"Design of non-viral gene delivery systems: input from live cell imaging studies"

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Nucleic acids delivery

* Basic and applied researches
* Therapeutic applications

Viral vectors

Adeno-Associated virus

Adenovirus

Retrovirus

Physical methods

Electrotransfer

Gene Gun

Ultrasound

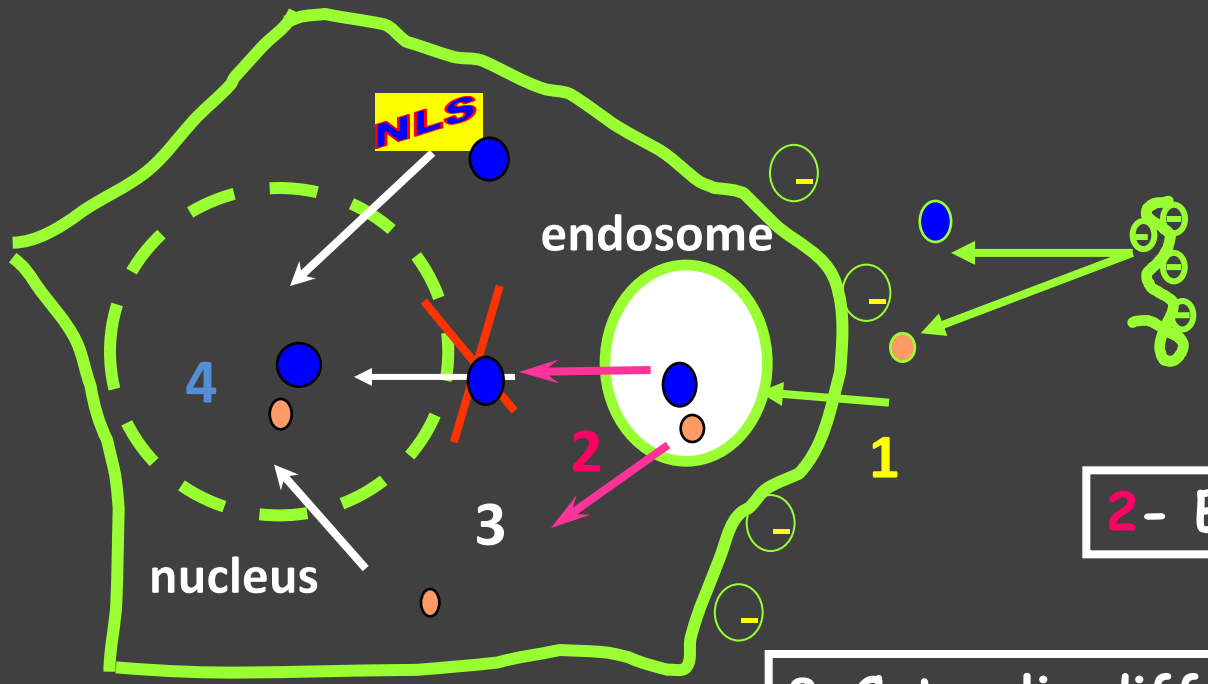
Laser

Chemical vectors

Lipides, Polymers

Biomaterials

Intracellular Barriers



1- Uptake
Protection
Targeting

2- Endosome escape

3- Cytosolic diffusion

4-Nuclear Import

NLS : Nuclear Localisation Signal

Pichon et al., 2010 Current Opin. Biotech.

Cell Imaging & intracellular investigations

-Endocytosis pathways investigation

-plasmid DNA & vector intracellular distribution

-pDNA dissociation from vectors

-Improving pDNA nuclear import with KB motifs

-Improving cytosolic diffusion with E3 14.7K peptide

- **Real time cellular imaging:** colocalization experiments, videomicroscopy

- **Fluorescence-based methods:**

-Fluorescence Recovery after Photo bleaching (FRAP)

-Förster Resonance Energy Transfer (FRET)

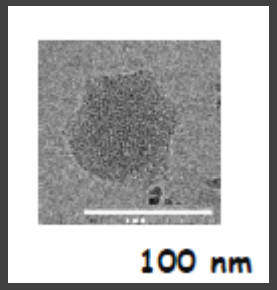
-Fluorescence life Time Imaging (FLIM)

Nucleic acids Formulations

A

DNA/RNA + Cationic Polymer → polyplex

50 - 150 nm



B

DNA/RNA + Cationic Liposome → lipoplex

200 nm

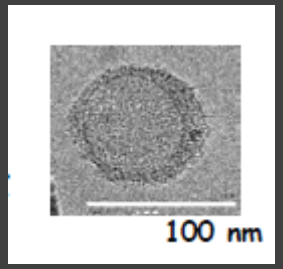
C

DNA/RNA + Cationic Polymer → + Liposome → Lipopolyplex

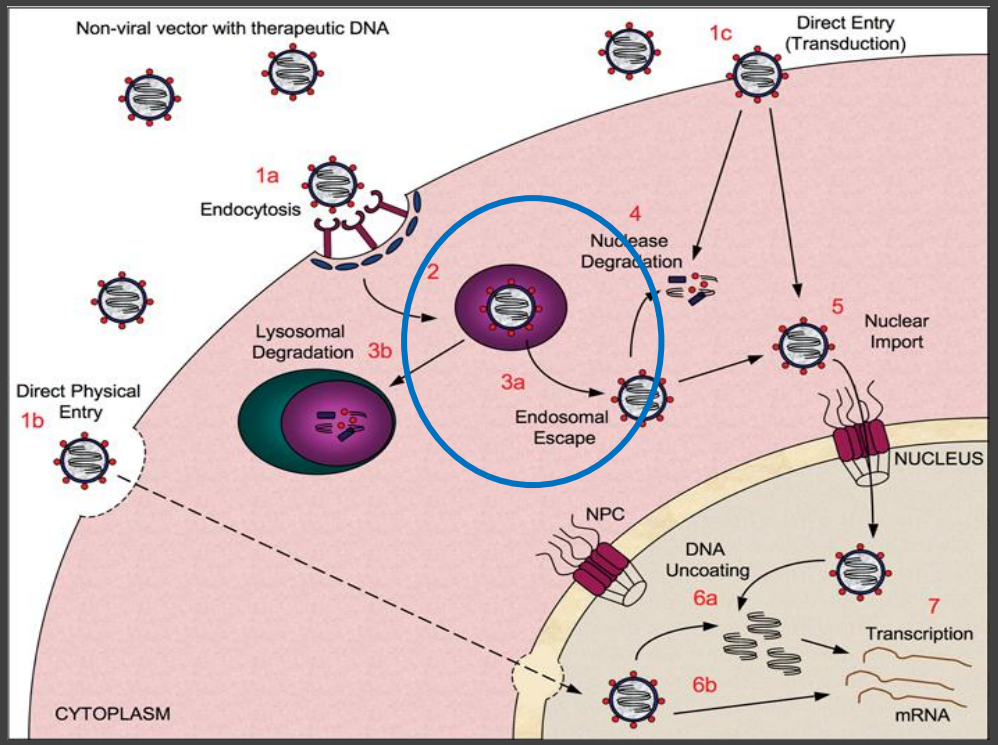
LPR: RNA

LPD: DNA

100 - 200 nm



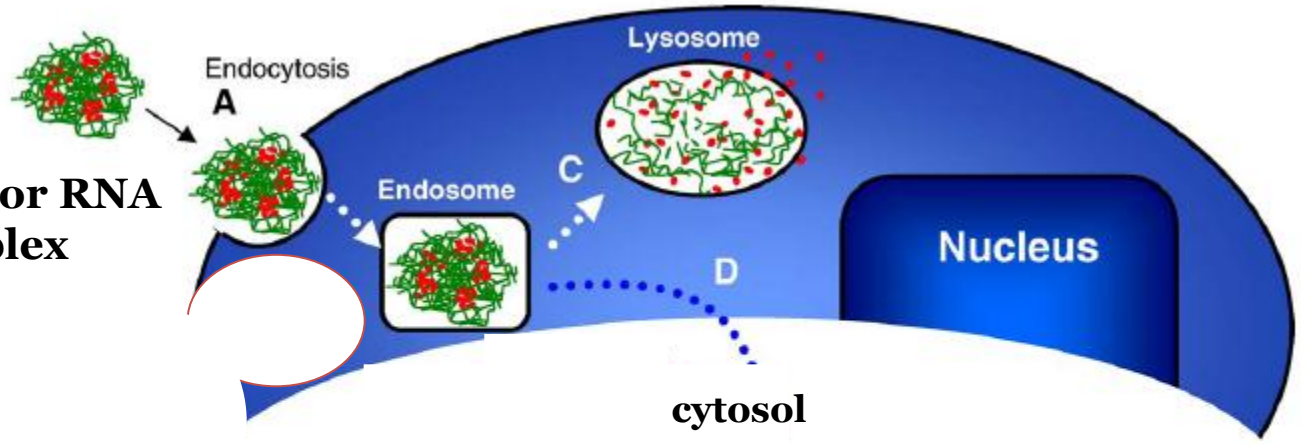
Histidine-based chemical vectors for endosomal escape



Midoux *et al.*, (2009) *Br. J. Pharmacol.* **157**, 166-178
Mével *et al.*, ChemBioChem 2008, 9: 1462-1471
Mével *et al.*, ChemComm. 2008, 21:3124-3126
Pichon et al., (2001) *Adv. Drug Delivery Rev.* **53**, 75-94.
Midoux & Monsigny (1999) *Bioconjugate Chem.* **10**, 406-411

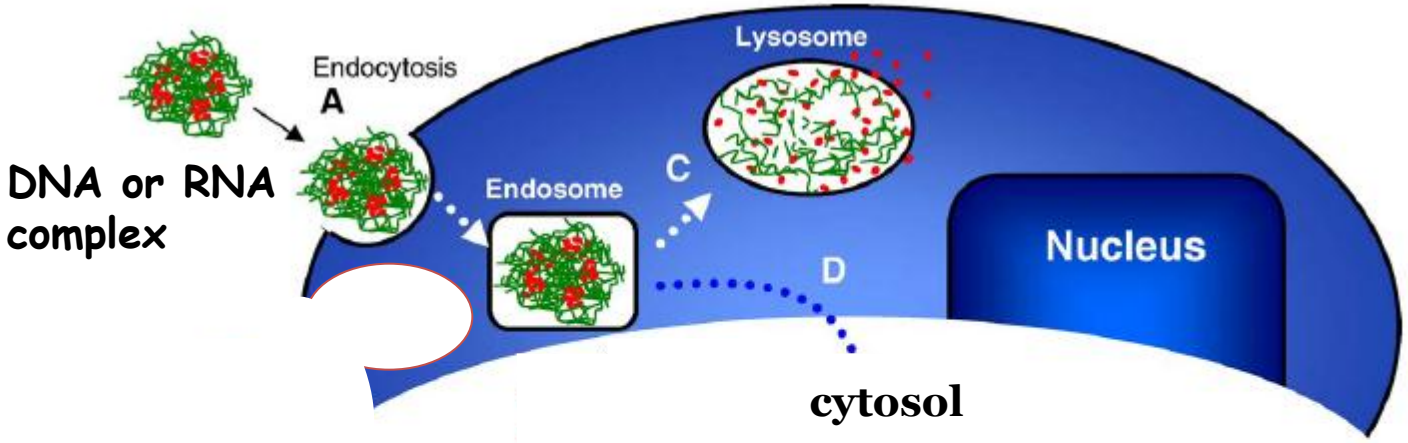
Mével, M et al., FR 07 57955. 28 Septembre 2007
Cheradame, et al. FR0851434 du 5 mars 2008.

