# Nanomaterials Enable Delivery of Genetic Material without Transgene Integration in Mature Plants

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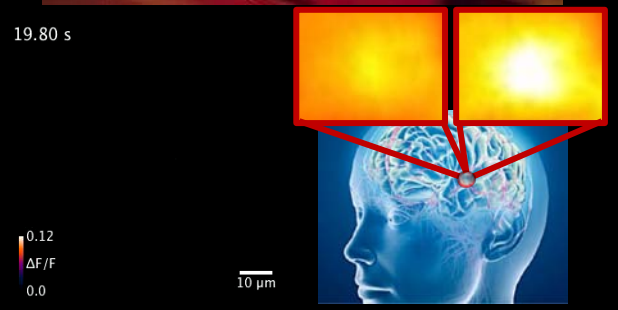
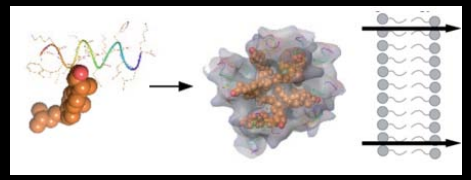
# LANDRY LAB

## NEUROTRANSMITTER IMAGING

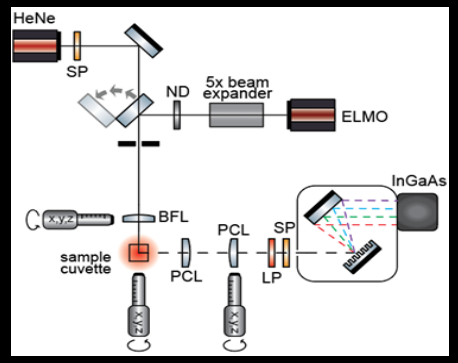

Beyene *et al. ACS Chem Neuro 2017*
Beyene *et al. Science Advances* 2019
Jeong *et al. Science Advances* 2019

## MEMBRANE INTERNALIZATION



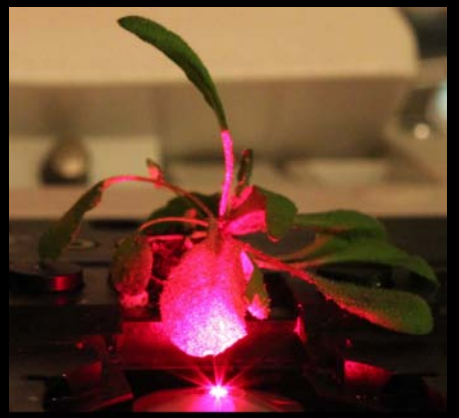
Li *et al. RSC Chemical Science* 2017
Zou *et al. ACS Biochemistry. 2018*

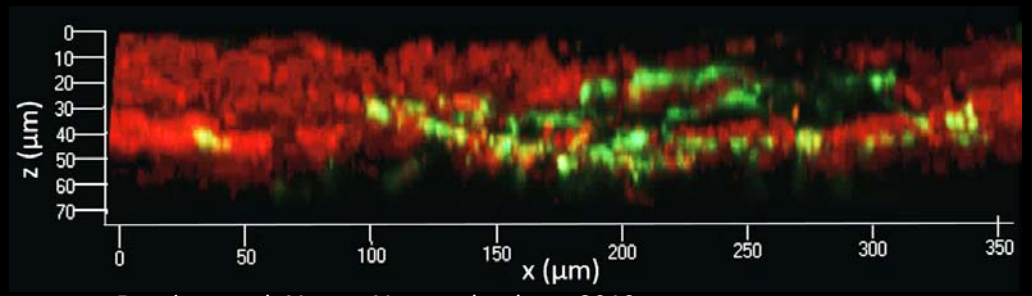
## DEEP-BRAIN MICROSCOPY




O'Donnell *et al. Adv. Funct. Mater. 2017*

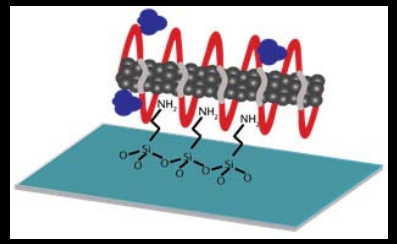
## PLANT & PLASTID TRANSFORMATION





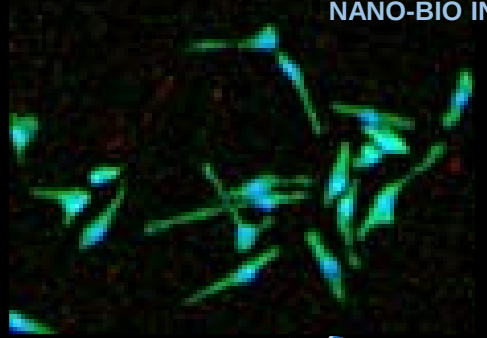

Demirer *et al. Nature Nanotechnology. 2019*
Zhang *et al. PNAS* 2019
Demirer *et al. Science Advances* 2020
Zhang *et al. Nature Protocols* 2020

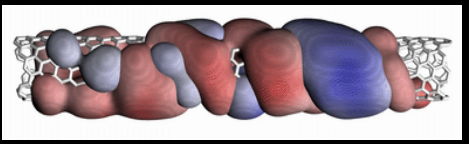
## NANOSENSORS



Luo *et al. ACS Sensors. 2017*
O'Donnell *et al. ACS Biochemistry. 2018*
Chio *et al. Nano Letters* 2019
Pinals *et al. JACS* 2020

## NANO-BIO INTERACTIONS





Beyene *et al. Nano Letters 2018*
Saleh *et al. NanoImpact. 2017*
Chio *et al. Adv. Funct. Mater.* 2020

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# In the year 2050, there will be about 9.7 billion people on Earth

We must increase food production by 70% to meet food security needs by 2050

# Genetic Engineering of Plants – At the Core of Agriculture, Medicine, Energy


Gozde S. Demirer
Markita P. Landry
Univ. of California, Berkeley

Huan Zhang

Gozde Demirer

## Delivering Genes to Plants

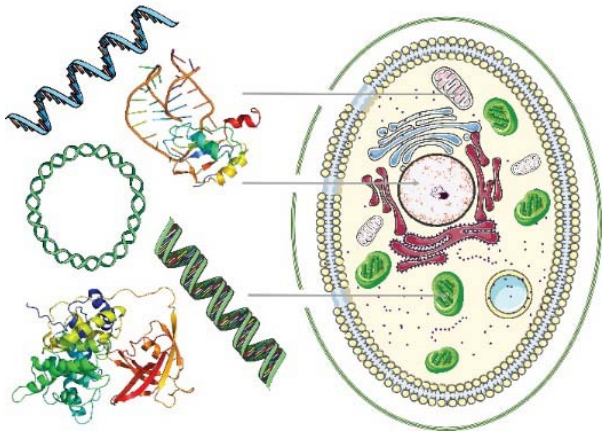
### Agriculture


### Pharmaceuticals


### Energy


**Biomolecular Cargos**

DNA, RNA, RNP, proteins



**Biological Domains**

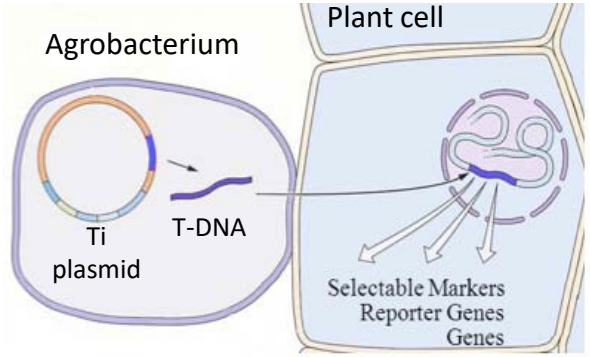
Nucleus, plastids, cytoplasm

↓ Gene delivery ↓ Gene silencing

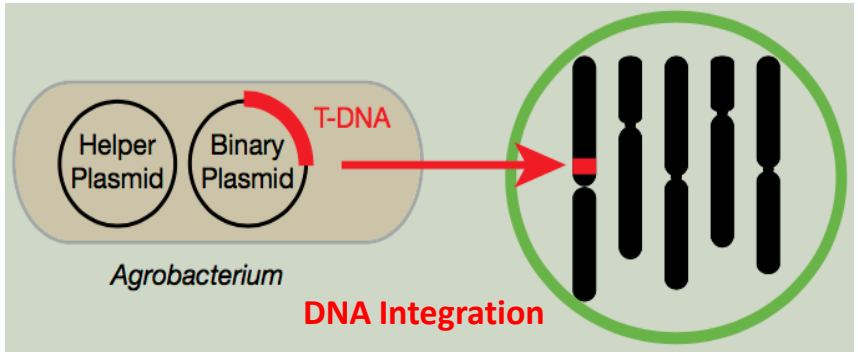
Demirer *et al. AIChE SBE* (2017)
Landry & Mitter, *Nature Nanotechnology* (2019)

# Current Methods for DNA Delivery to Plants

## Agrobacterium-Mediated Delivery

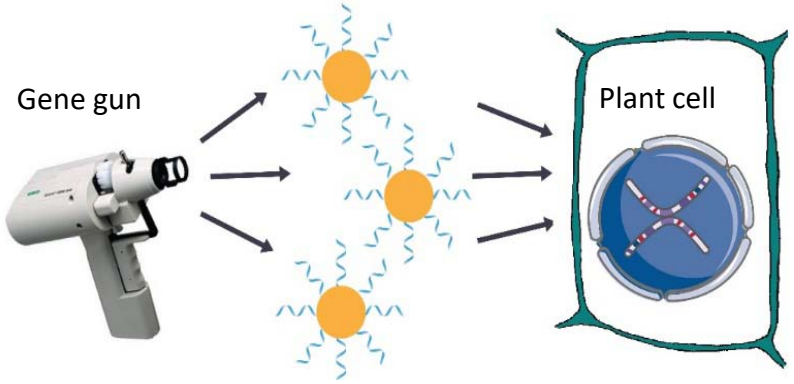


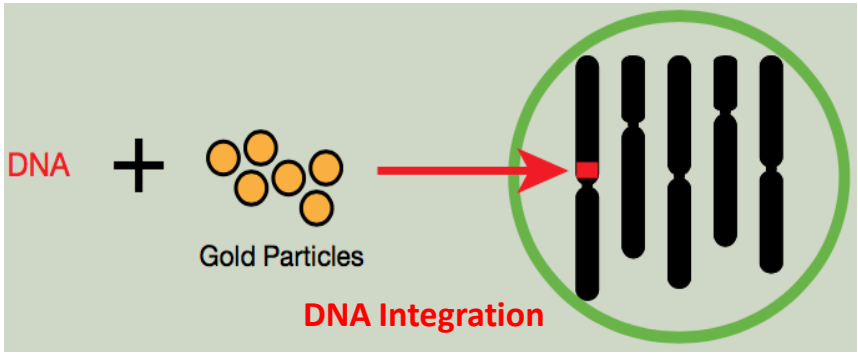

Martha Hawes,
University of Arizona




Host range limitation

## Biolistic Particle Delivery



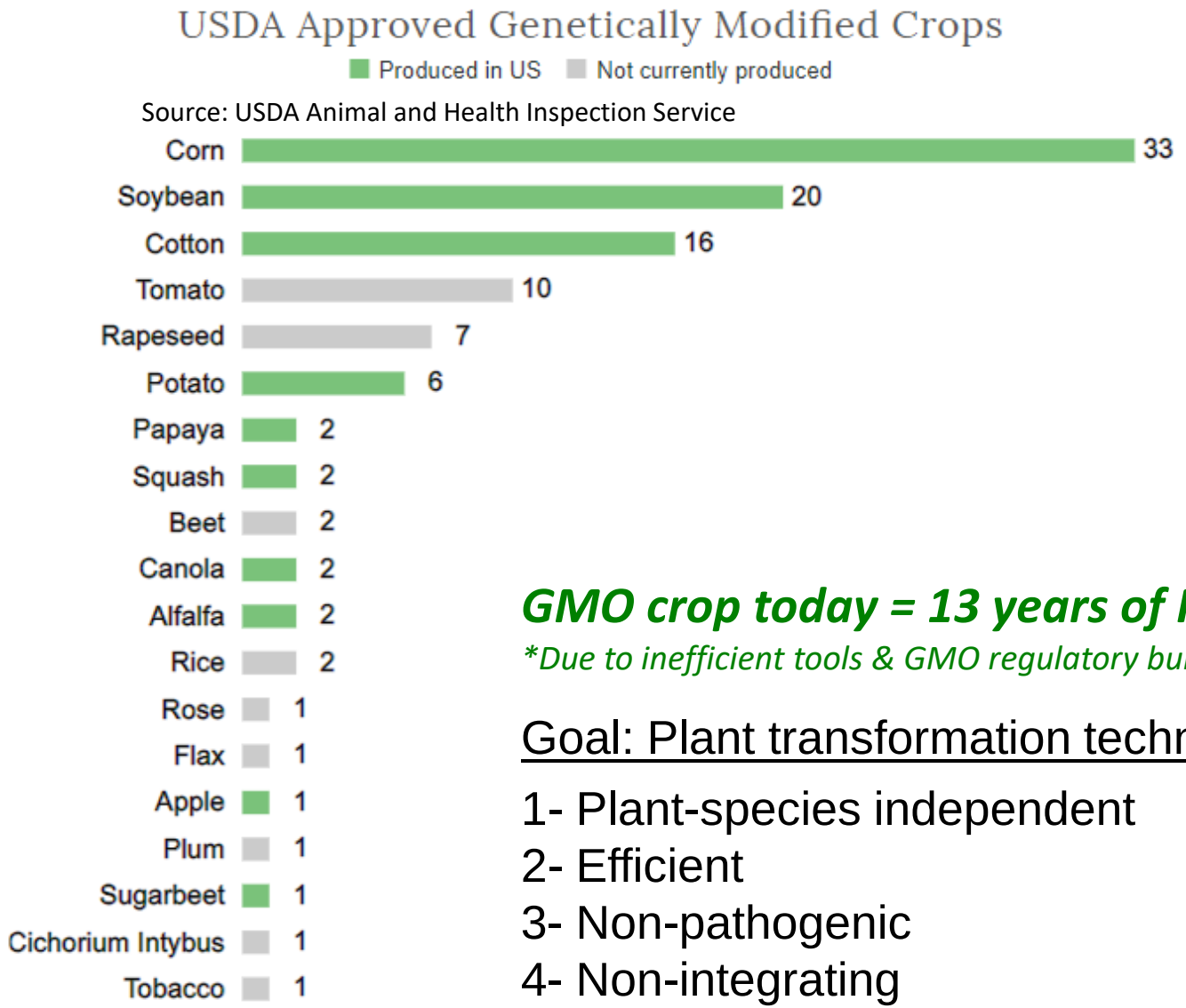




Low transformation efficiency
Cell damage

*Lack of control over location & frequency of transgene integration*

Landry & Mitter, *Nature Nanotechnology* (2019)

# GM Successes, But Contributions to the GM Cost




Golden Rice in Bangladesh - 2018

***GMO crop today = 13 years of R&D at a cost of $136M***

**Due to inefficient tools & GMO regulatory burden*

Goal: Plant transformation technology that is:

1- Plant-species independent
2- Efficient
3- Non-pathogenic
4- Non-integrating

*Inefficient tools*

*GMO regulatory burden*

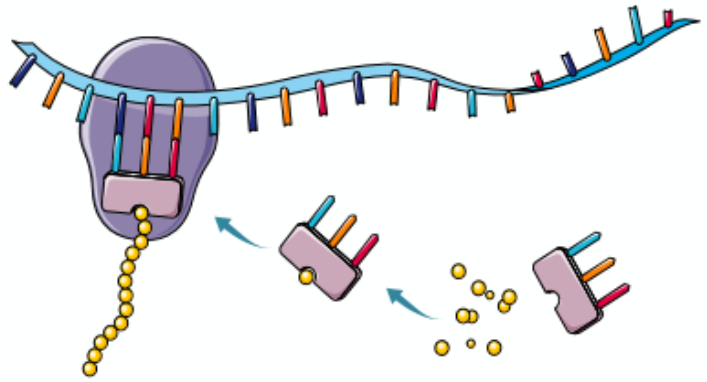


Landry & Mitter, *Nature Nanotechnology* (2019)

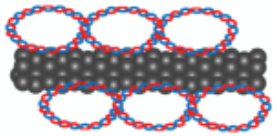
# Nanoparticles to Enable & Control Transgenic Protein Expression in Plants

*Develop a delivery tool that can transfer biomolecules into various plant species in a force-independent manner*

## Gene Expression Platform

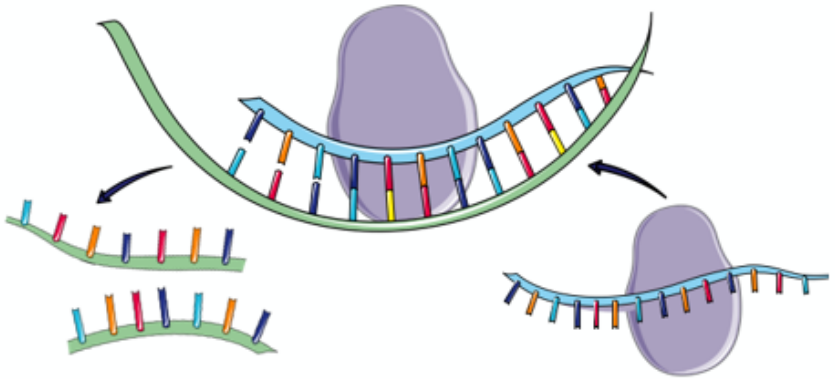


**Cargos:** plasmid DNA
linear DNA



**Targets:** arugula protoplasts and leaves
wheat leaves

## Gene Silencing Platform



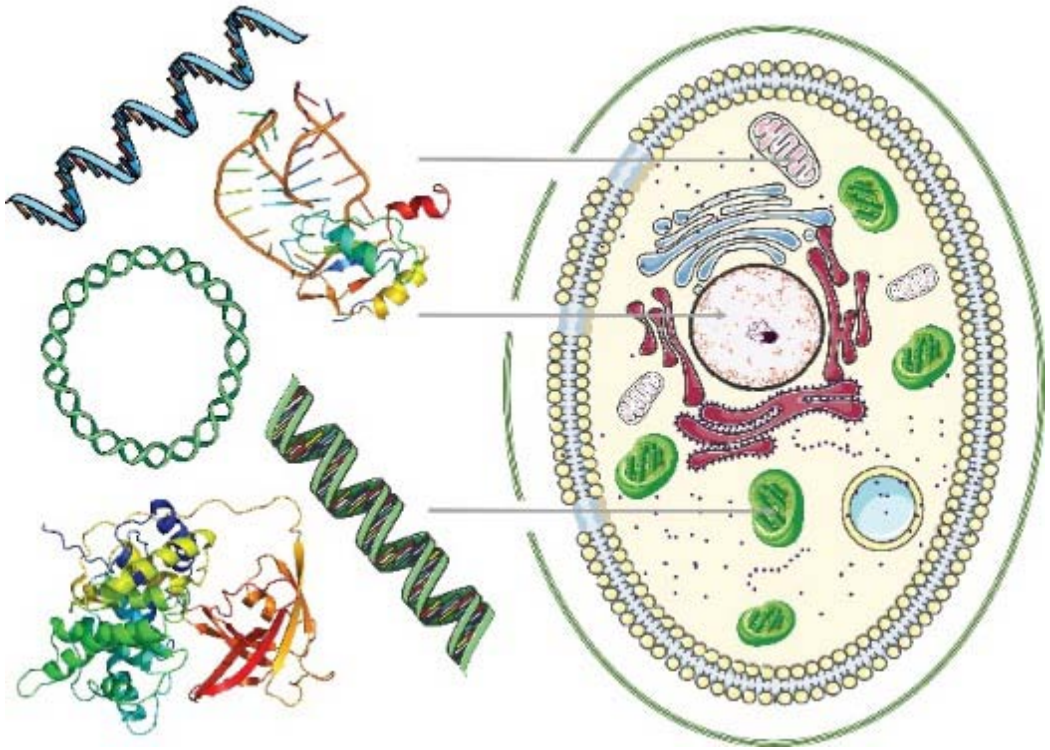
**Cargos:** small interfering RNA (siRNA)
synthetic guide RNA (sgRNA)

