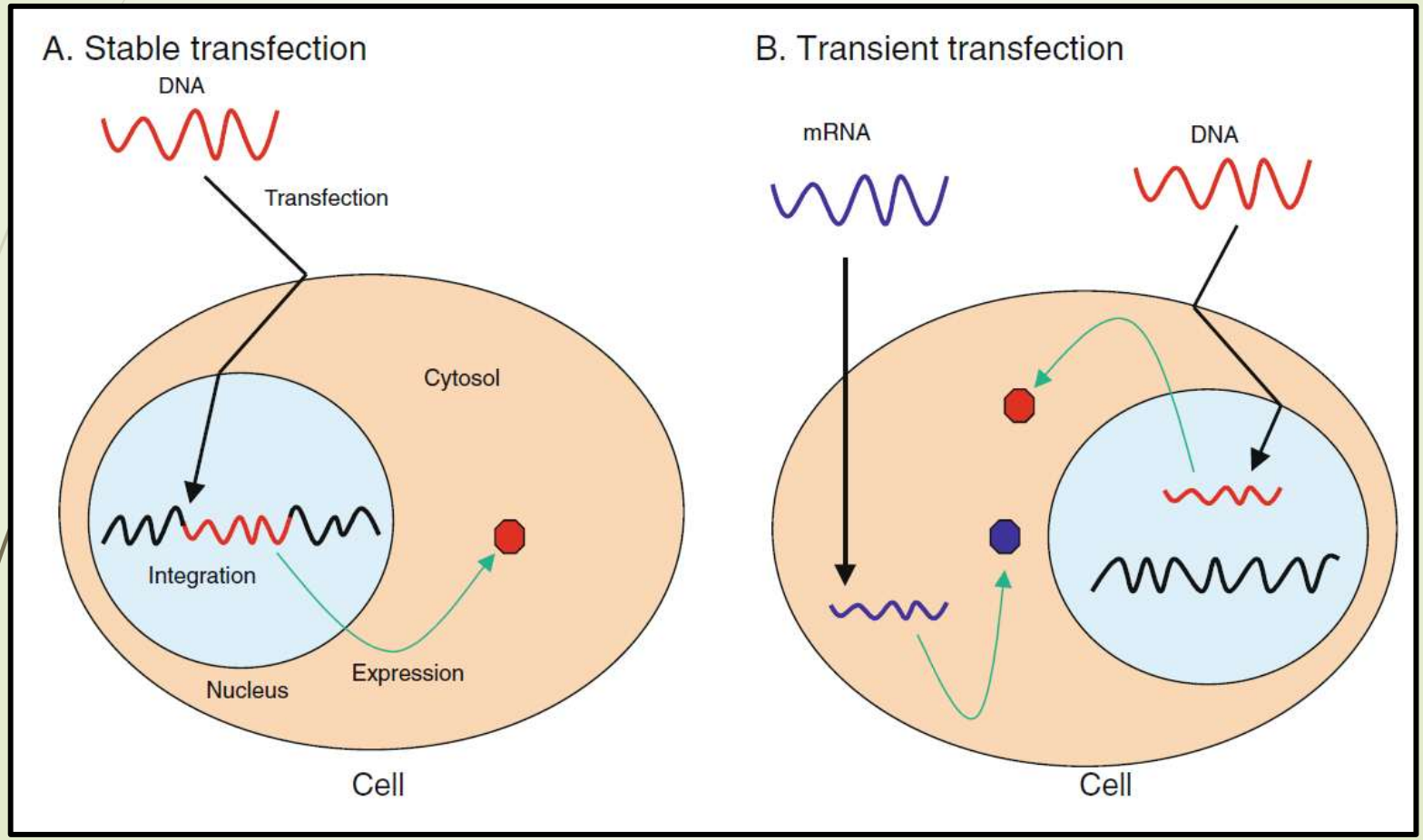
- Use high-quality plasmid DNA that is free of proteins, RNA, and chemicals for transfections; endotoxin removal should be part of the preparation procedure
- Typically, DNA is suspended in sterile water or TE buffer to a final concentration of 0.2–1 mg/ml
- The optimal amount of DNA to use in the transfection will vary widely depending upon the type of DNA, transfection reagent/method, target cell line, and number of cells

Transfection Workflow




Transfection
Biological
Herpes simplex virus
Adenovirus
Adenovirus-associated virus
Lenti virus
Chemical
DEAE-dextran
Calcium phosphate
Liposome
Physical
Microinjection
Electoporation
Biolistic Particle Delivery

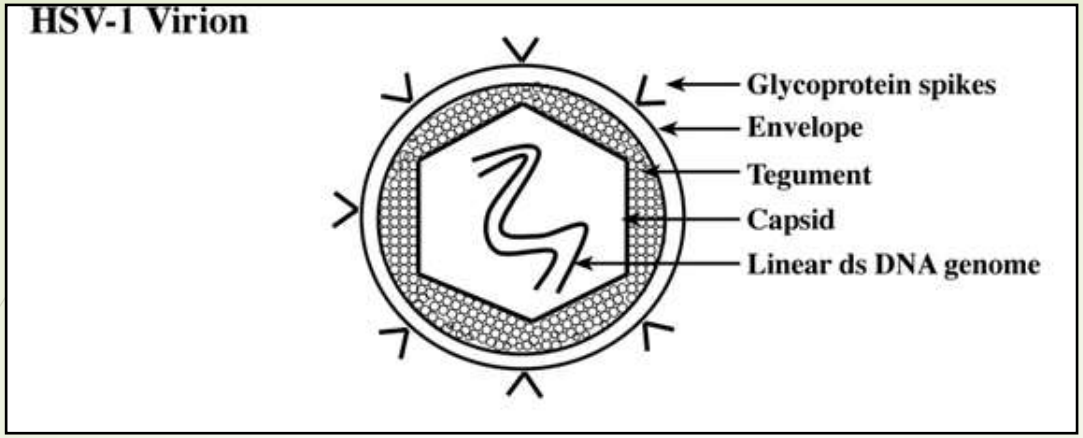
Two Different Transfections

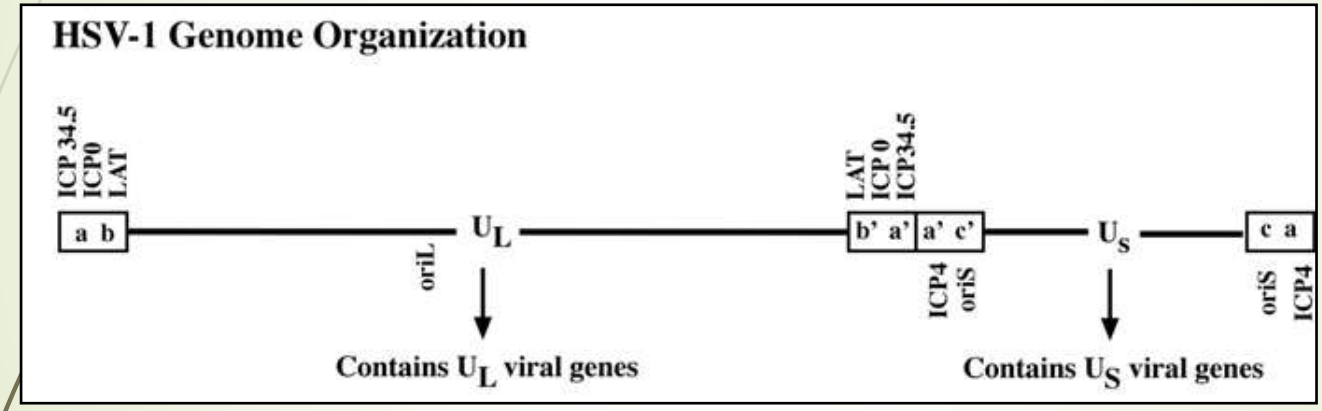


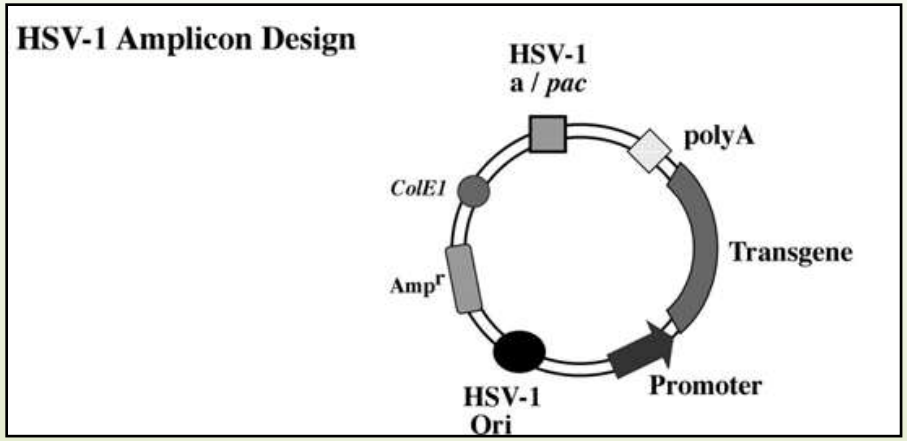
Class	Methods	Advantages	Disadvantages	Examples
Biological	• Virus-mediated	- High-efficiency	- Potential hazard to laboratory personnel	Herpes simplex virus, Adeno virus, Adeno-associated virus, Vaccinia virus, Sindbis virus
		- Easy to use		
		- Effective on dissociated cells, slices, and in vivo	- Insertional mutagenesis	
			- Immunogenicity	
			- DNA package size limit	
Chemical	• Cationic polymer	- No viral vector	- Chemical toxicity to some cell types	DEAE-dextran, polyethyleneimine, dendrimer, polybrene, calcium phosphate, lipofectin, DOTAP, lipofectamine, CTAB/DOPE, DOTMA
	• Calcium phosphate	- High-efficiency	- Variable transfection efficiency by cell type or condition	
	• Cationic lipid	- Easy to use	- Hard to target specific cells	
		- Effective on dissociated cells and slices		
		- Plenty of commercially available products		
		- No package size limit		
Physical	• Direct injection	- Simple principle and straightforward	- Needs special instruments	Micro-needle, AFM tip, Gene Gun, Amaxa Nucleofector, phototransfection, Magnetofection
	• Biolistic particle delivery	- Physical relocation of nucleic acids into cell	- Vulnerable nucleic acids	
	• Electroporation	- No need for vector	- Demands experimenter skill, laborious procedure	
	• Laser-irradiation	- Less dependent on cell type and condition		
	• Sonoporation	- Single-cell transfection		
	• Magnetic nanoparticle			