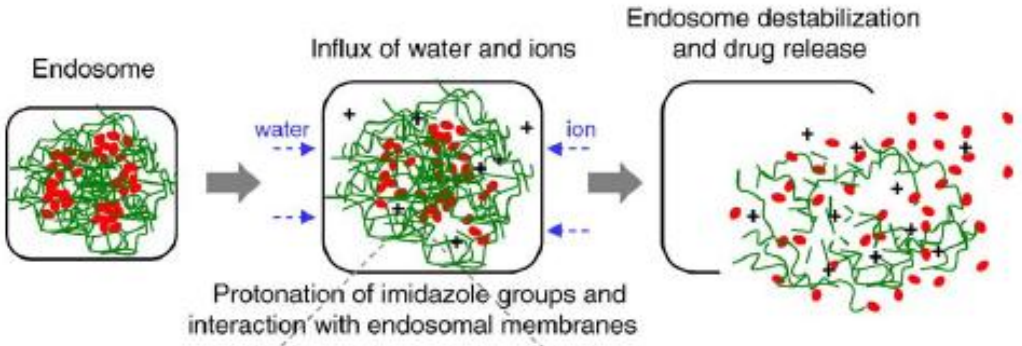
Cellular Uptake & Endosomal escape



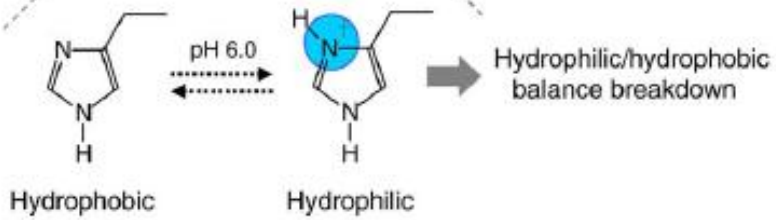
DNA or RNA complex

Endosomal Escape

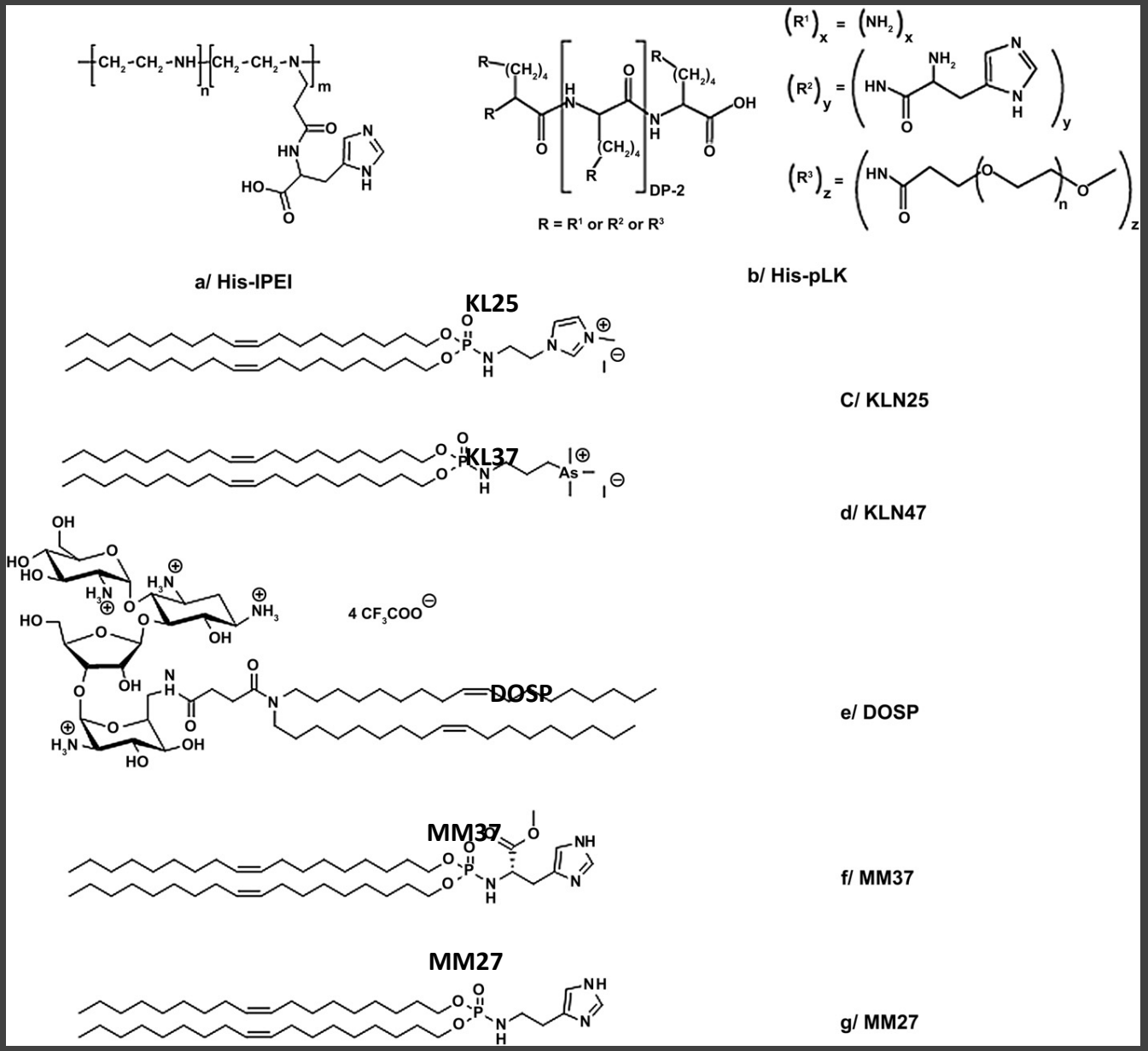




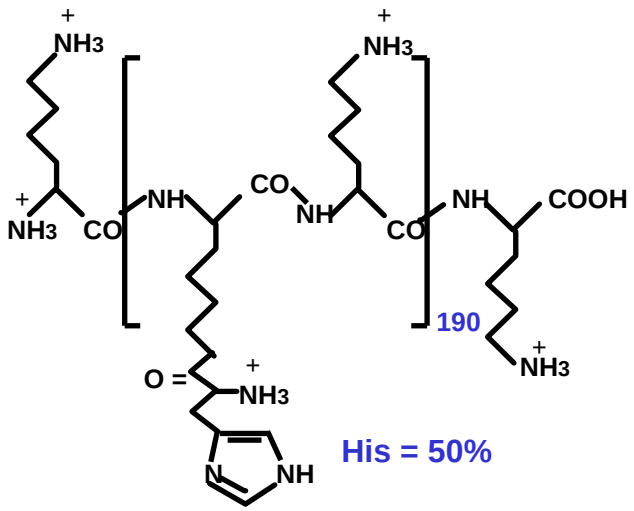
PROTON SPONGE EFFECT



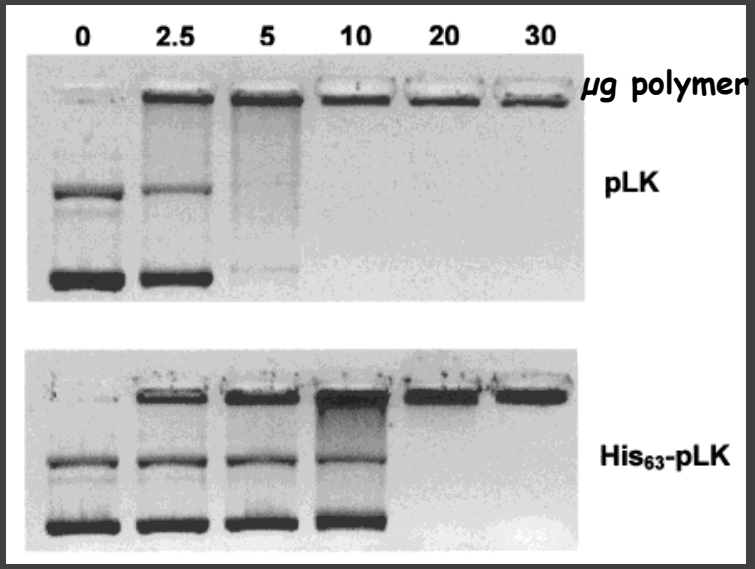
Histidylated chemical vectors



Histidylated polylysine



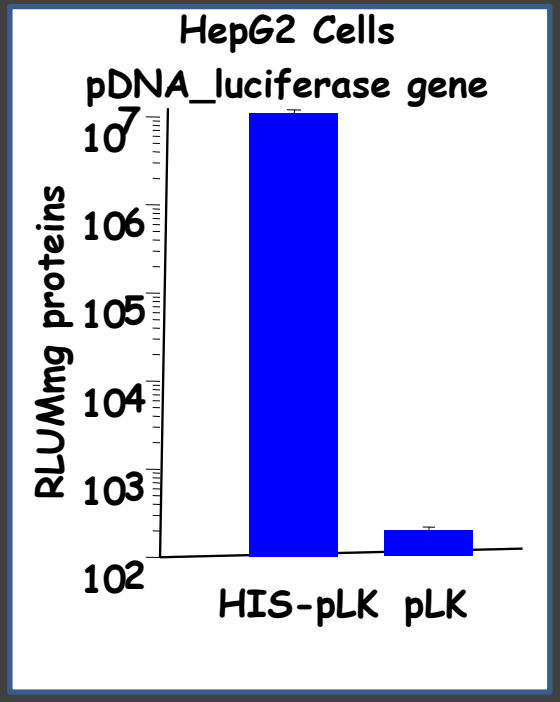
Gel retardation assay (10μg pDNA)



~100 nm, 20 mV

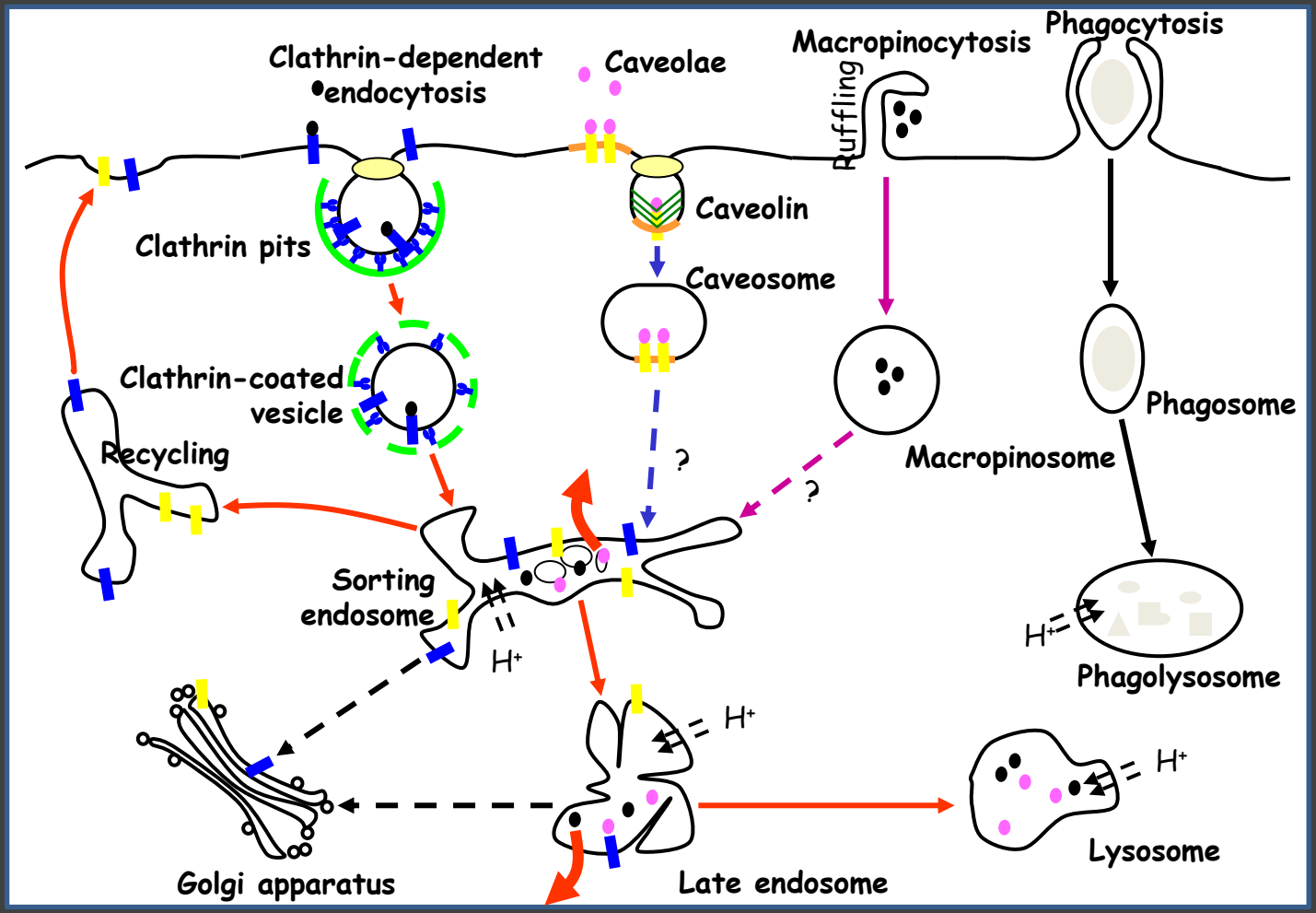
Table 1. Size and ζ Potential of Histidylated Polyplexes[a]

	polymer/pDNA weight ratio (μg/μg)					
	ζ potential (mV)			size (nm)		
	2	3	4	2	3	4
His_{109}-pLK	+17.5 ± 3 (+32)*	+18 ± 2 (+33)	+17 ± 3 (+34)	140 ± 10	110 ± 10	110 ± 10
$AcHis_{100}$-pLK	−18 ± 2 (+32)	−5 ± 3 (+34)	+14 ± 3 (+37)	179 ± 10	170 ± 10	165 ± 15
pLK	+10 ± 3 (+12)	+17 ± 3 (+18)	+22 ± 3 (+22)	100 ± 10	100 ± 10	90 ± 10



Do polyplexes reach acidic compartments once uptaken by cells?

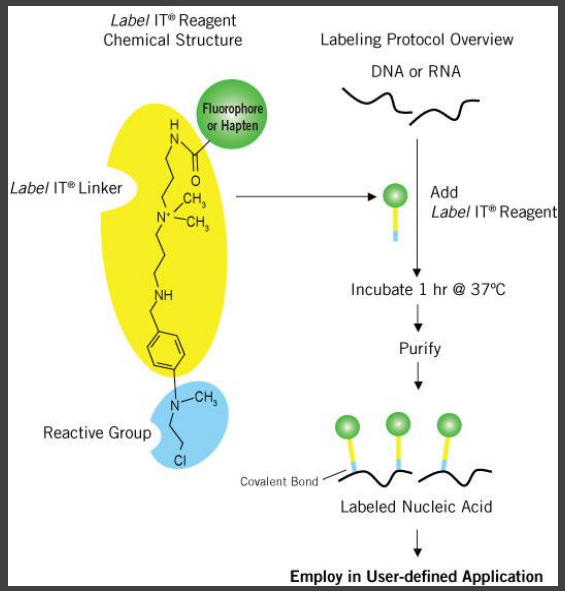
Endocytosis pathways

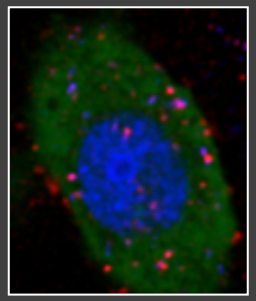


Zeiss LSM 510 Meta Confocal microscopy
Flow cytometry BD LSR

Tools to investigate the ntracellular routing

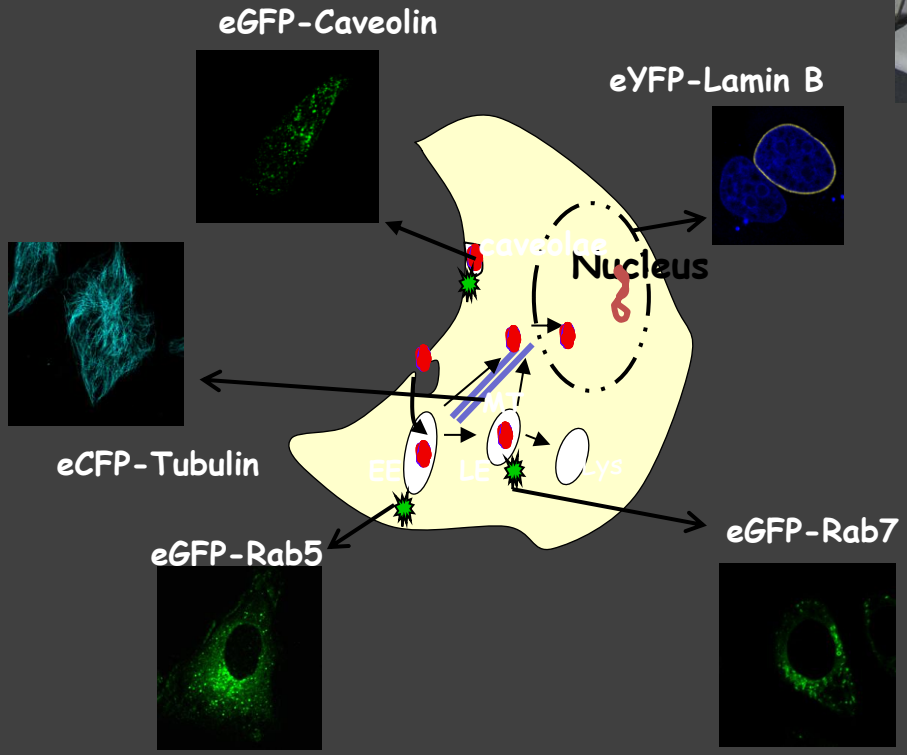
- Fluo-labeled pDNA





- Fluo-labeled vectors

- Pharmacological inhibitors of endocytosis pathways
- Immunostaining/Cellular tools

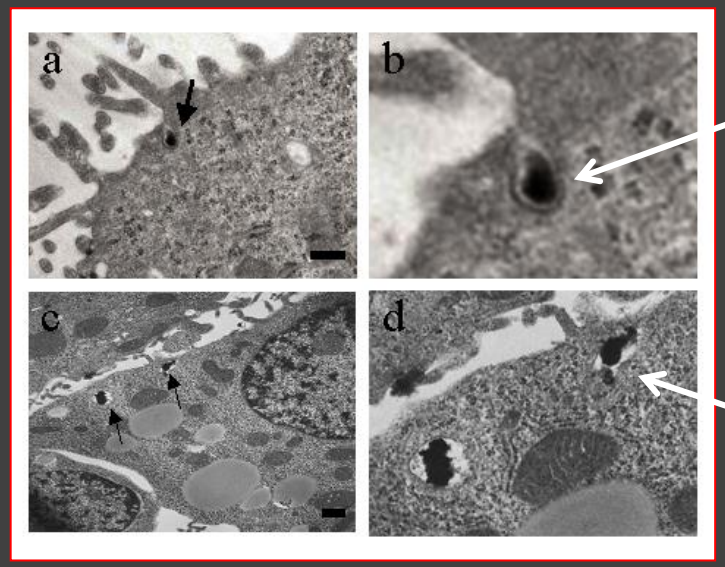


LSM 510 Meta (ZEISS), Argon (458nm, 477nm, 488nm & 514nm) + Hélium-Néonlasers (543nm et 633nm)

