Characterizing CRISPR-Cas ribonucleoprotein delivery with bioreducible polymer nanoparticles

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CRS 2025 ANNUAL MEETING & EXPOSITION
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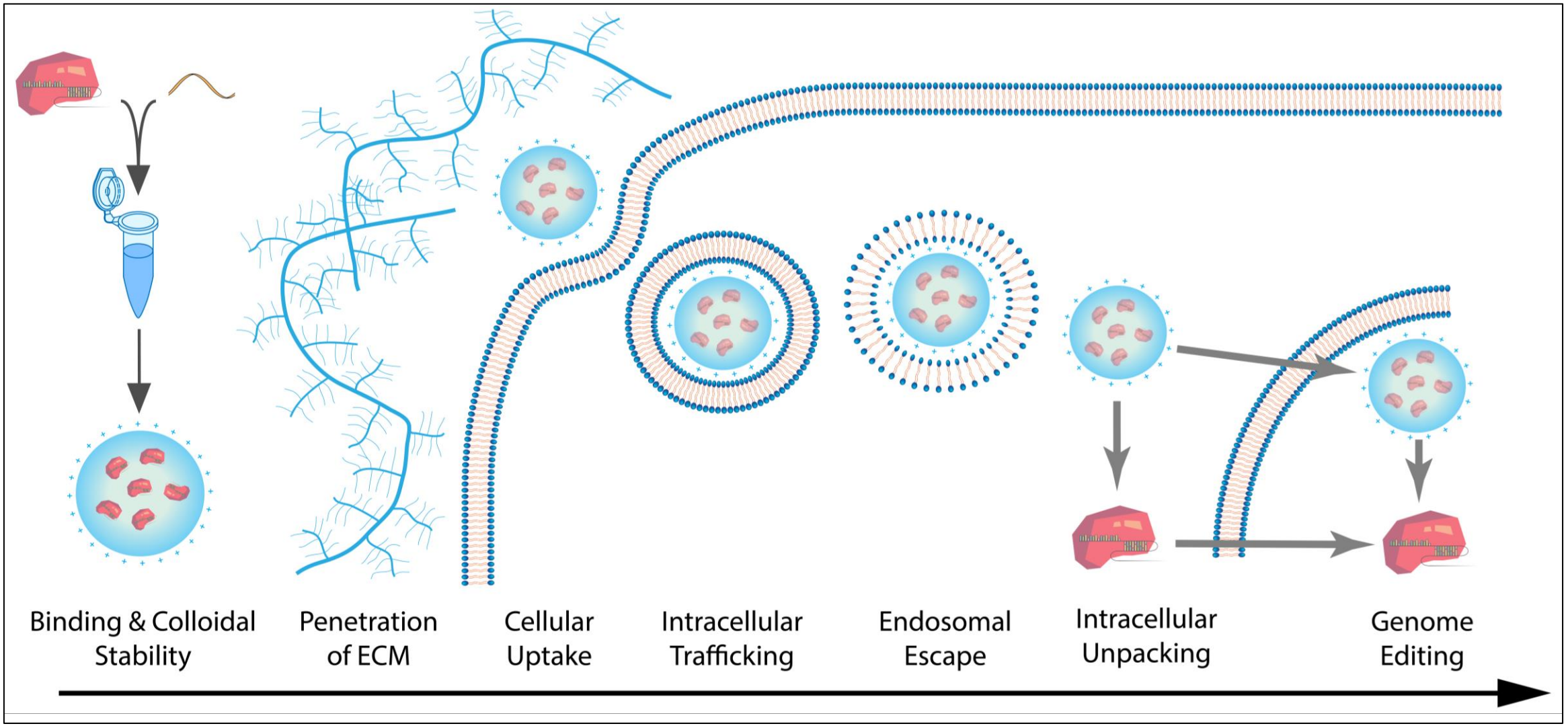
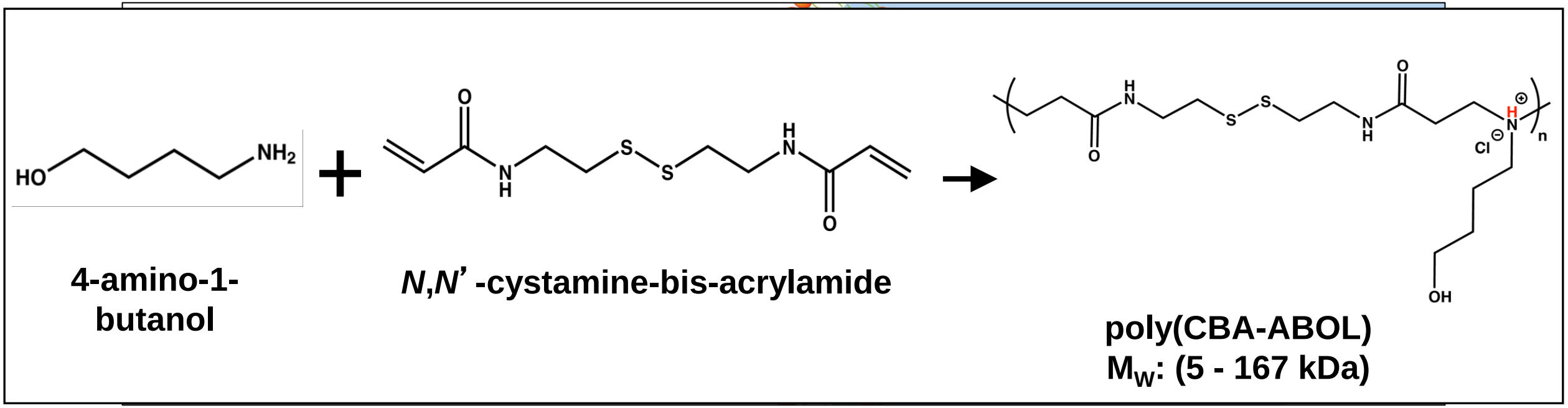
NEXT-GENERATION DELIVERY INNOVATIONS