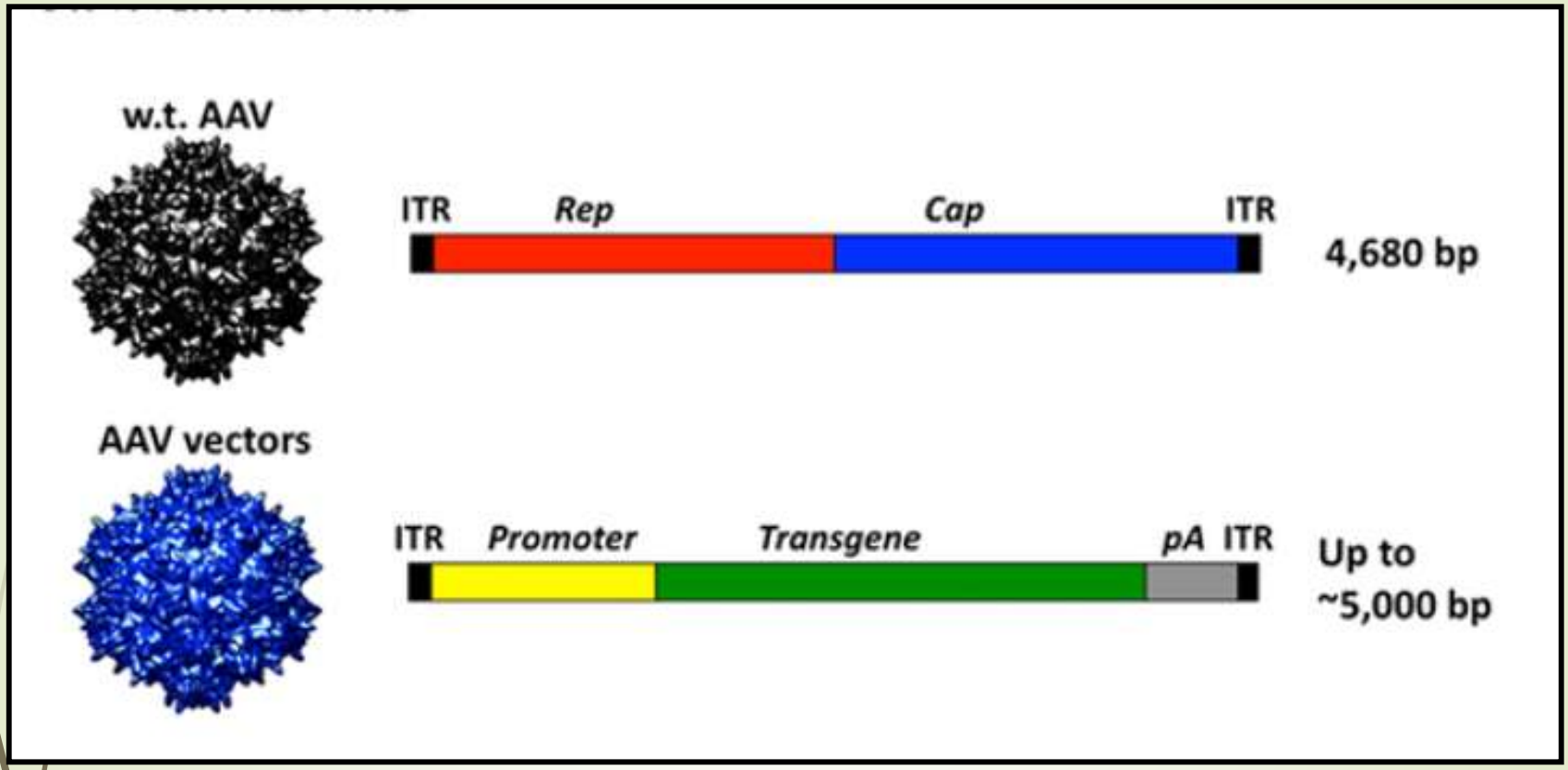
Viruses in Use

Viral Vector	DNA Insert Size	Expression	Pitfalls
Retro viral	8 kb	Stable	Random insertion site
Lenti viral	9 kb	Stable	Random insertion site
Adeno Virus	8 kb	Transient	Highly immunogenic
Adeno associated Virus	5 kb	Stable, site specific location	Requires helper virus and difficult to remove
Herpes Simplex Virus	30-40 kb	Transient	No gene expression during latent infection
Vaccina Virus	25 kb	Transient	Potential cytopathic effects

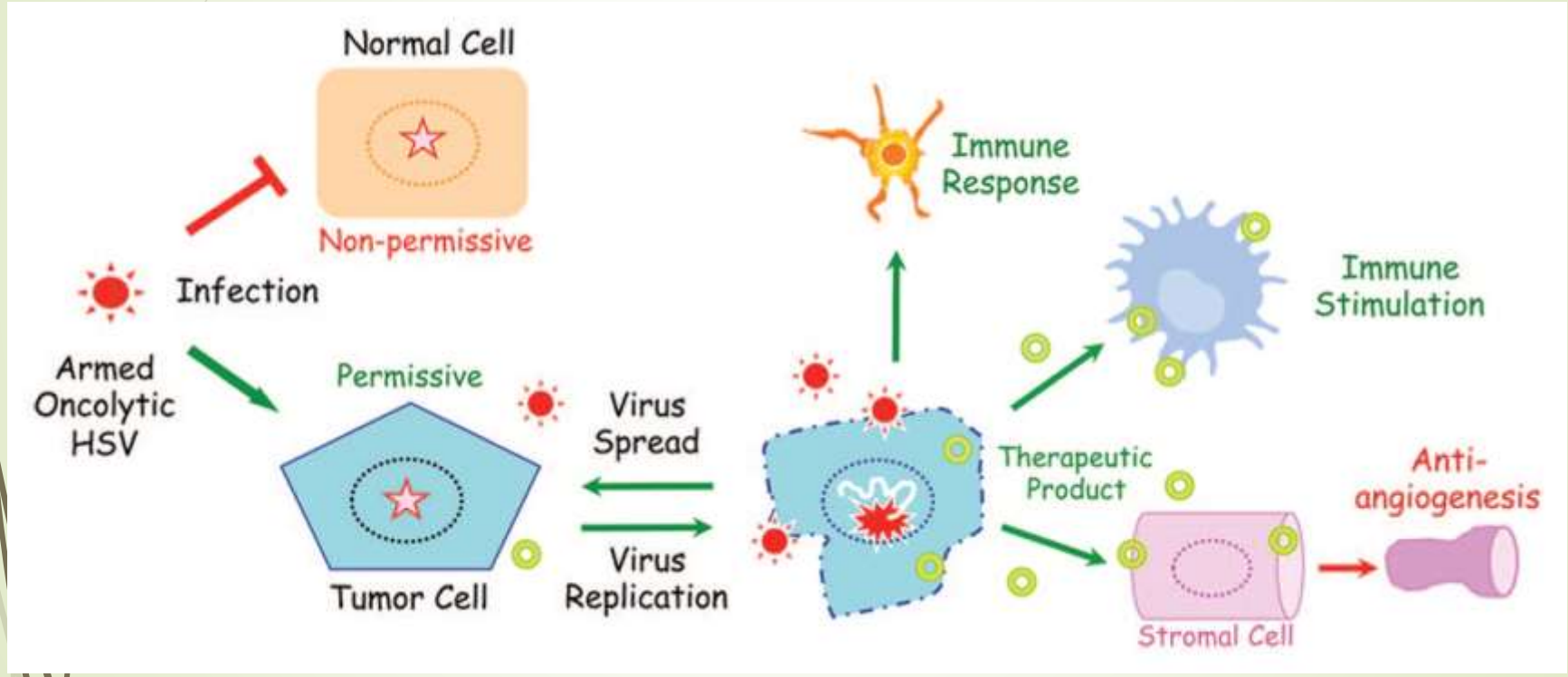

HSV-1 Virion
Glycoprotein spikes
Envelope
Tegument
Capsid
Linear ds DNA genome


HSV-1 Genome Organization
ICP 34.5
ICP0
LAT
a b
oriL
UL
LAT
ICP 0
ICP34.5
b' a'
a' c'
ICP4
oriS
US
c a
oriS
ICP4
Contains UL viral genes
Contains US viral genes


HSV-1 Amplicon Design
HSV-1
a / pac
polyA
ColE1
Transgene
Ampr
HSV-1
Ori
Promoter


w.t. AAV
ITR
Rep
Cap
ITR
4,680 bp
AAV vectors
ITR
Promoter
Transgene
pA
ITR
Up to
~5,000 bp