Co-localization experiments

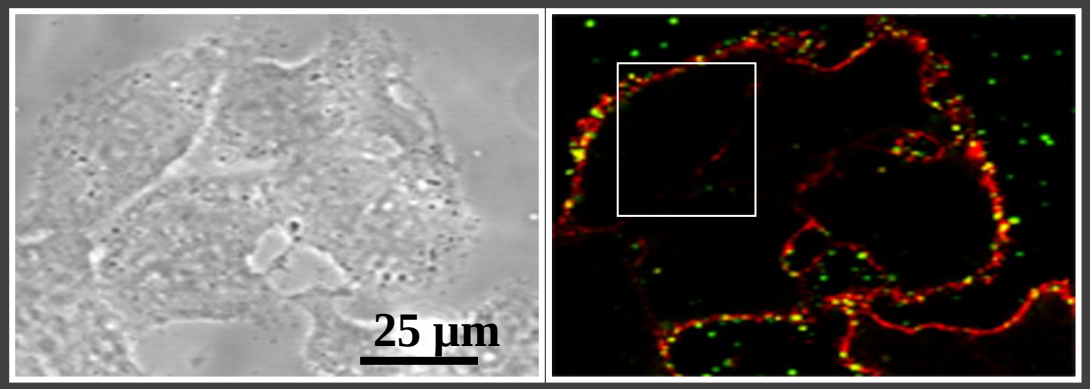
Endocytosis pathways

HepG2 cells: transfection with His-pLK/pDNA, 30 min 37°C

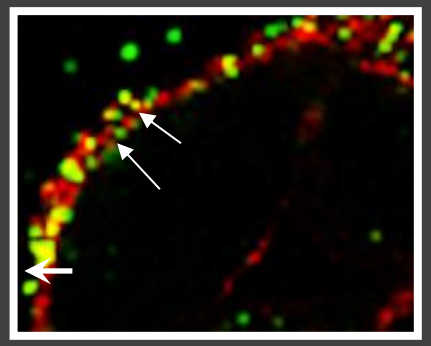


Coated vesicles

Uncoated vesicles



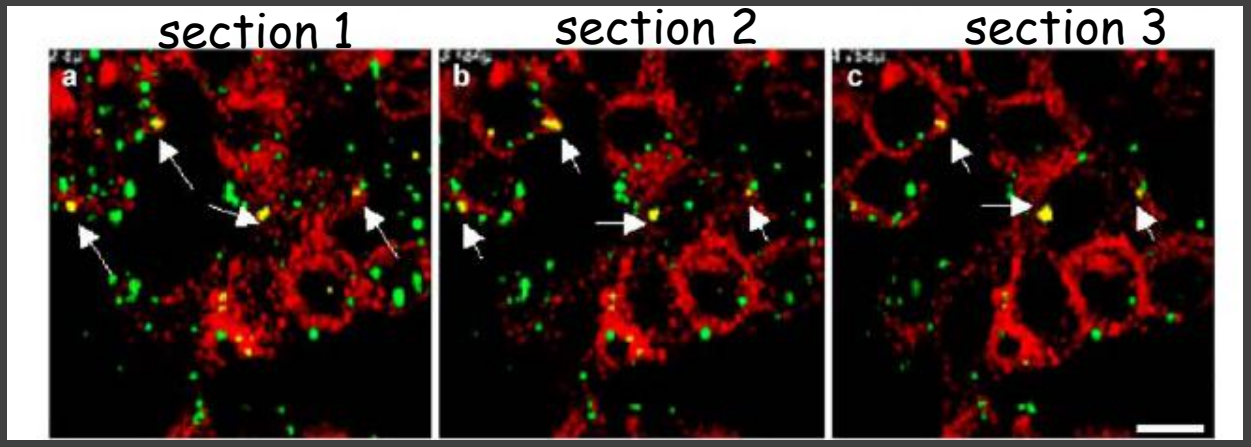
F-DNA/R-Cholera toxin B



Cholera toxin B binds to GM1 ganglioside

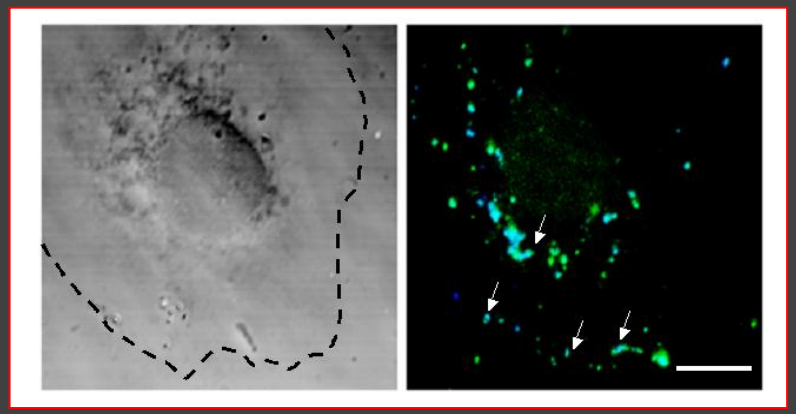
Goncalves *et al., Mol. Ther. 2004*, 10: 373-385.

Colocalization experiments: EEA1, Transferrin receptor,LAMP1, Rab11



2h at 4°C,
30 min at 37°C

Rho-Transferrin receptor
FITC-pDNA



30 min 37°C, 30 min chase

Rab 11 F-pDNA/His-vector
Rab 11⁺ endosomes: Recycling endosomes

Epitope specific flow cytometry sorting : organelles preparation

TfR- and EEA1-positive vesicles containing pDNA (%)

	TfR^+			$TfR^{++}/EEA1^{++}$			$TfR^-/EEA1^{++}$			$TfR^-/EEA1^+$		
Chase	U	N	N+C	U	N	N+C	U	N	N+C	U	N	N+C
5 min	20 ± 9	9.5 ± 2	0	18 ± 0.5	12 ± 0.5	ND	0	10	0	0	0	ND
30 min	28 ± 5	9.5 ± 1	0	20 ± 1	14 ± 0.5	8.6 ± 0.2	0	11	0	0	0	29 ± 4
120 min	25 ± 5	15 ± 4.5	0	19.5 ± 1	17	8 ± 0.3	0	11	0	0	0	33 ± 5

HepG2 cells

TABLE 1: Involvement of clathrin lattices, cytoskeleton, and macropinocytosis on polyplex uptake

	His-polyplex uptake (% of control)	Transferrin uptake (% of control)
Hypertonic medium	45 ± 5	36 ± 6
Cytosolic acidification	45 ± 5	–
Chlorpromazine	50 ± 5	–
MBC + lovastatin	50 ± 8	–
Filipin III	53 ± 11	80 ± 5
Chlorpromazine + Filipin III	0 ± 5	–
CytD*	45 ± 1	–
Noc*	65 ± 2.5	–
CytD* + Noc*	45 ± 1	–
PMA	130 ± 5	90 ± 5
DMA	58 ± 5	–
Wortmannin	69.5 ± 5	81 ± 2
Genestein	81 ± 12	76 ± 2
DMA + PMA	45 ± 3	–
PMA + filipin III	71 ± 8	–

Clathrin-dependent

Cholesterol

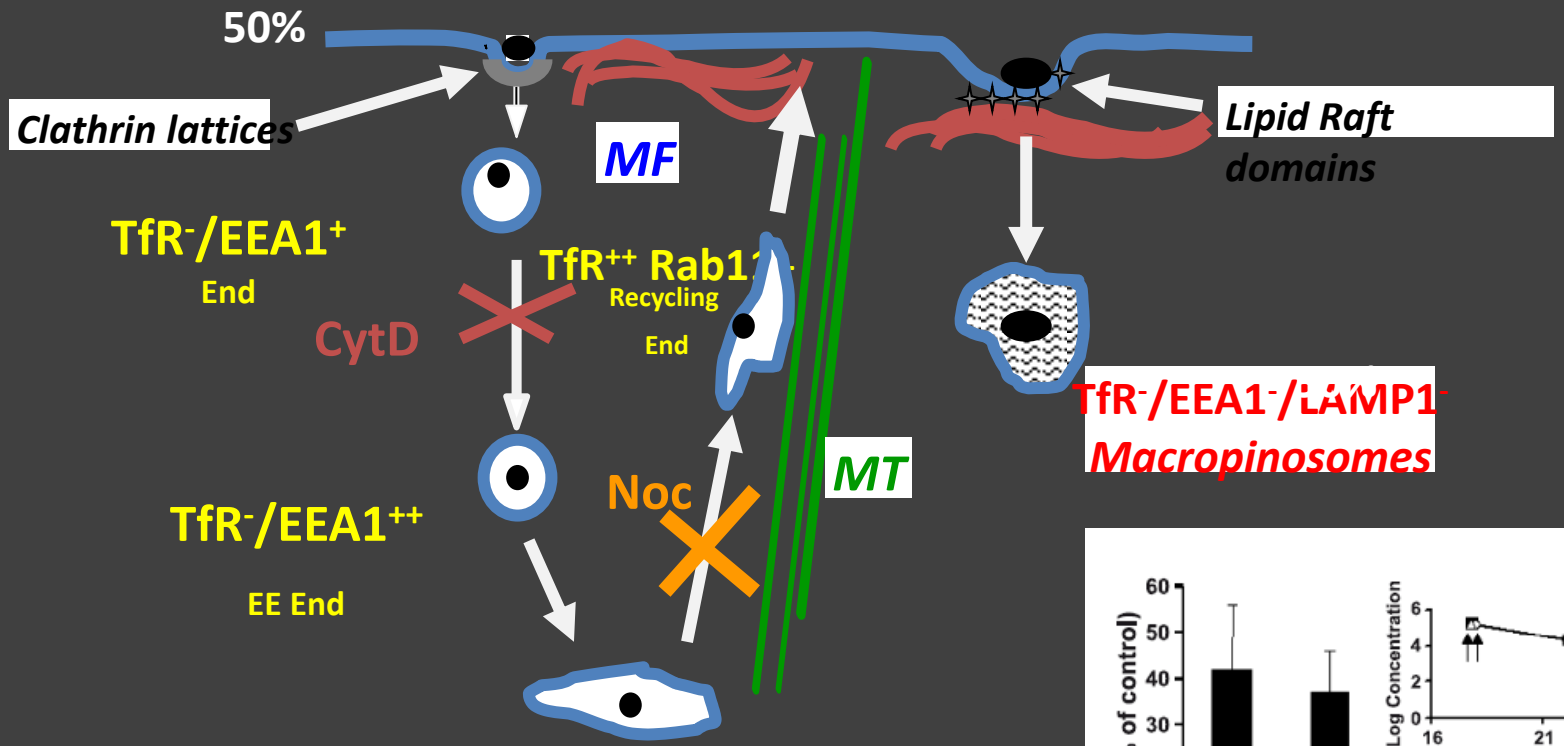
Cytoskeleton

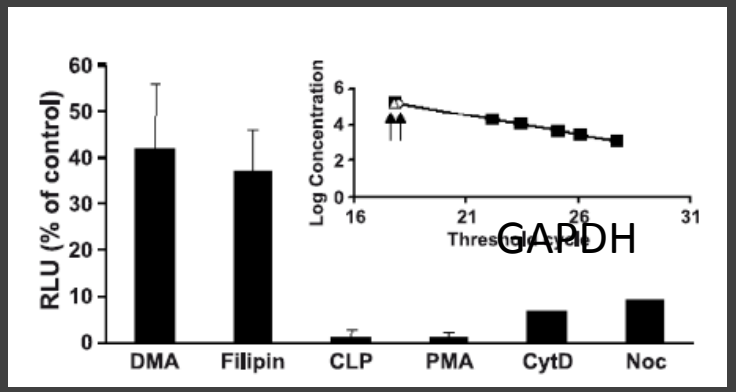
Macropinocytosis

Flow cytometry of Flu-DNA/His-pLK HepG2 cells

Goncalves 2004 Mol Ther

Schematic model of the uptake and intracellular routing of 1st His-polyplexes





Histidylated lipids
Histidylated Polyethyleneimine

Investigation of intracellular pDNA condensation state during endocytosis

FRET and Photobleaching experiments

To overcome the limits of confocal optics

Limit of lateral résolution in microscopy

0.15µm

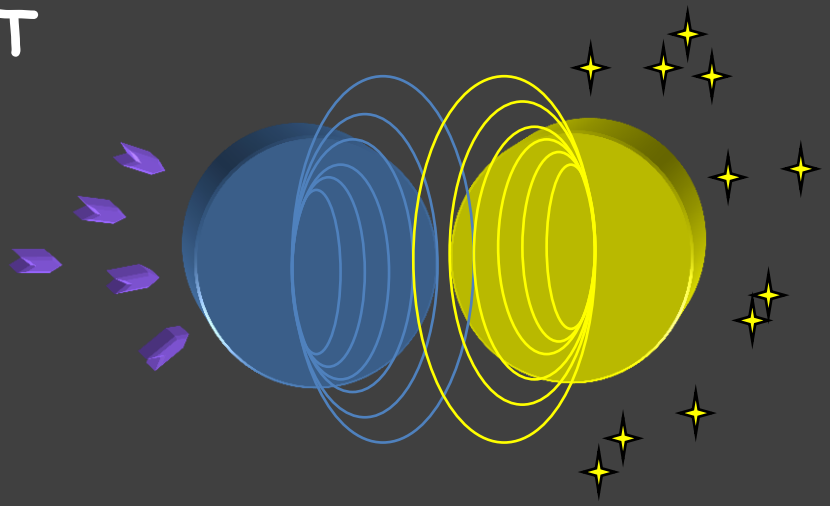
Resolution needed to observe molecular interactions

<100 Å

FRET

Distance donor–acceptor

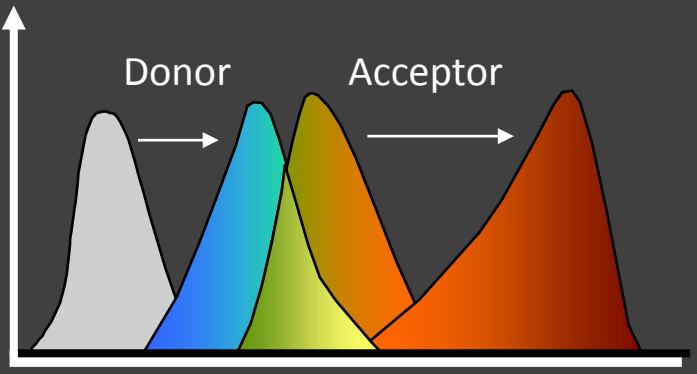
<100 Å



Forster resonance energy transfer (FRET)

Spatial resolution (0.01μm) >> conventional microscopy (λ/2)

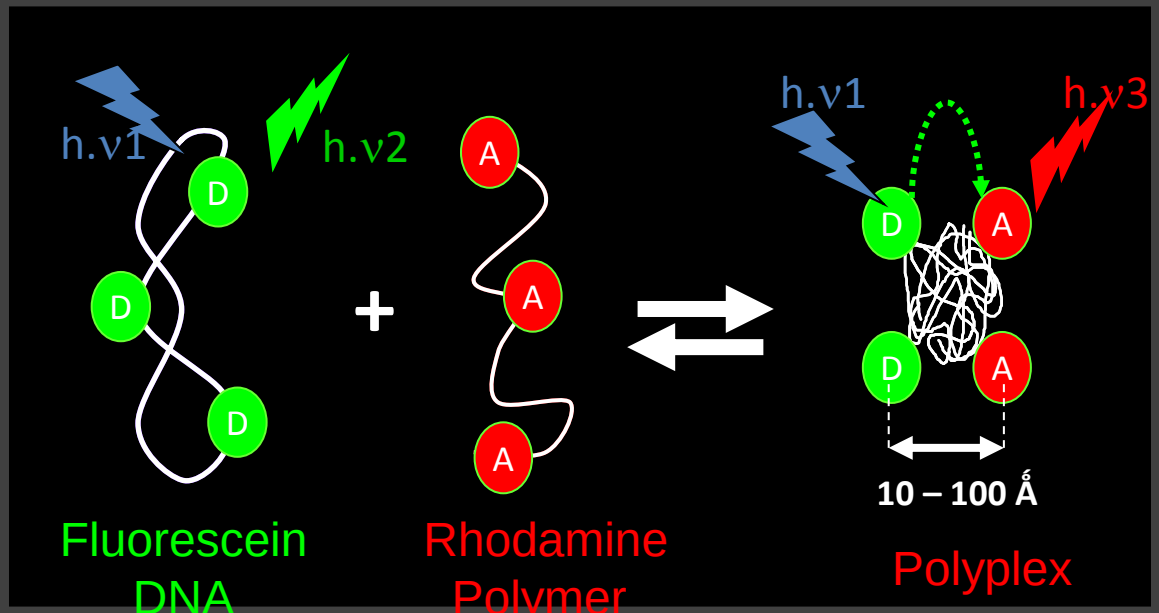
⇨ Molecular interaction



-Non radiative

-Requires overlap of the emission band of the donor and the absorption band of the acceptor