Nonviral Delivery



Grigsby and Leong *J R Soc Interface* (2010)

poly(CBA-ABOL)



4-amino-1-butanol

***N,N'*-cystamine-bis-acrylamide**

poly(CBA-ABOL)
M_W: (5 - 167 kDa)

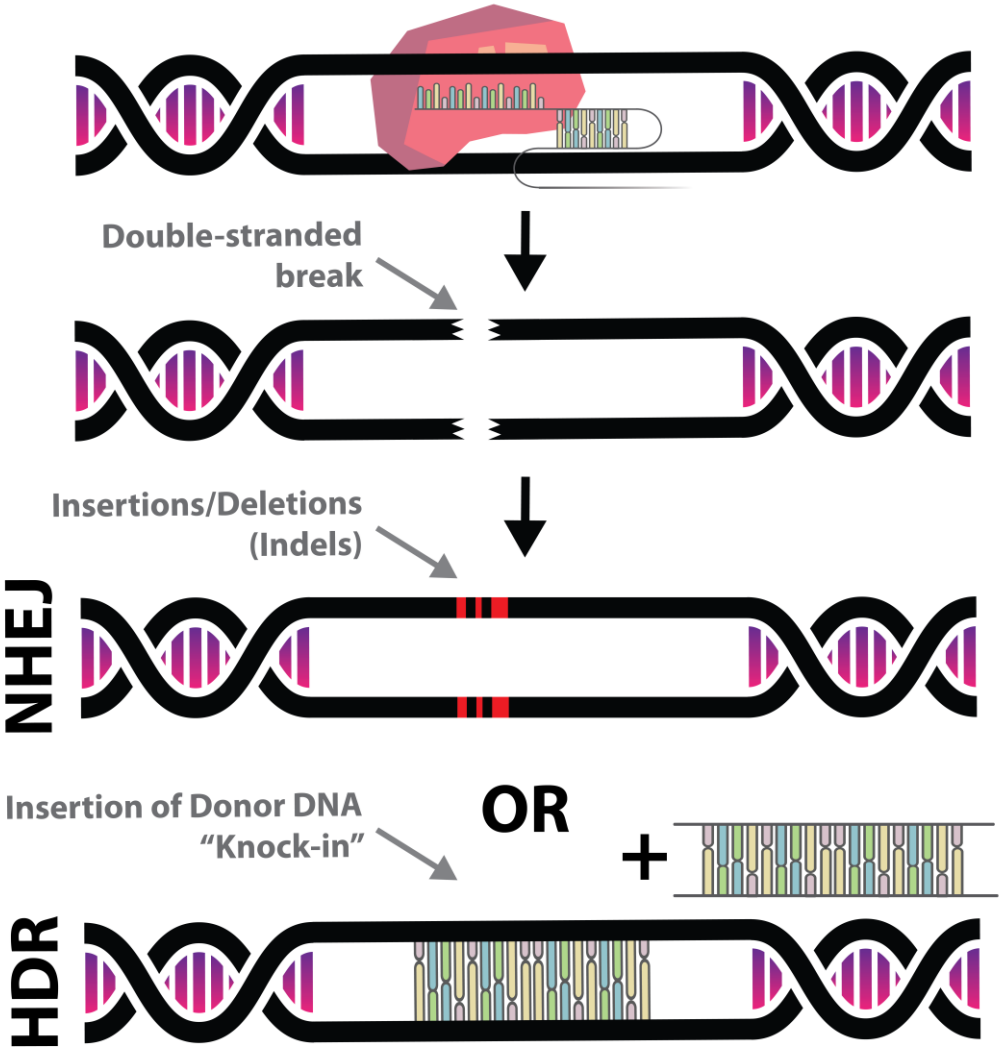
[GSH] = 2 – 20 μM [GSH] = 0.5 – 10 mM

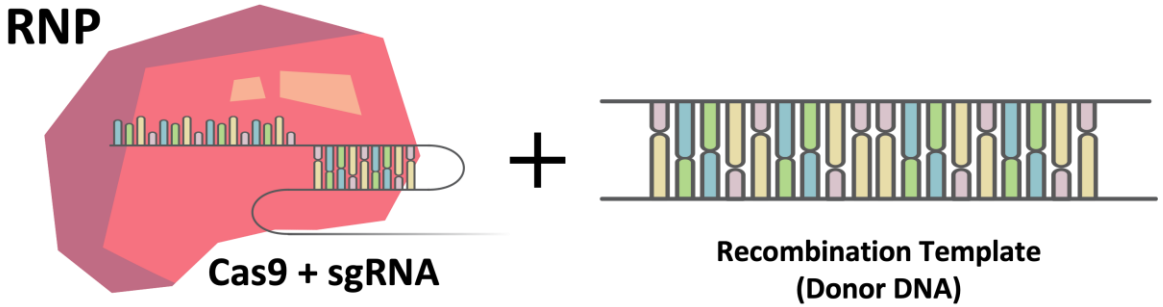
Peptidomimetic linear polymer with bioreducible disulfide bonds incorporated into the backbone

Unpackaging: Bioreducibility promotes intracellular unpacking while diminishing cytotoxicity

Endosomal escape: High buffering capacity promotes endosomal escape via the proton sponge effect