Armed oHSV used in specific targeting cancer cell

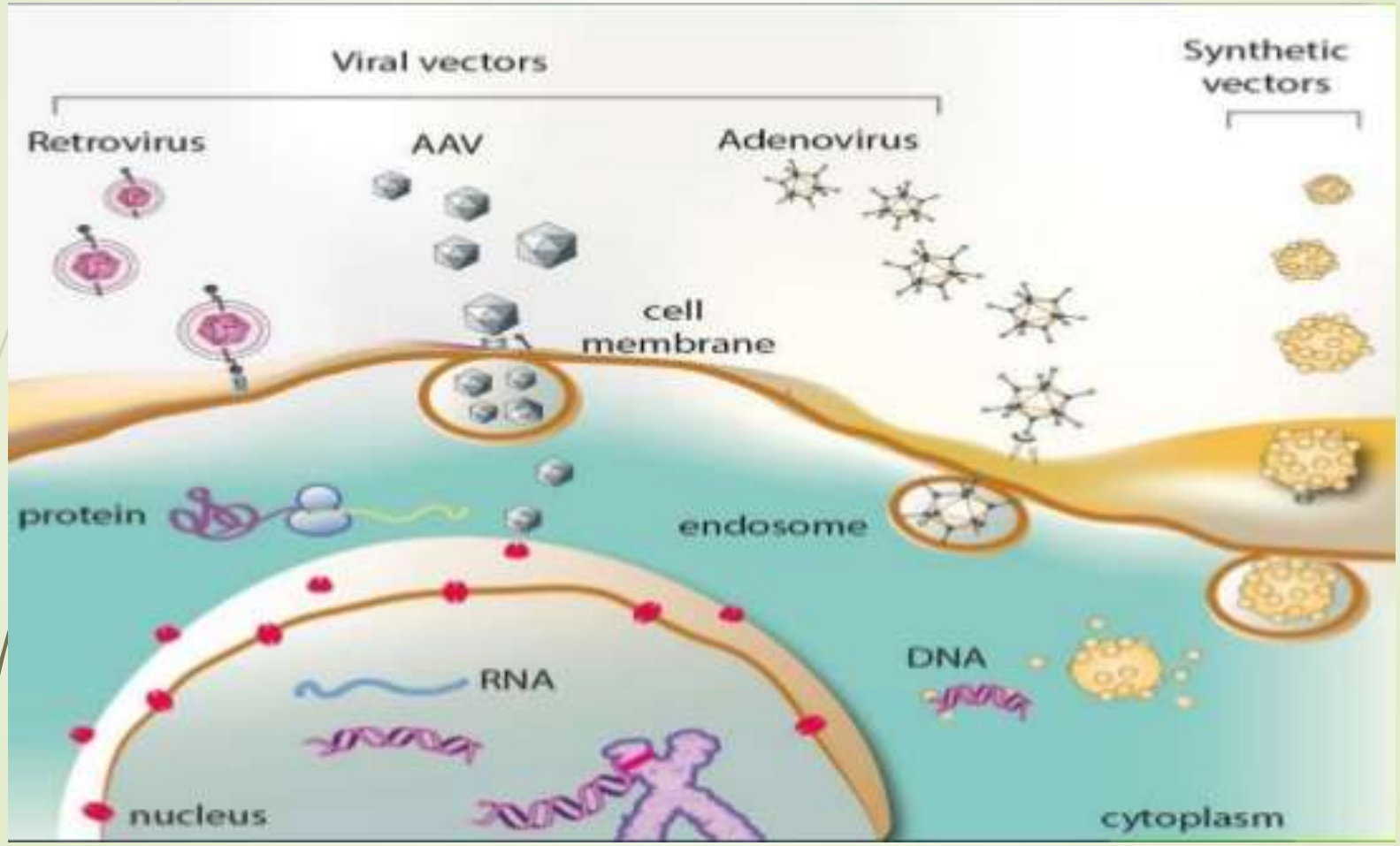


Viral Gene Deletions to Achieve Tumor Selective Replication

Table 2 Examples of viral gene deletions to achieve tumor-selective replication

Parental strain	Agent	Genetic alteration	Genetic/phenotypic target within tumours	In clinical trials
Adenovirus	ONYX-015	E1B-55kd deletion	p53 null/mutant or/and inactivated p53-pathway	I–III
	Ad-Δ24	E1A CR2 deletion	pRb null/mutant or/and loss of cell cycle control	No
	CB-1	E1A CR2 deletion; E1B-55kd deletion	pRb and p53 defective pathways or/and loss of cell cycle control	No
	*dl*922-947	E1A CR2 deletion	pRb null/mutant or/and loss of cell cycle control	No
	*dl*331	VA-1 gene deletion	Ras-activated pathway	No
Herpes simplex virus-1	*dl*sptk	Thymidine kinase gene deletion	Replication	No
	R3616	Deletion of both γ34.5 genes	Loss of neurovirulence	No
	HrR3	ICP6 gene deletion	Replication	No
	G207	Deletion of both γ34.5 genes; ribonucleotide reductase disruption	Replication; loss of neurovirulence	I–II
	NV1020	Deletion of one γ34.5 gene; deletion in tk gene; insertion of exogenous copy of tk gene	Replication, loss of neurovirulence	I
Vaccinia virus	VV-TK$^-$	Thymidine kinase gene deletion	Replication	No
	VV-SPI-1/2$^-$	Deletion of SPI-1 and SPI-2 genes	Replication	No
	WDD	Thymidine kinase gene deletion; deletion of VGF gene	Replication	No
Polio virus	PV1(RIPO)	IRES element replaced by IRES from HRV2	Loss of neurovirulence; replication	No
Influenza virus	IVA-NS1$^-$	NS1 gene deletion	IFN-pathway deficiency	No

tk, thymidine kinase; IRES, internal ribosomal entry site; HRV2, human rhinovirus type 2; IFN, interferon.


Viral vectors
Synthetic vectors
Retrovirus
AAV
Adenovirus
cell membrane
protein
endosome
RNA
DNA
nucleus
cytoplasm

HSV-based vectors for neurological diseases

Pathological disturbances	Application	Genes or proteins involved	Mechanism	Ref.
Neurodegenerative disorders	Alzheimer's disease	NMDAR-NR1	Relationship between NMDAR and Aβ oligomers. Silencing of NR1-NMDAR subunit expression through delivery of NR1 antisens sequences	Decker (2010)
		Aβ peptide Tau, Alpha-synuclein,	Vaccination against Aβ peptides to prevent or remove peptide deposition.	Frazer (2008), Peruzzi (2009)
	Parkinson's Disease	TH, GTP-CH-I, AADC, VMAT-2 Hexokinase II	Gene replacement in 6-hydrodopamine-lesioned or rotenone–treated rats	Sun (2004), Corona (2010)
	Narcolepsy	Pre-pro-orexin	Gene replacement in a KO model for orexin	Liu (2008)
Ataxias	Friedreich's ataxia	FRDA locus Frataxin	Gene replacement	Gimenez-Cassina (2011), Cortes (2006, 2008)
	Ataxia telangectasia	ATM cDNA		
Neuroprotection	Different types of neuroprotection	BDNF, NT-3 GDNF, Bcl-2, HSP72 Catalase, Peroxidase	Gene overexpression or replacement in lesioned or drug-treated model animals	Garrido (1998), Bowers (2002), Sun (2005), Arvanian (2006), Hoehn (2001), Zemliak (2006)
Cancer	Glioblastomas	Prodrugs HSV-1 ICP0	Cell toxicity	Rainov (1998), Cuchet (2005), Hoshi (2000), Ho (2010)
		Inhibitors of metalloproteinases	Inhibition of invasive activity	Saydam (2005), Shah (2003, 2005), Ho (2006, 2010)
		EGFR	Gene silencing (RNAi)	
		FasL, FADD, TRAIL	Induction of apoptosis in cancer cells	
	Shwannomas	Caspase-1	Selective apoptosis in cells infected with amplicons expressing the apoptosis-inducing enzyme, caspase-1 (ICE) driven by the Schwann cell-specific promoter P0	Prabhakar (2010)
Behavioral traits. Learning and memory	Inhibitory avoidance.	NMDA-NR1	Inhibition of NR1 subunit expression	Adrover (2003), Cheli (2006)
	Auditory reversal.	PKC beta II	Activation of PKC pathways	Neill (2001)
	Fear conditioning.	GluR1	AMPAR mobilization	
	Social transmission of food preference; Anxiety.	CREB	Manipulation of cAMP function in different regions of the brain	Rumpel (2005)
	Alcoholism.	GABA	Inhibition (iRNA) of GABA expression in the amygdala	Han (2007, 2008), Brightwell (2005, 2008), Barrot (2005), Liu (2011)

Virus like particles(vlp)

Alternative approach to classical methods

- **Viral capsid-** without viral genetic information.

Eg: Pappilloma viruses: L1 and L2 proteins.