Ro: Förster distance
The distance for which the energy transfer efficiency is equal to 50%, Ro <100 **Å**



h.ν1: 488nm, h.ν2: 520nm
h.ν3: 620 nm

Fluo/Rho: Ro= 51.3 **Å**

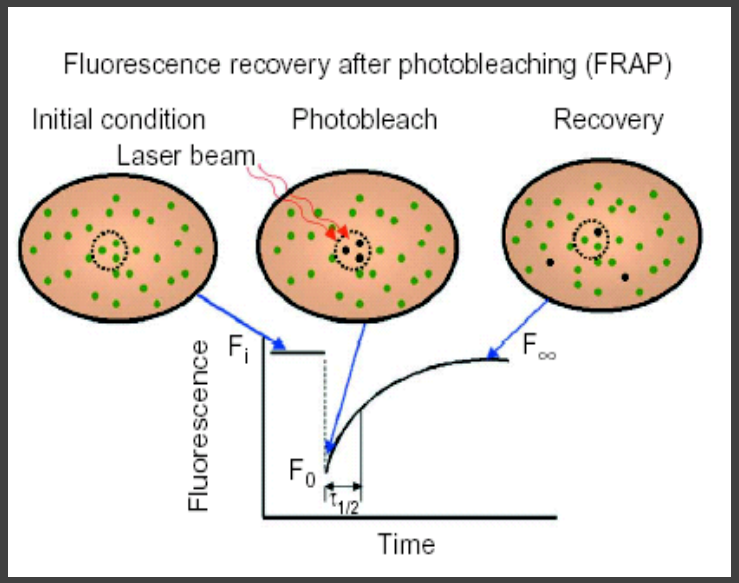
Transfer = pDNA condensation

No transfer = no condensation

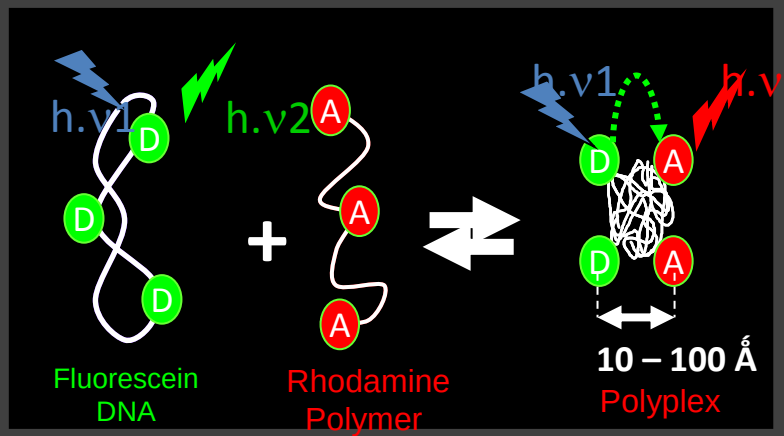
FRAP : Fluorescence Recovery After Photobleaching

Measurement of transports and molecules exchange between compartments

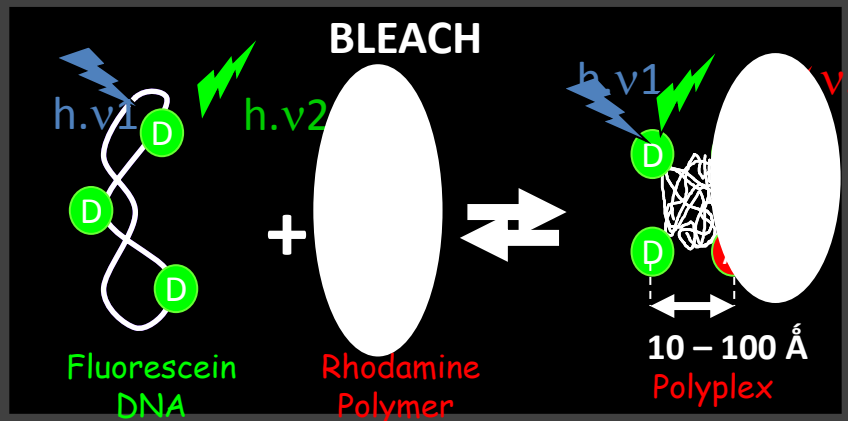
1976: originally used in studies of plasma membranes
-diffusion rates
-mobile and bound fractions



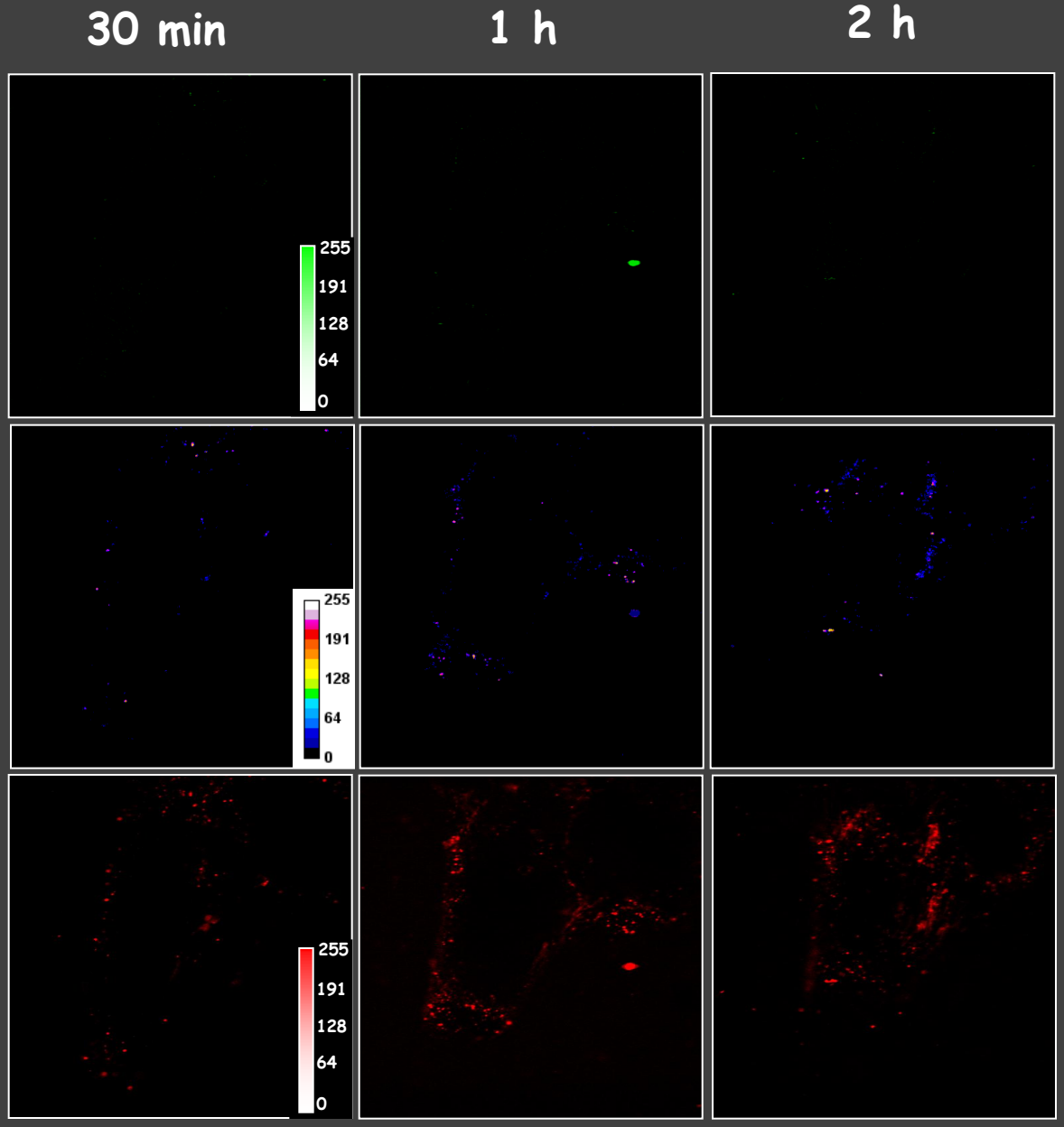
NATURE CELL BIOLOGY VOL 4 APRIL 2002
Partha Roy*, et al.,

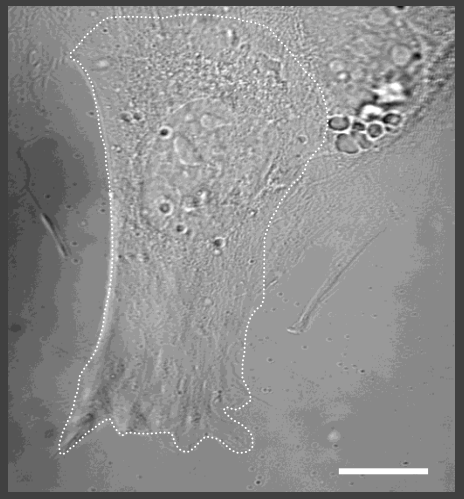


Photobleaching of acceptor: Validation of FRET



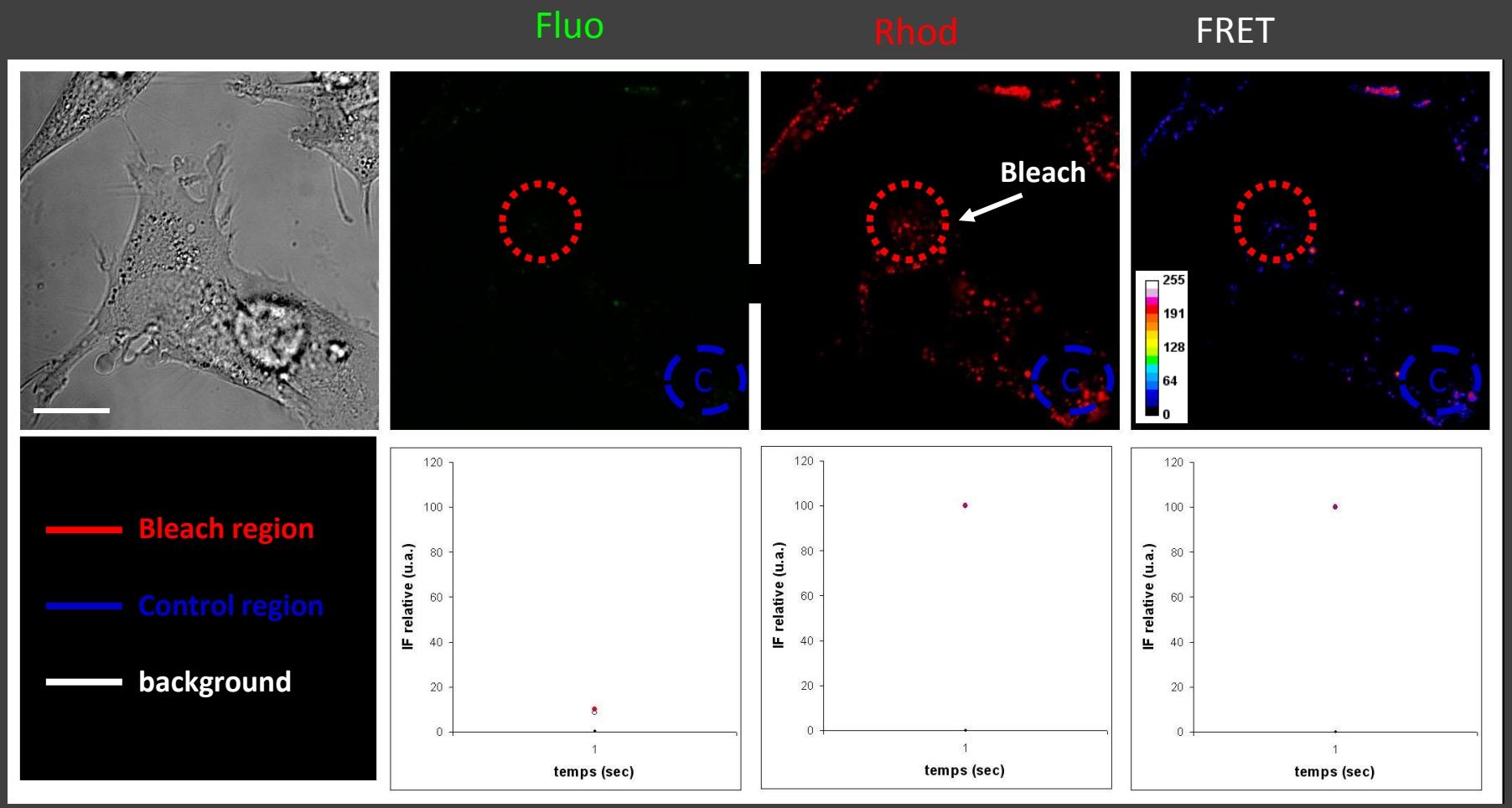
Kinetic study





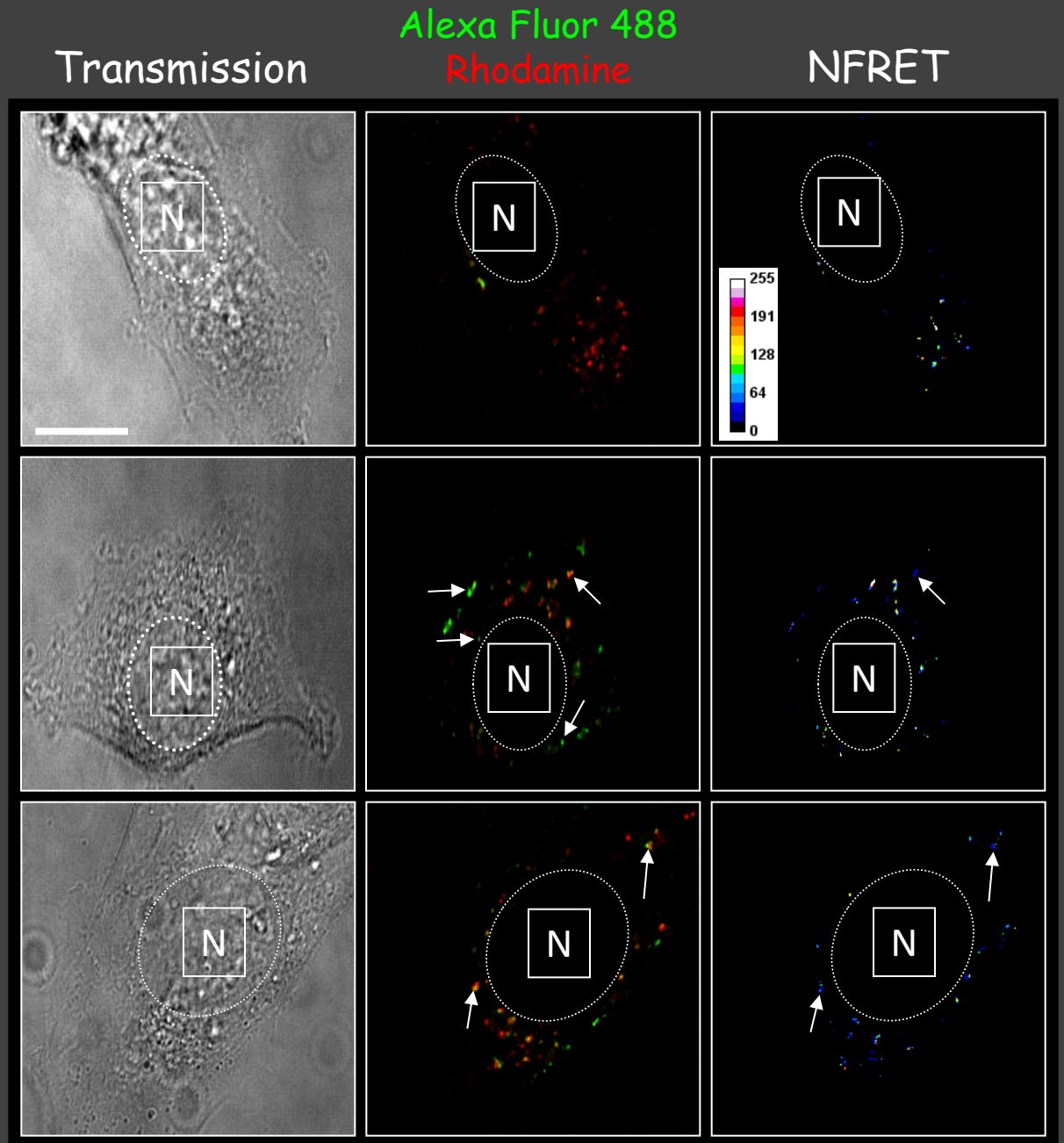
Photobleaching of the acceptor to validate the FRET

Cells transfected with F-DNA / HIS polyplexes. Photobleaching of rhodamine by 800 pulses at 543 nm