CRISPR-Cas Mechanisms

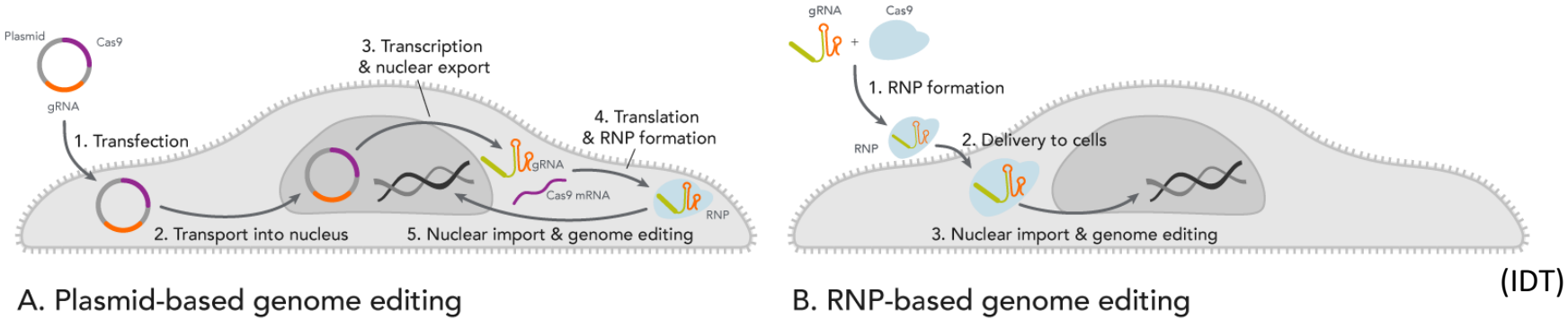




Also…

- Base editors
- Prime editors
- Gene activators/repressors
- Etc., etc., etc….

Why RNPs?



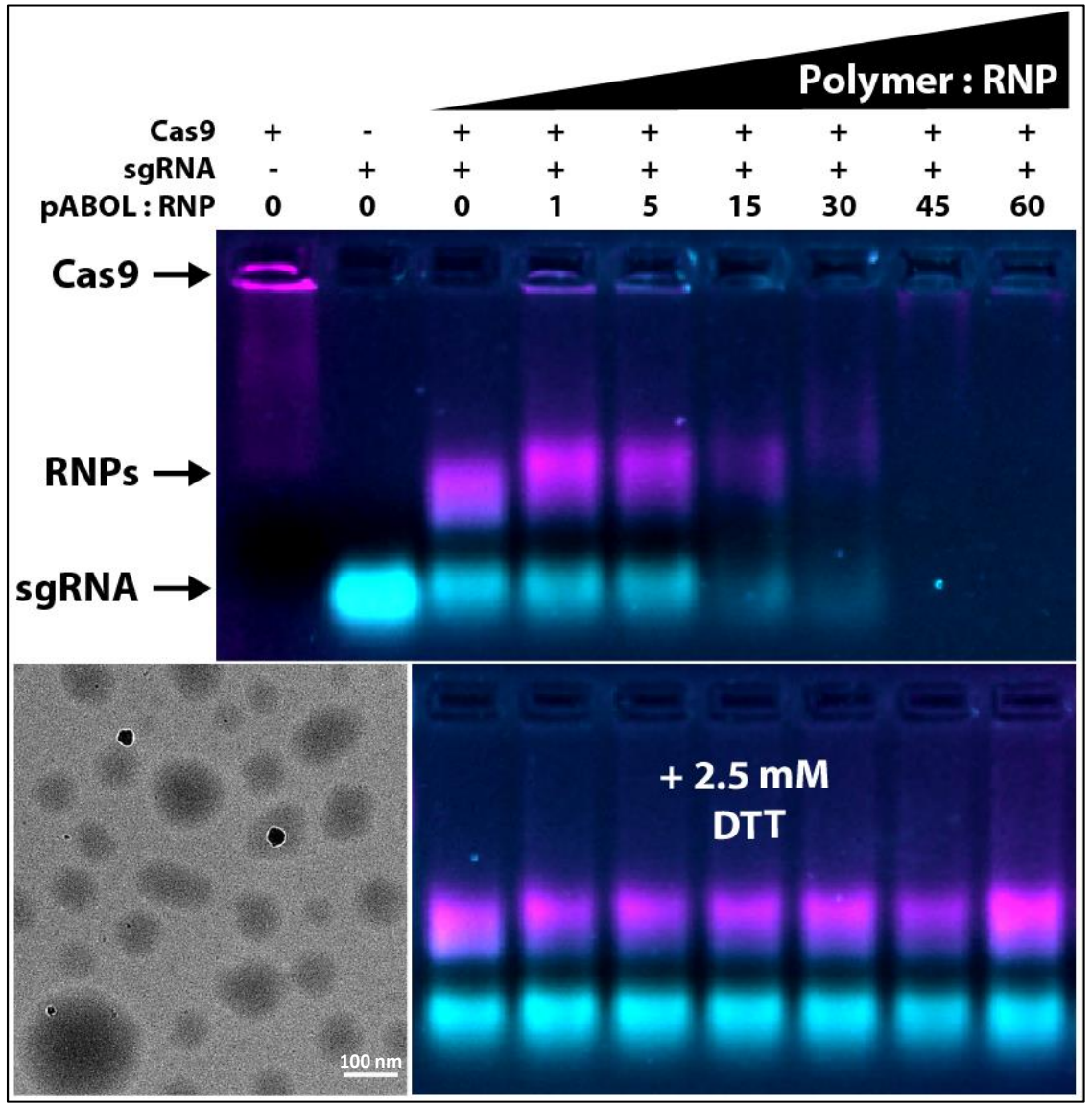
A. Plasmid-based genome editing

B. RNP-based genome editing

(IDT)

- RNP = ribonucleoprotein → Cas9 + sgRNA
- Eliminates potential for plasmid recombination
- Shorter period of activity may limit off-target effects
- Typically more efficient in slow growing cells – NLS?
- 45 CRISPR trials ongoing: 24 ex vivo (e.g. CAR-T), 4 in vivo (1 DNA in gel, 1 AAV, 2 mRNA in LNPs), in vitro diagnostics