**Target:** GFP-mutant *N. benthamiana* leaves

# Which Nanomaterials Would Enable Crossing of Plant Cell Wall?

## Barriers to Cross

1. Cell Wall
2. Plasma Membrane
3. If plastid (Organelle Membrane)



## Carrier Design Consideration

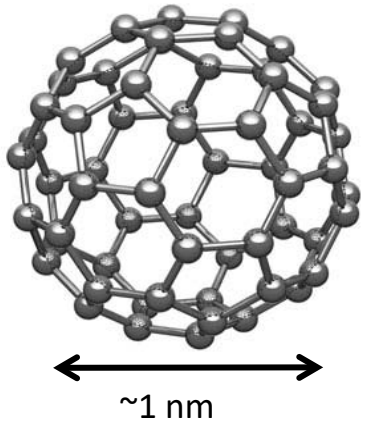
NP uptake and transport limited by pore diameters → size exclusion limit (SEL)

**Cell wall** – SEL ~ 5-20 nm
**Cell membrane** – SEL > 500 nm
**Plasma, nuclear, & plastid membranes –** additional considerations if cytosolic or nuclear localization is necessary to affect gene function

Cell wall poses the dominant barrier to exogenous biomolecule delivery

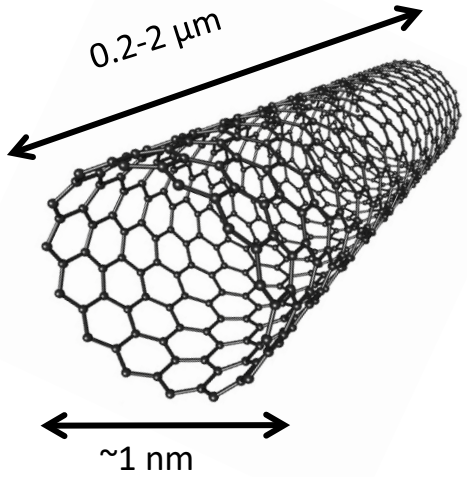
# Nanomaterialsfor Gene Delivery – Optimizing Cargo Loading vs. Effective Size

**0-D**



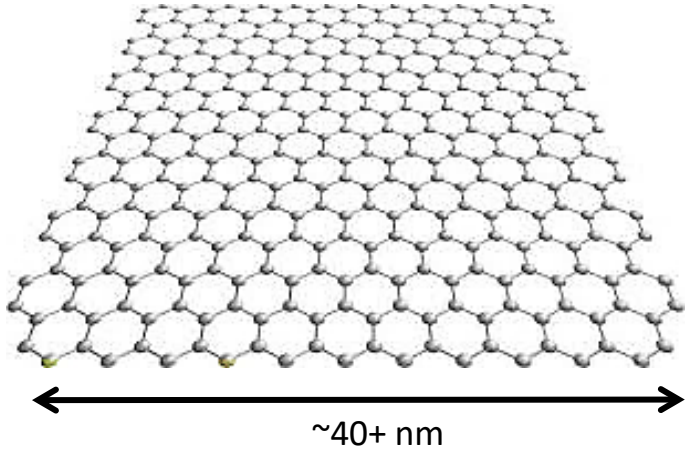

Buckminsterfullerene $C_{60}$

**1-D**




SWNT

**2-D**




Graphene

**1-D SWNTs**

Large surface area (A:V Ratio)

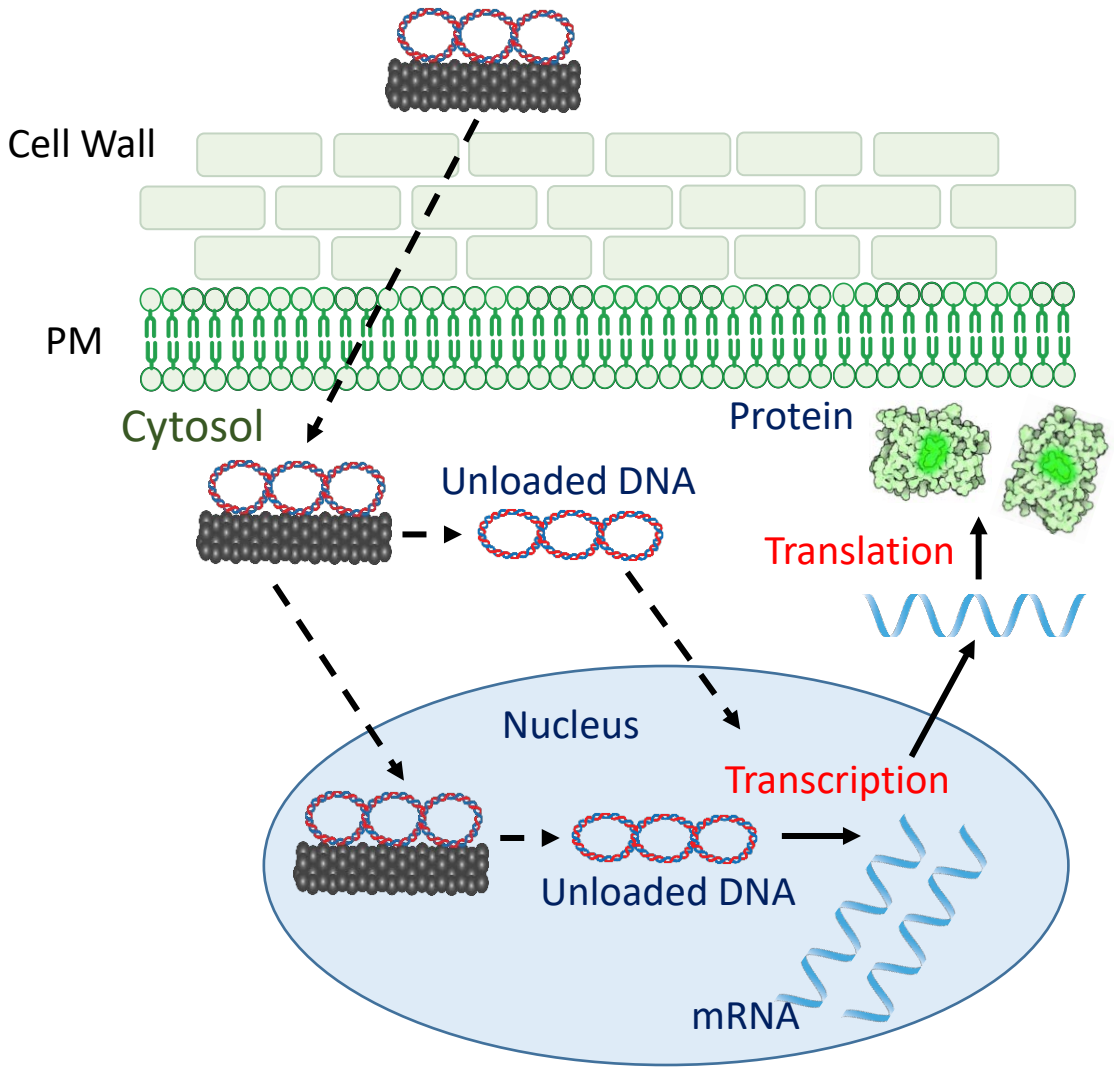
1 nm smallest dimension

Non-toxic

Passive Entry – cell wall pore

# Workflow: CNT-Based Plasmid Delivery & Gene Expression

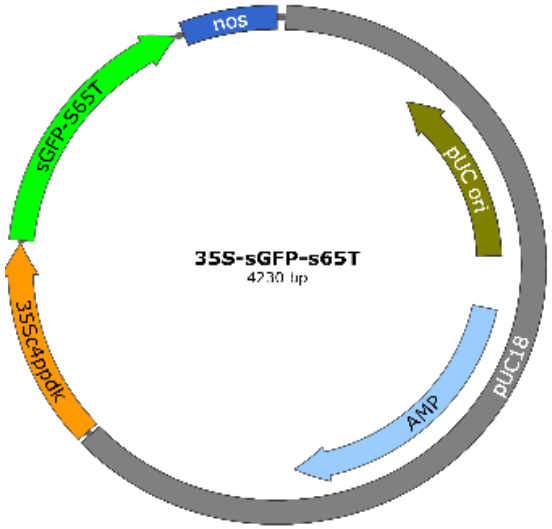
## Exogenous Gene Expression Pathway

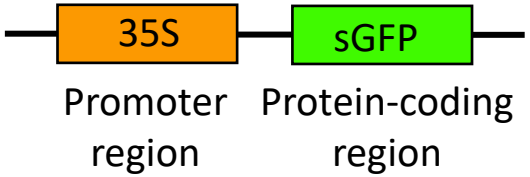



Trafficking

Gene Expression

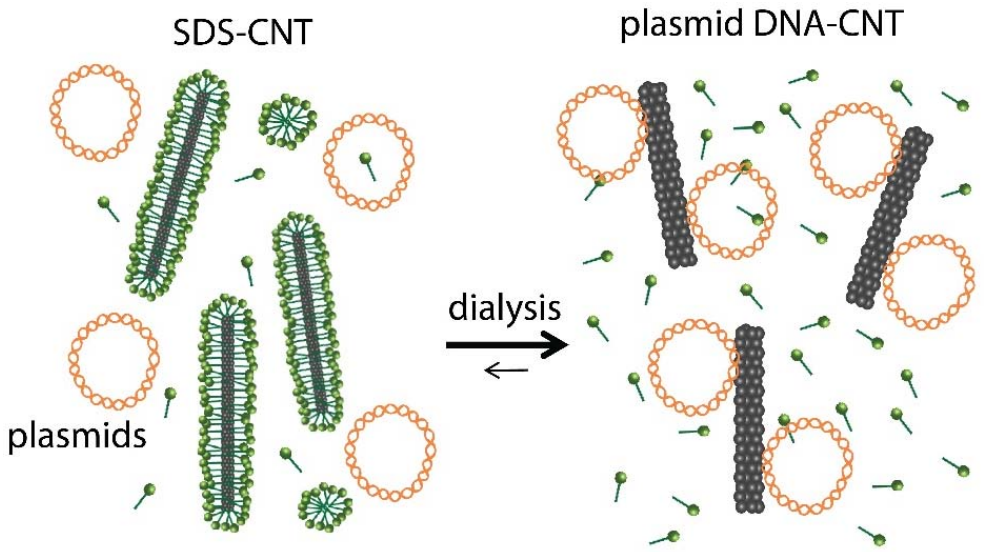
## Reporter Gene (GFP) Plasmid Construct



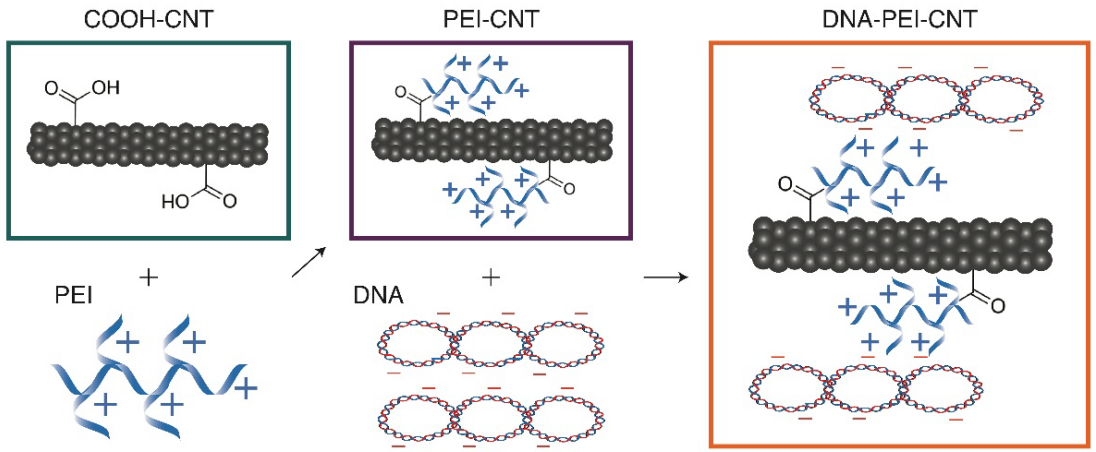



# Conjugation Chemistries for Loading Plasmid DNA Onto Nanotubes

## Loading of DNA on SWNT Nanoparticles

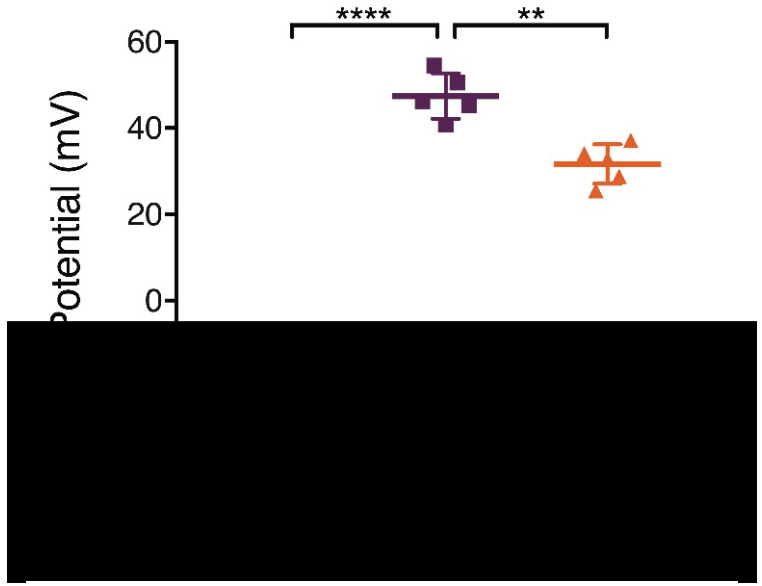


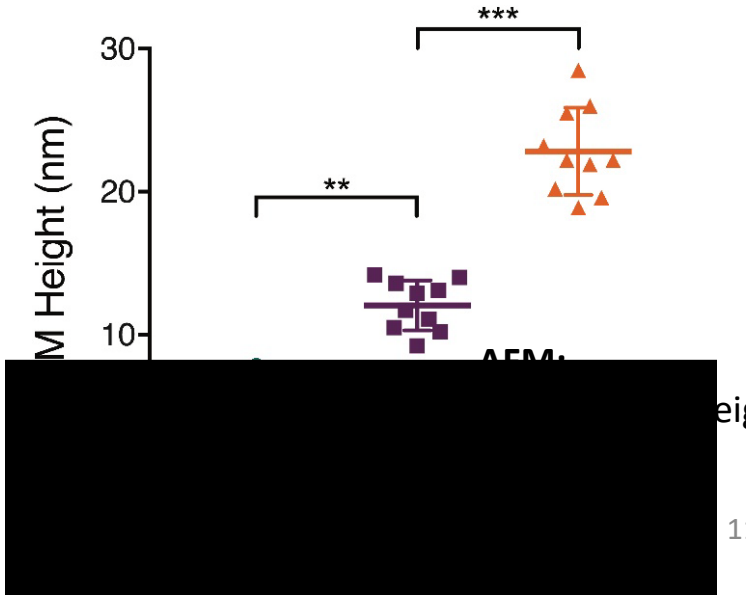

**π-π stacking:**
Low efficiency, large $K_d$




**Electrostatic grafting:**
High efficiency, low $K_d$

## Confirmation of DNA Loading

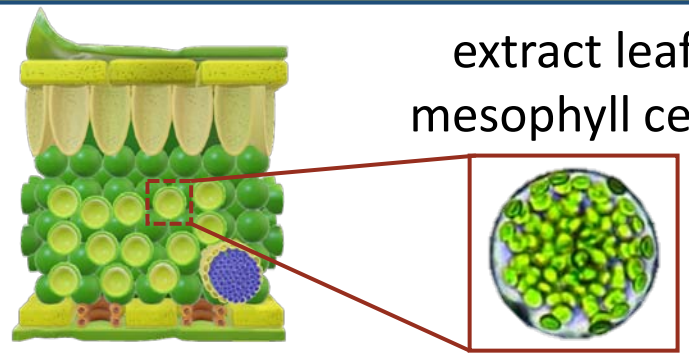



eight

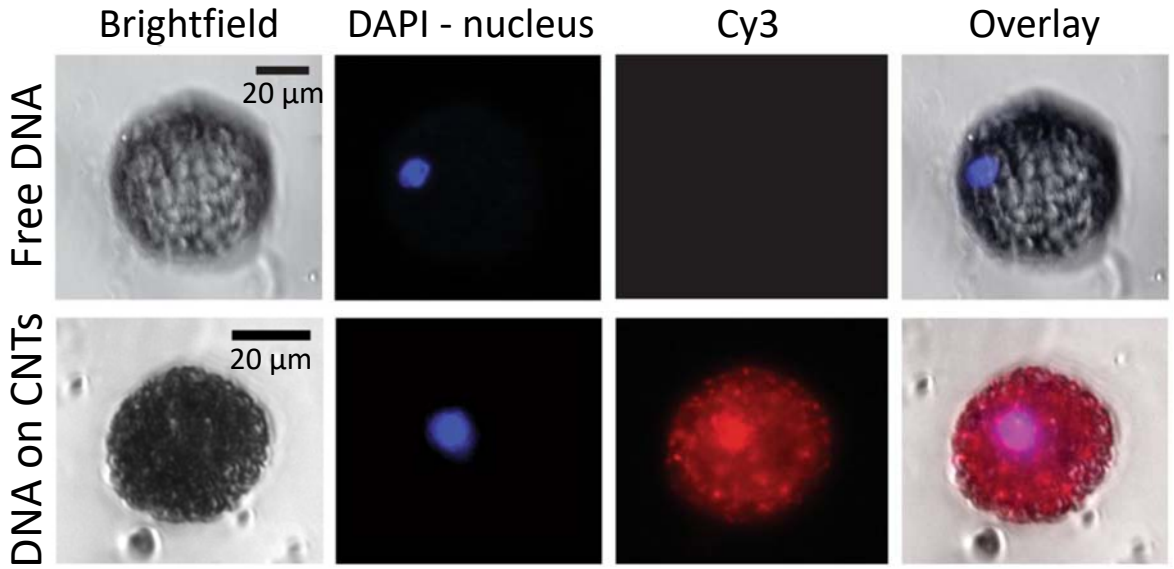
# DNA-CNTs can enter intact plant leaf cells