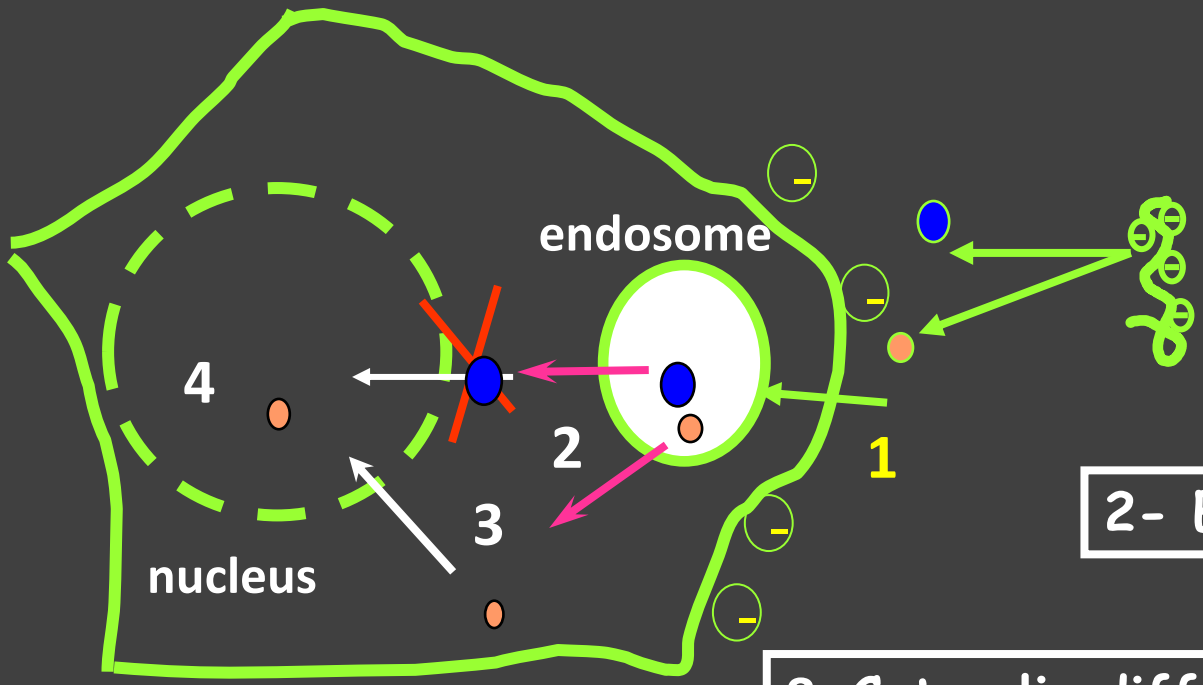


Photobleaching of Rhodamine → increases the fluorescence of Fluorescein due to the destruction of Rhodamine acceptor

Intracellular trafficking of His-polyplexes



Improvement of pDNA nuclear import



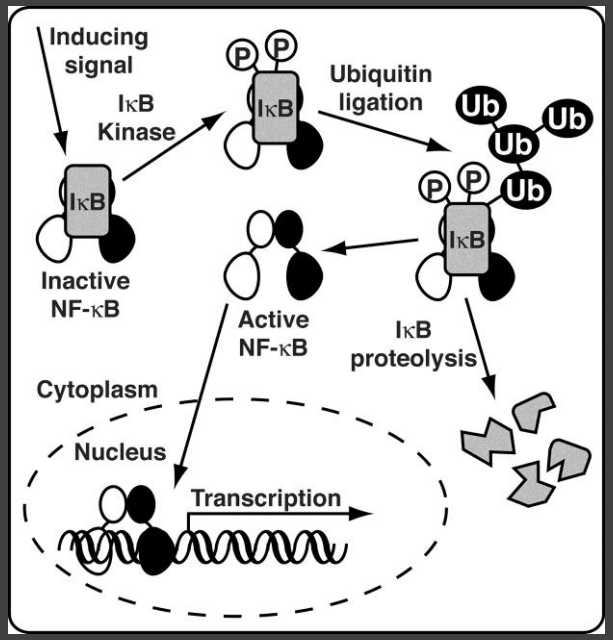
1- Uptake
Protection
Targeting

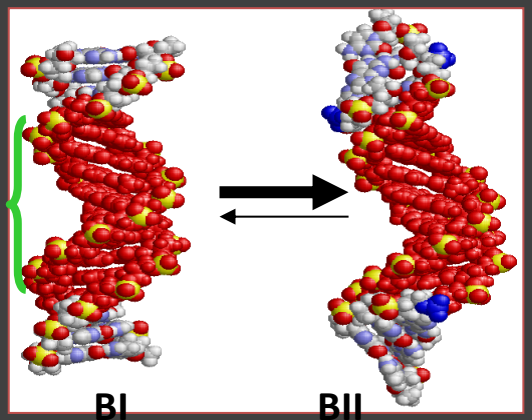
2- Endosome escape

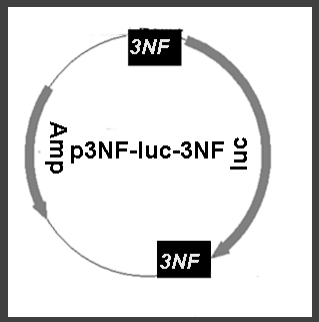
3- Cytosolic diffusion

Optimized κB sites to enhance pDNA nuclear import

5'-CTGGGGACTTTCCAGCTGGGGACTTTCCAGCTGGGGACTTTCCAGG-3

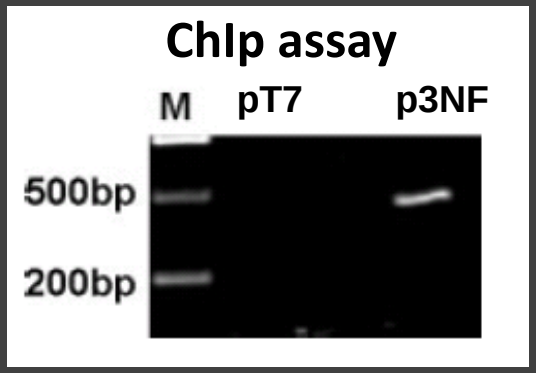






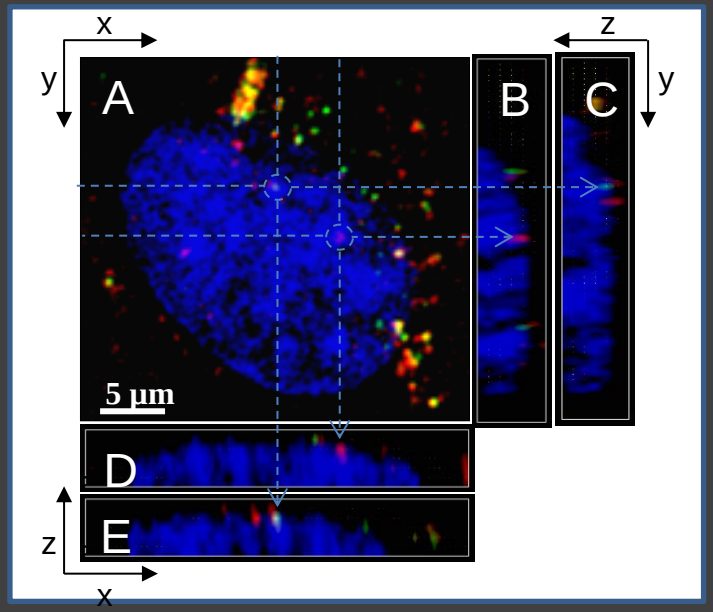
www.rohan.sdsu.edu/

Hela cells: transfection with 3NF bearing pDNA
Crosslinking
Immunoprecipitation with anti-NFkB,
PCR with luciferase primers



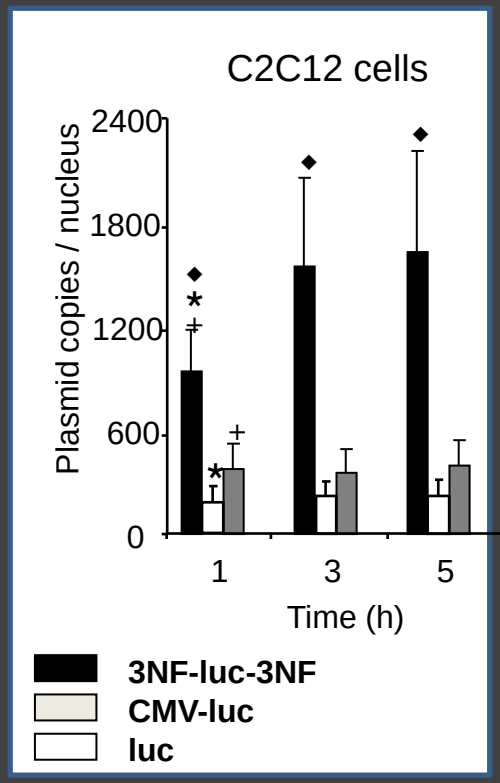
Quantification of pDNA spots localized inside the nucleus

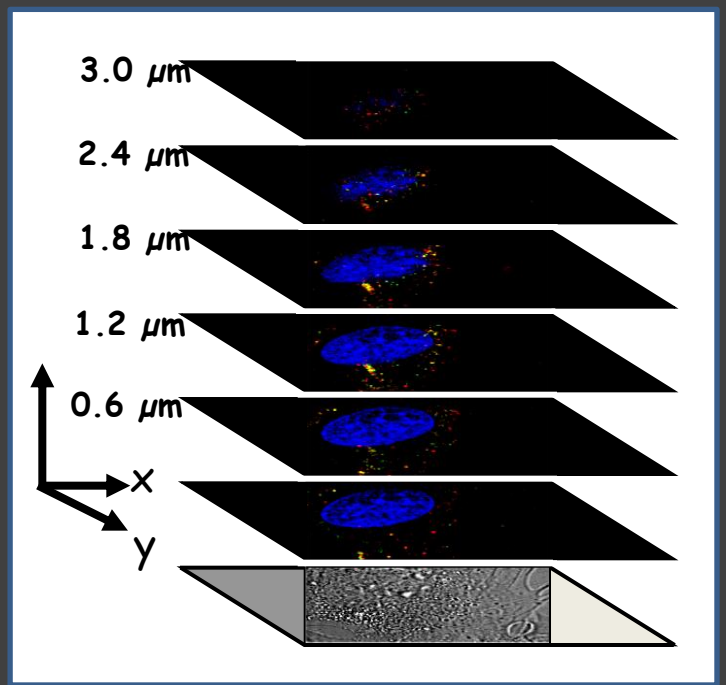
Fluorescence emissions collected in a multi-tracking mode.

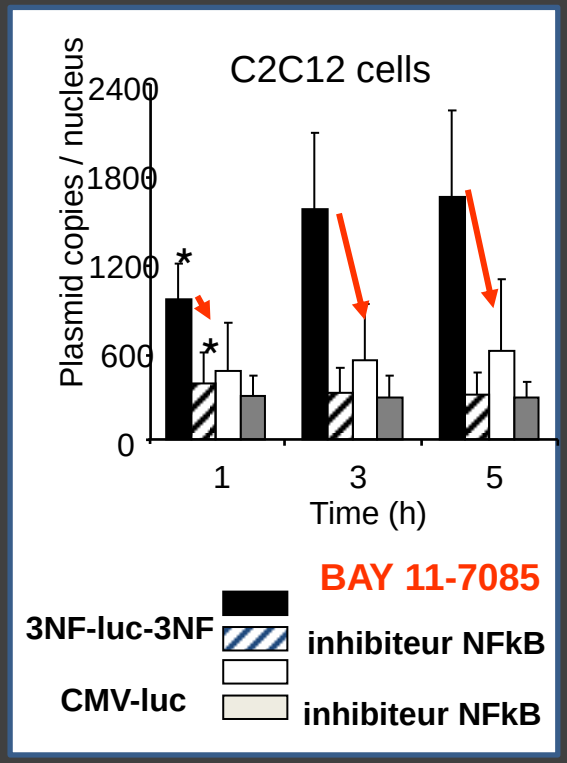


pDNA-Flu/LPEI-Rho

Nucleus= Draq 5





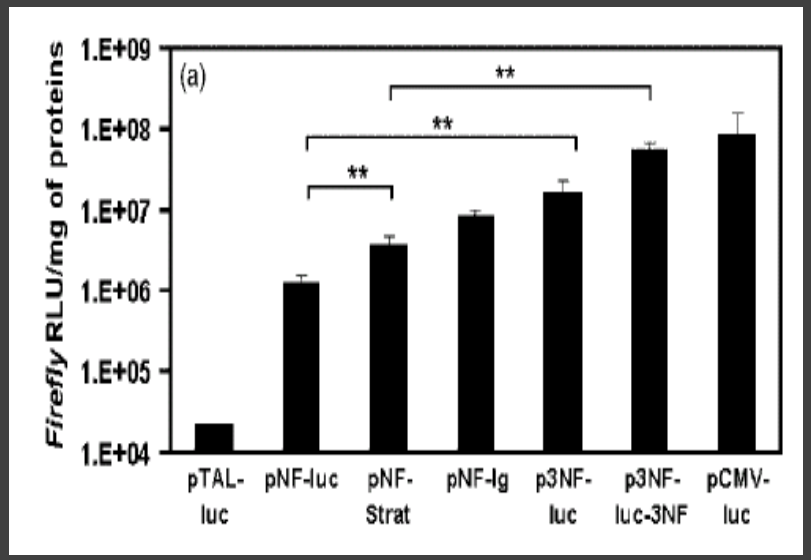


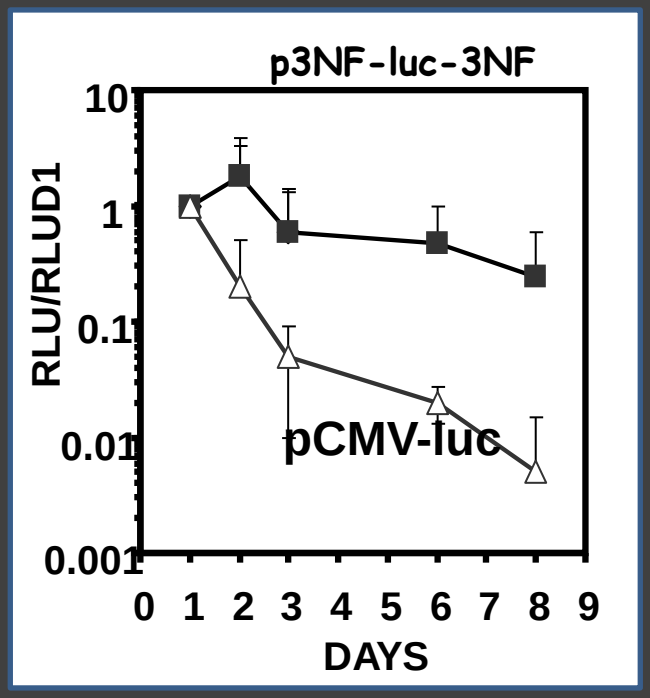
Breuzard et al., NAR 2008

Hela cells transfected with His-vectors and pDNA bearing NFKB sequences

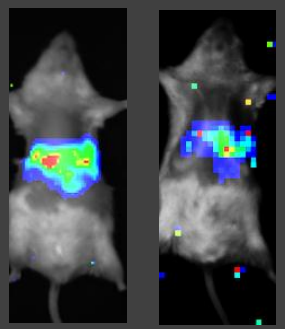
Table 1. κB sites sequences

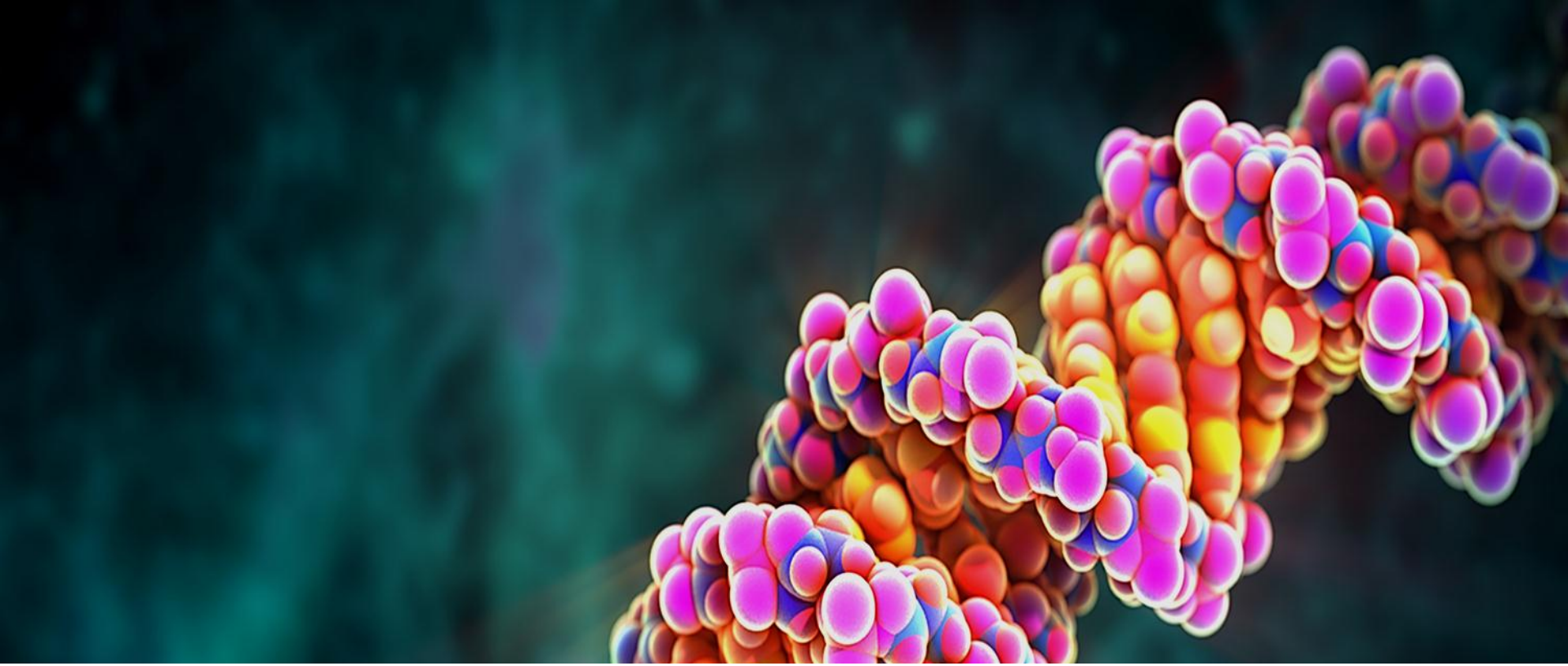
Name	Nucleotide sequences
3NF	5′-CTGGGGACTTTCCAGCTGGGGACTTTCCAGCTG-GGGACTTTCCAGG-3′
NF	(5′-GGGAATTTCC-3′)$_4$
NF-Strat	(5′-GGGAATTTCC-3′)$_5$
NF-Ig	5′-TGGGGACTTTCCGCTGGGGACTTTCCGCTG-GGGACTTTCCGC-3′