Complexation & Release

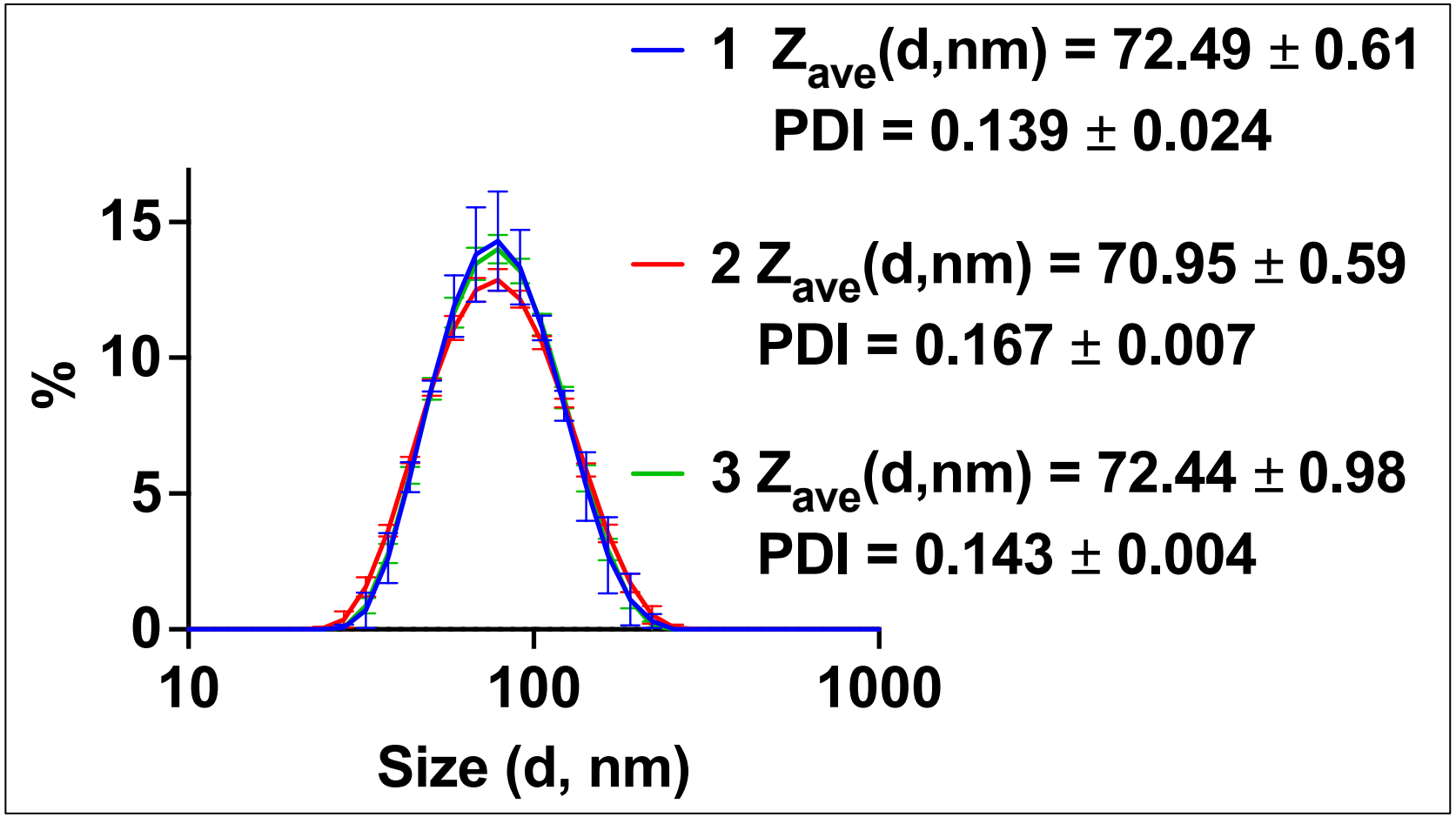


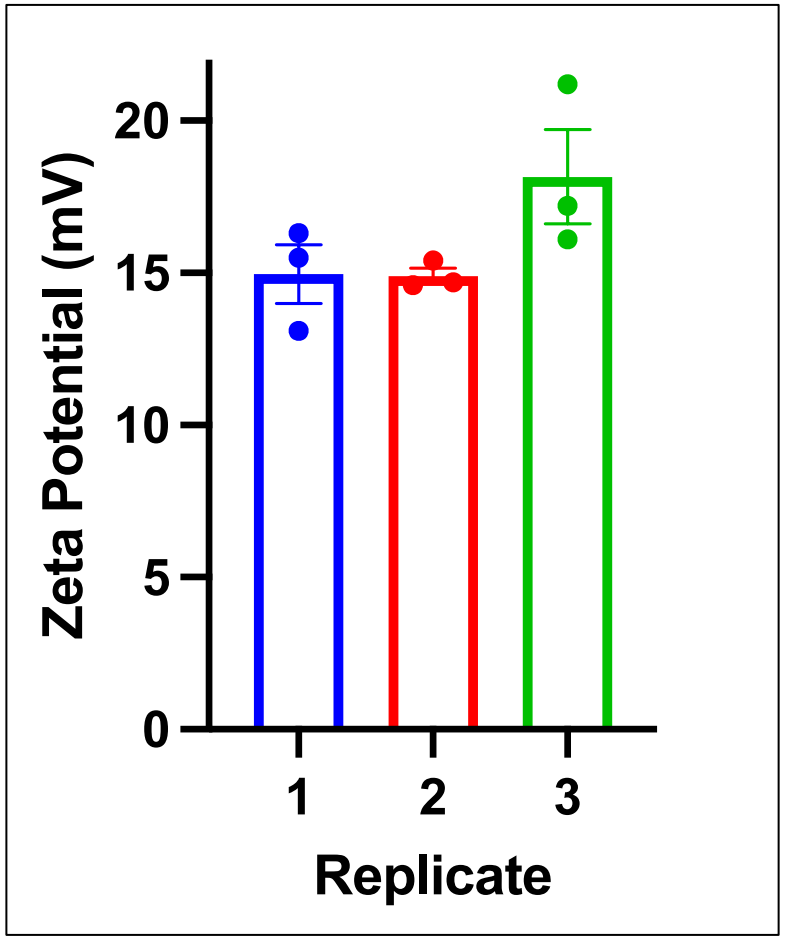
Polymer fully complexes RNPs with net negative charge @ 45:1 mass ratio