## Internalization into plant leaf protoplasts

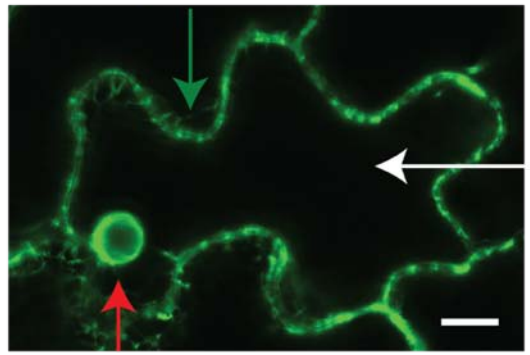


extract leaf mesophyll cells

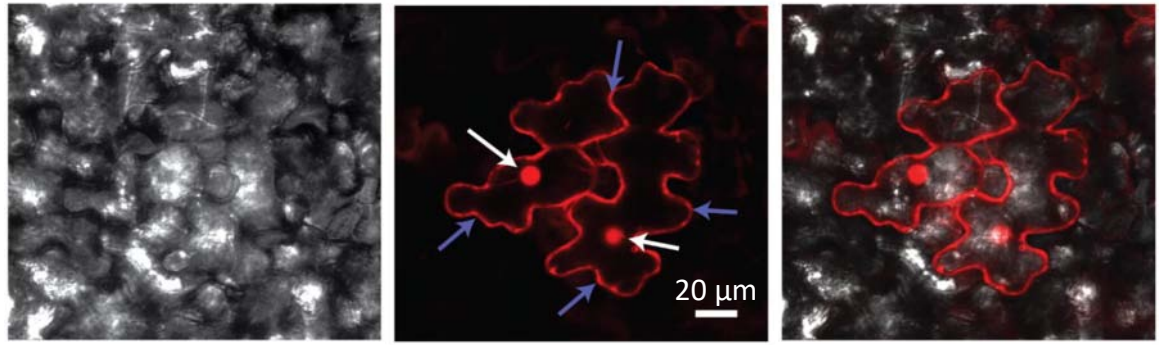
**no plant cell wall**



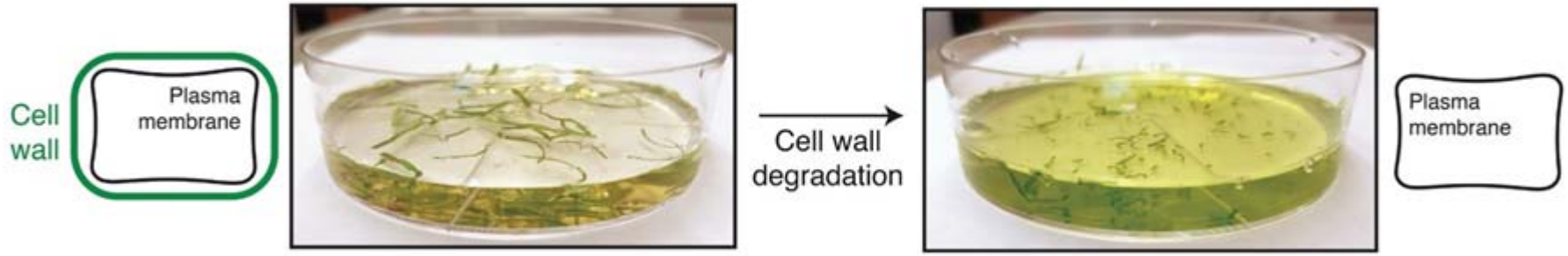

## Internalization into leaf intact cells

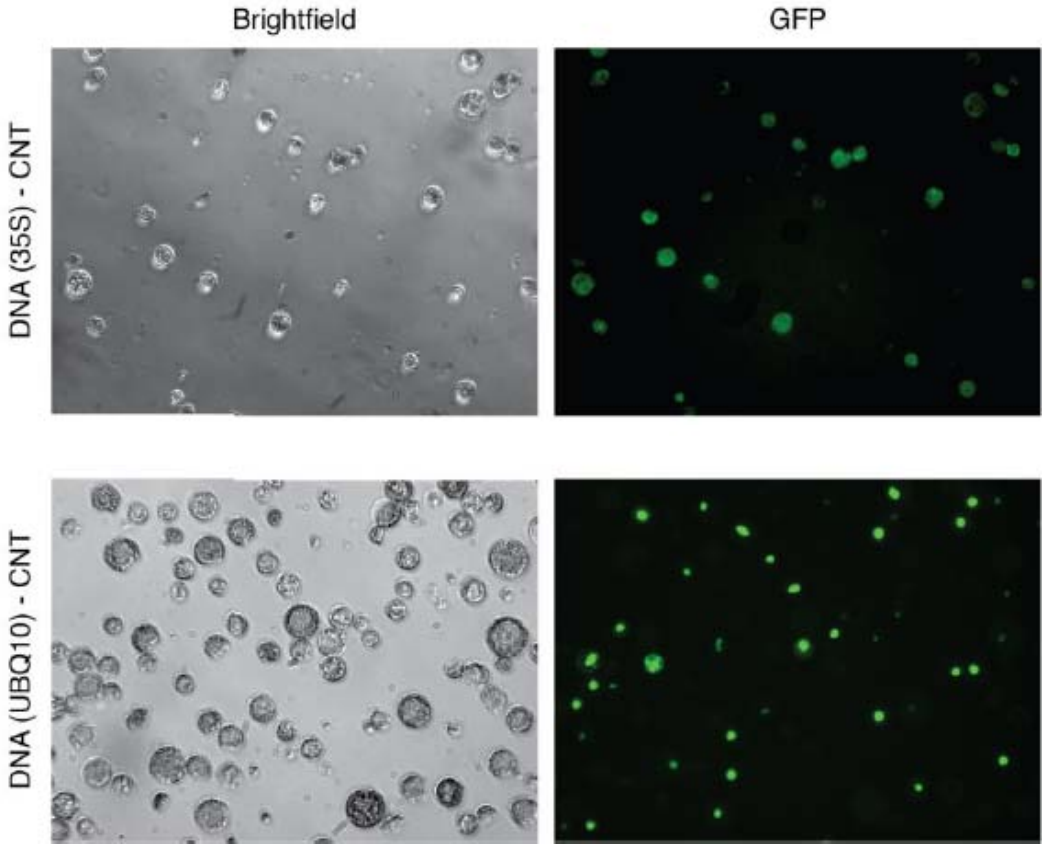


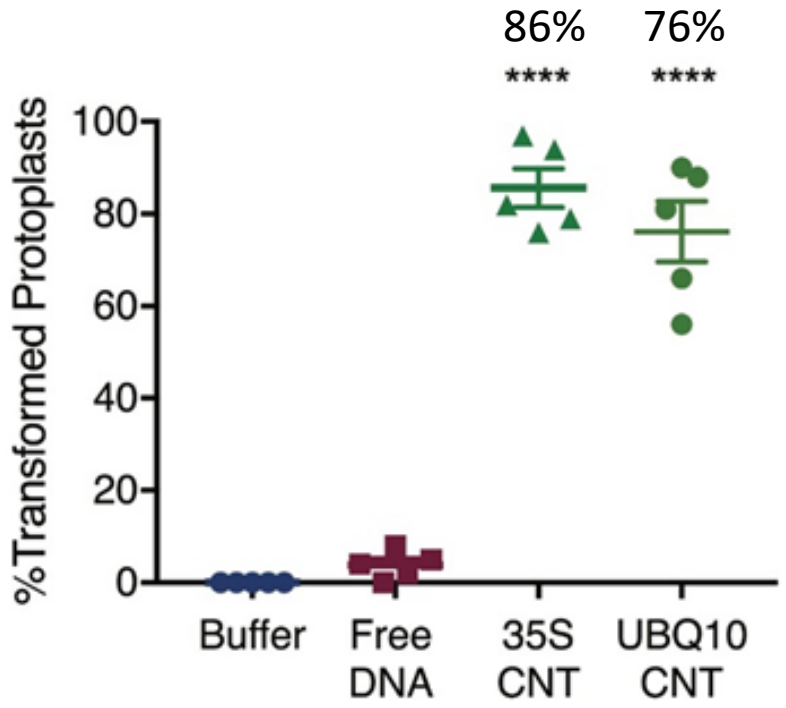

**with plant cell wall**



# Testing Protoplast Transformation with GFP Plasmid-Nanotube Conjugates

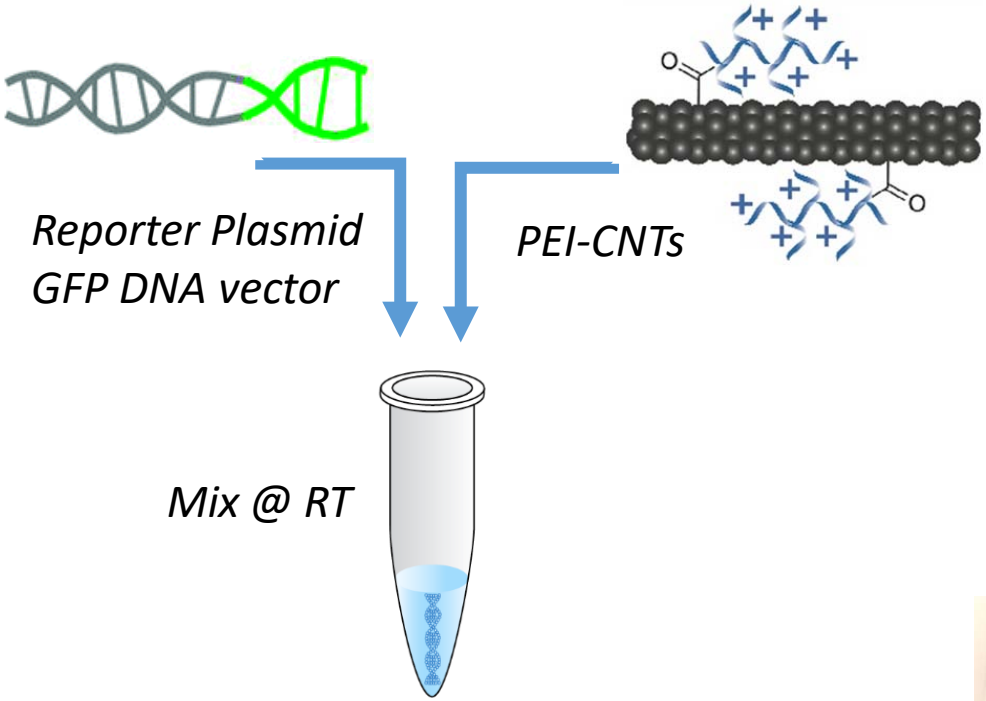



*Comparable to PEG-based protoplast transformation*

# Workflow for Testing DNA Plasmid Delivery in Plants




**Model species**

*Arabidopsis*

*Nicotiana benthamiana*

Infiltration of DNA-CNTs
(1-5 mg/L = 1-5 ppm)

72 hour
incubation

Confocal
imaging

**Non-model species**

Spinach

Arugula

Sorghum

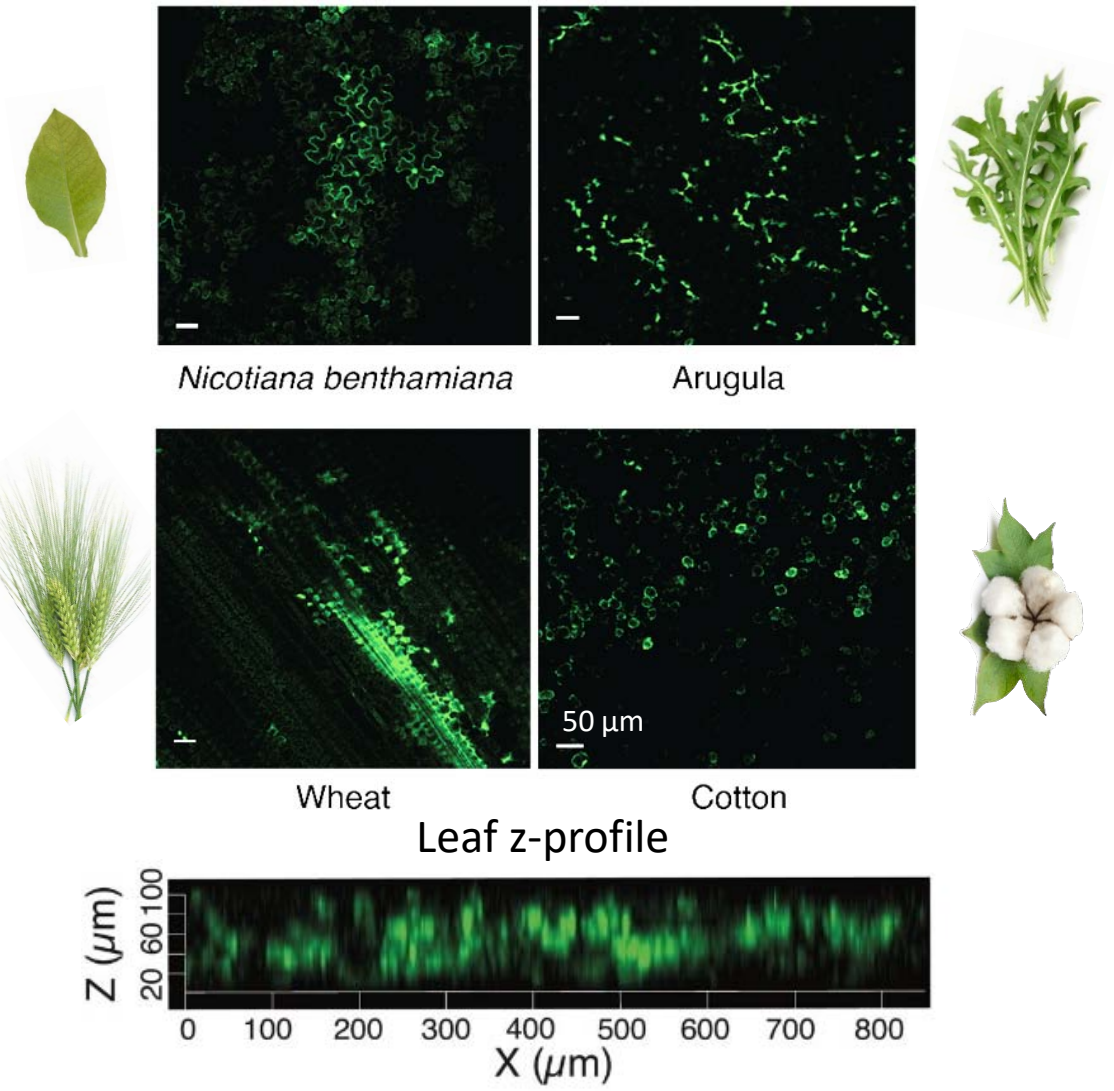
Cotton

Wheat

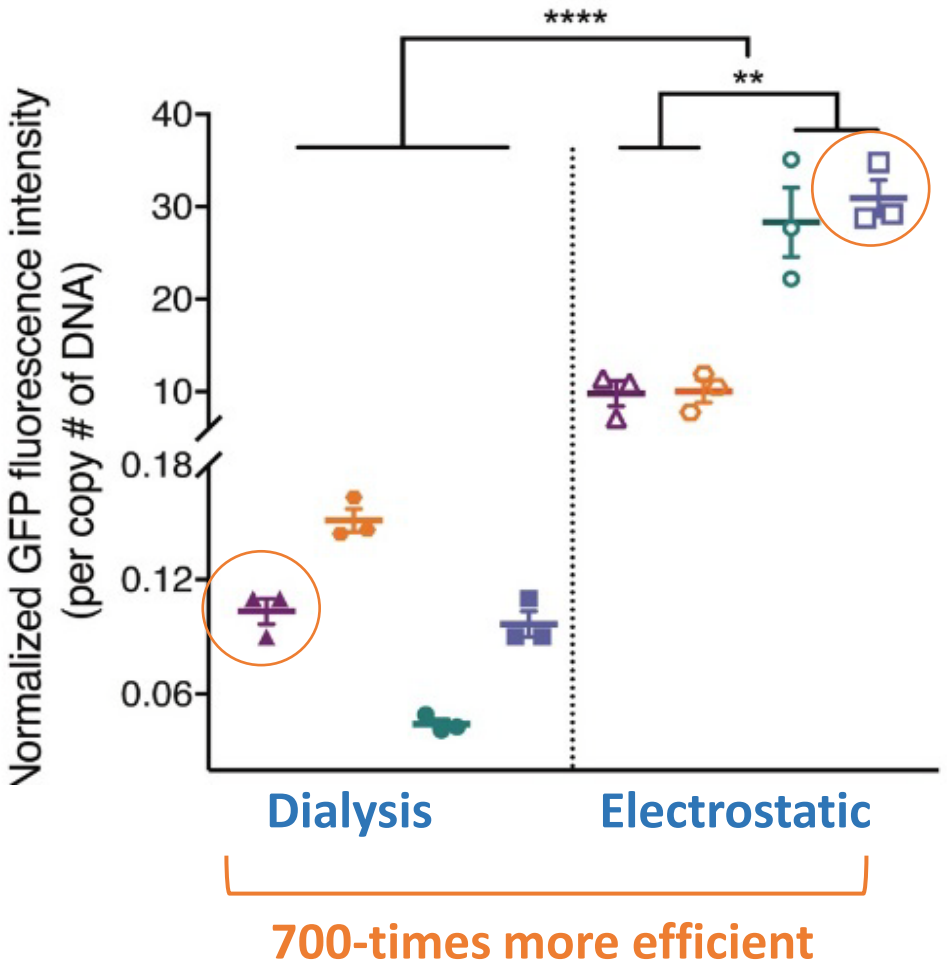
Demirer, Zhang *et al. Nature Protocols* (2019)

# Transgene Expression in Monocot and Dicot Plants with Nanomaterial Delivery

## GFP Expression Through Leaf Profile




## Effect of nanomaterial chemistry

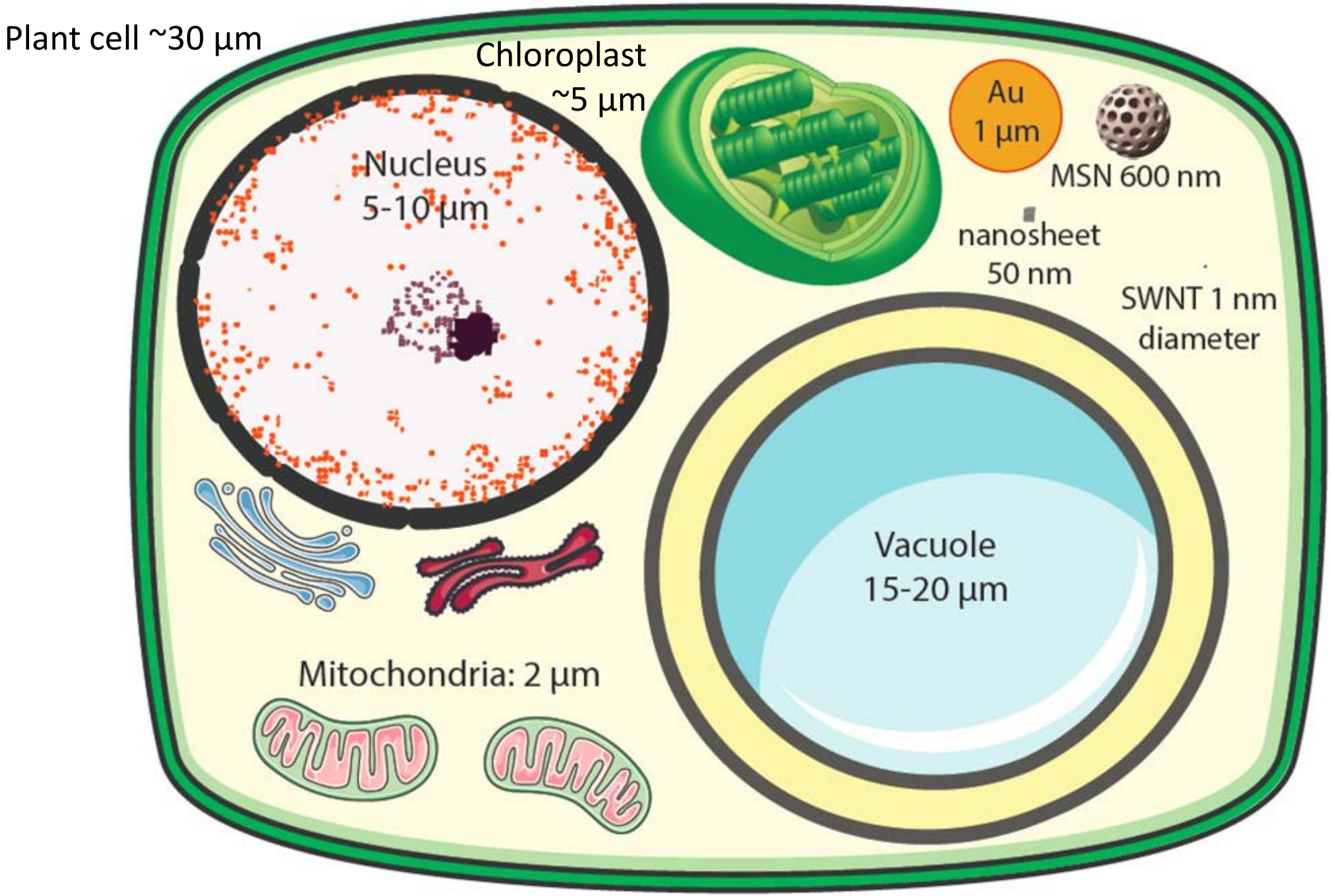



**π-π stacking:**
Low efficiency, large $K_d$

**Electrostatic grafting:**
High efficiency, low $K_d$

Demirer *et al. Nature Nanotechnology* (2019)