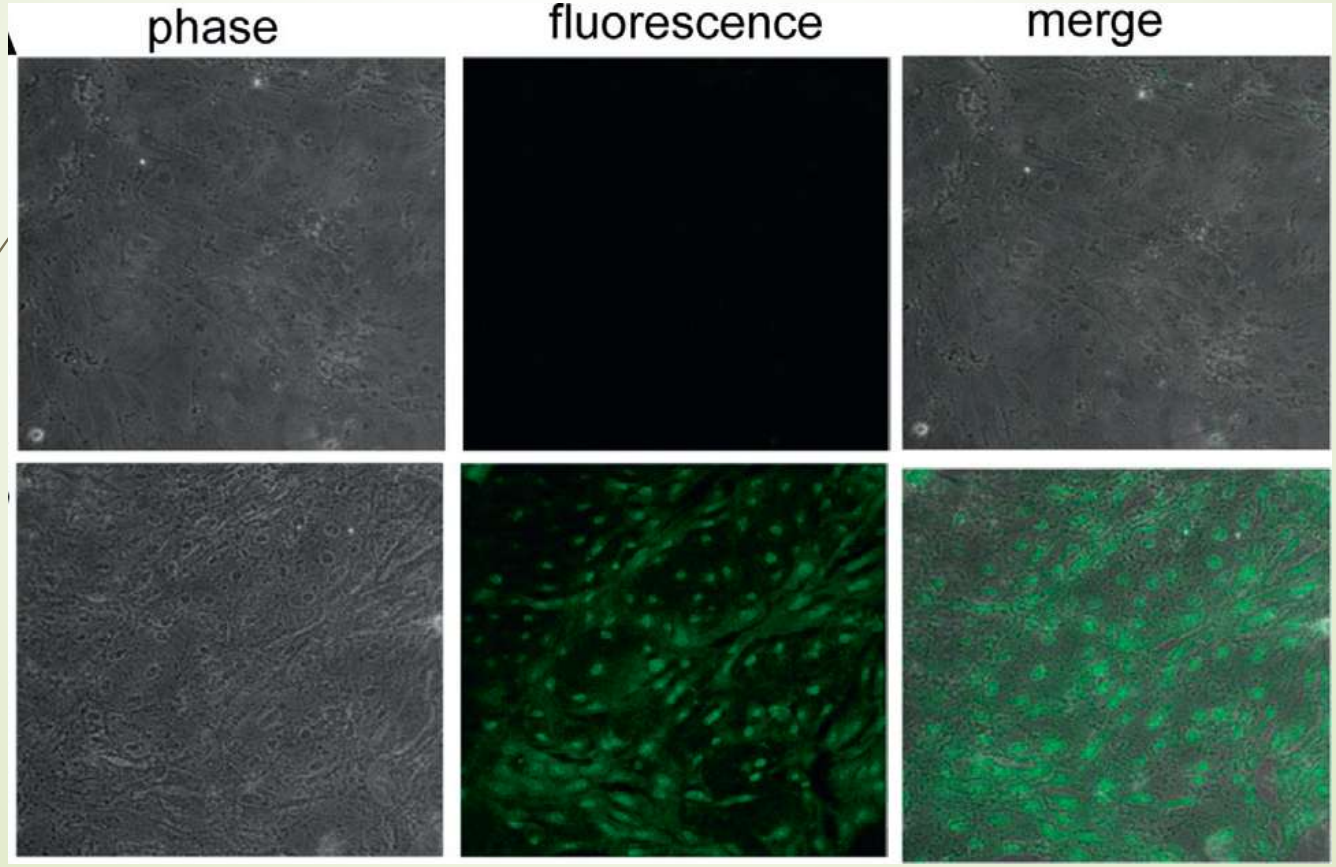
- Predominantly use - **vaccination**

Gene delivery - human polymo JC virus, murine polymovirus, pappilomaviruses and AAV- based VLPs.

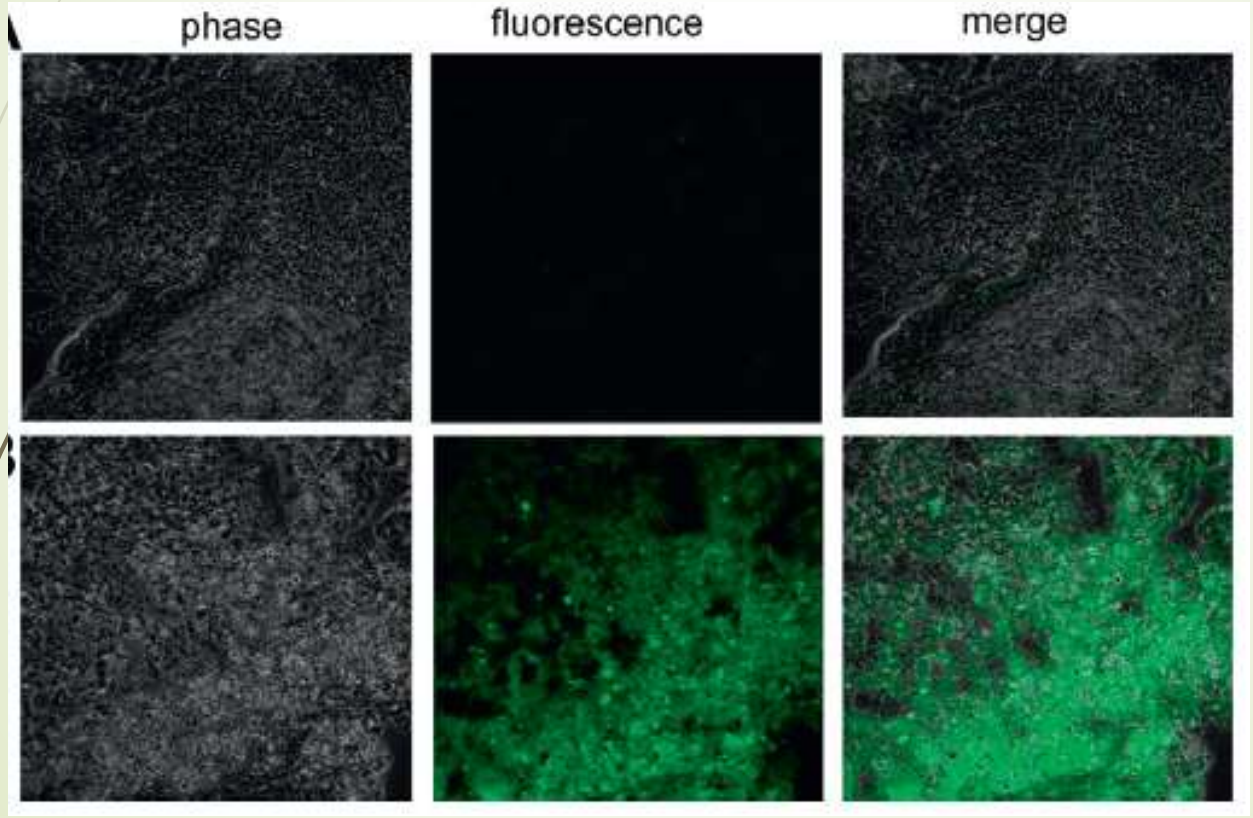
Inhibition of Human Bladder Cancer Growth by a Suicide Gene Delivered by JC Polyomavirus Virus-like Particles in a Mouse Model

Chiung-Yao Fang,* Yi-Da Tsai,* Mien-Chun Lin, Meilin Wang, Pei-Lain Chen, Chun-Nun Chao, Yih-Leh Huang, Deching Changt and Cheng-Huang Shent

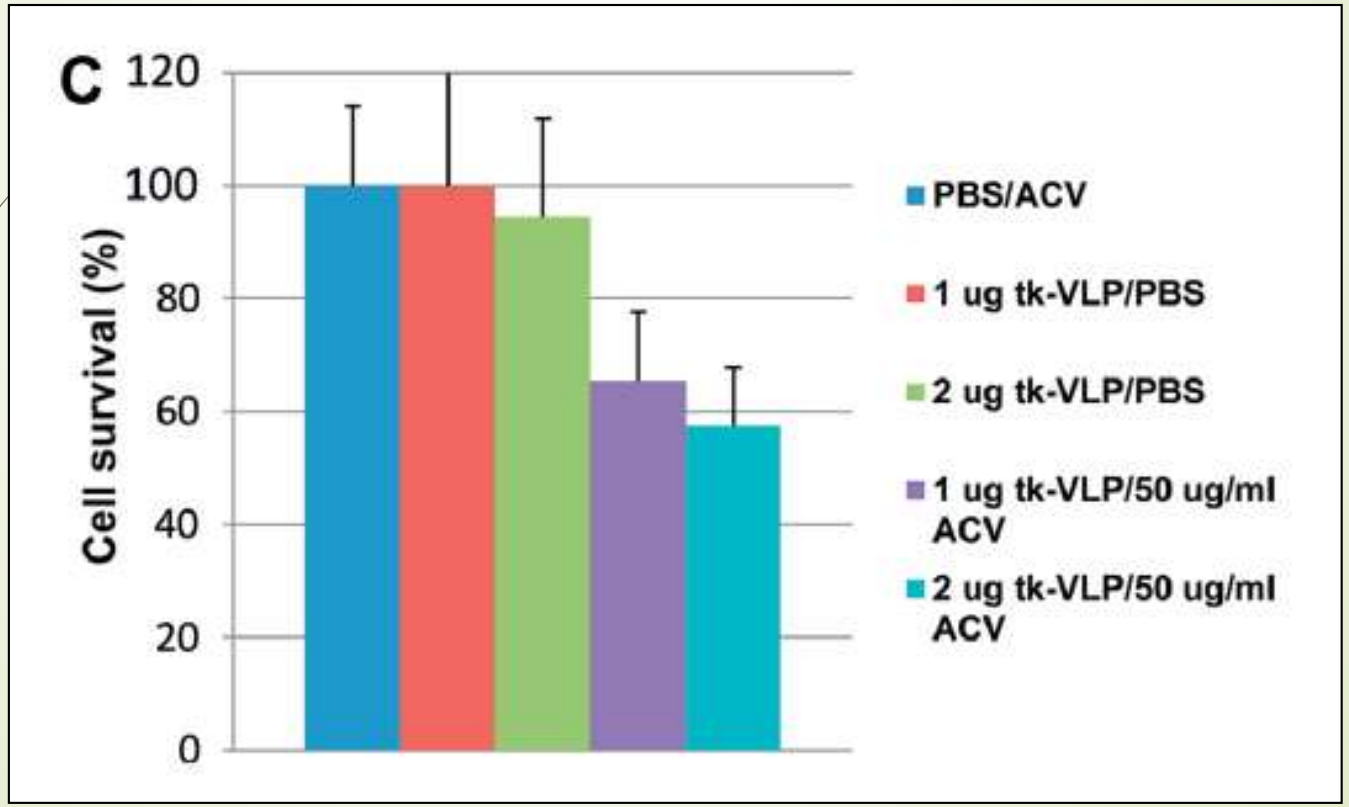
THE JOURNAL OF UROLOGY® Vol. 193, 2100-2106, June 2015