Upon reduction, rapid and complete release of RNP payload is triggered

Self-assembly produces homogeneous nanoparticles ~100 nm

Size & Charge

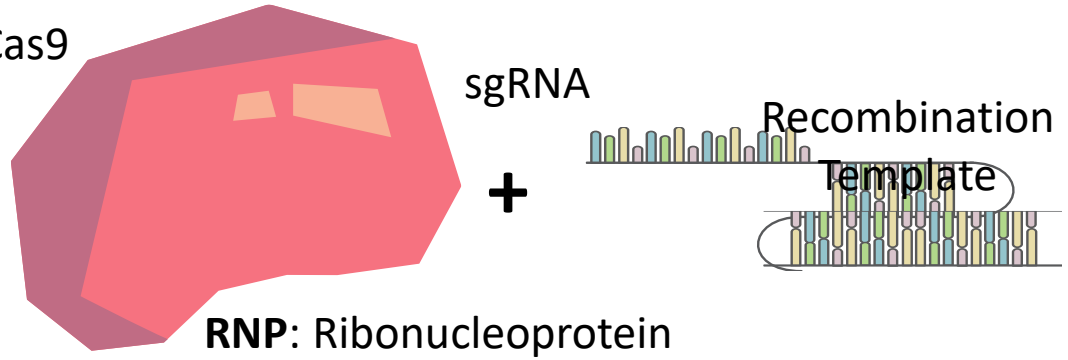


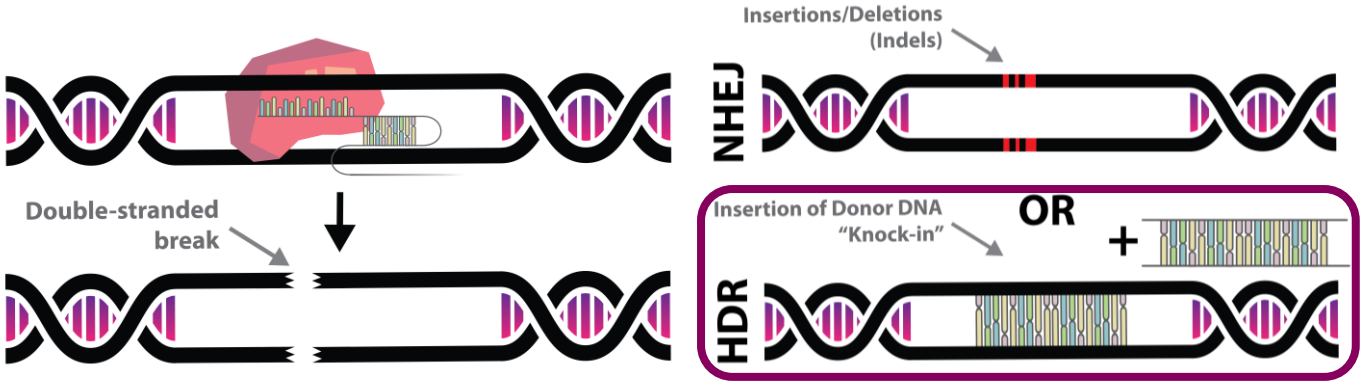


Cas9 alone → 15 nm and +2 mV (isoelectric point ∽ pH = 8)

RNPs → 27 nm and -30 mV

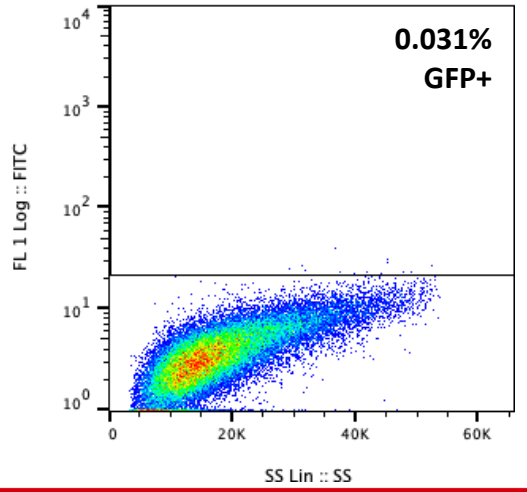
Generating Reporter Cells



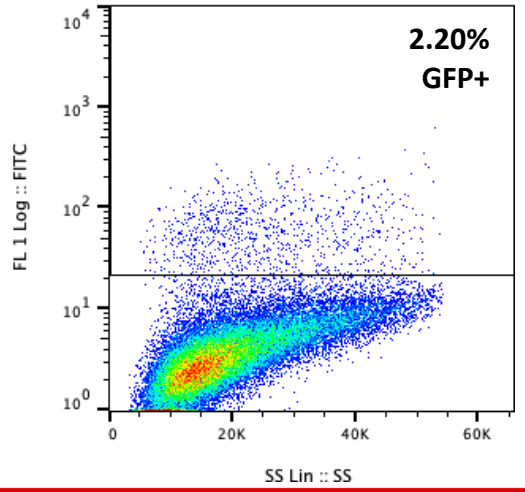


Goal: Knock in a copy of GFP to the C-terminus of human 60S ribosomal protein L24 (*Rpl24*) via homology-directed repair (HDR) in SK-N-BE(2) cells