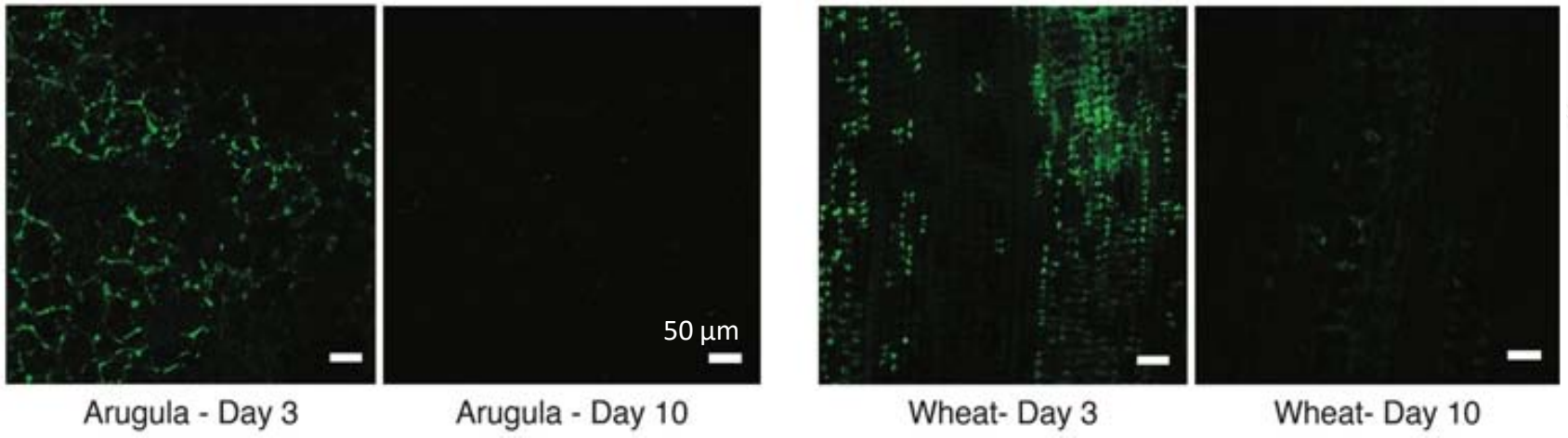
Demirer, Zhang, *et al. Nature Protocols* (2019)


Plant cell ~30 µm
Chloroplast
~5 µm
Au
1 µm
MSN 600 nm
nanosheet
50 nm
SWNT 1 nm
diameter
Nucleus
5-10 µm
Vacuole
15-20 µm
Mitochondria: 2 µm

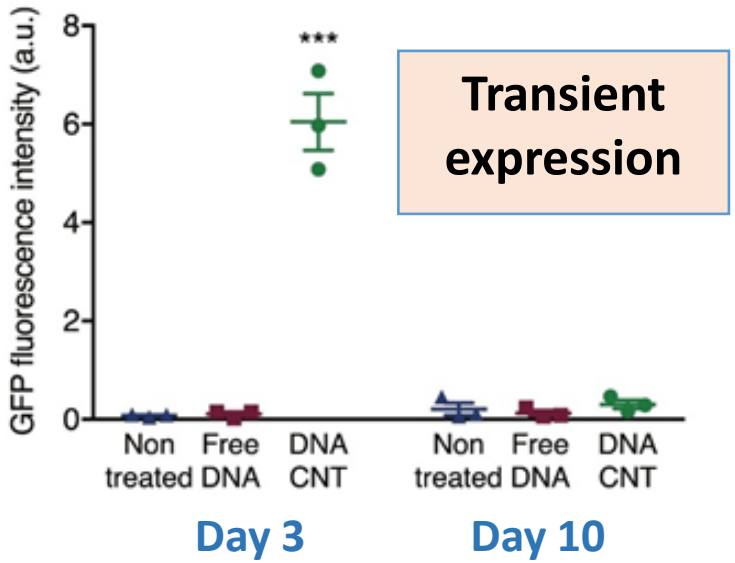
# Gene Expression is Transient with Nanotube-Based Delivery

## *Transient GFP Gene Expression with Carbon Nanotubes*

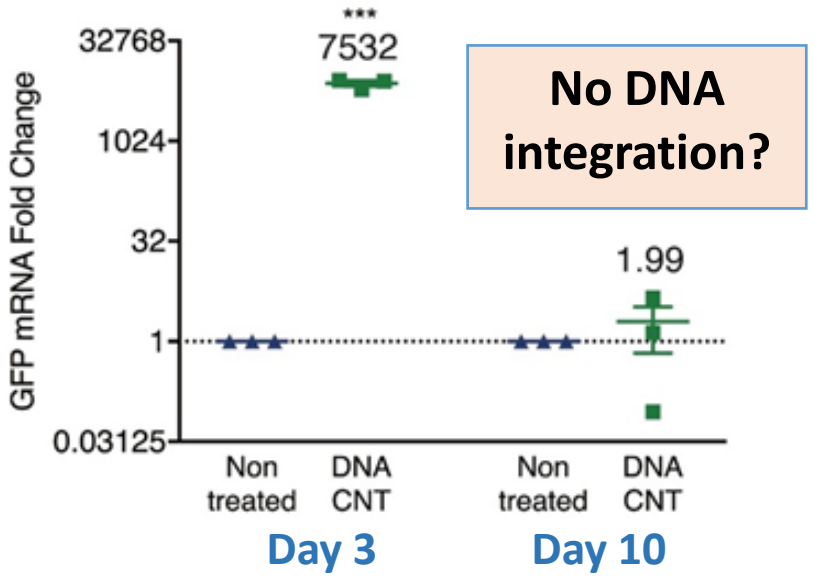
*Suggests no transgene integration into plant genome*




**Expression (protein)**




**Transient expression**




**No DNA integration?**

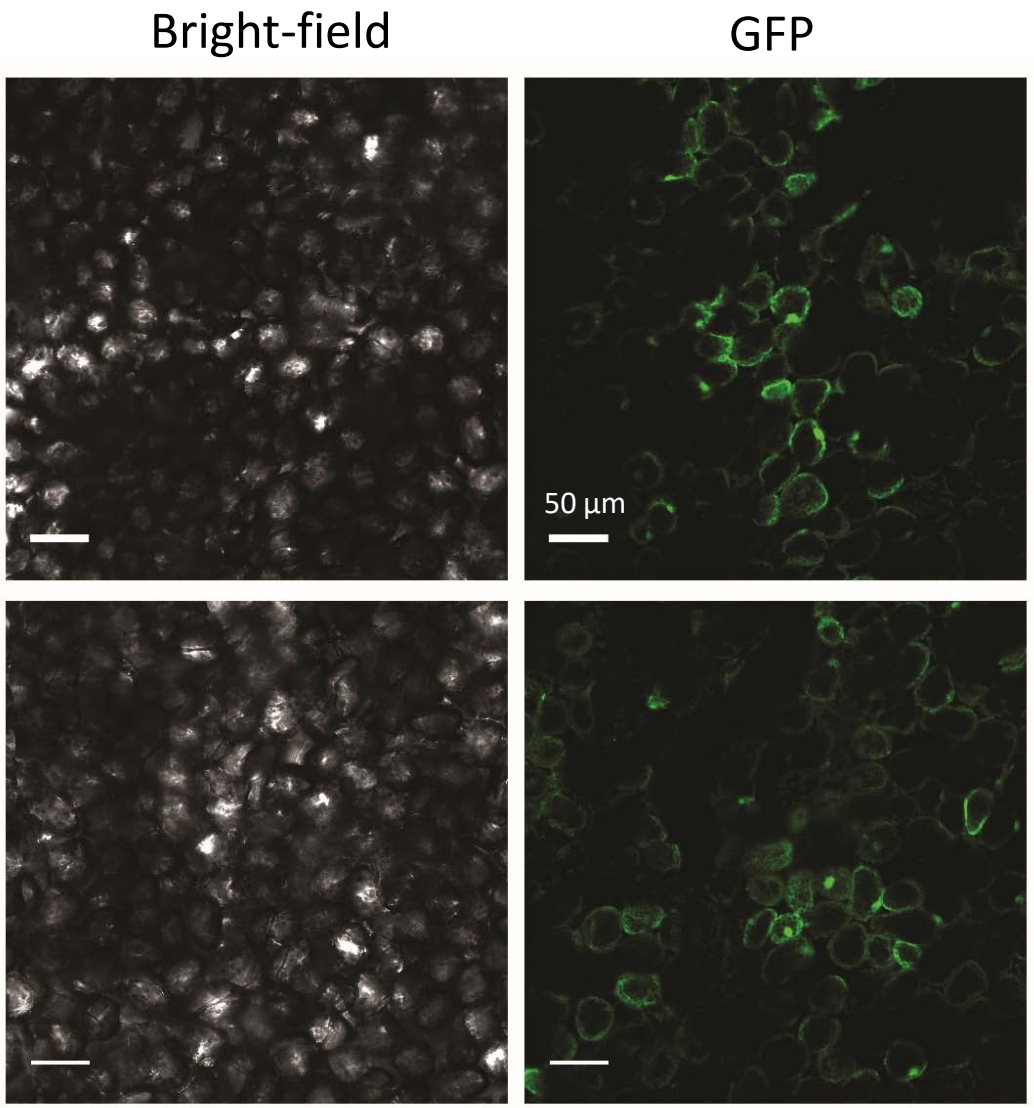
**Expression (mRNA)**

Demirer *et al. Nature Nanotechnology* (2019)

# In Contrast, Agrobacterium-Mediated Gene Expression is Not Transient

Agrobacterium containing GFP vector – **Day 3**

Agrobacterium containing GFP vector – **Day 10**

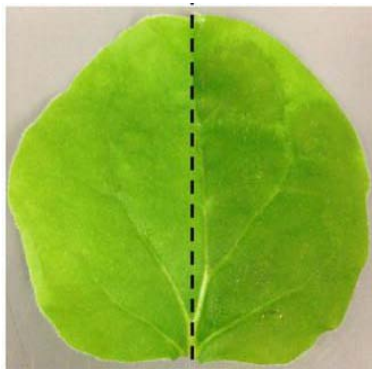



**Constitutive transgene expression**

**DNA integration into plant nuclear genome**

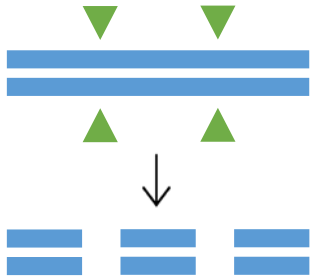
Demirer *et al. Nature Nanotechnology* (2019)

# Digital Droplet PCR to Test Transgenic DNA Integration into Plant Genome

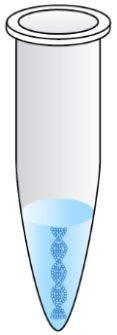


1. Treat leaves - control and sample

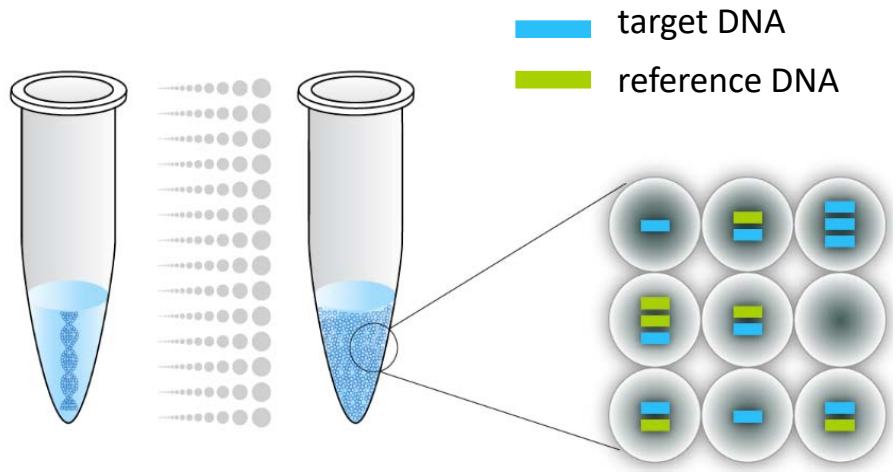
2. Extract genomic DNA



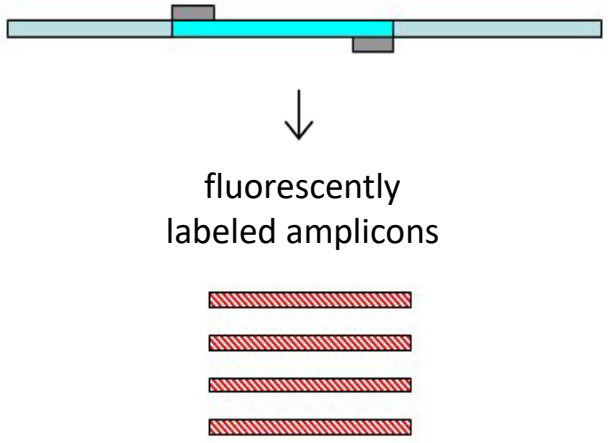
3. genomic DNA digestion



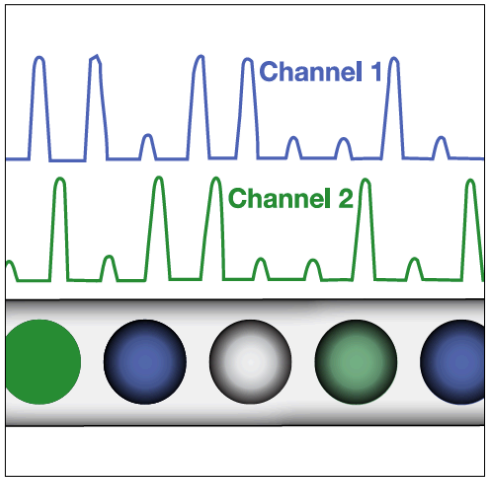
4. Mix genomic DNA fragments with probes for i) transgene and ii) reference gene




5. Partition sample into 20,000 nanoliter-sized droplets




6. PCR amplification of target and reference genes



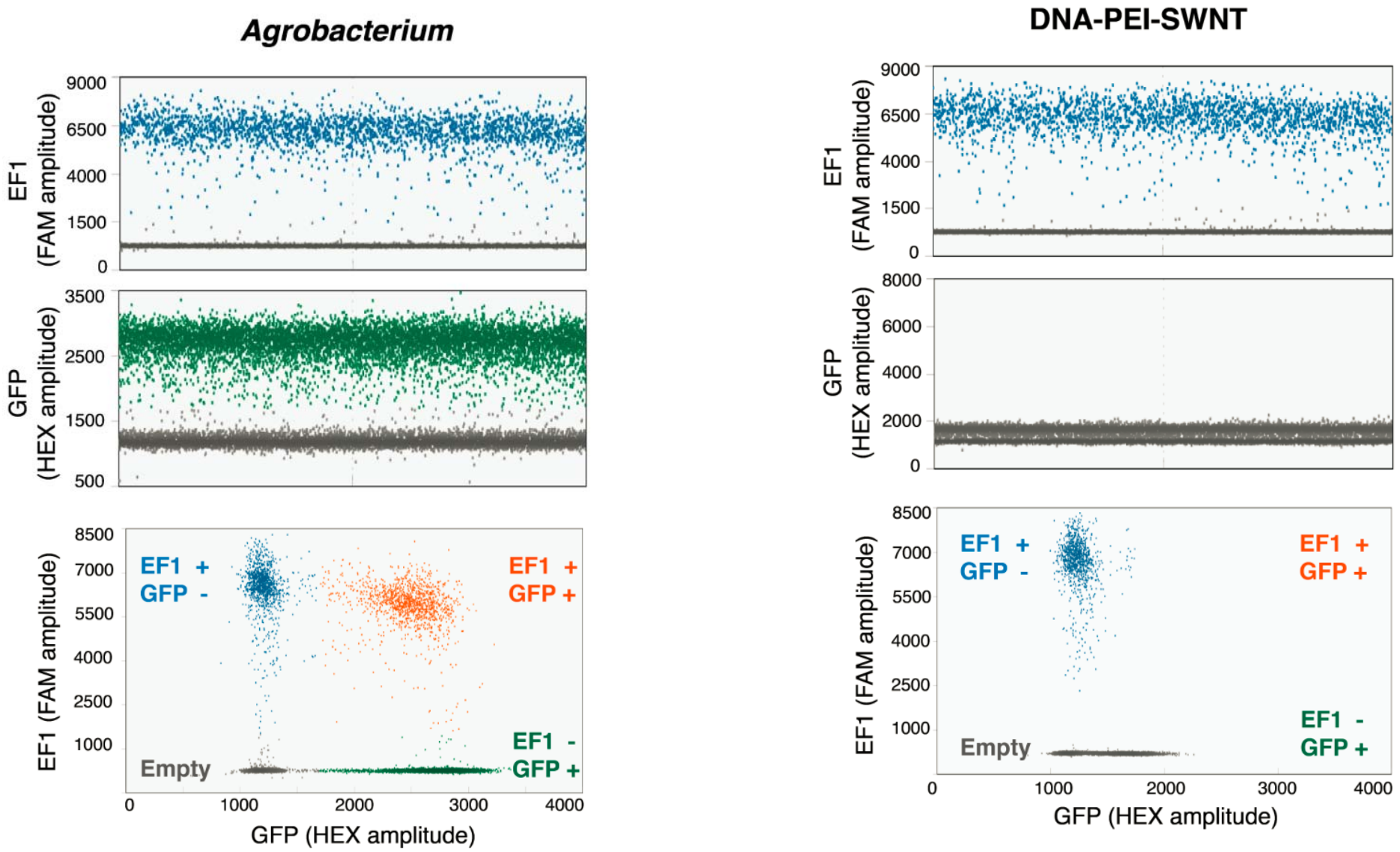

7. Read droplet fluorescence for target and reference genes



Demirer *et al. Nature Nanotechnology* (2019)

# ddPCR Shows No Transgene Integration into Plant Genome

***Digital Droplet PCR confirms no target DNA integration with 1:100,000 sensitivity***




Demirer *et al. Nature Nanotechnology* (2019)