Hydrodynamic injection



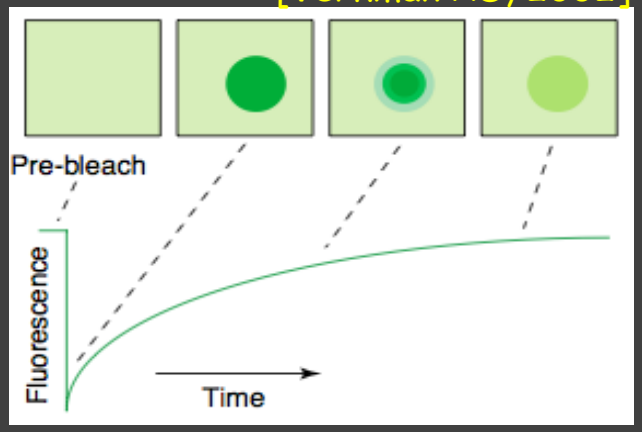


Improving the cytosolic diffusion of pDNA

Cytosolic diffusion

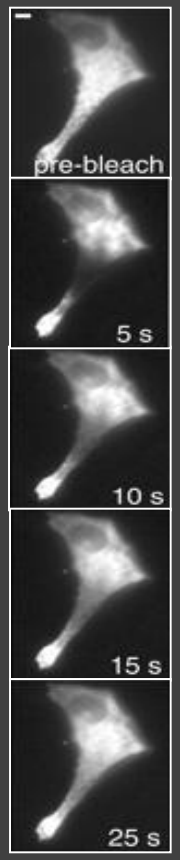
FRAP : Fluorescence recovery after photobleaching

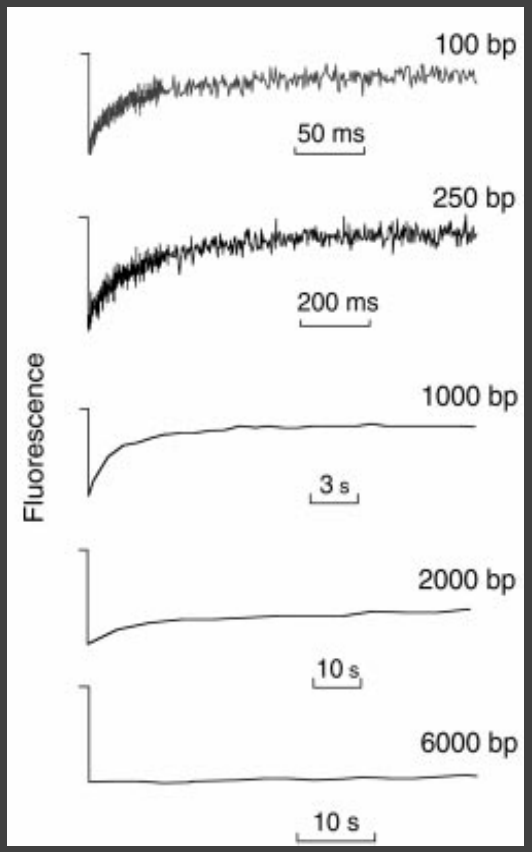
[Verkman AS, 2002]



Principle of FRAP experiment

FRAP : Fluorescein-labeled DNA diffusion in microinjected HeLa cells.





[Lukacs GL *et al.*, 1993]

DNA fragments larger than 2000 bp are immobile in the cytosol

Dynein and cytosolic diffusion towards nucleus

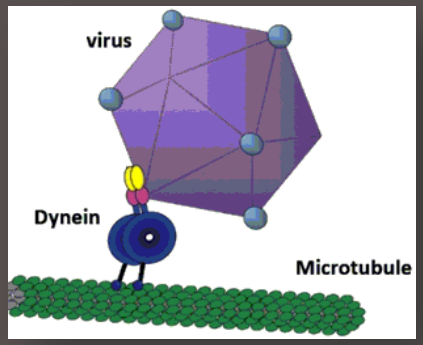
Virus	Protein that binds to a dynein polypeptide	Dynein protein
	UL34	LIC
Herpes simplex	UL9	LC8
	UL35(VP26)	TCTEL1
Herpes virus 7	UL19	LC8
African swine fever	p54	LC8
Mokola	Viral phosphoprotein	LC8
Rabies	Viral phosphoprotein	LC8
Papillomavirus	Capsid protein L2	TCTEL1
	Protein E4	LC8
Borna disease	Viral glycoprotein G	LC7
Mason-Pfizer monkey	Viral Matrix	TCTEL1
Adenovirus	Viral capsid hexon	LIC
Ebola Virus	Viral phosphoprotein	LC8

Dynein molecular motor

- Molecular protein complex (1.2 MDa)
- Walks along microtubules toward the minus end (toward the centrosome).
- 3 Homodimers Light Chains: TCTEL1 - LC8 - LC7

Interaction with cargo

- TCTEL1
- LC8

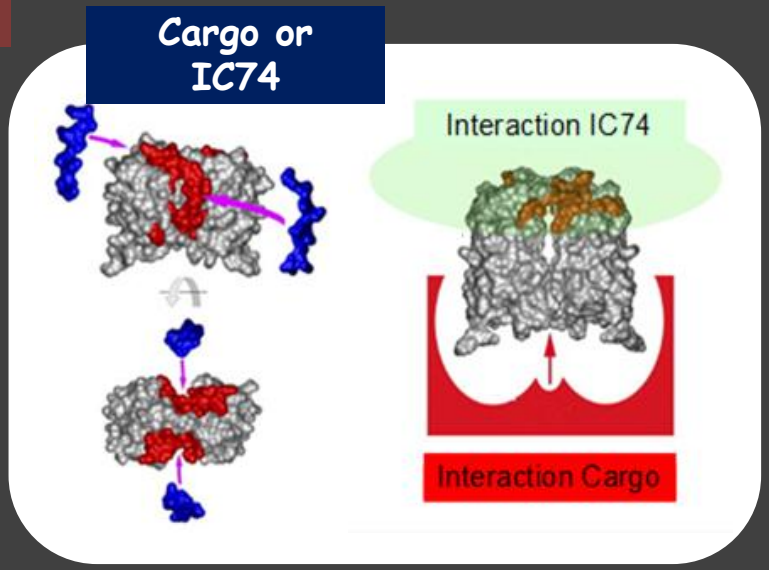


Dynein Light chains: LC8 or TCTEL1

LC8

-Interaction with cargo and IC74 at same site.

-Problem of competition and specificity.

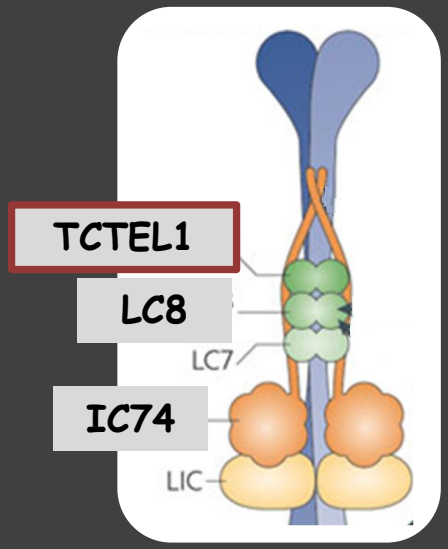


[Wu et. al., 2005; Rapali et al, 2011]

TCTEL1

- Interaction with cargo and IC74 at opposite site.

- Less competition and more specificity.



[Kardon & Vale, 2009]

TCTEL1 is a target of choice to exploit the movement of dynein toward cell nucleus

E3 14.7K Protein and FIP-1

[Hortwitz *et al.*, 2004]

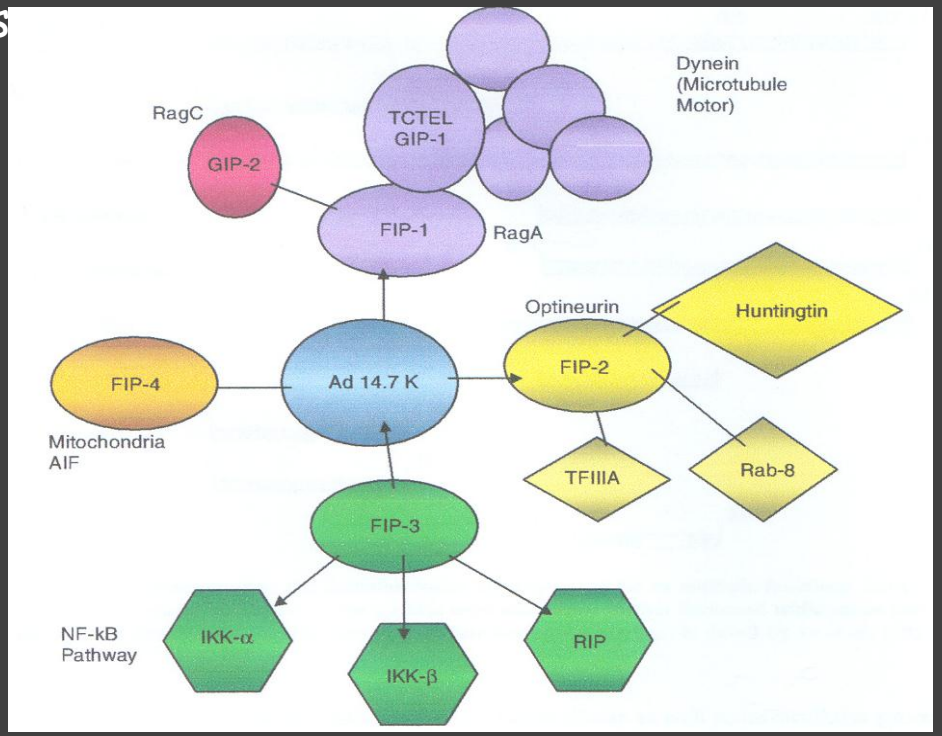
FIPs : Fourteen 7K -Interacting Proteins

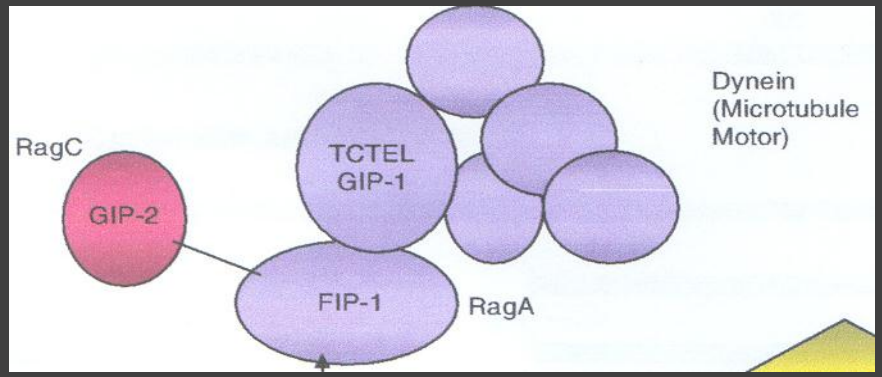
FIP-1 (RagA)

FIP-2 (Optineurin)

FIP-3 (NEMO, IKKY)

FIP-4 (AIF)





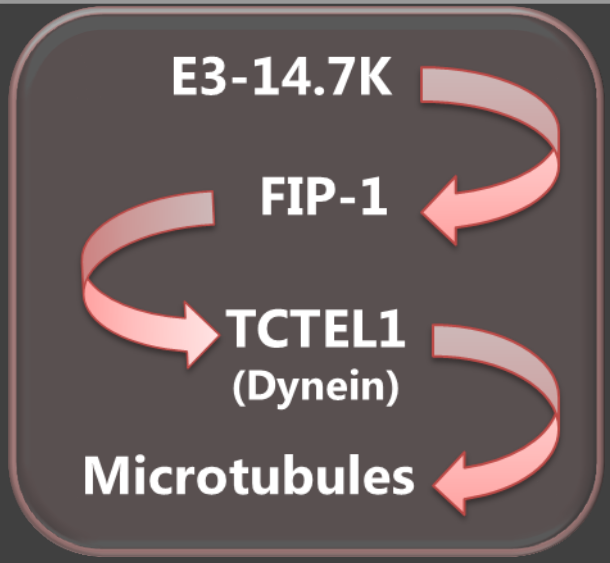
E3-14.7K adenoviral protein strategy

E3-14.7K

- Adenoviral protein
- Early phase - E3
- 14.7kDa
- Four partners FIPs (Fourteen.7K interacting protein)

Step One

Interaction network



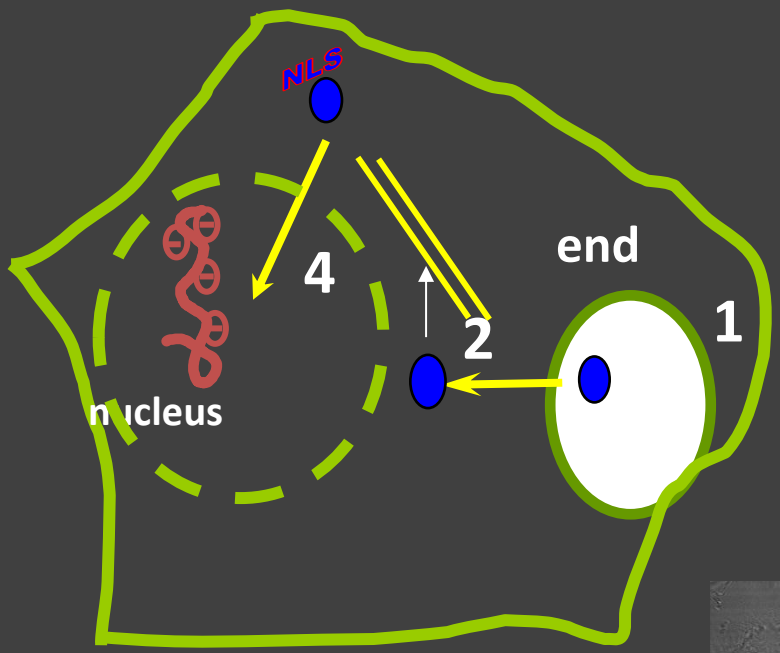
Step two

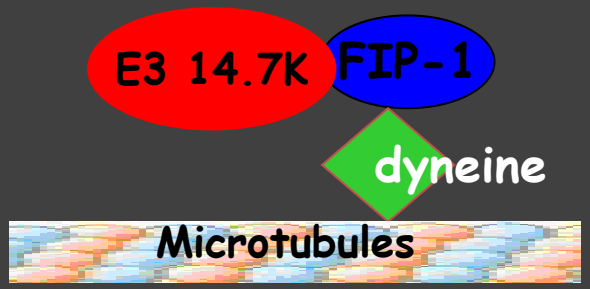
Determine the Interacting peptide

[Foster & Kim, 2002; Wold et al., 1999; Li et al., 1998]