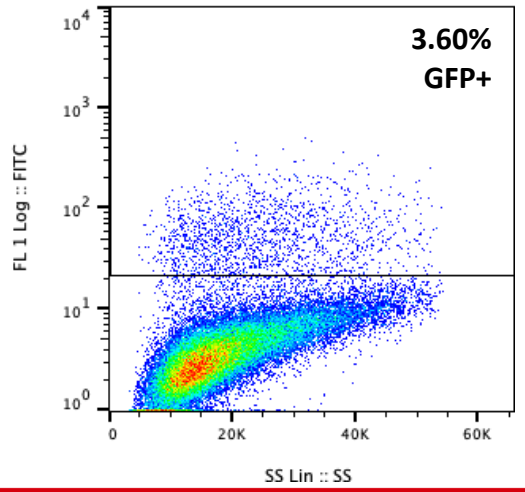
Control

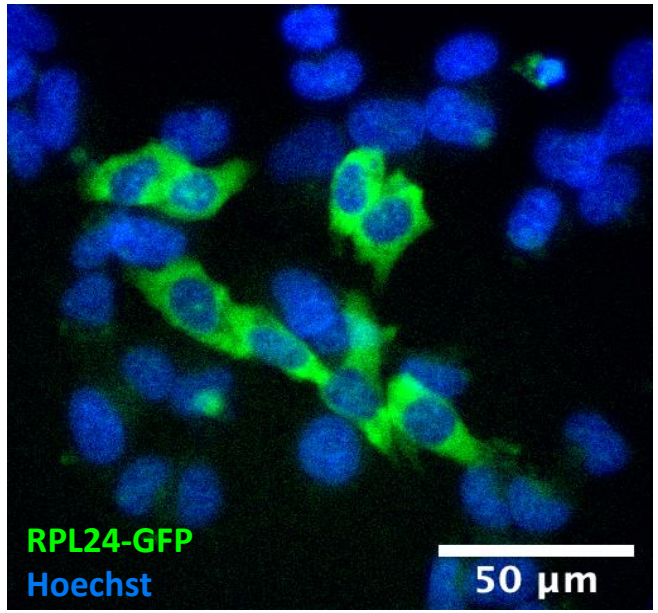


CRISPRMAX™

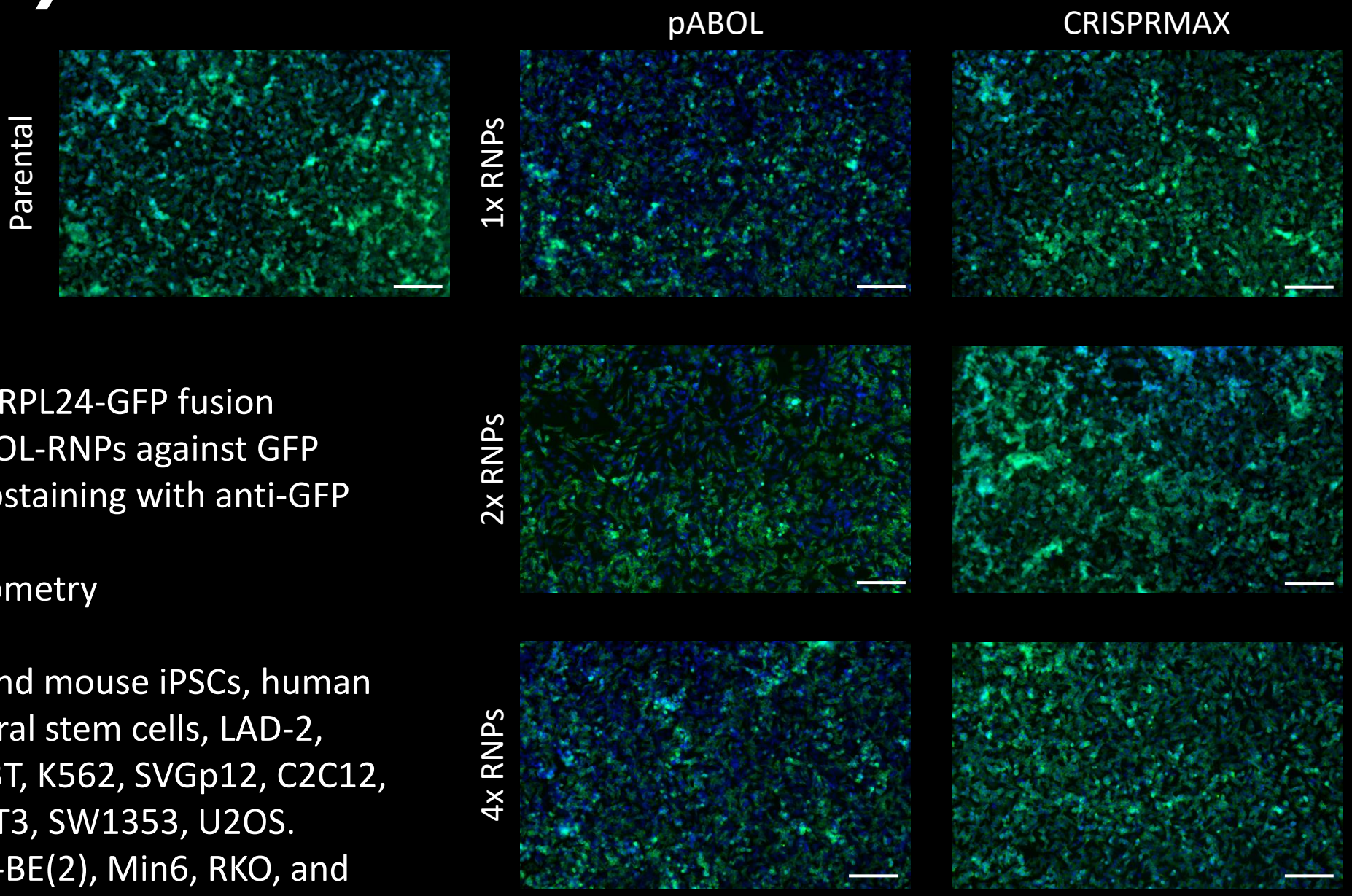


pABOL





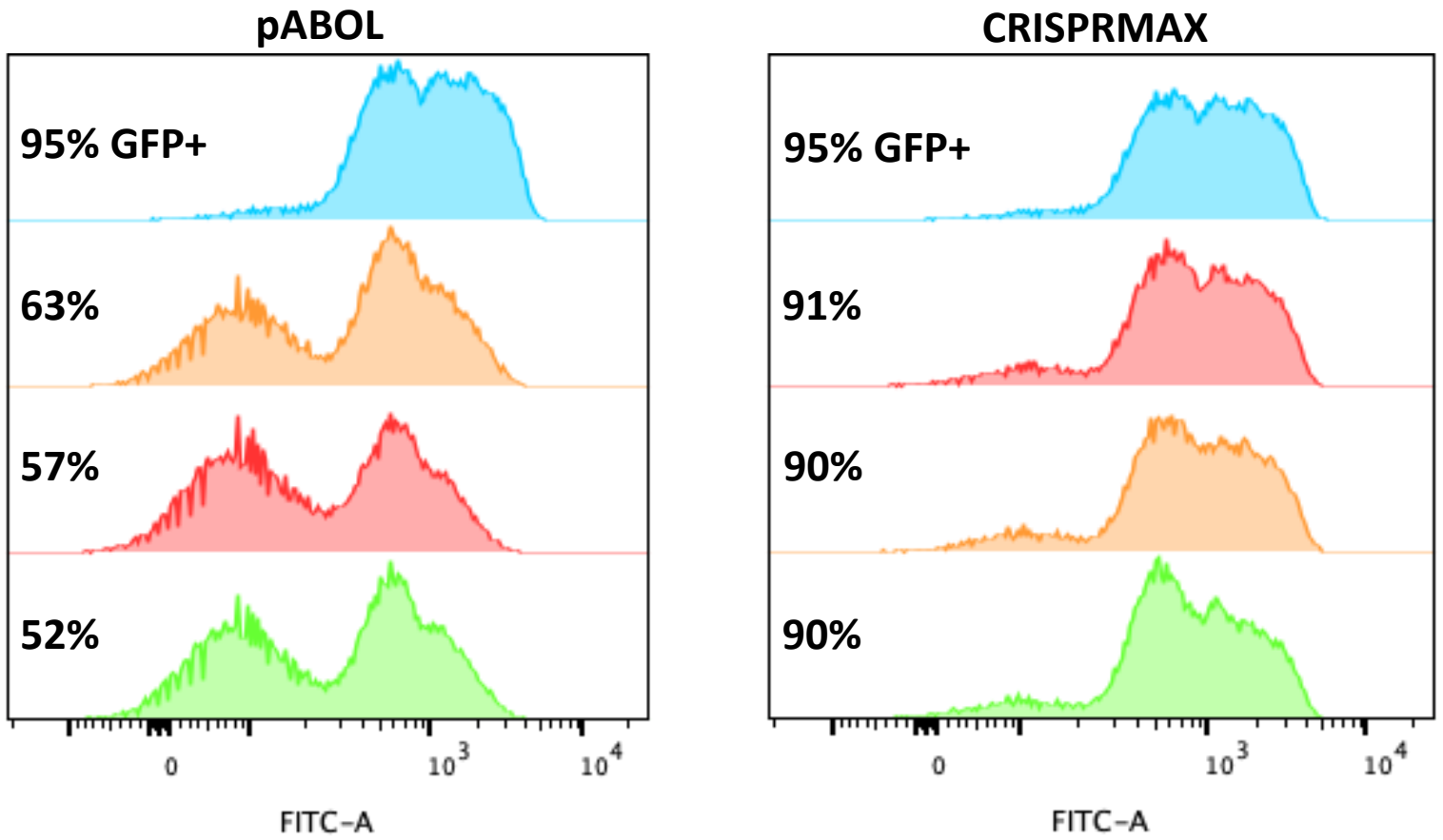
SK-N-BE(2) Cells