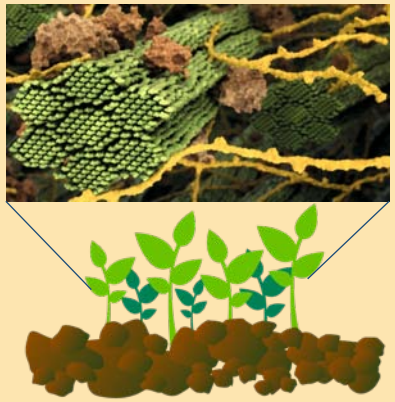
# Next Directions

## Plant genetic engineering can address critical global problems

### Food

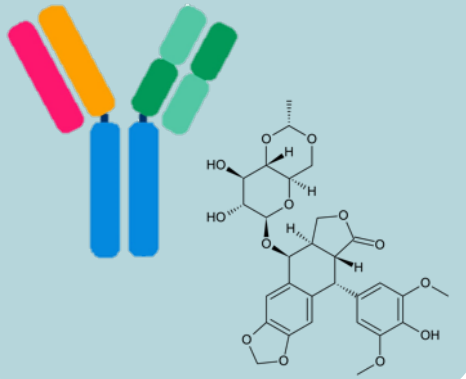
Disease & climate resistant crops

### Energy



Advanced biofuels

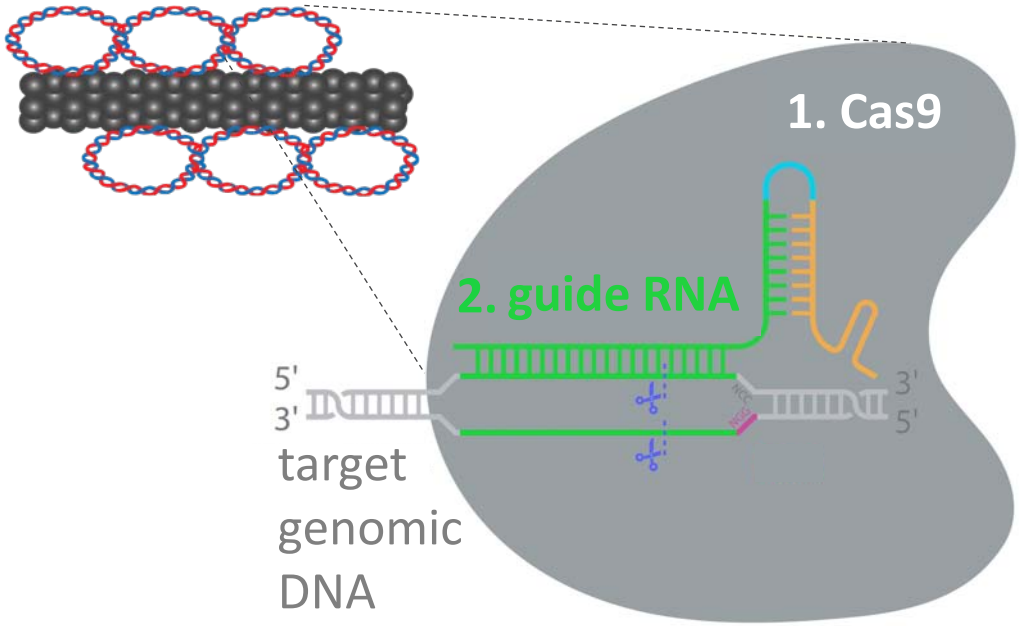
### Medicine

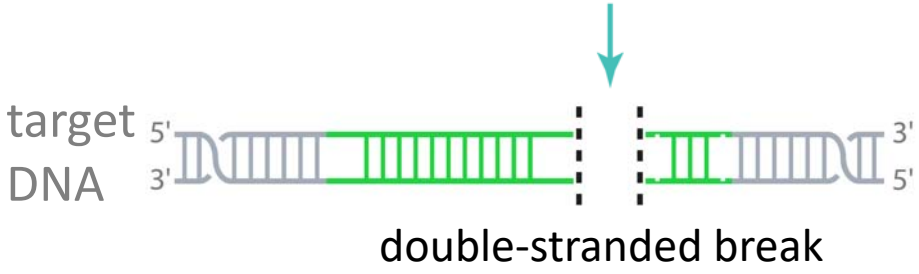


Synthesis of therapeutics

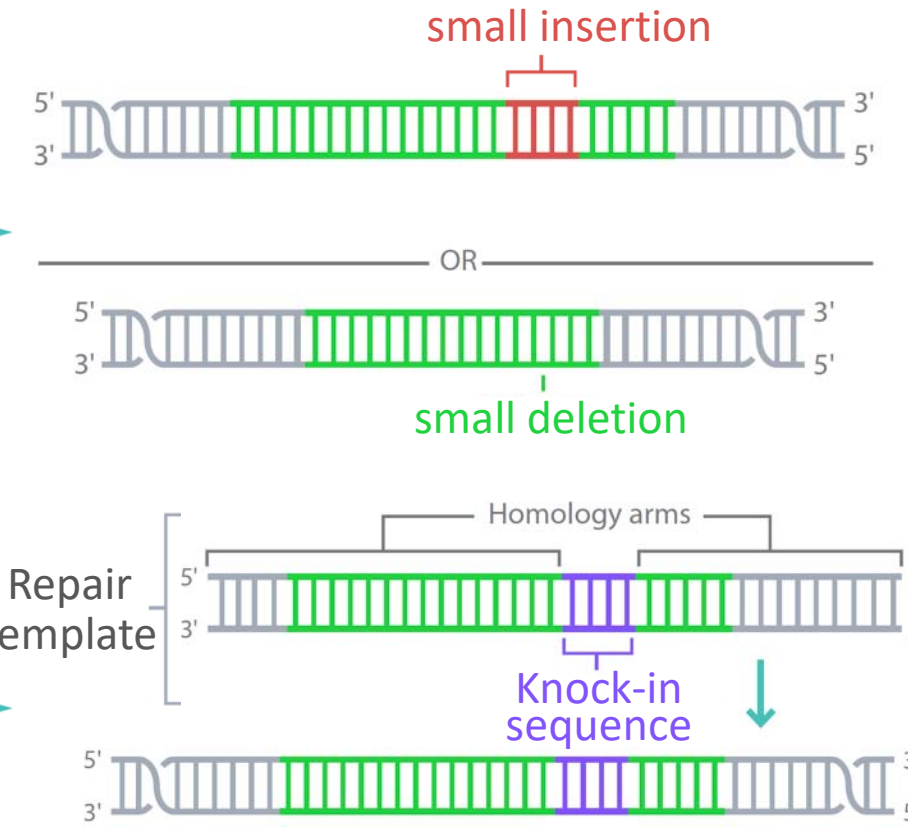
## Identify and modify plant genes with practical relevance

# CRISPR gene editing can enable targeted and fast improvements




Endogenous repair of double-stranded break:

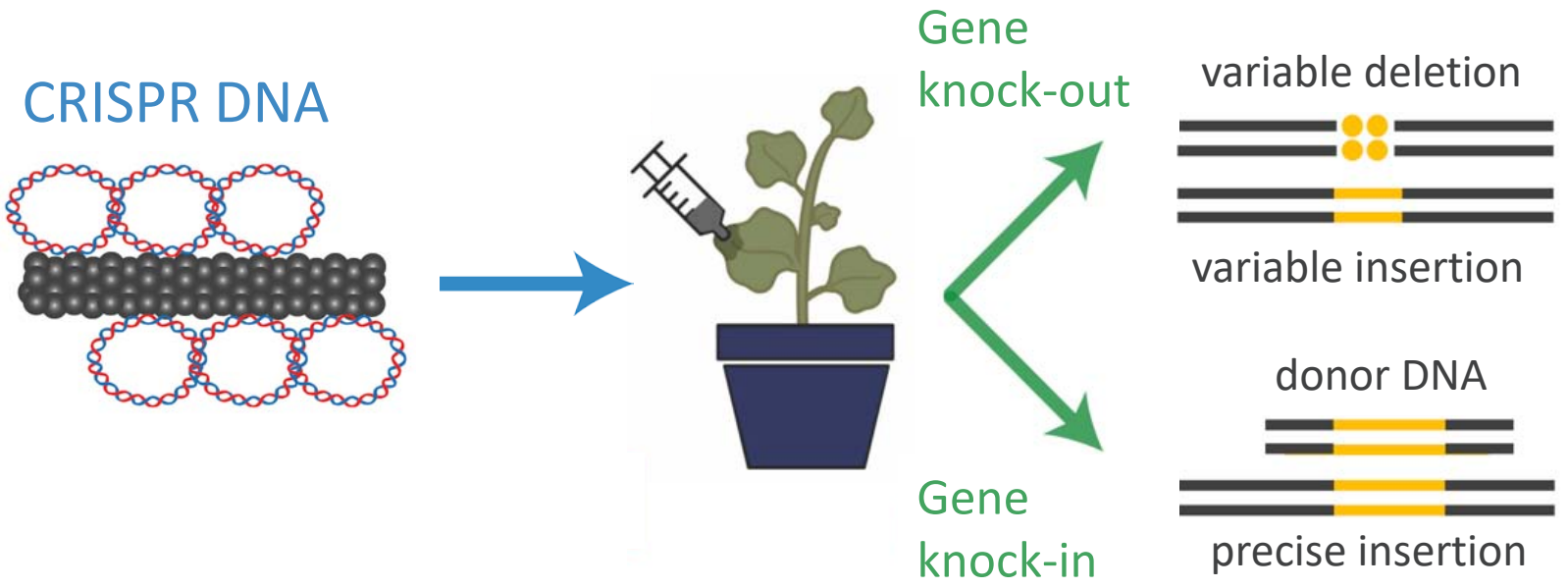



Gene knock-out

Gene knock-in

Adapted from Synthego

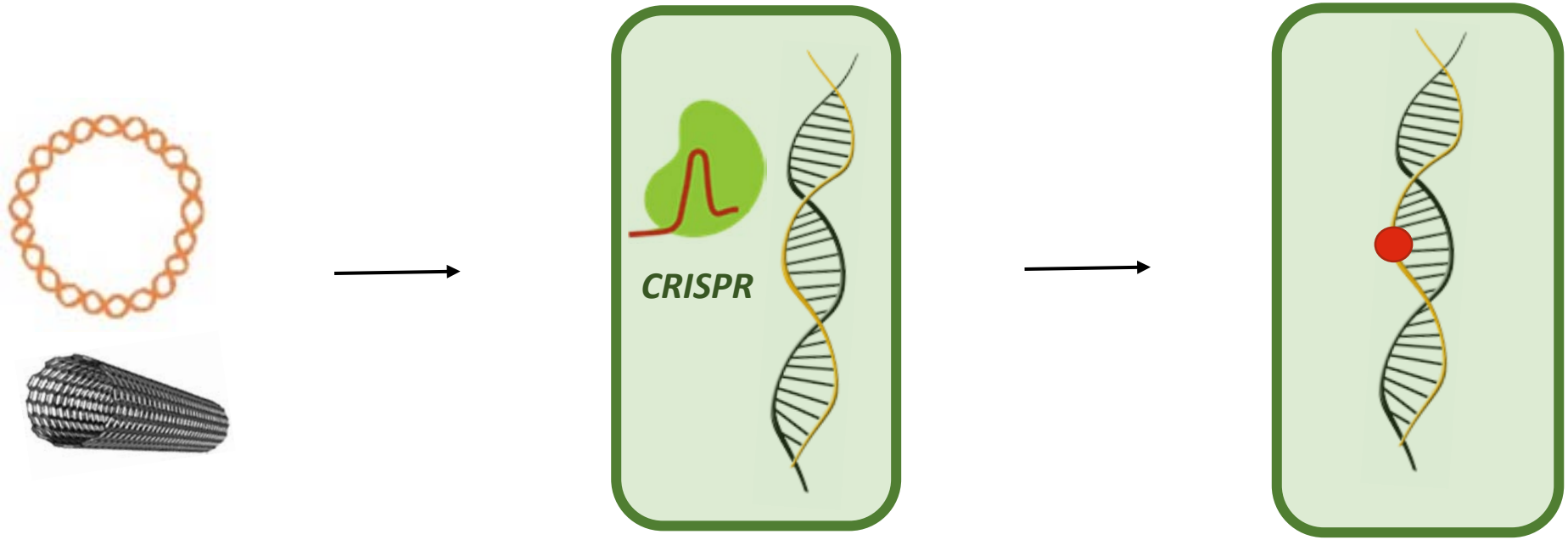
## Developing integration-free delivery platforms for plant genome editing




Plant genome editing approaches that do not induce CRISPR DNA integration for gene *knock-out* studies

# CRISPR in Plants – Genome Editing Without Transgene Cross-Out

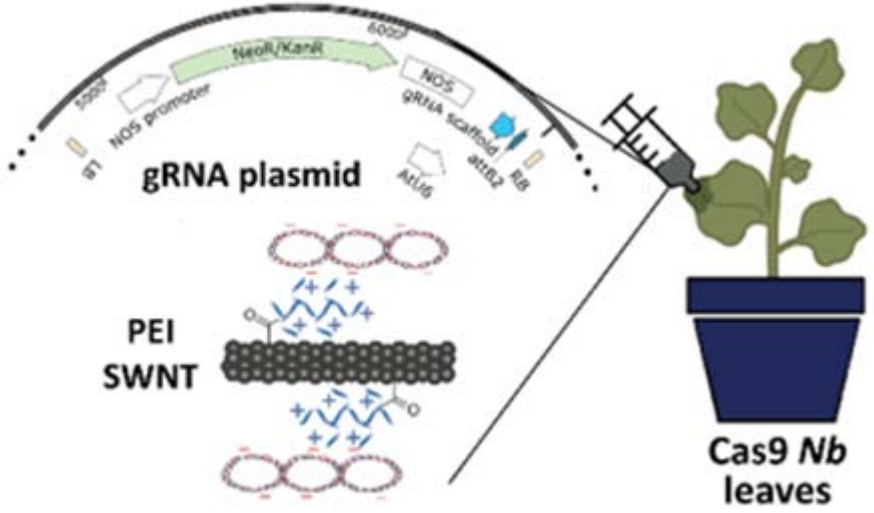
***Transient expression of Cas9 & sgRNA, permanent genome edit***

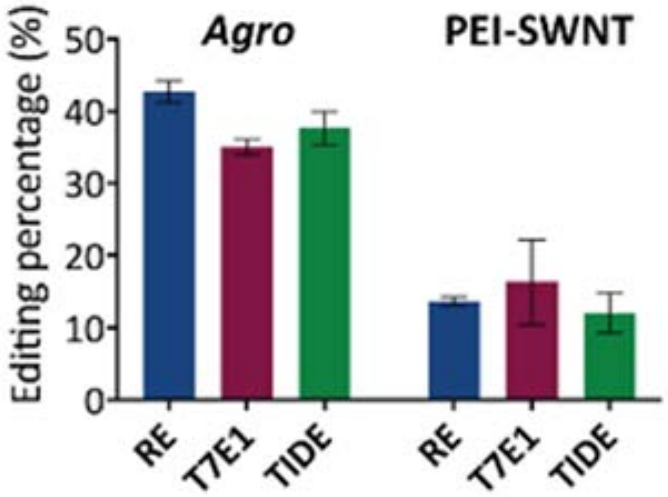



Vegetatively propagated crops

# CRISPR with Carbon Nanotube Plasmid Delivery

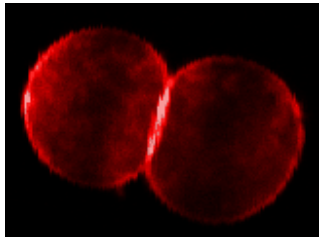
***Transient expression of Cas9 & sgRNA***




Leaves

Seeds



Pollen

Flower

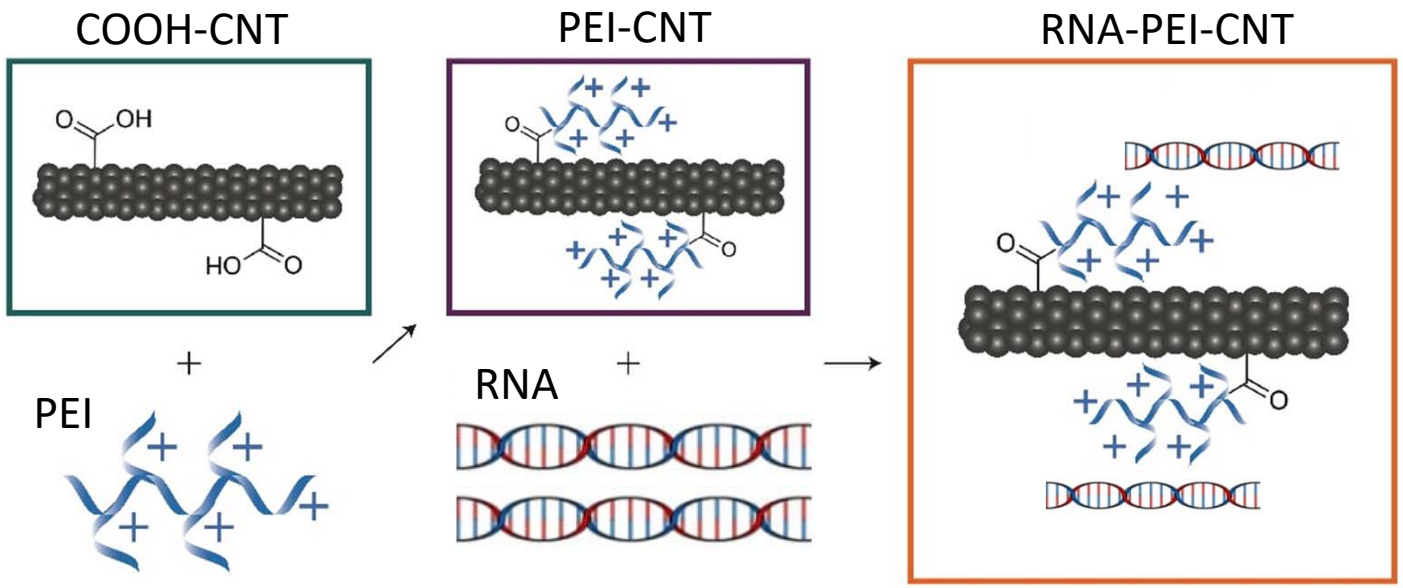
Callus



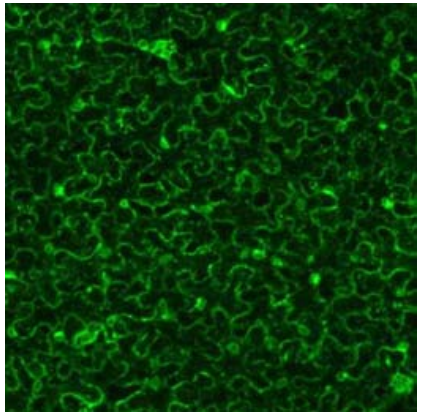
*Unpublished*

# RNAi with CNT Nanoparticles

## Nanoparticle-Mediated Transient Gene Expression and Silencing




mGFP5
*Benthamiana*



mGFP5 + siRNA-CNT

*No silencing observed*

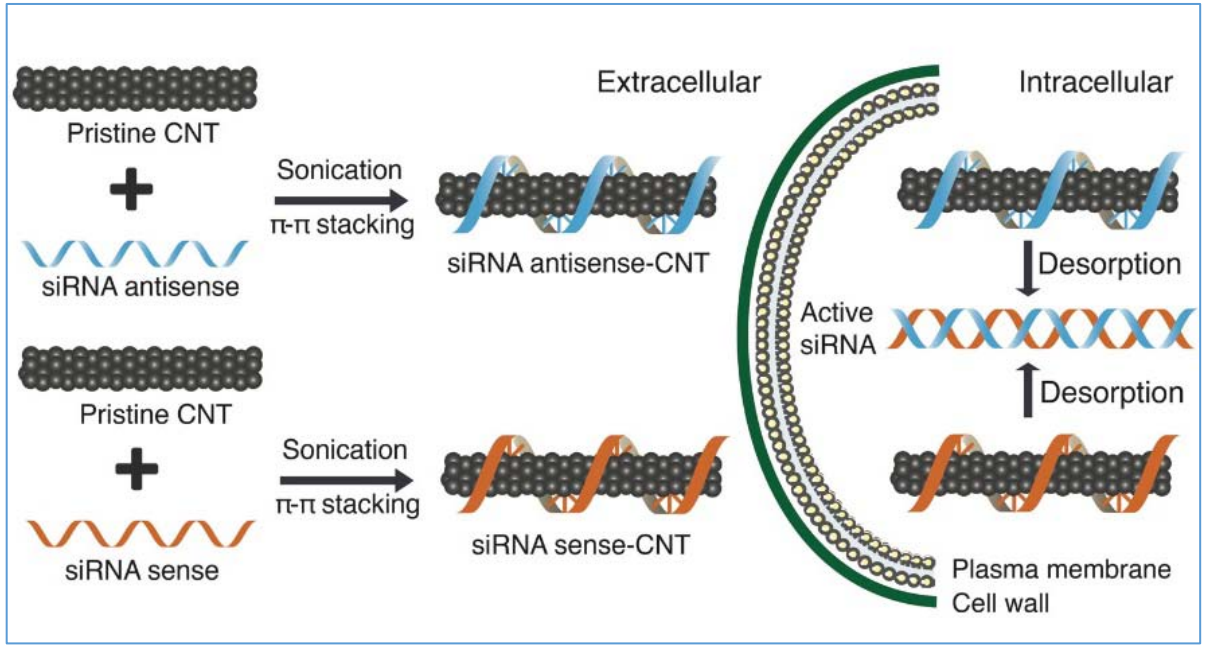
Demirer *et al. Science Advances* 2020

# Gene Silencing Through Activatable siRNA Cargoes

mGFP5 *benthamiana*

siRNA-CNT

Silenced GFP




$$\Delta G_{extracell} = +511.2 \frac{kcal}{mol}$$

***In vitro* desorption unlikely**

$$\Delta G_{intracell} = -200.8 \frac{kcal}{mol}$$

**Intracellular desorption favored**

$$\Delta G_{hyb} = (G_{dsRNA-SWCNT} + G_{SWCNT}) - (G_{ssRNA-SWCNT} + G_{cRNA-SWCNT})$$

$$\Delta G_{des} = (G_{dsRNA} + 2G_{SWCNT}) - (G_{dsRNA-SWCNT} + G_{SWCNT})$$

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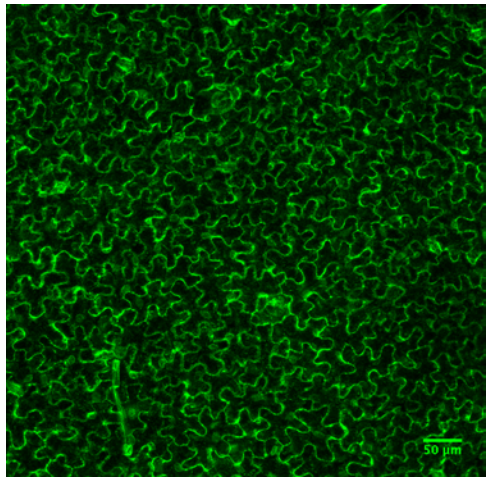
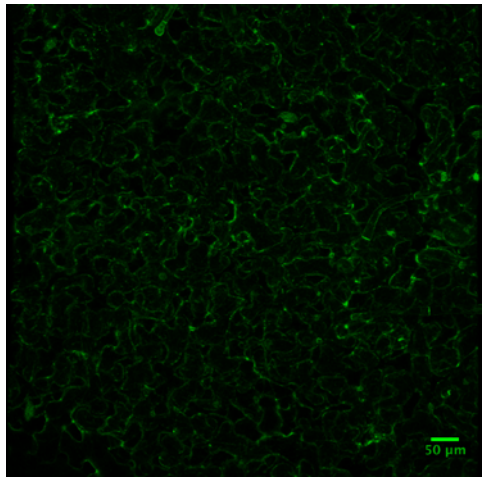
# Methodology for siRNA Delivery into Transgenic Benthamiana mGFP5