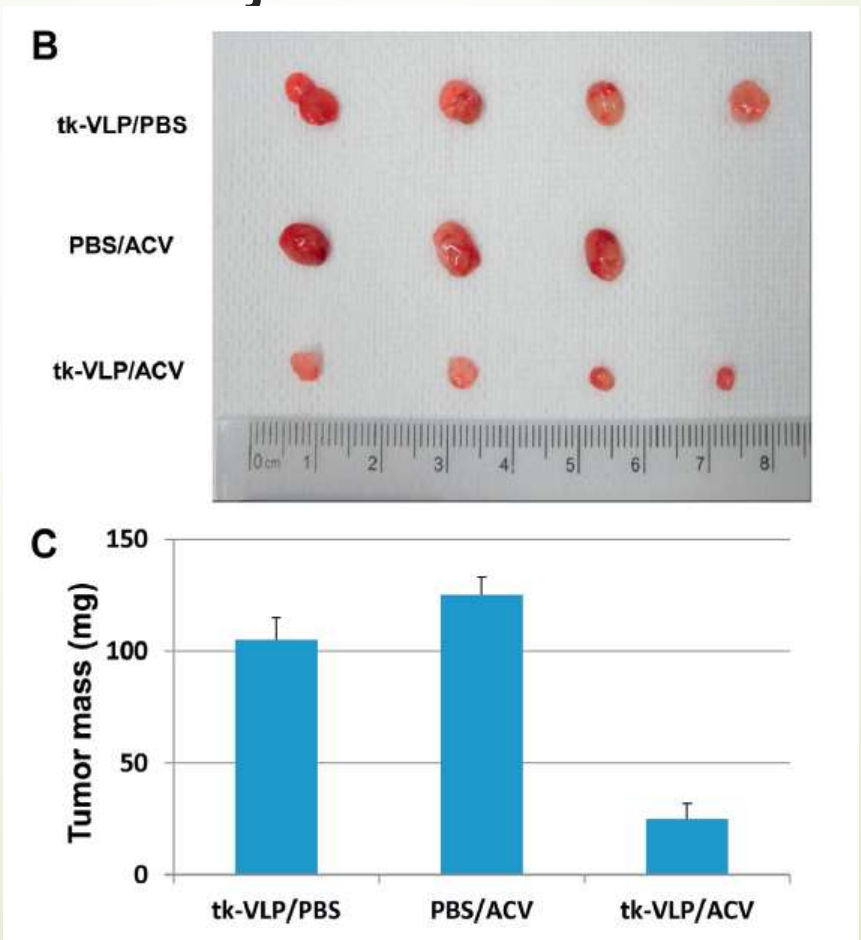
Gene transduction activity of JCPy VLPs in nude mice inoculated subcutaneously with HT-1197 cells and injected with control VLPs



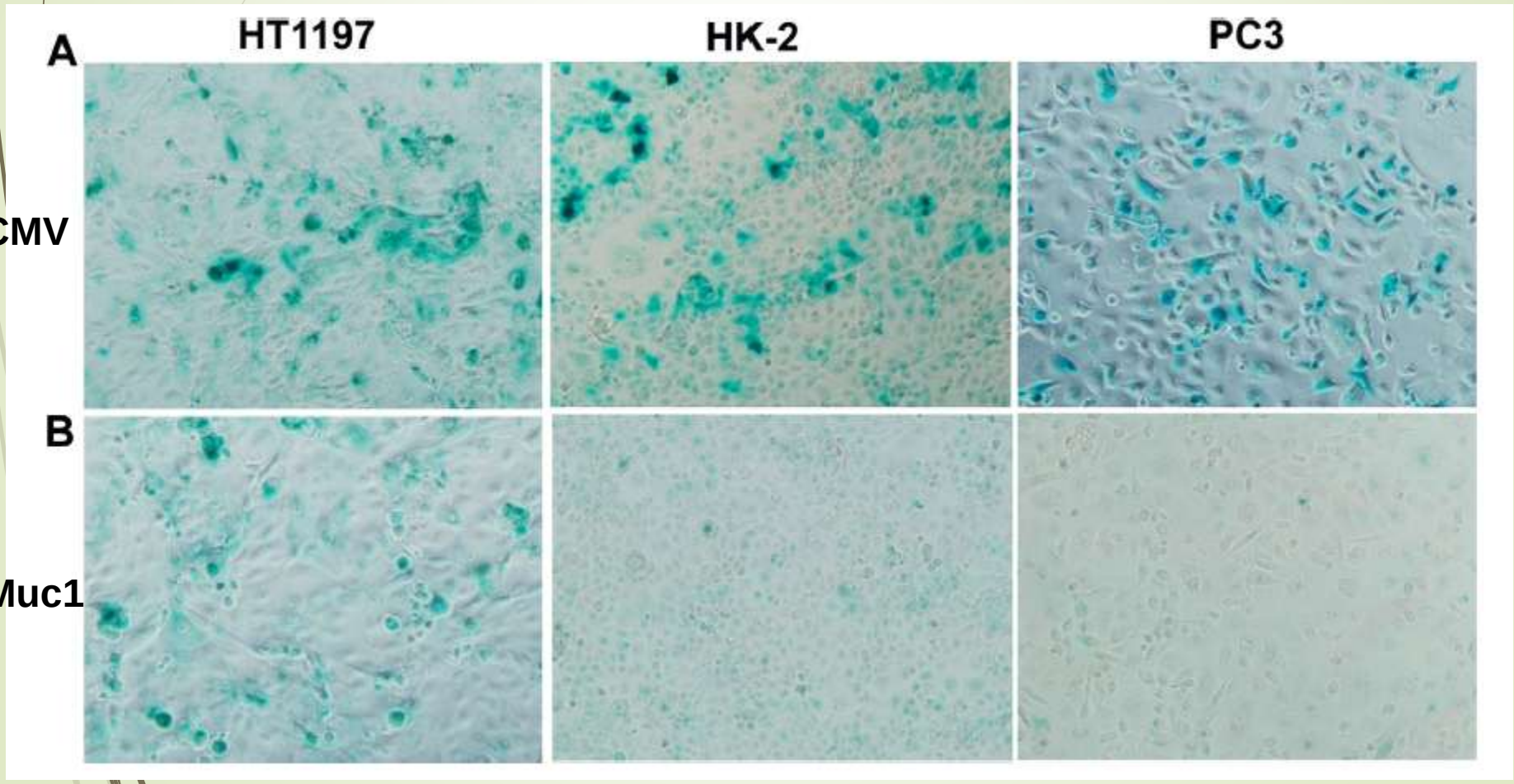
Cytotoxic effect of tk-VLPs on HT-1197 human bladder carcinoma cells infected with tk-VLPs and treated with ACV



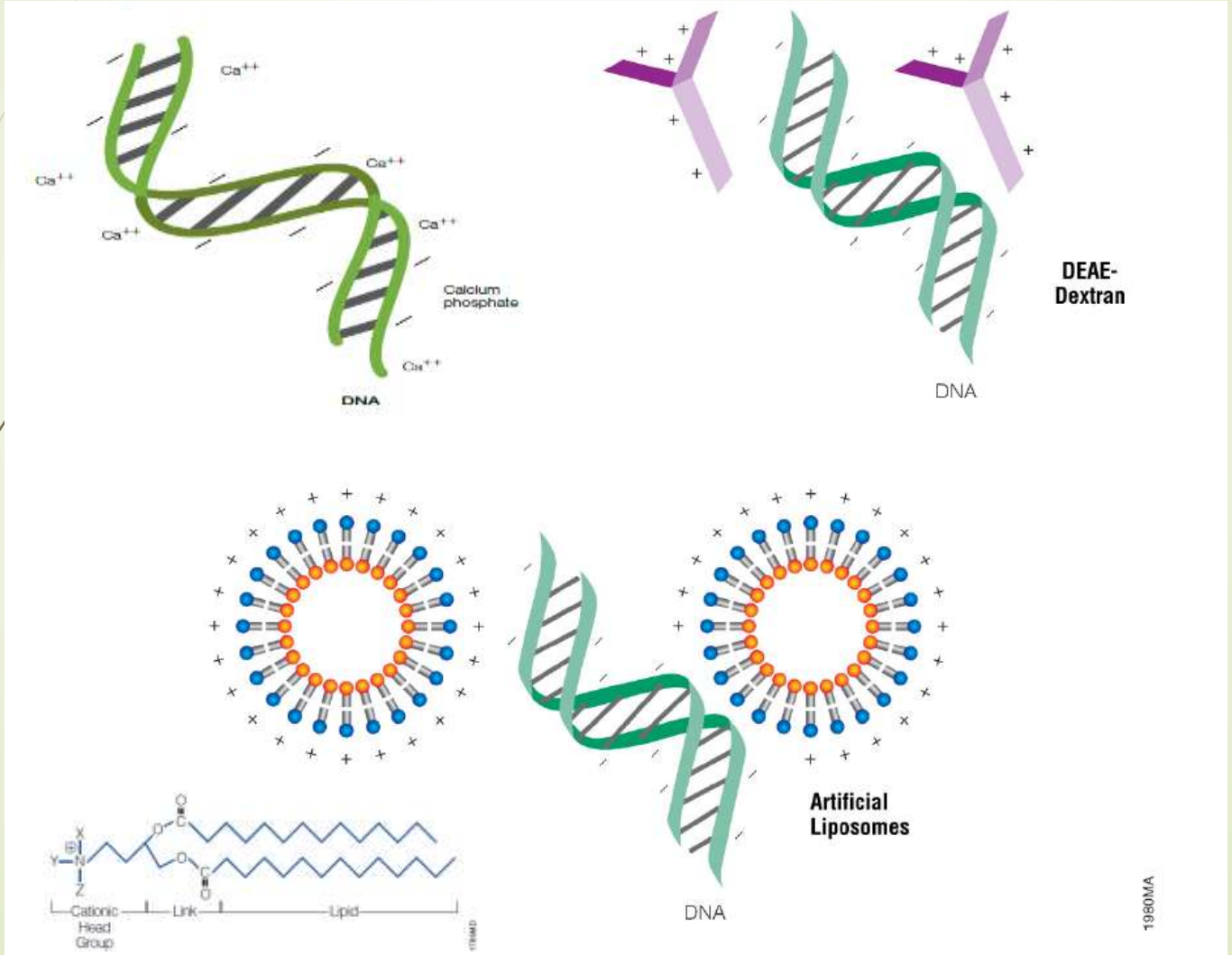
Monitored tumor nodule growth inhibition by tk-VLPs in mice bearing HT-1197 derived tumors injected with tk-VLPs or PBS followed by intraperitoneal injection of ACV or PBS