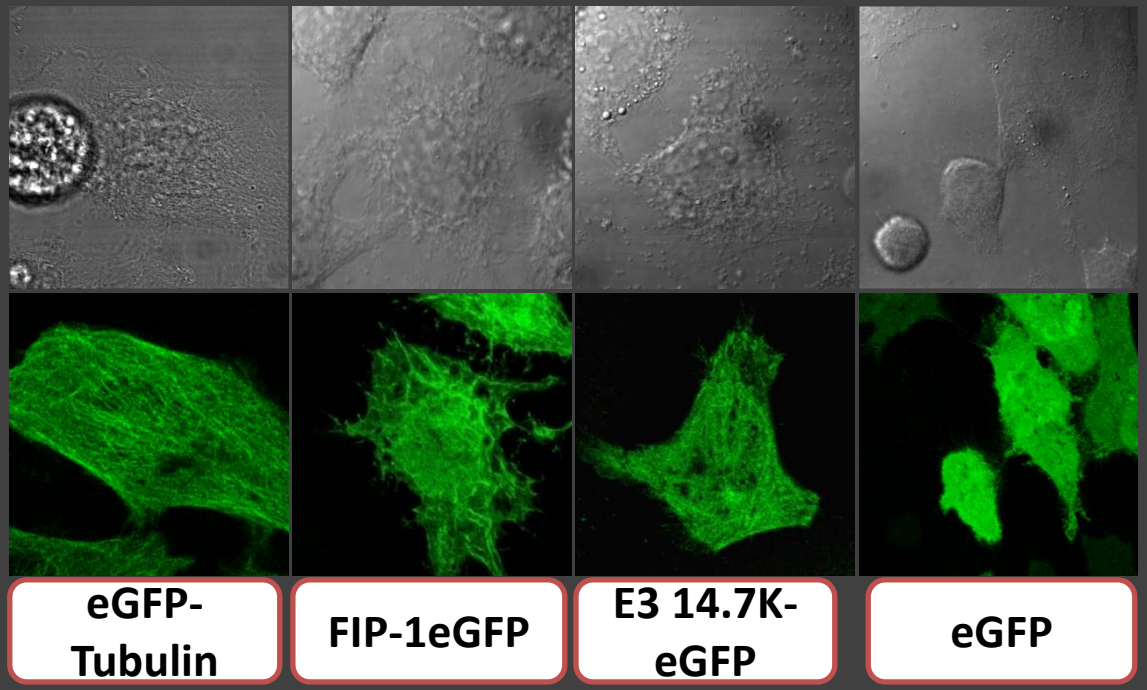
Step Three

Exploit the peptide for active cytosolic diffusion of pDNA





Hela cells expressing:
-eGFP-tubulin
-FIP1-eGFP
-E3 14.7K-eGFP

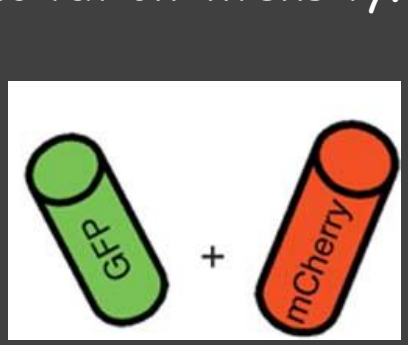


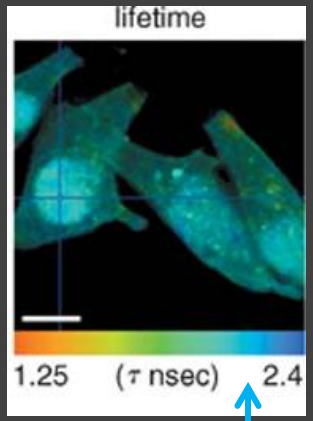
➢ Similar intracellular distribution

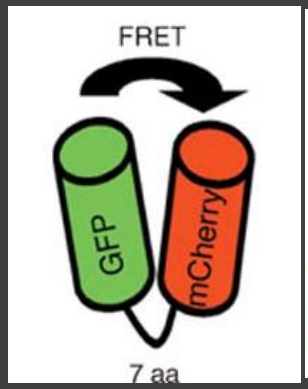
Fluorescence Lifetime Measurement

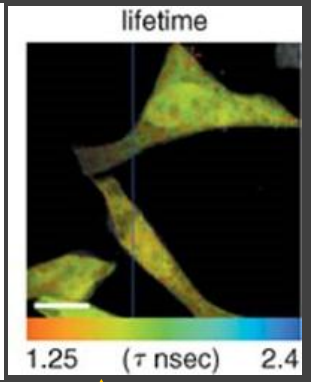
FLIM: - presence of FRET visualized by the lifetime of the excitation state of spatially distributed fluorescent molecules

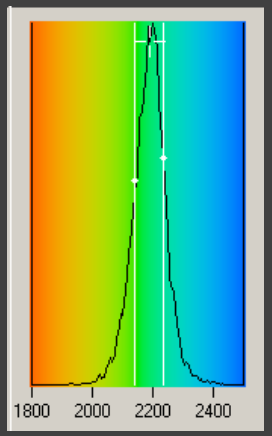
-independent of the local concentrations of fluorescence molecules and the excitation intensity.











Llères D, et *al.* (JCB 2007)

2.2ns

1.75ns

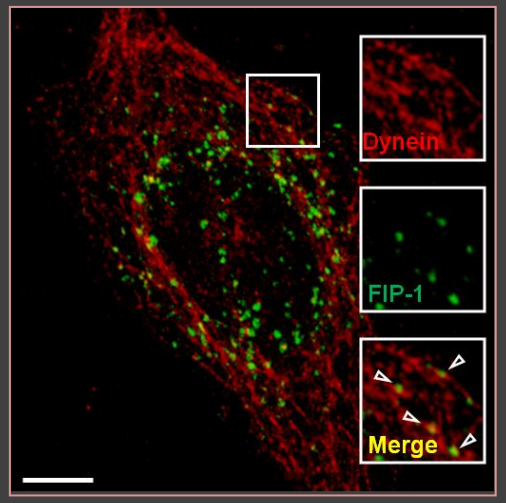
Color coded fluorescence lifetime image, distribution of lifetimes and lifetime decay curve .

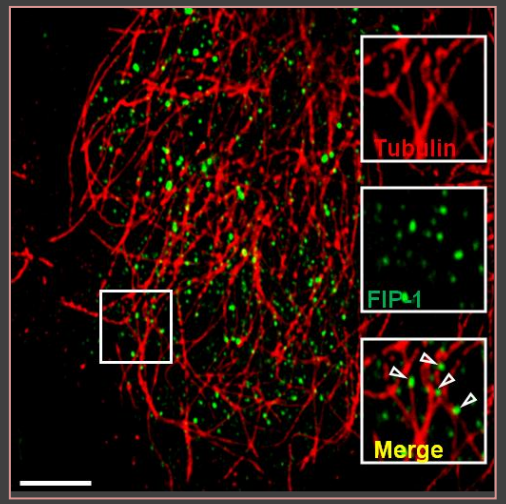
- Inverted Leica SP2 confocal microscope coupled to a 80-MHz mode-locked Mai-Tai® Ti:Sapphire tunable laser (720-920 nm, 100 fs laser pulse; Spectra Physics) for two-photon excitation.
- Time-resolved fluorescence intensity: time-correlated single-photon counting approach.
- Donnor: eGFP; Acceptors : td-Tomato and DsRed2

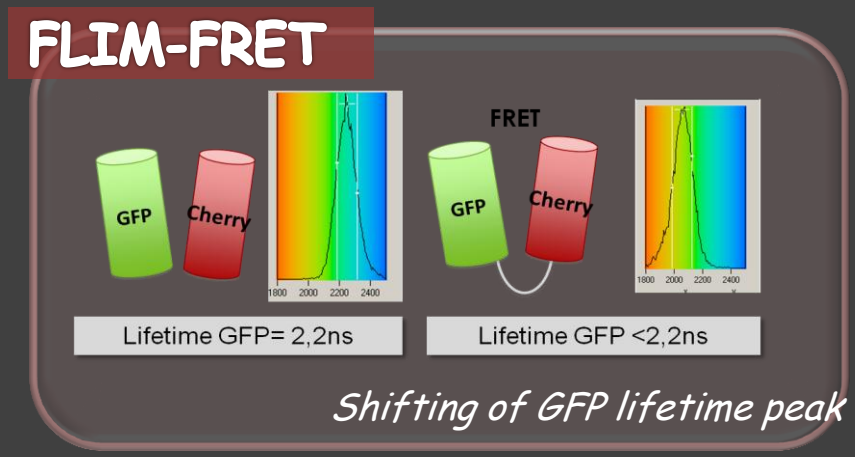
E3-14.7K interaction network

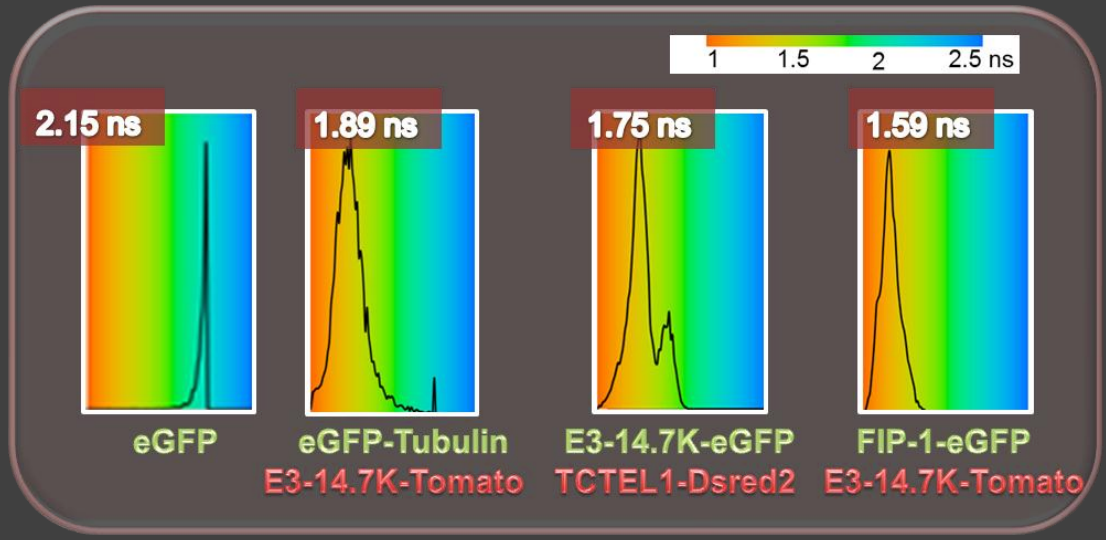
FIP-1 interacts with TCTEL1 (Dynein LC) / Microtubules

E3-14.7K ?









E3-14.7K → FIP-1 → TCTEL1 (Dynein) → Microtubules

Indentification of E3-14.7K/FIP-1 interacting peptide

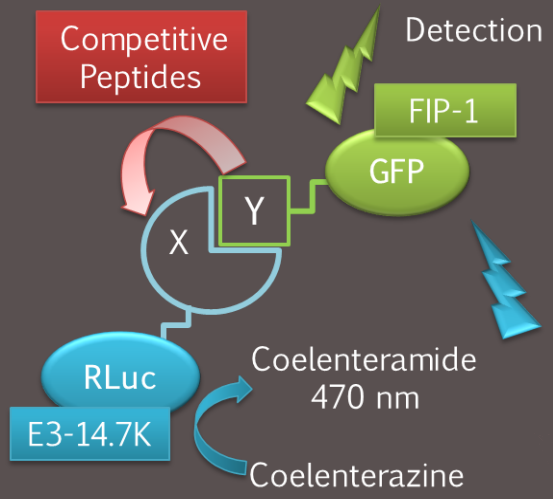
Screening of five overlapping peptides of E3-14.7K:

P38-57 P65-84 P106-125

VNLHQCKRGIFCLVKQAKVTYDSNTTGHRLSYKLPTKRQKLVVMVGEKPITITQHSVETEGCIHSPCQGPEDLCTLIKTLCGLKDLIPFN

P50-69 P79-98

Bioluminescence Resonance Energy Transfer (BRET)



- Fixed amount of cytosolic extracts of Hela cells expressing E3 14.7K + increasing amount of FIP-1-eGFP recombinant protein
- Mithras LB 940 with MikroWin 2000 software (RLuc filter, 485 ± 10 nm; YFP filter, 530 ± 12 nm
- BRET ratio is the emission signals at 530 nm divided by emission signals at 485 nm.

Indentification of E3-14.7K/FIP-1 interacting peptide

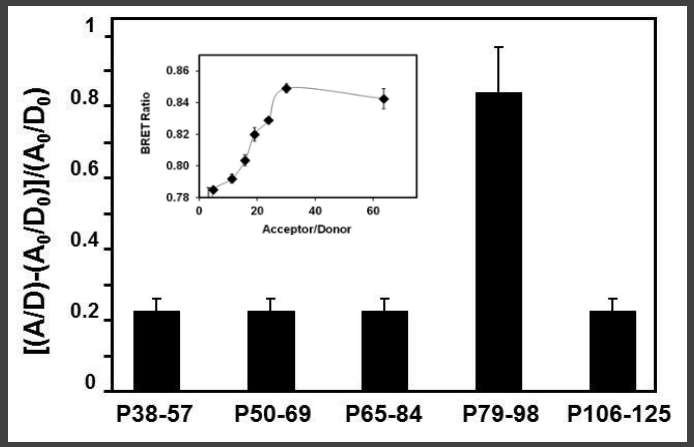
Screening of five overlapping peptides of E3-14.7K:

P38-57 P65-84 P106-125

VNLHQCKRGIFCLVKQAKVTYDSNTTGHRLSYKLPTKRQKLVVMVGEKPITITQHSVETEGCIHSPCQGPEDLCTLIKTLCGLKDLIPFN

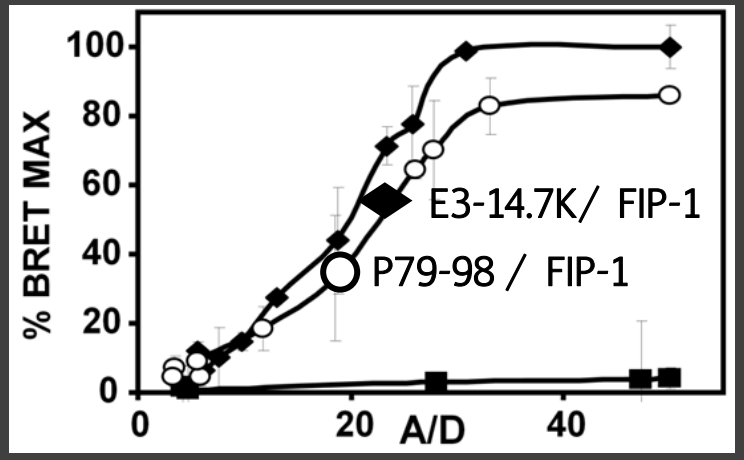
P50-69 P79-98

BRET competition in vitro



P79-98 inhibits Energy transfer between E3-14.7K / FIP-1

BRET: Interaction in cellulo

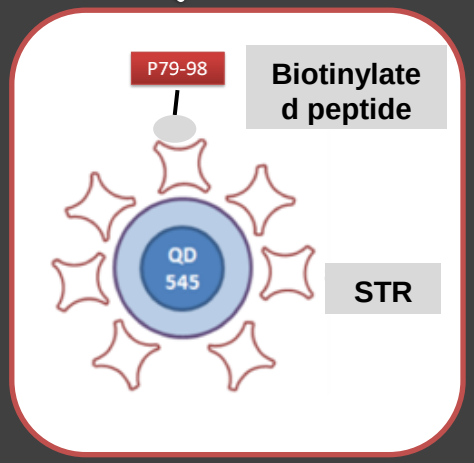


Energy transfer in live cells between E3-14.7K /FIP-1 and P79-98 /FIP-1

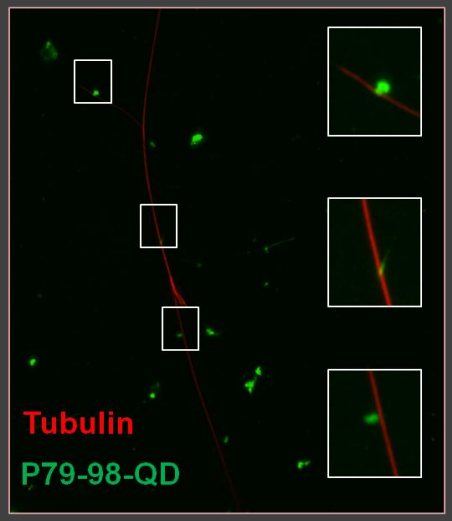
[Pigeon et. al., 2013]