## Infiltration into leaves


CNT-only treated leaf

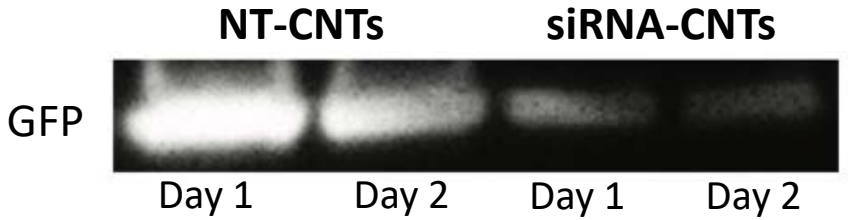



siRNA-CNT silenced leaf

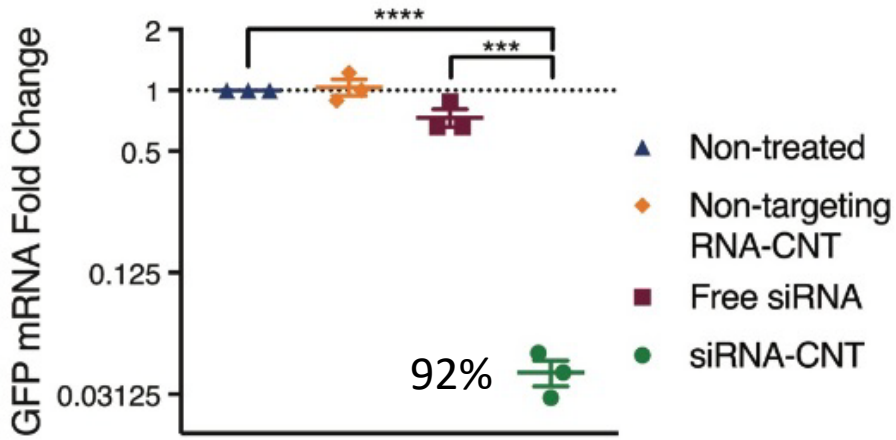



(GFP channels)

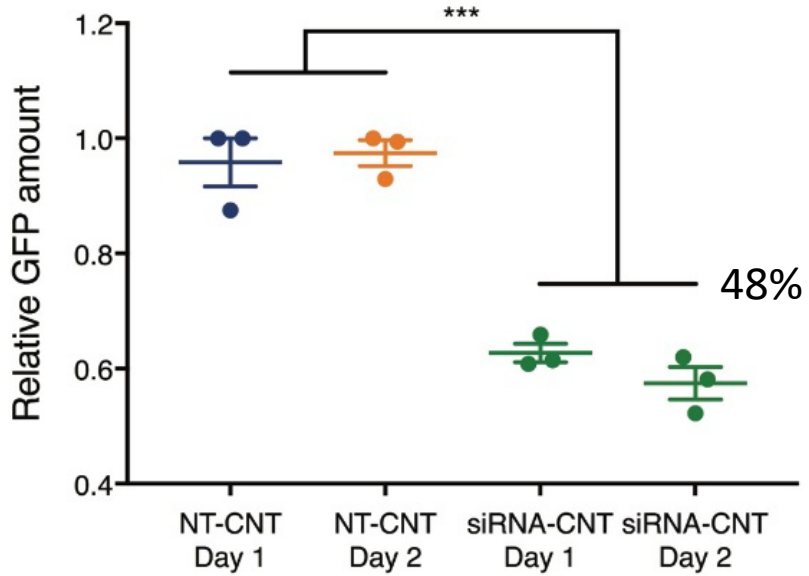
## Western blot




## Silencing (mRNA)




## Silencing (protein)

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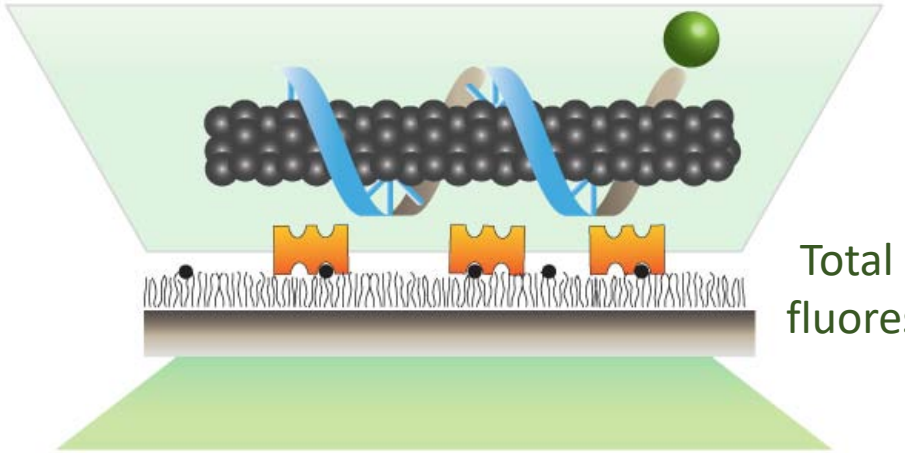
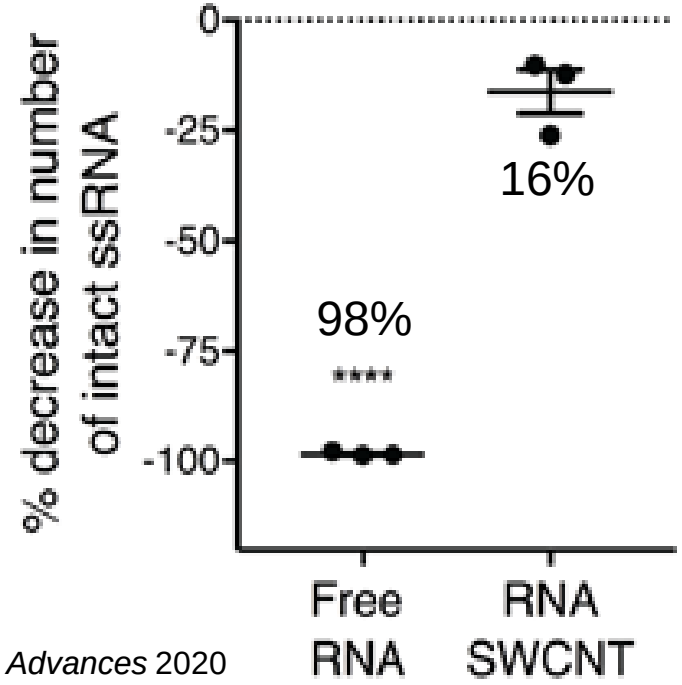
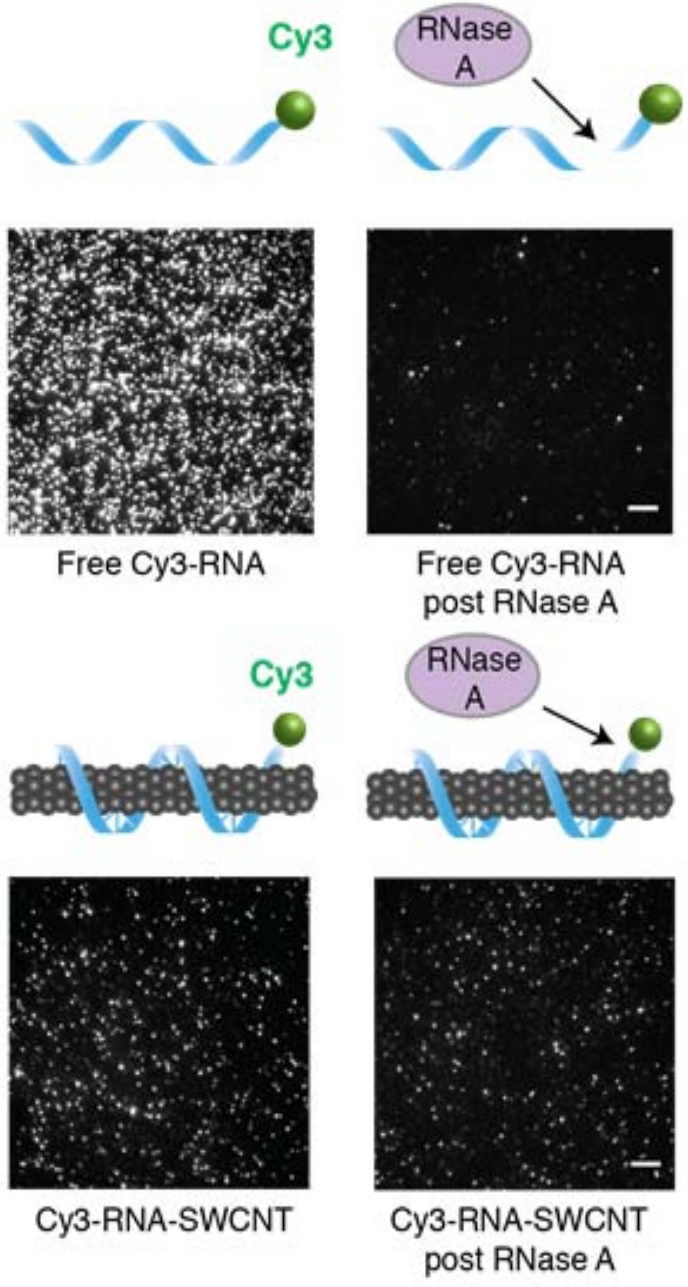
# RNA Adsorption on CNTs Protects RNA From Nuclease Degradation



Total internal reflection fluorescence microscopy

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# A More Fundamental Question

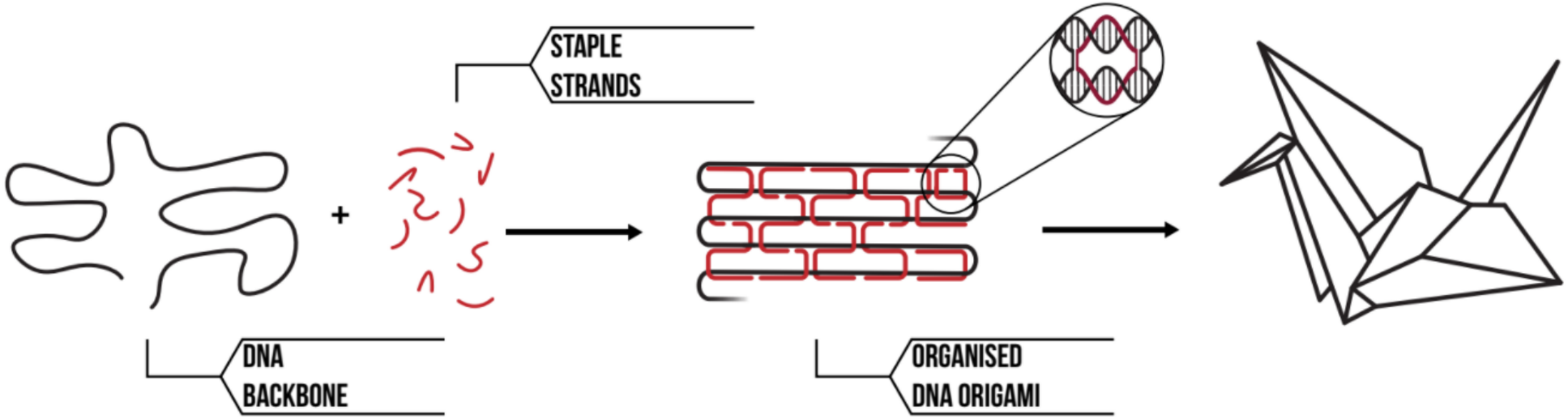

How do nanoparticle parameters affect plant cellular uptake?

Dr. Huan Zhang

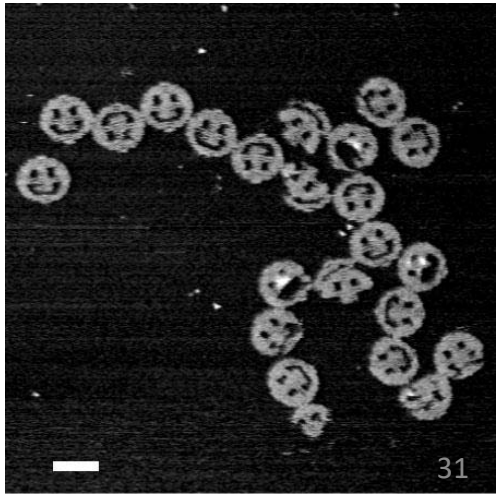
# Does Delivery Extend to Other Nanoparticle Types?

*2006 - Paul Rothemund shows long ssDNA template can be 'stapled' into a 3D structure with shortDNA staple strands*

→ Generation of 3D nanostructures with excellent control over size, shape, rigidity

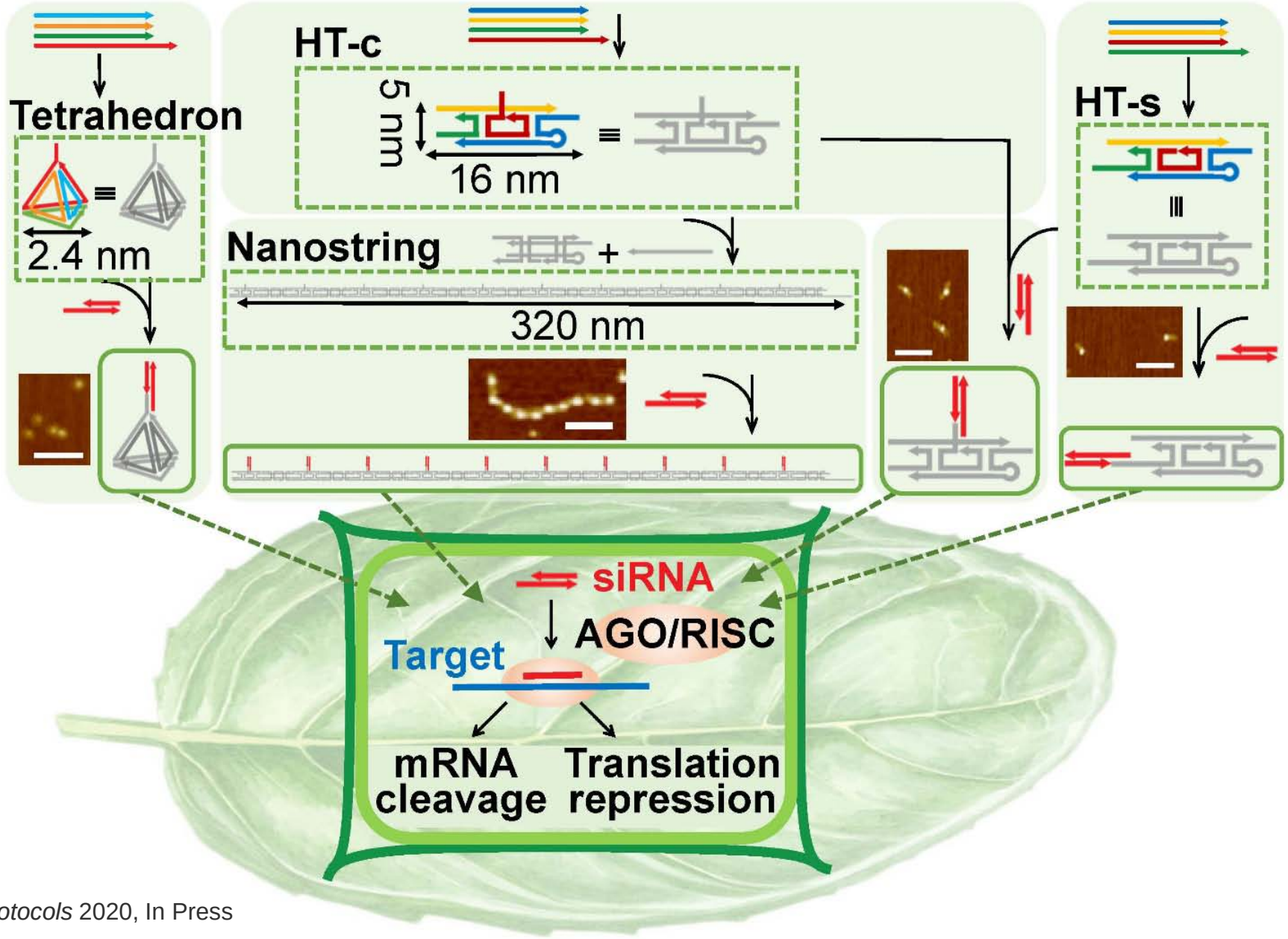






Rothemund, *Nature* (2006)