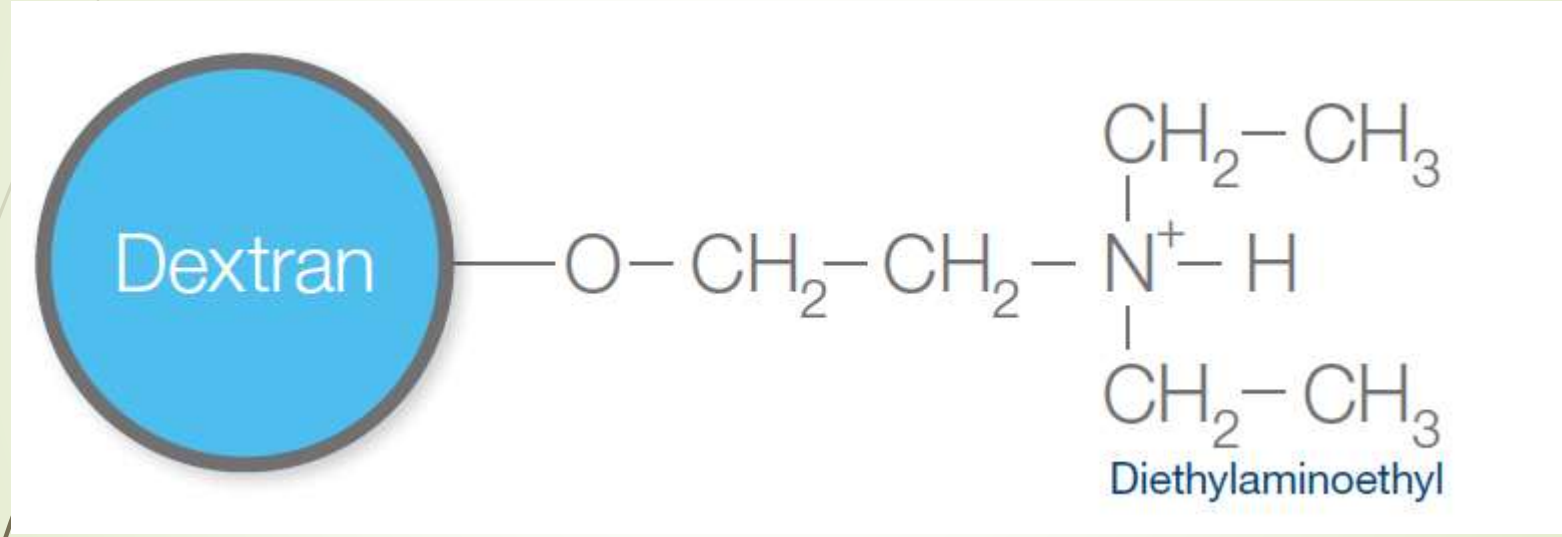
Selective gene transduction by JCPy VLPs in human bladder cancer HT-1197, kidney HK-2 and prostate cancer PC3 cells



Chemical Transfection



DEAE-Dextran



Method Overview

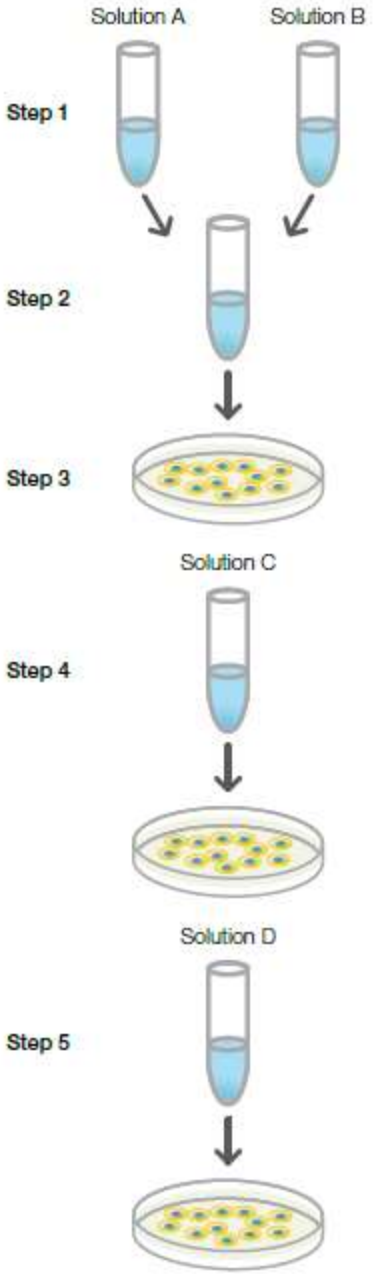
Solution A: DNA (~1–5 µg/ml) diluted into 2 ml of growth medium with serum containing chloroquine

Solution B: DEAE-dextran solution (~50–500 µg/ml)

Solution C: ~5 ml of DMSO

Solution D: Complete growth medium

1. Add solution A to solution B, then mix gently.
2. Aspirate cell medium and apply the mixed A and B solutions to the subconfluent cell culture. Incubate the DNA mixture for ~4 hr.
3. Aspirate supernatant.
4. Add solution C to induce DNA uptake.
5. Remove DMSO and replace with complete growth medium; assay for transient gene expression.