Indentification of E3-14.7K/FIP-1 interacting peptide

Quantum Dot



P79-98: VVMVGEKPITITQHSVETEG



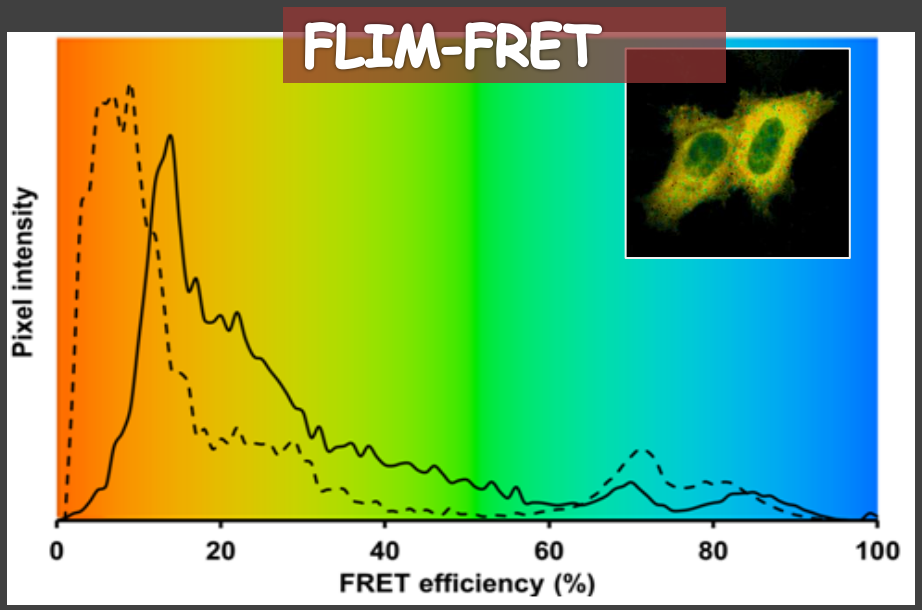
P38-57: VNLHQCKRGIFCLVKQAKVT

Peptide P79-98 of E3-14.7K interacts with microtubules *in cellulo* and *in vitro*

— eGFP-Tubulin P79-98-Tomato

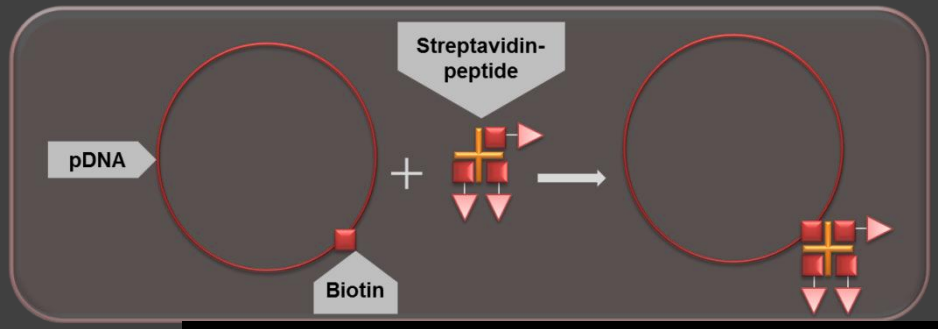
--- eGFP-Tubulin P38-57-Tomato

FRET efficiency quantified using the SPCImage software(Becker & Hickl GmbH).

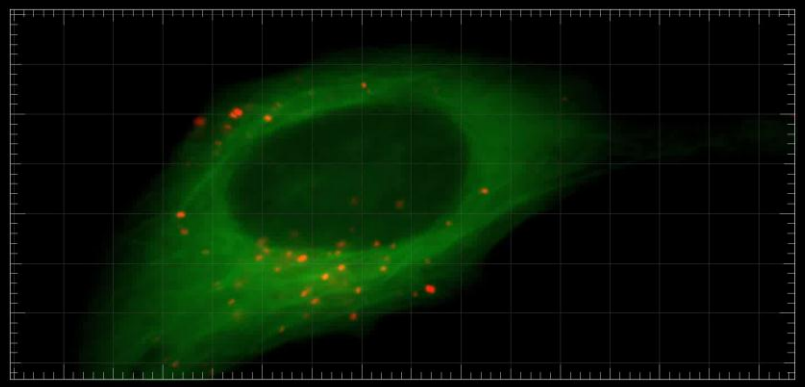


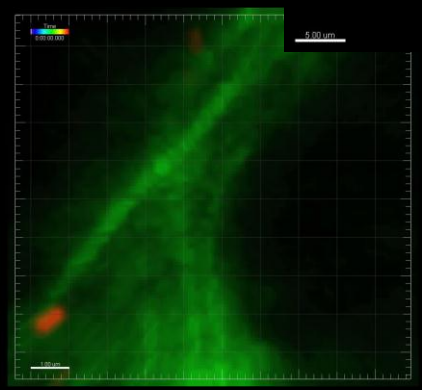
Intracellular dynamic of p79-98/pDNA

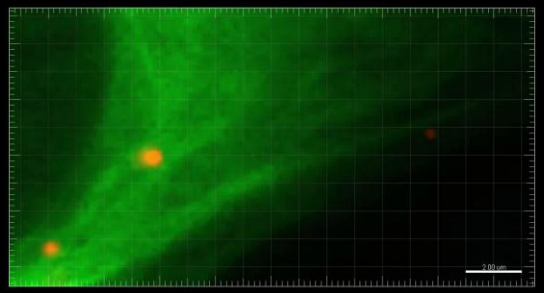
Vidéo ObserverZ1 - Zeiss



HeLa eGFP-Tubulin
Transfection
His-lPEI polyplexes
Cy3-pDNA-P79-98
2 hours after transfection







Polyplexes

P79-98 conjugated to pDNA induces microtubule-mediated transport of pDNA *in cellulo*.

[Pigeon et. al., 2013]

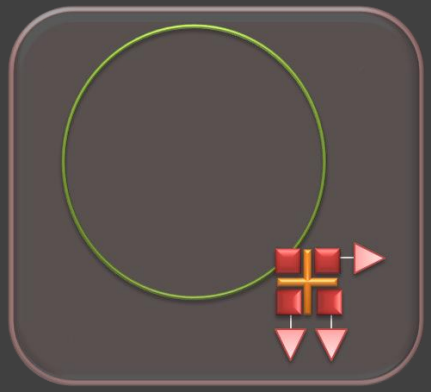
Impact of p79-98 on transfection efficiency

HeLa cells

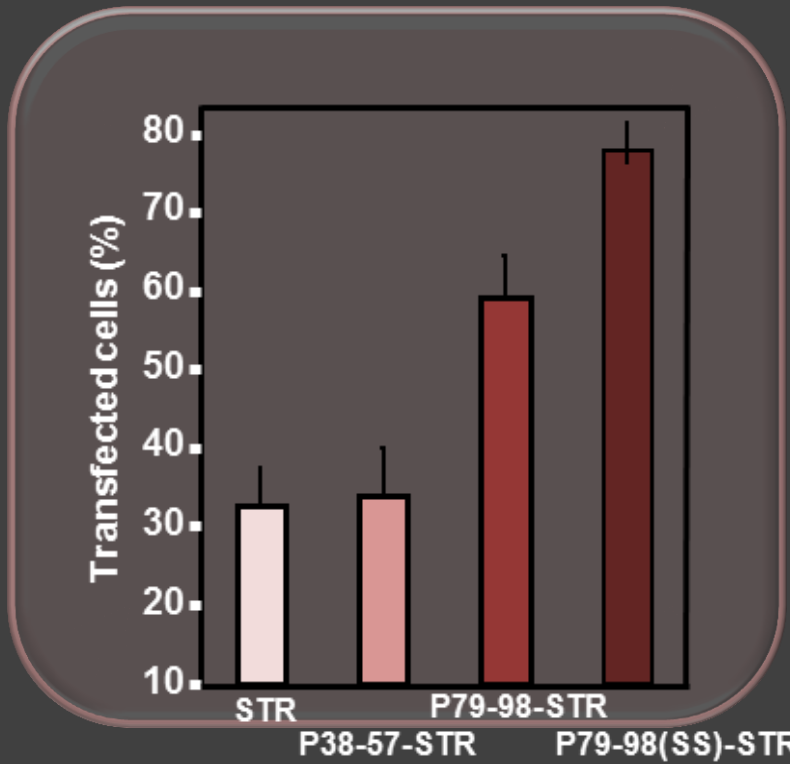
Transfection

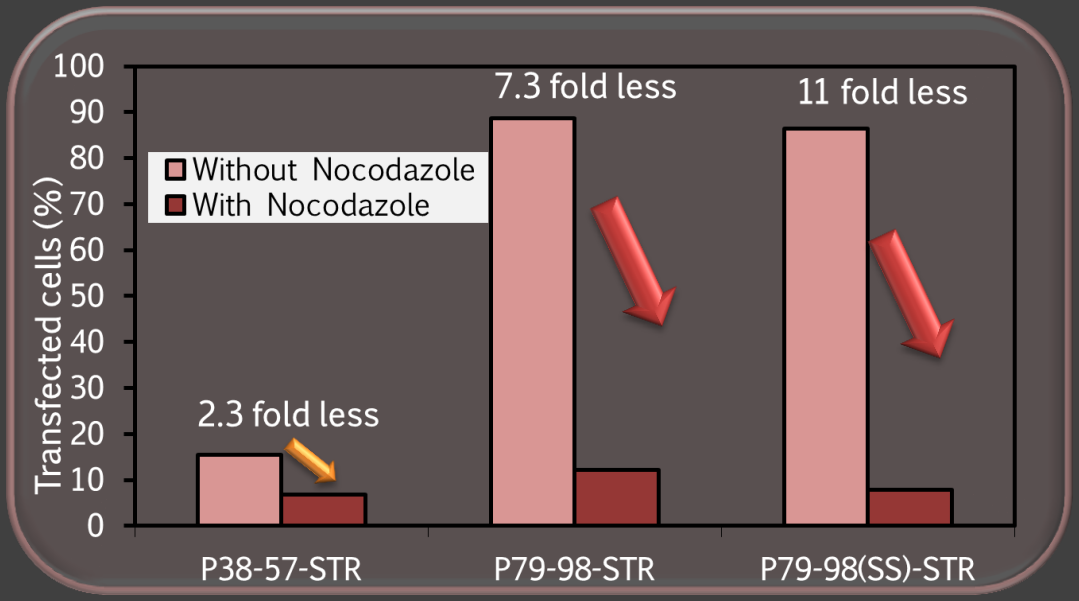
His-lPEI polyplexes

pDNA-P79-98



pCMV-eGFP-P79-98



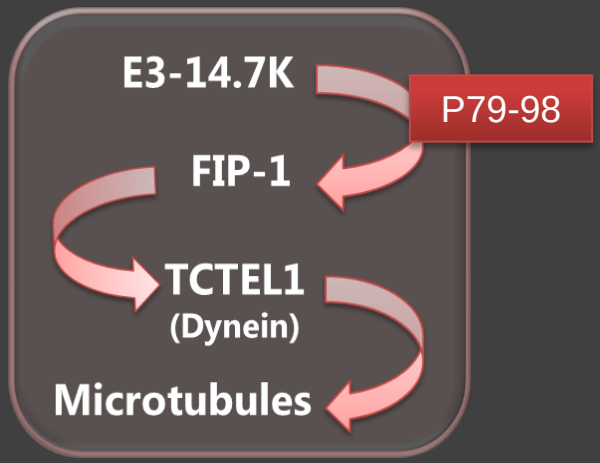


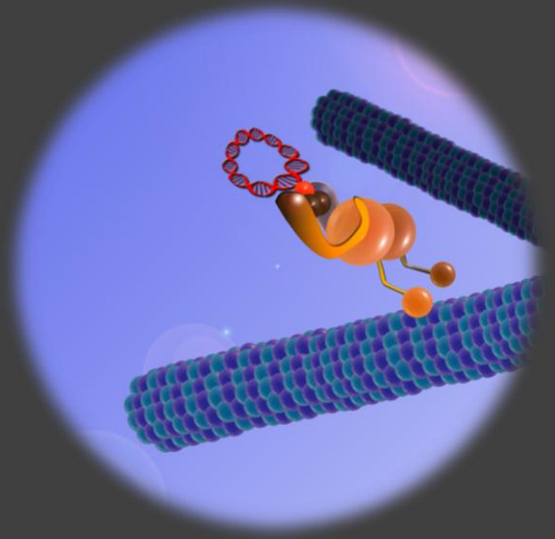
Improvement: 30% vs 80%.

Enhancement :dependence on microtubules integrity

Conclusions

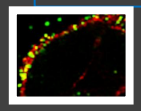
- Identification of P798-98 peptide of E3-14.7K that interacts with FIP-1
- FIP-1 binds to TCTEL1 light chain of dynein: movement along microtubules
- P79-98 conjugated to pDNA induces microtubule-mediated transport of pDNA *in cellulo*
- Single particle tracking needs to be performed to understand the process
- pDNA-P79-98 drastically increases by 150% the number of transfected cells.
- P79-98 on pDNA or on the vector: which option is the best?

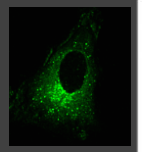


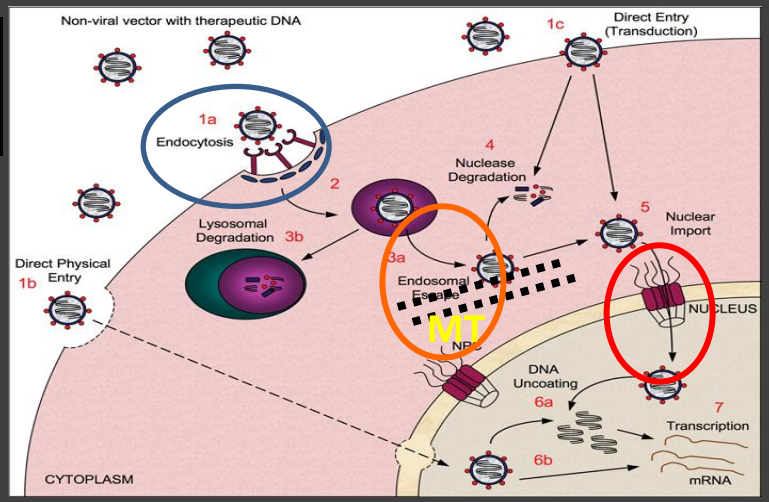


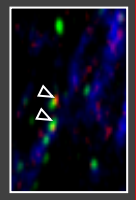
Summary

Trafficking:
Colocalization experiments & epitope-specific flow cytometric sorting allowed us to delineate the endocytosis pathways and intracellular routing of pDNA complexes



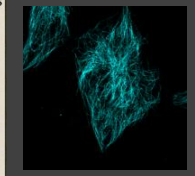






Nuclear import:
Improvement of transfection efficiency by inserting 2x3NF KB sites in the backbone of pDNA.
More resolutive technique is required to follow the pDNA entry in the nucleus.

FP-Tubulin



Cytosolic migration:
Confocal microscopy, FLIM BRET and videomicroscopy experiments clearly validated that P79-98 from E3 14.7K promotes microtubules binding of pDNA and its transport toward the nuclear envelope upon polyplexes transfection.
Enhancement of transfection efficiency

Segregation, condensation state:
FRET, Photobleaching are powerful tools to determine the state of pDNA complexes during their intracellular routing

Building artificial virus

Team: « Nucleic acids by non viral systems »

Patrick Midoux, Chantal Pichon, Jean Marc Malinge, Jean-Pierre Gomez Patrick Baril, Anthony Delalande
Cristine Goncalves, *Loic Lebegue*, Virginie Mallard, Rudy Clémençon, Chloé Leduc,
Postdocs: *Gilles Breuzard, Benoit Maunit, Ludivine Billiet*, *Julie Lodewick*, Laure Magreangeas, Lily Mongin
Ph.D.students: Thomas Thibault, Federico Perche, Lucie Pigeon, Marie-Pierre Gosselin
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ENS Cachan
Patric Tauc
Eric Deprez
Brigitte Hartmann