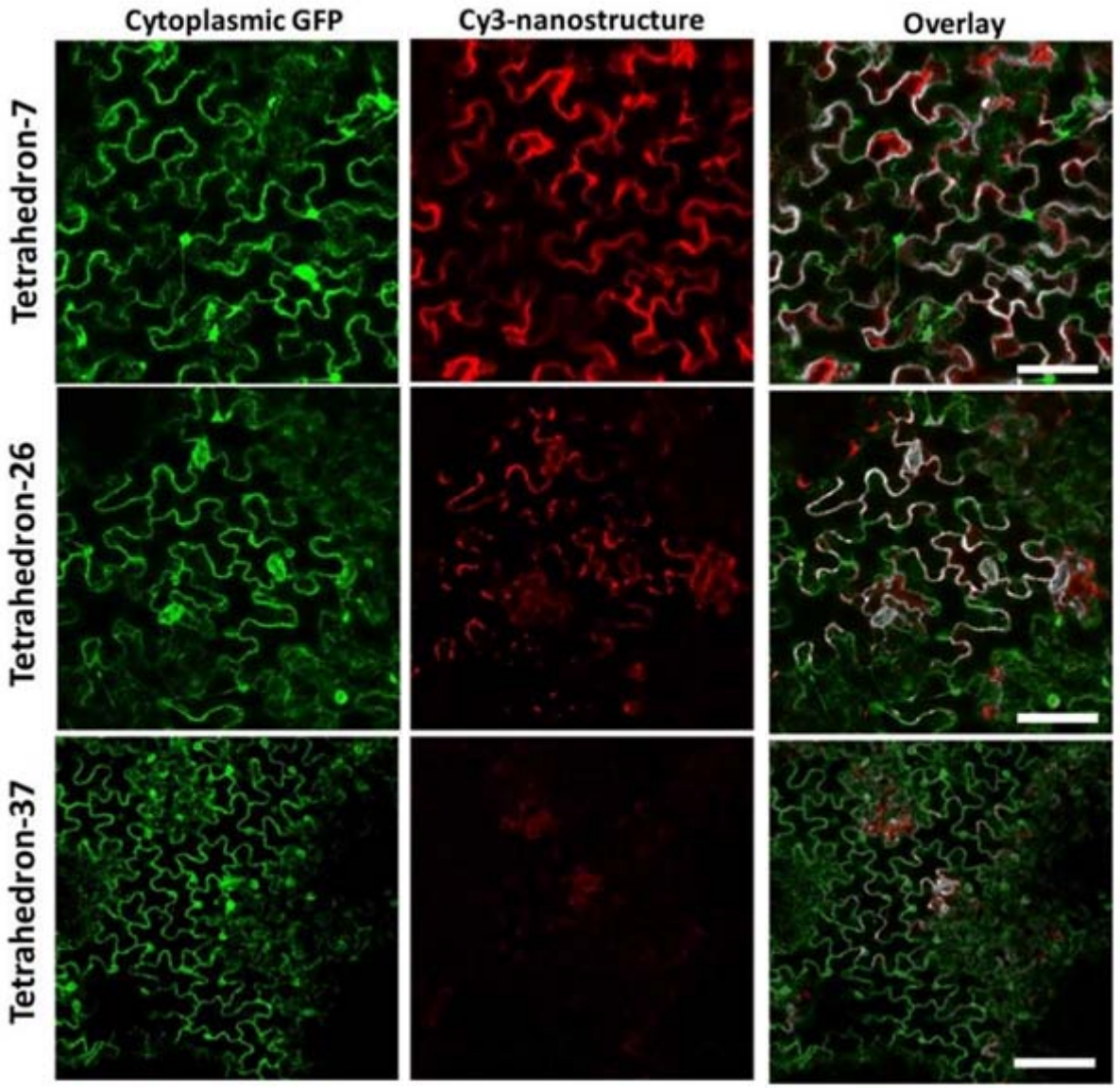
# DNA Nanostructures to Probe Plant Cell Wall Internalization Mechanism