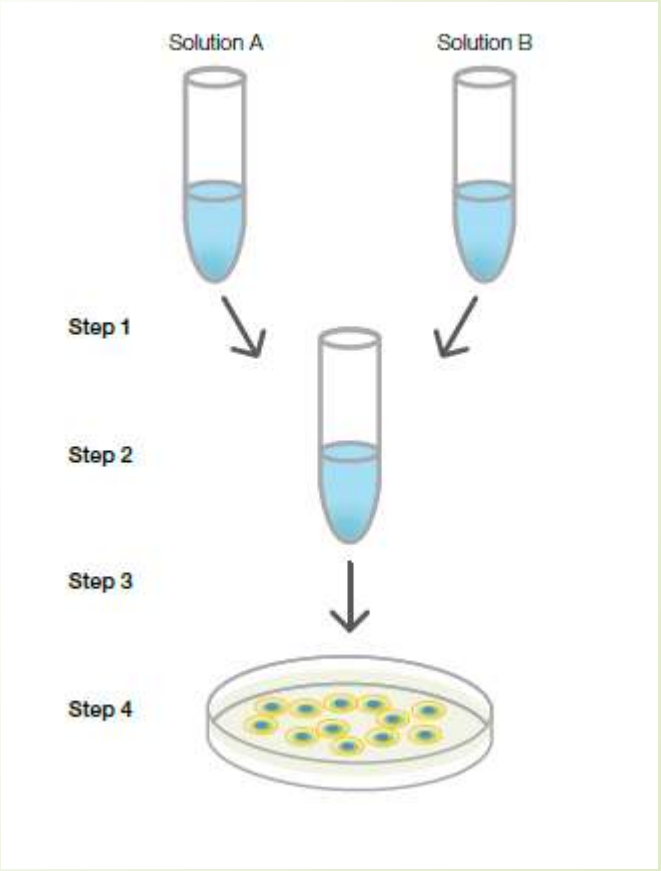
Calcium Phosphate Co-Precipitation

Method Overview

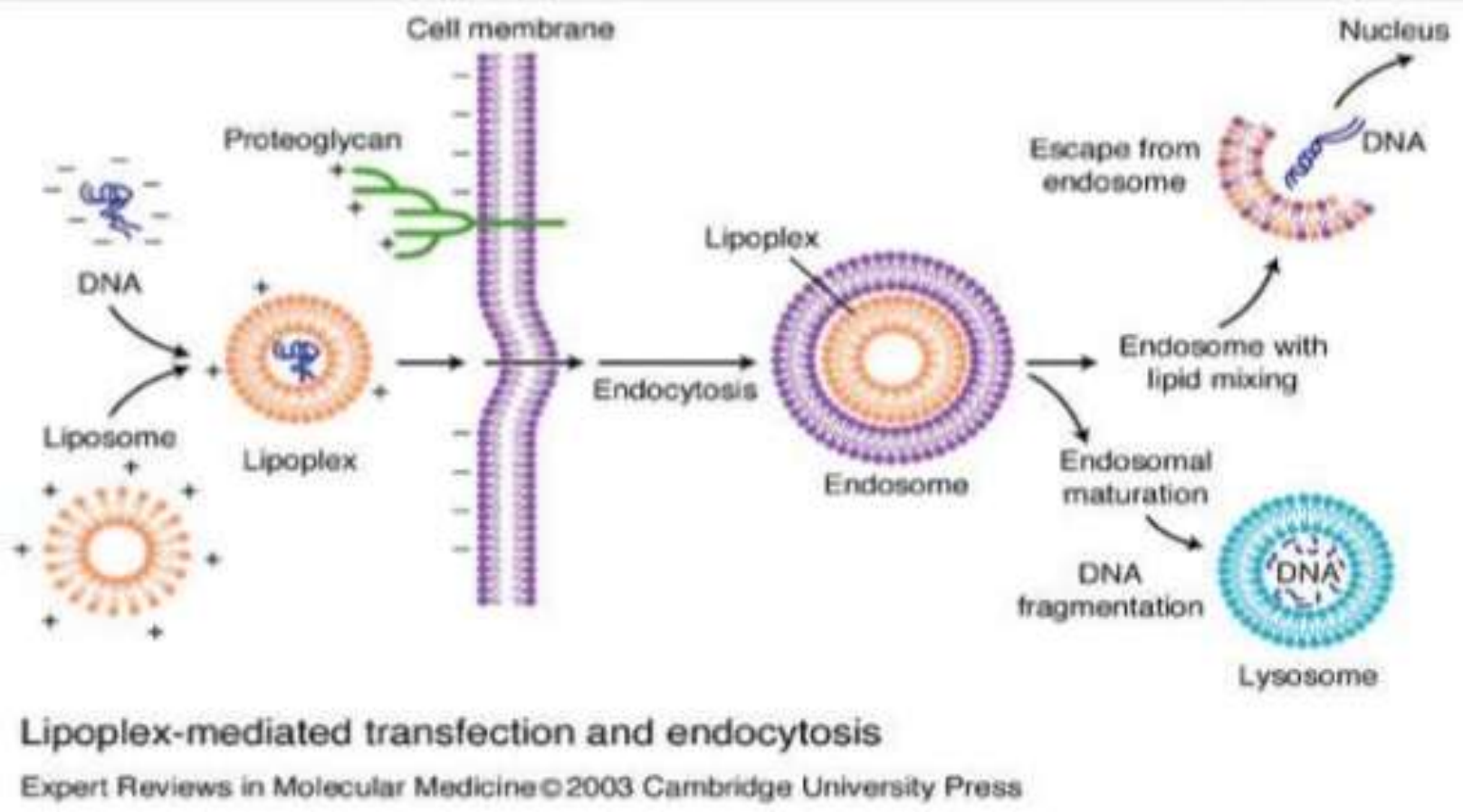
Solution A: DNA in calcium solution

Solution B: 2x Hanks buffered saline solution

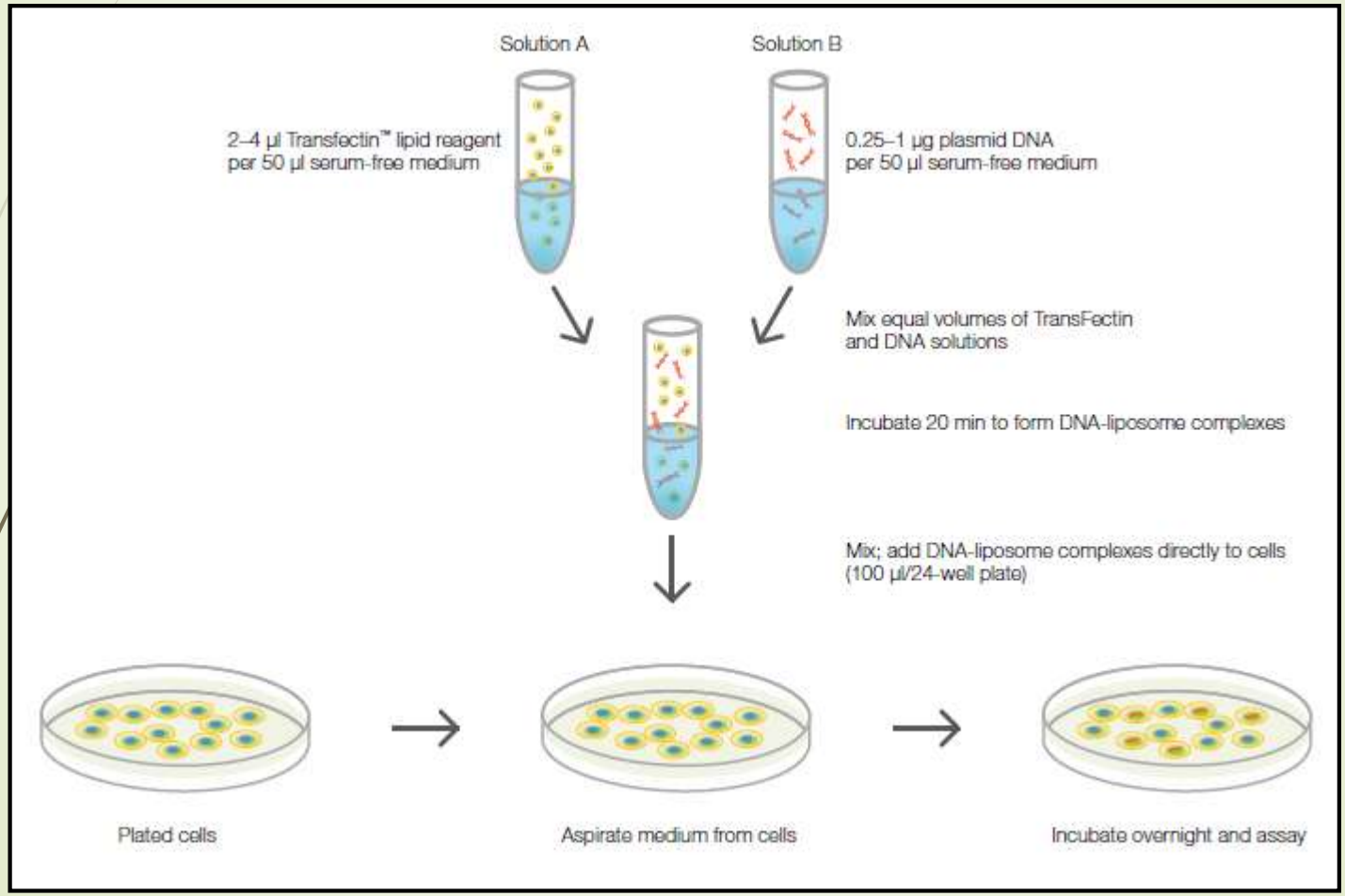
1. Add solution A to solution B while vortexing.
2. Incubate 20–30 min. Apply the solution to a subconfluent cell culture.
3. Incubate 2–12 hr. Replace the solution with complete growth medium.
4. Assay for transient gene expression or begin selection for stable transformation time.



Liposomes and lipoplexes



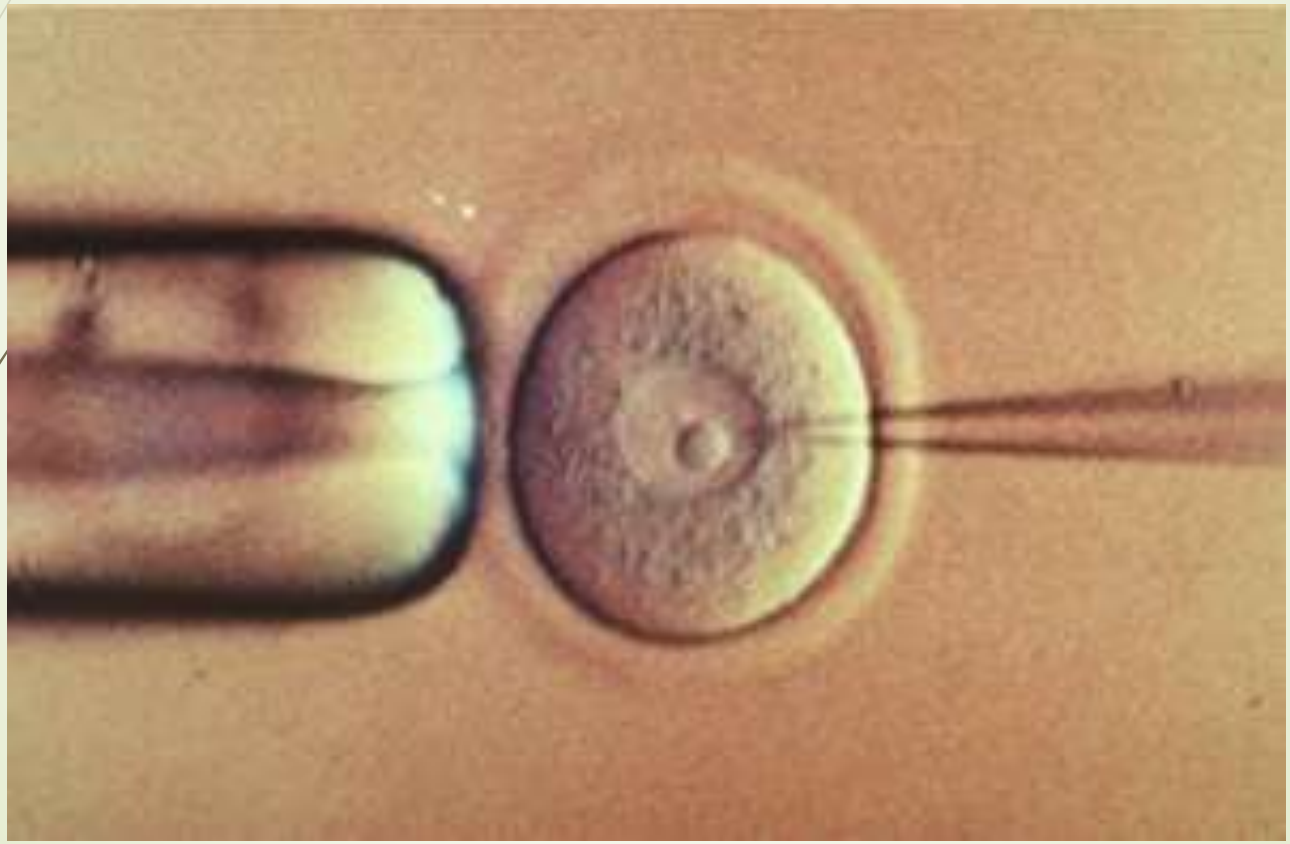
Lipoplex-mediated transfection and endocytosis


Solution A
Solution B
2–4 µl Transfectin™ lipid reagent per 50 µl serum-free medium
0.25–1 µg plasmid DNA per 50 µl serum-free medium
Mix equal volumes of TransFectin and DNA solutions
Incubate 20 min to form DNA-liposome complexes
Mix; add DNA-liposome complexes directly to cells (100 µl/24-well plate)
Plated cells
Aspirate medium from cells
Incubate overnight and assay