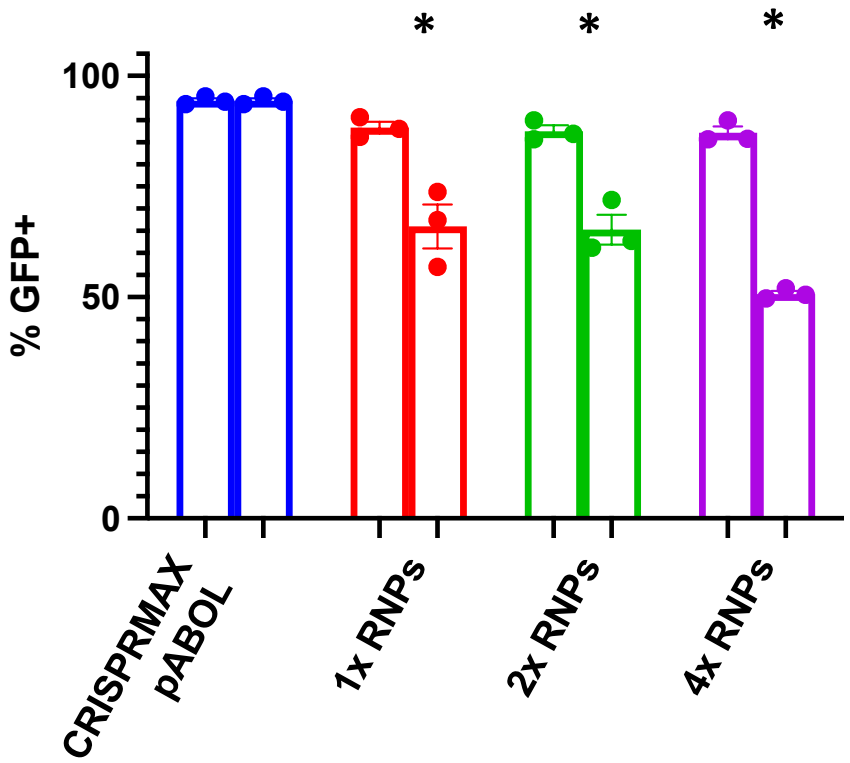


- SK-N-BE(2) cells with RPL24-GFP fusion transfected with pABOL-RNPs against GFP
- Imaged after immunostaining with anti-GFP antibody and DAPI
- Analyzed by flow cytometry