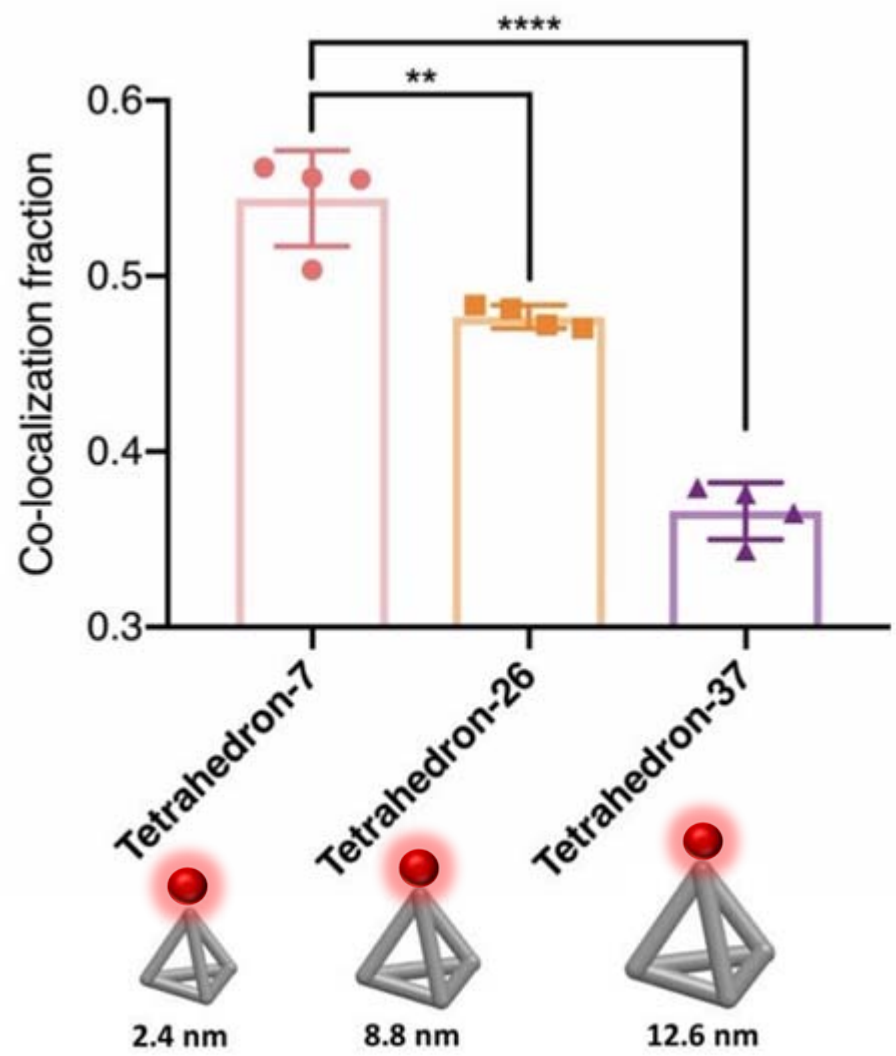

Zhang *et al. Nature Protocols* 2020, In Press

# Size-Dependent DNA Nanostructure Internalization

## Transgenic mGFP5 Benthamiana

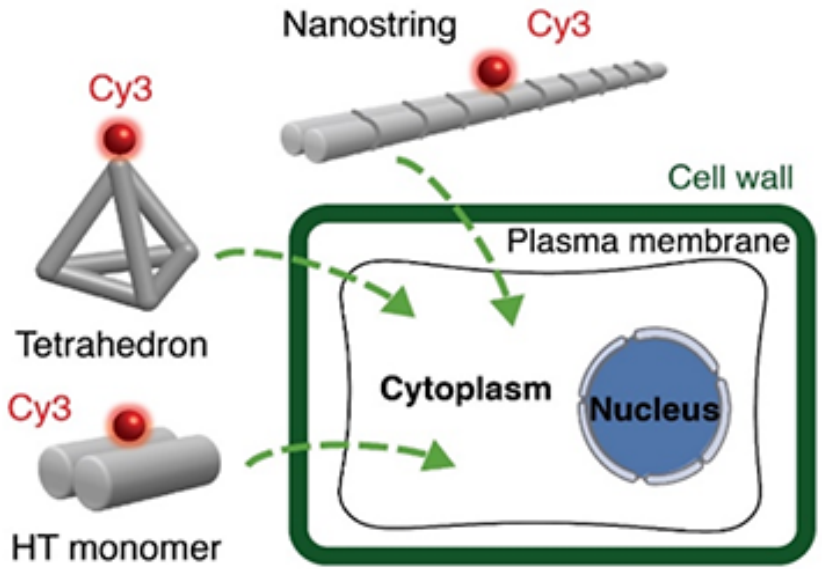


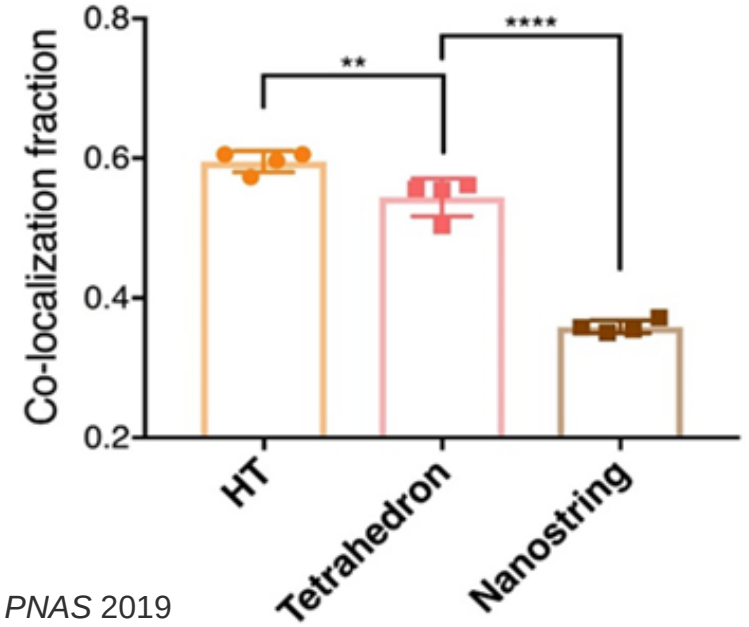

Zhang *et al. PNAS* 2019

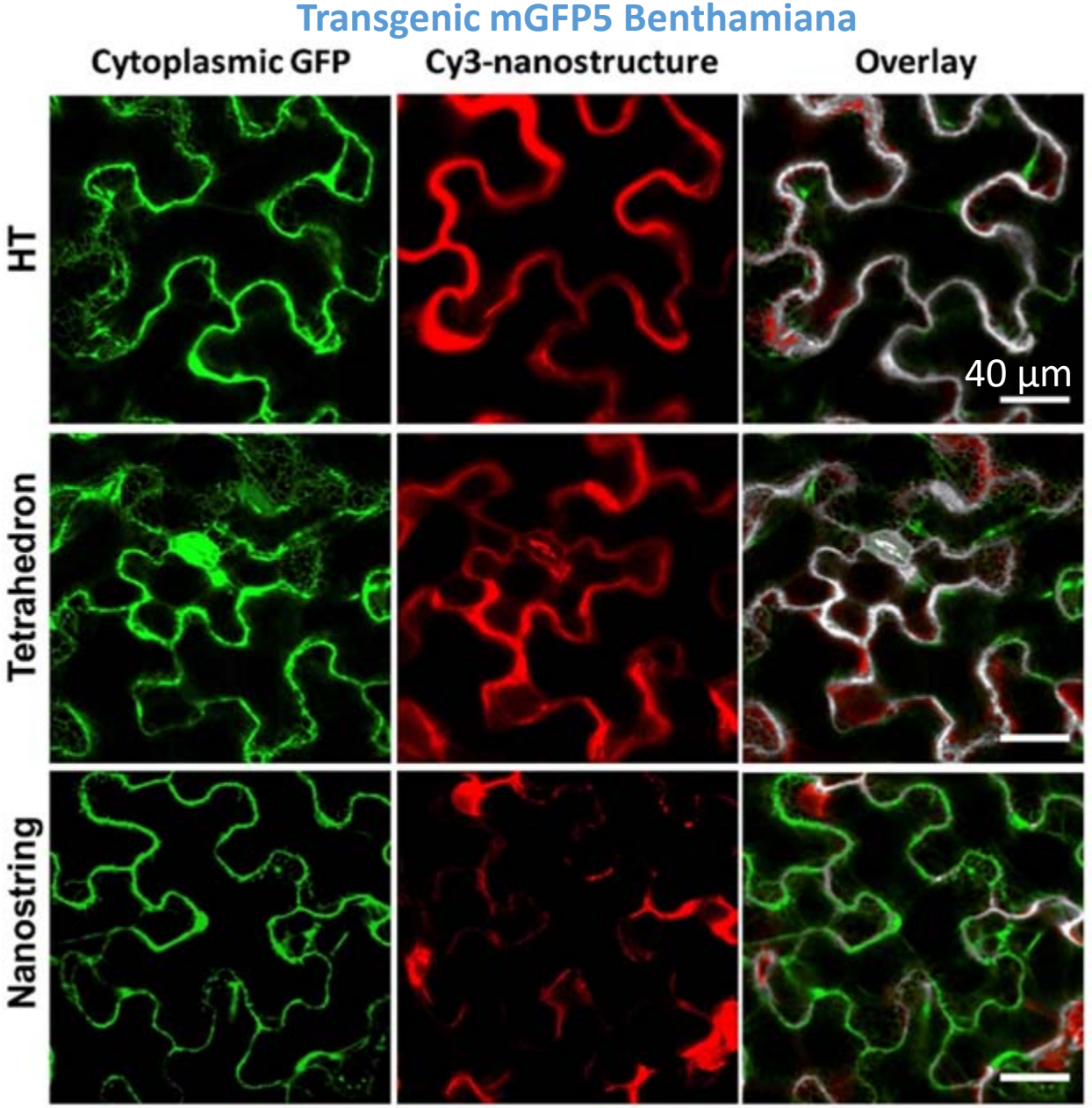



Zhang *et al. Nature Protocols* 2020, In Press

# Monomer and Tetrahedron Internalize into Plant Cells – But Not Nanostrings

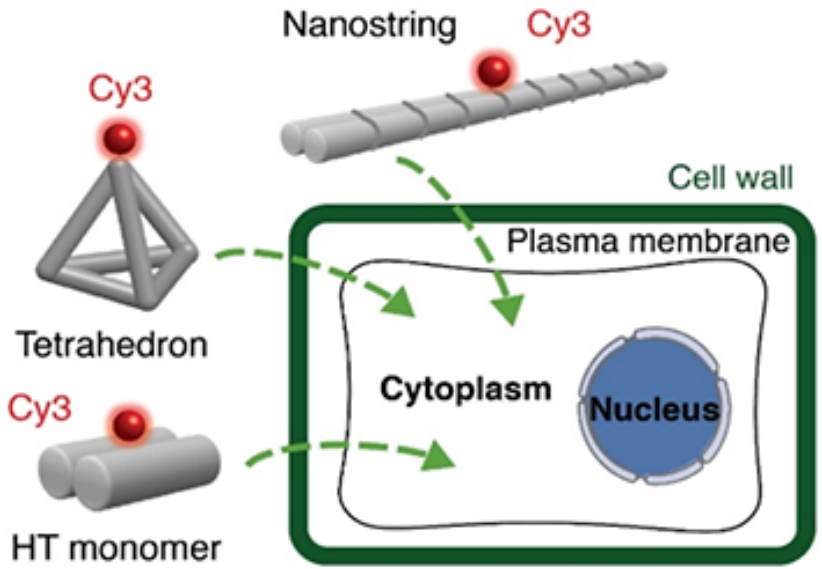


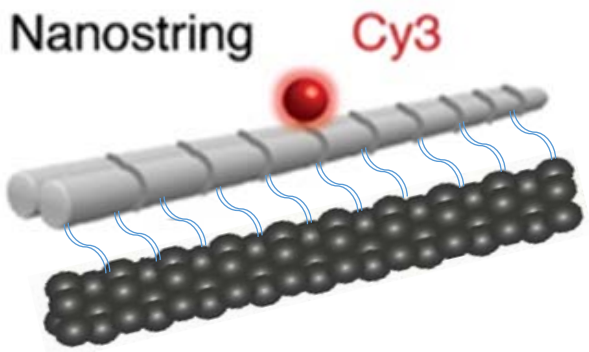



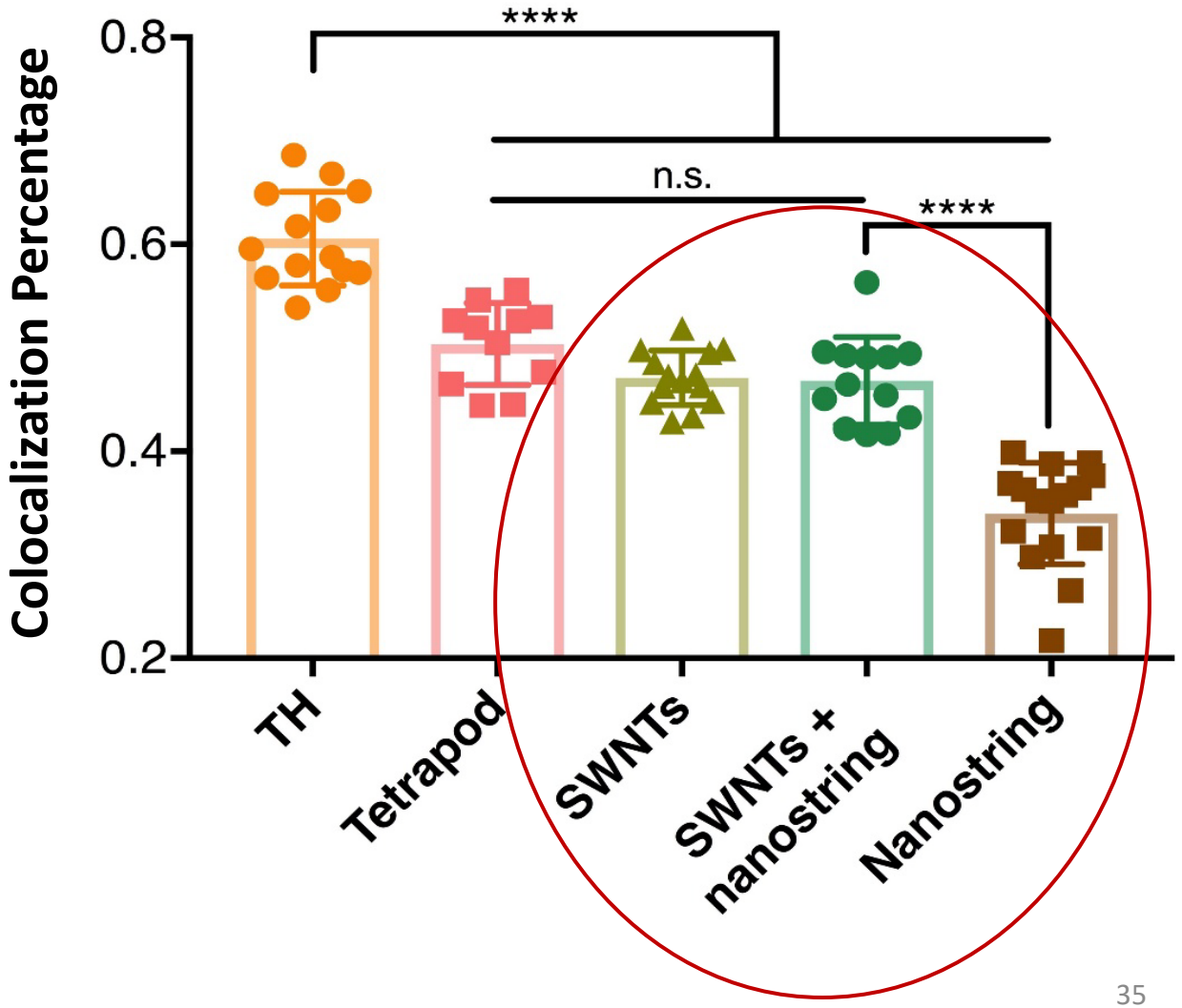




Zhang *et al. PNAS* 2019

# Nanostrings Internalize if Tethered to a Rigid Single Wall Carbon Nanotube (SWNT)

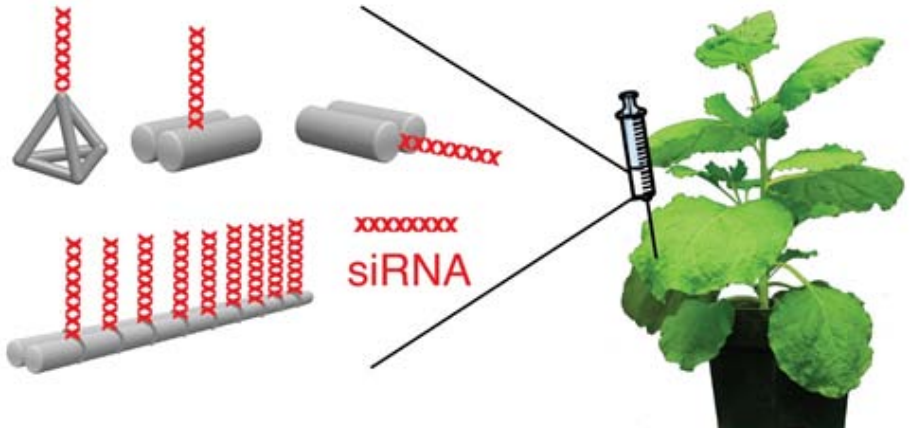


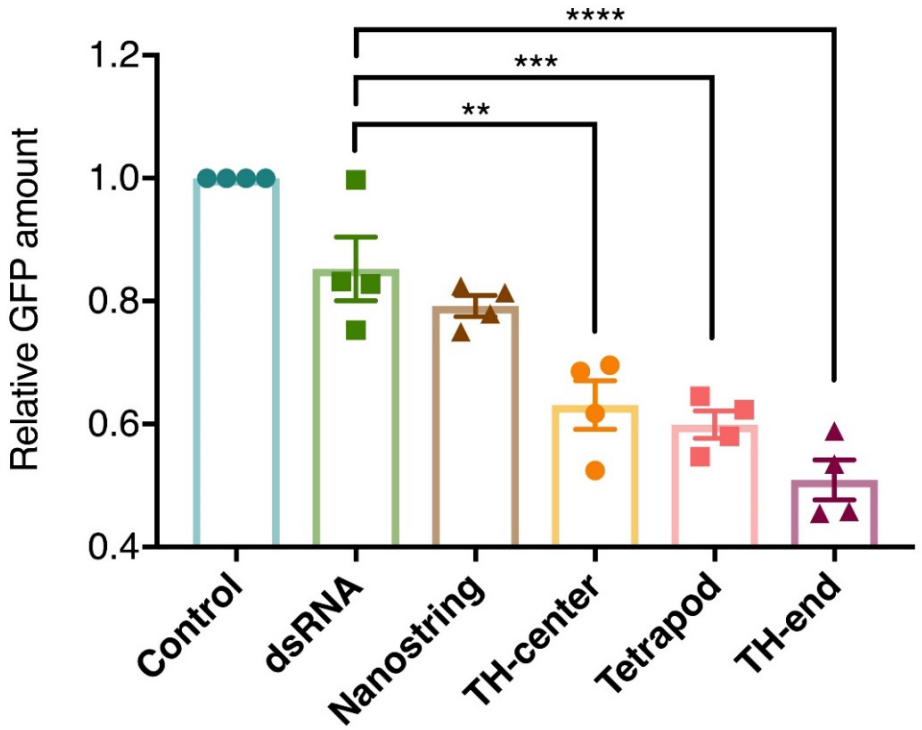



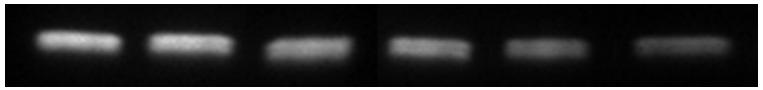




Zhang *et al. PNAS* 2019

# DNA Nanostructure Geometry Affects siRNA Silencing Efficiency & Pathway

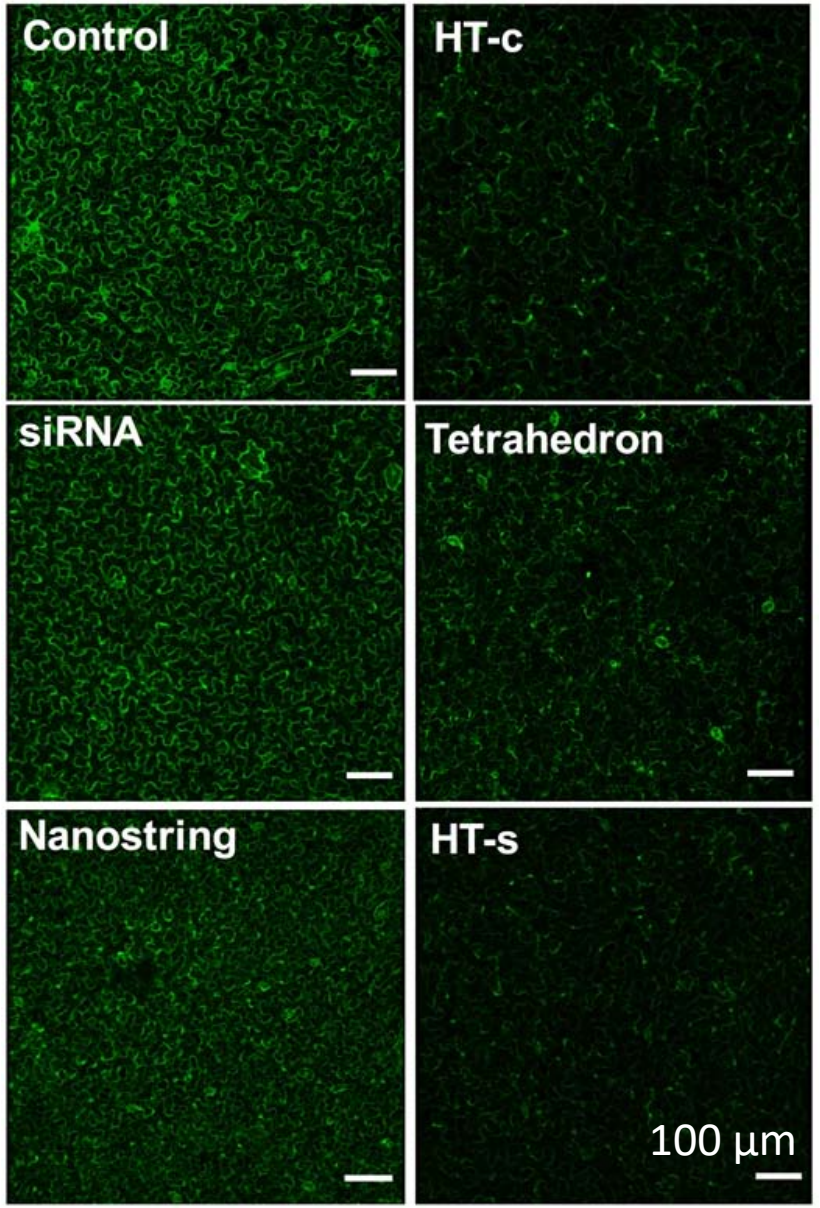


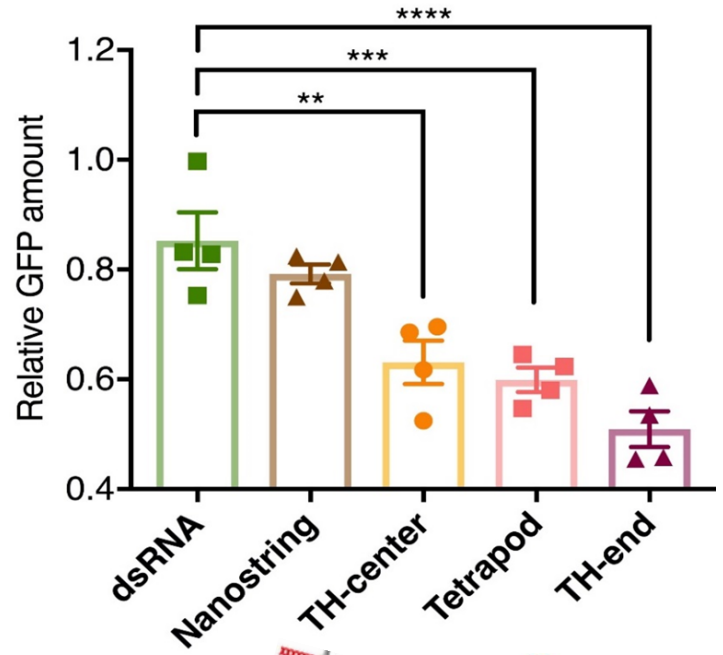



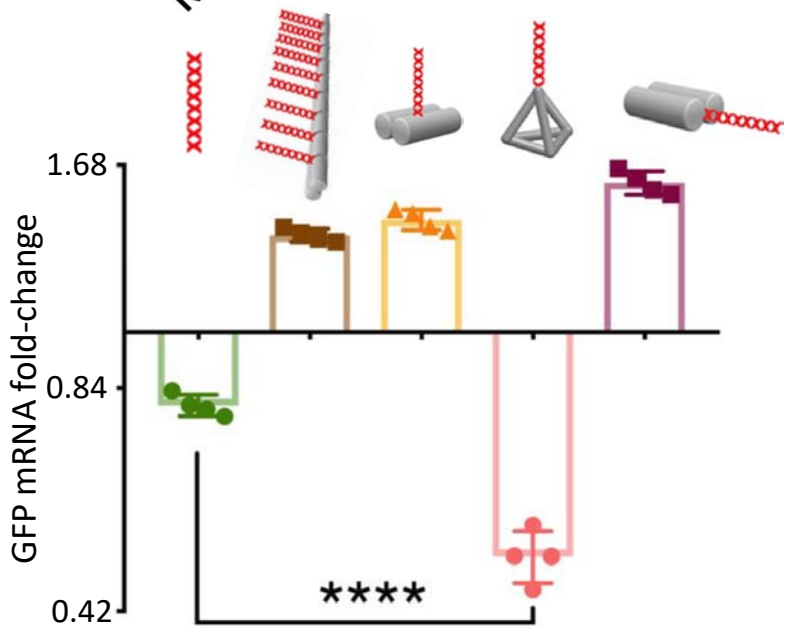
Zhang *et al. PNAS* 2019

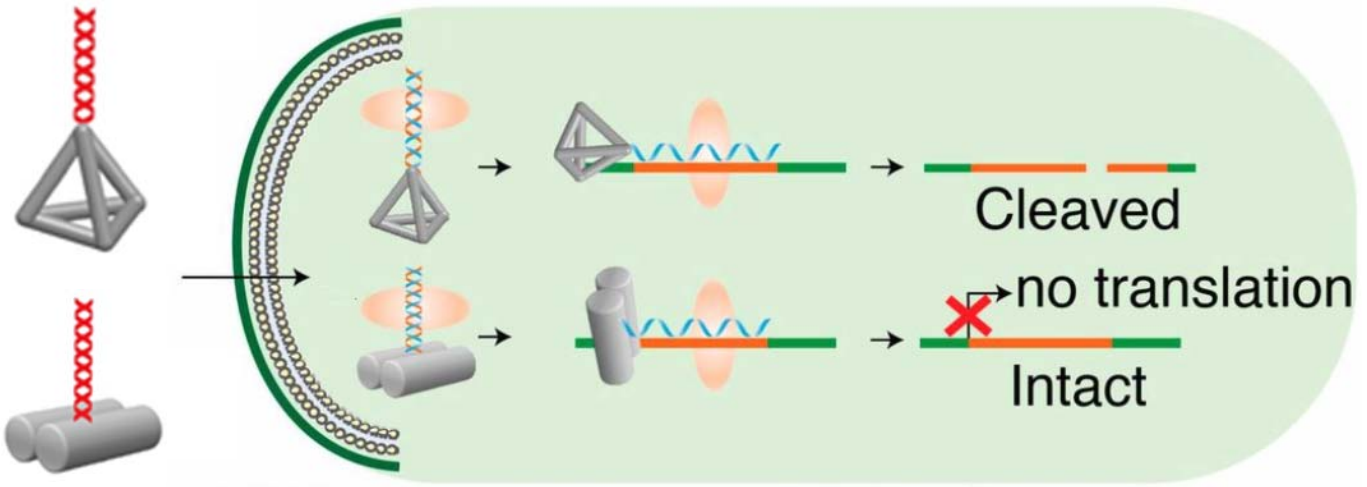
*GFP silencing in mGFP5 Benthamiana*



# Attachment Locus Affects Gene Silencing Pathway



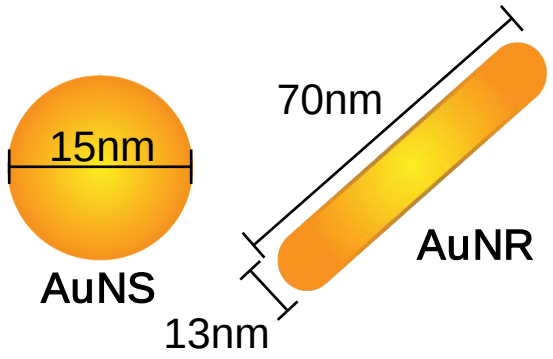



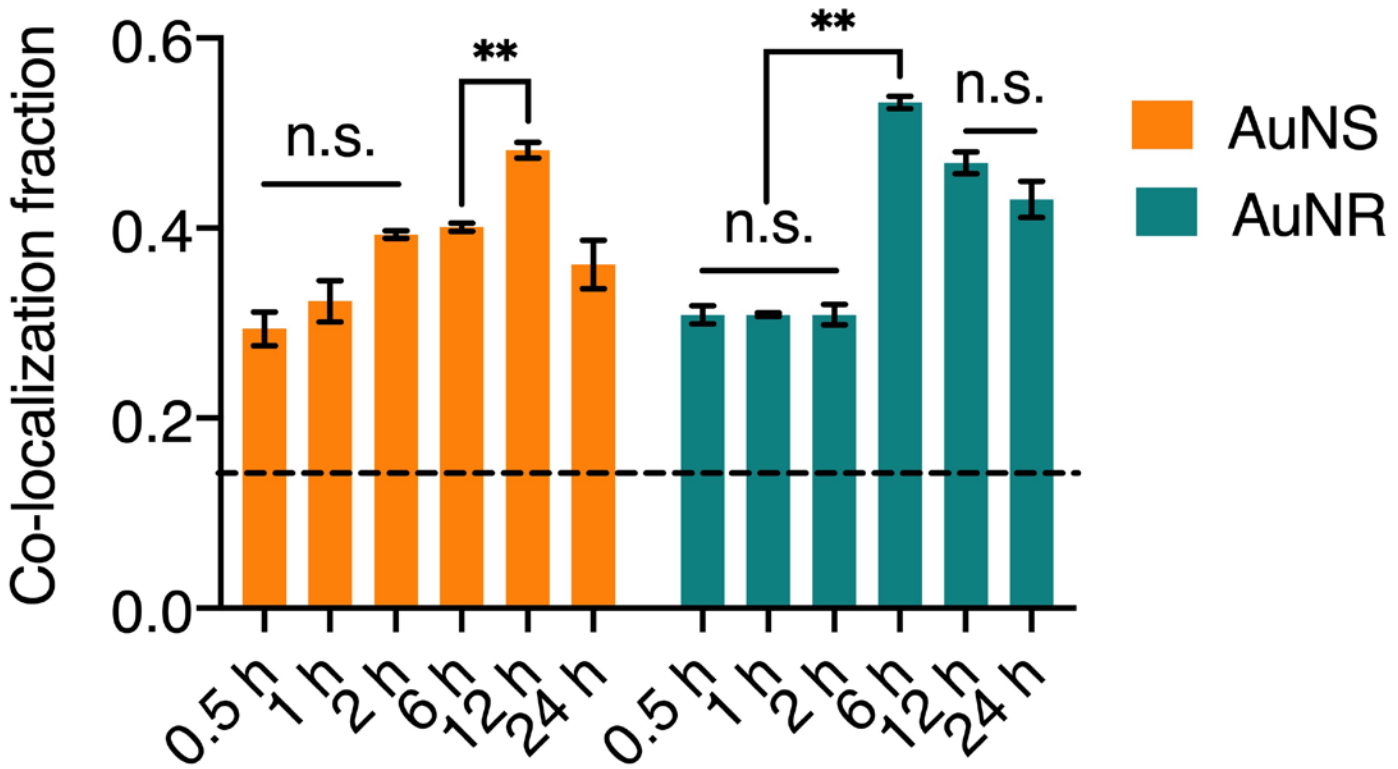

← *Post-transcriptional gene silencing*

← *Transcriptional gene silencing*

Zhang *et al. PNAS* 2019

# What About Internalization Timescales? Effect of Shape & Size

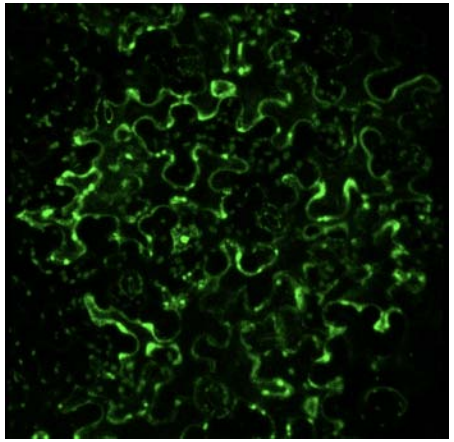


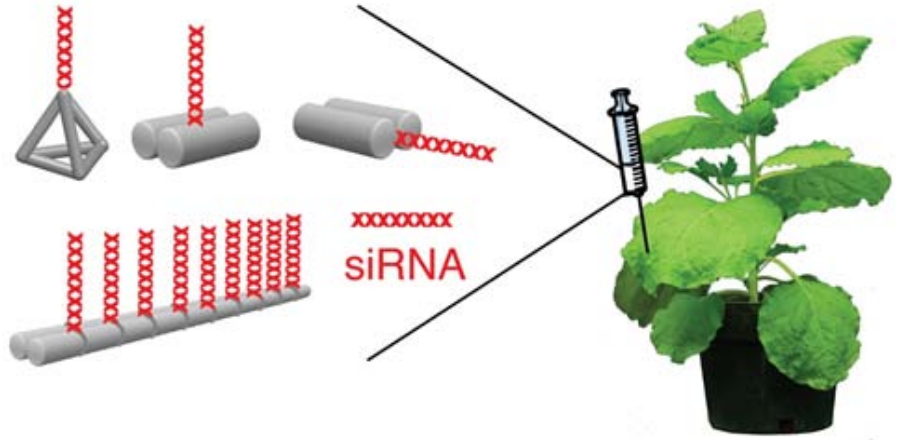

*Unpublished*

# Summary of DNA and RNA Delivery to Plants

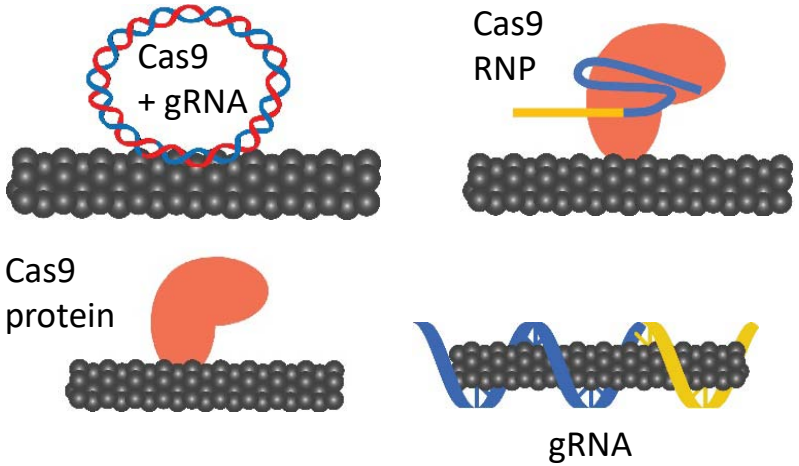
## DNA Delivery



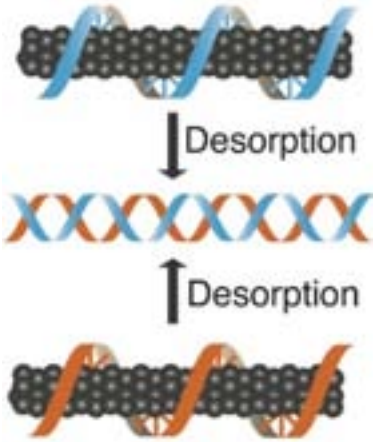
## RNA Delivery




### Force-independent nanotube transformation




### DNA-Free Gene Silencing




Demirer, Zhang *et al. Nature Protocols* (2019)

Zhang *et al. Nature Protocols* 2020, In Press

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Summer 2022


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Ami Thakrar

Cindy Zhou

BASF We create chemistry

BIOHUB

hhmi Howard Hughes Medical Institute

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BRAIN & BEHAVIOR RESEARCH FOUNDATION Awarding NARSAD Grants

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NIH MIRA R35

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Berkeley Neuroscience HELEN WILLS NEUROSCIENCE INSTITUTE

GORDON AND BETTY MOORE FOUNDATION



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