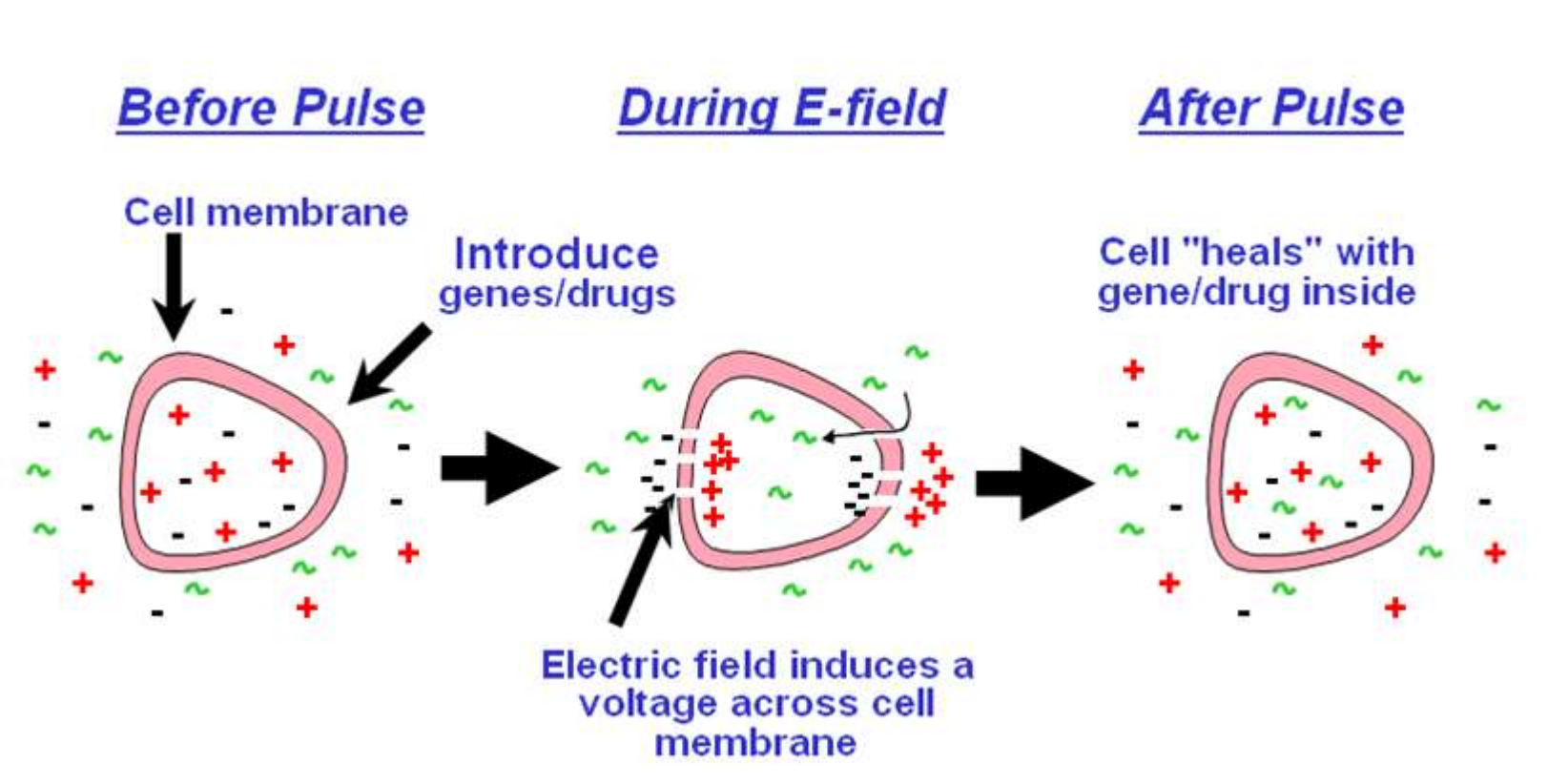
Physical Methods

- Direct microinjection
- Electroporation
- Biolistic particle delivery

Microinjection



Electroporation



Biolistic Particle Delivery

Helios® Gene Gun

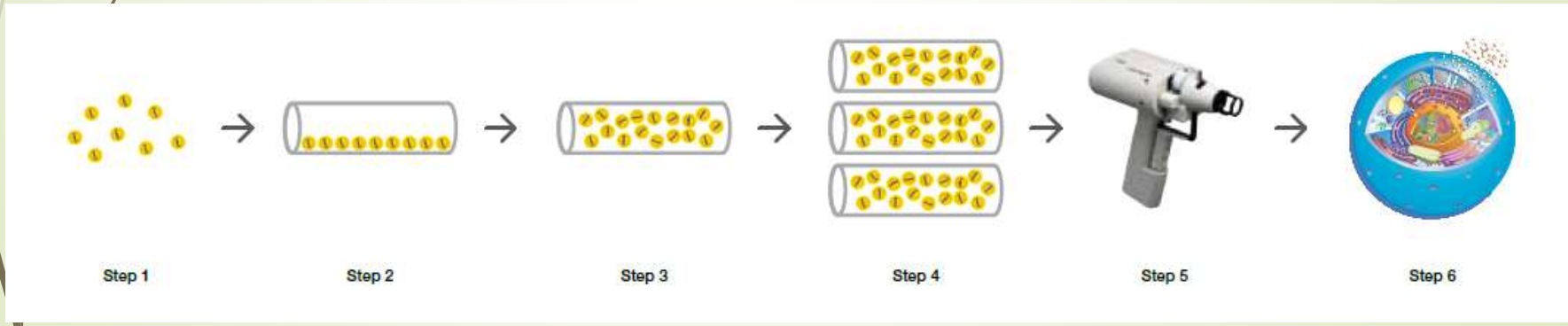
- For in situ, in vivo, and in vitro transformations
- Applications for animals, plants, cell culture, nematodes, yeast, and bacteria
- Pressure range 100–600 psi enables fine-tuning of penetration
- Highly portable — can be used in the field
- Small target area for accurate targeting

PDS-1000/He™ Biolistic Particle Delivery System

- For in vitro, ex vivo, and in vivo (for some plants and microbes) transformations
- Applications for animal cell and organ cultures, plant cell cultures and explants, pollen, insects, algae, fungi, and bacteria
- Pressure range 450–2,200 psi gives flexibility and penetration — ideal for plant applications
- Large target area — more cells can be transformed

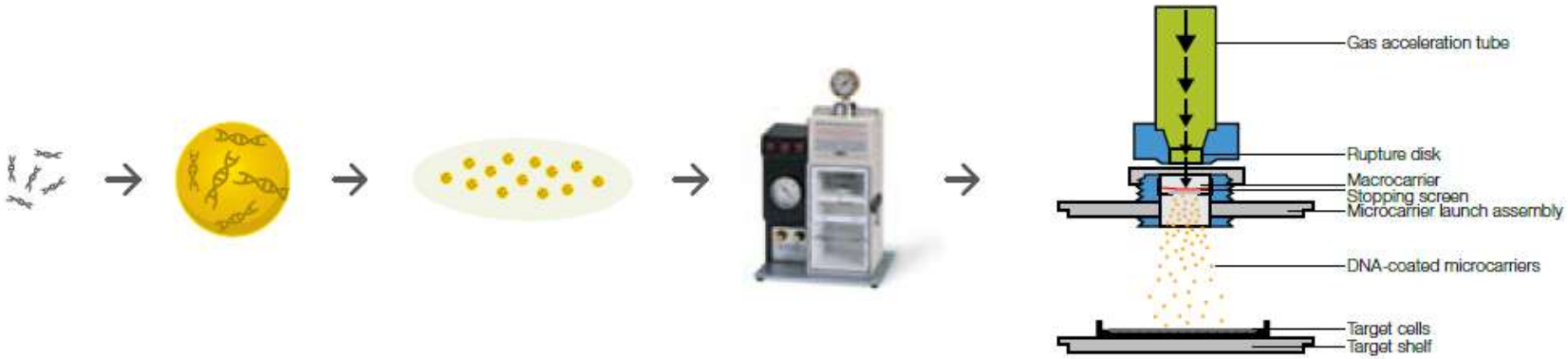
Helios® Gene Gun — Process Overview

1. Precipitate DNA onto gold particles.
2. Load DNA/gold into tubing.
3. Rotate tubing to coat DNA gold over inside surface.
4. Cut tubing into cartridges.
5. Load cartridges into gene gun.
6. Deliver DNA into target cells.



PDS-1000/He™ System — Process Overview

1. DNA-coated gold particles (microcarrier) are spread over the central area of a thin plastic disk (macrocarrier).
2. Disk loaded with the DNA-gold particles is placed into a holder inside the PDS-1000/He system.
3. The system uses high-pressure helium, released by a rupture disk, and partial vacuum to propel the macrocarrier loaded with microcarrier toward the target cells.
4. Macrocarrier is stopped after a short distance by a stopping screen.
5. DNA-coated gold particles continue travelling toward the target to penetrate the cells.
6. Sample chamber is subjected to partial vacuum, from 15 to 29 inches of mercury, depending on the target cells.



Take Home Message

Class	Methods	Advantages	Disadvantages	Examples
Biological	• Virus-mediated	- High-efficiency - Easy to use - Effective on dissociated cells, slices, and in vivo	- Potential hazard to laboratory personnel - Insertional mutagenesis - Immunogenicity - DNA package size limit	Herpes simplex virus, Adeno virus, Adeno-associated virus, Vaccinia virus, Sindbis virus
Chemical	• Cationic polymer • Calcium phosphate • Cationic lipid	- No viral vector - High-efficiency - Easy to use - Effective on dissociated cells and slices - Plenty of commercially available products - No package size limit	- Chemical toxicity to some cell types - Variable transfection efficiency by cell type or condition - Hard to target specific cells	DEAE-dextran, polyethyleneimine, dendrimer, polybrene, calcium phosphate, lipofectin, DOTAP, lipofectamine, CTAB/DOPE, DOTMA
Physical	• Direct injection • Biolistic particle delivery • Electroporation • Laser-irradiation • Sonoporation • Magnetic nanoparticle	- Simple principle and straightforward - Physical relocation of nucleic acids into cell - No need for vector - Less dependent on cell type and condition - Single-cell transfection	- Needs special instruments - Vulnerable nucleic acids - Demands experimenter skill, laborious procedure	Micro-needle, AFM tip, Gene Gun, Amaxa Nucleofector, phototransfection, Magnetofection

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