- Also tested: human and mouse iPSCs, human and mouse ESCs, neural stem cells, LAD-2, hTERT-RPE1, HEK-293T, K562, SVGp12, C2C12, Neuro-2A, C6, BALB3T3, SW1353, U2OS. HCT116, PC-3U, SK-N-BE(2), Min6, RKO, and SW480 cells

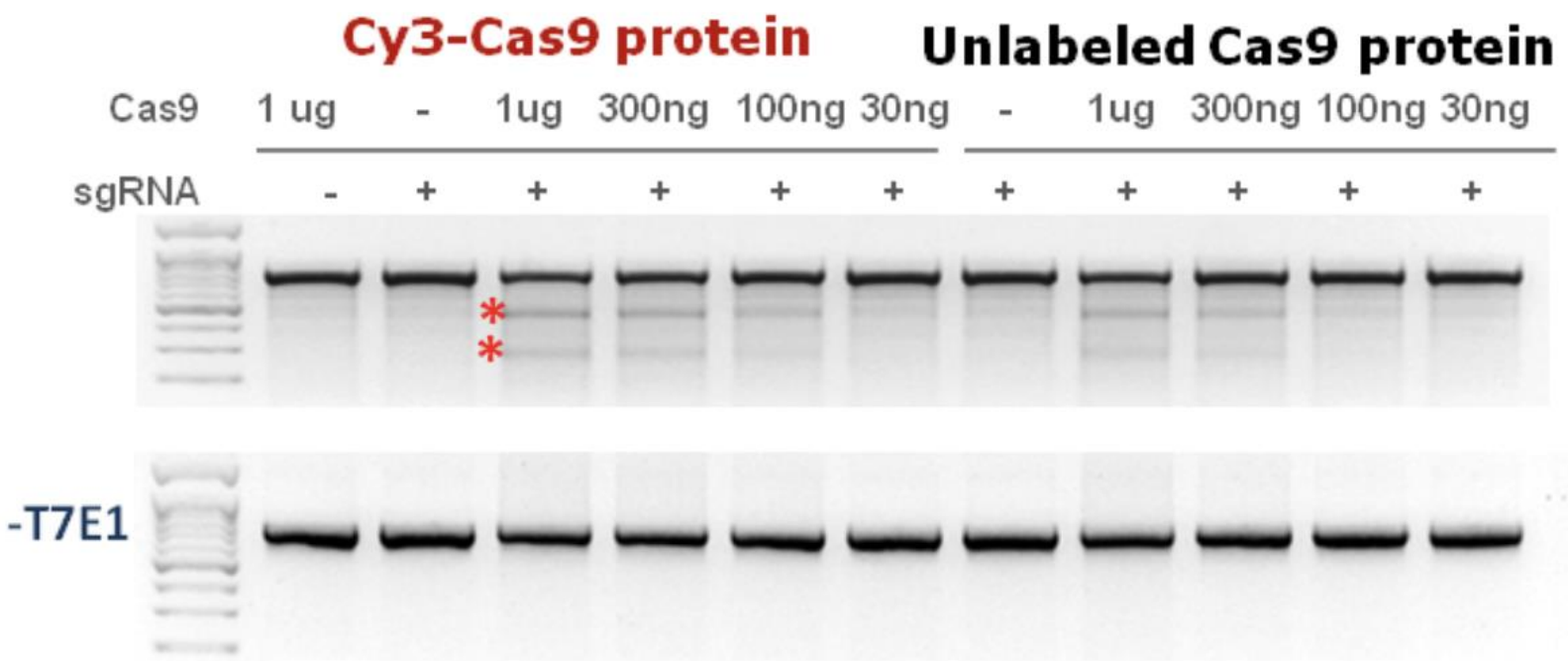
Flow Cytometry

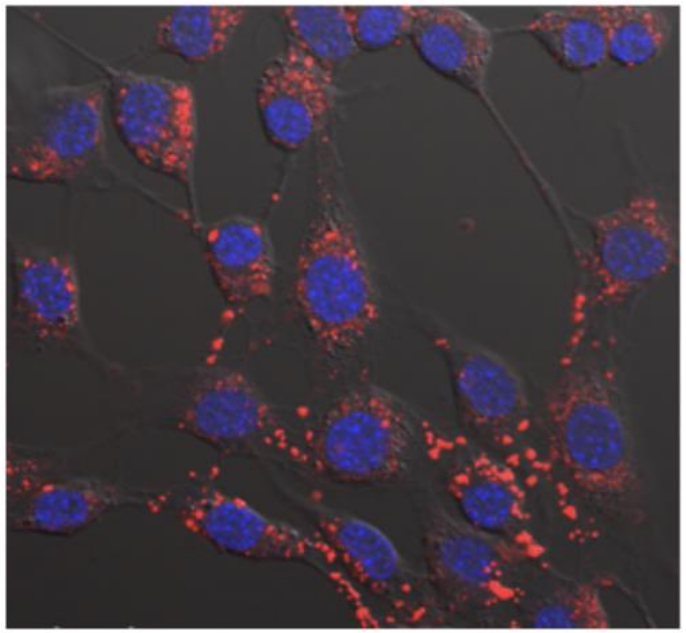




- pABOL outperforms lipoplex CRISPRMAX at each dose with increasing efficacy
- No dose dependence in this range for CRISPRMAX

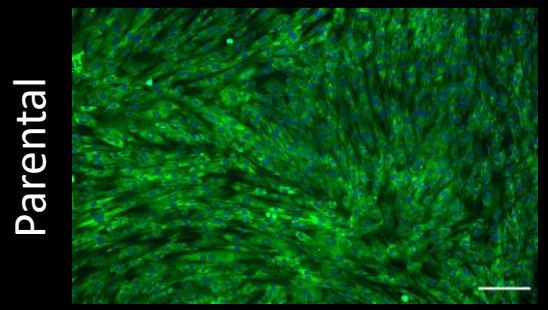
Delivery Mechanisms

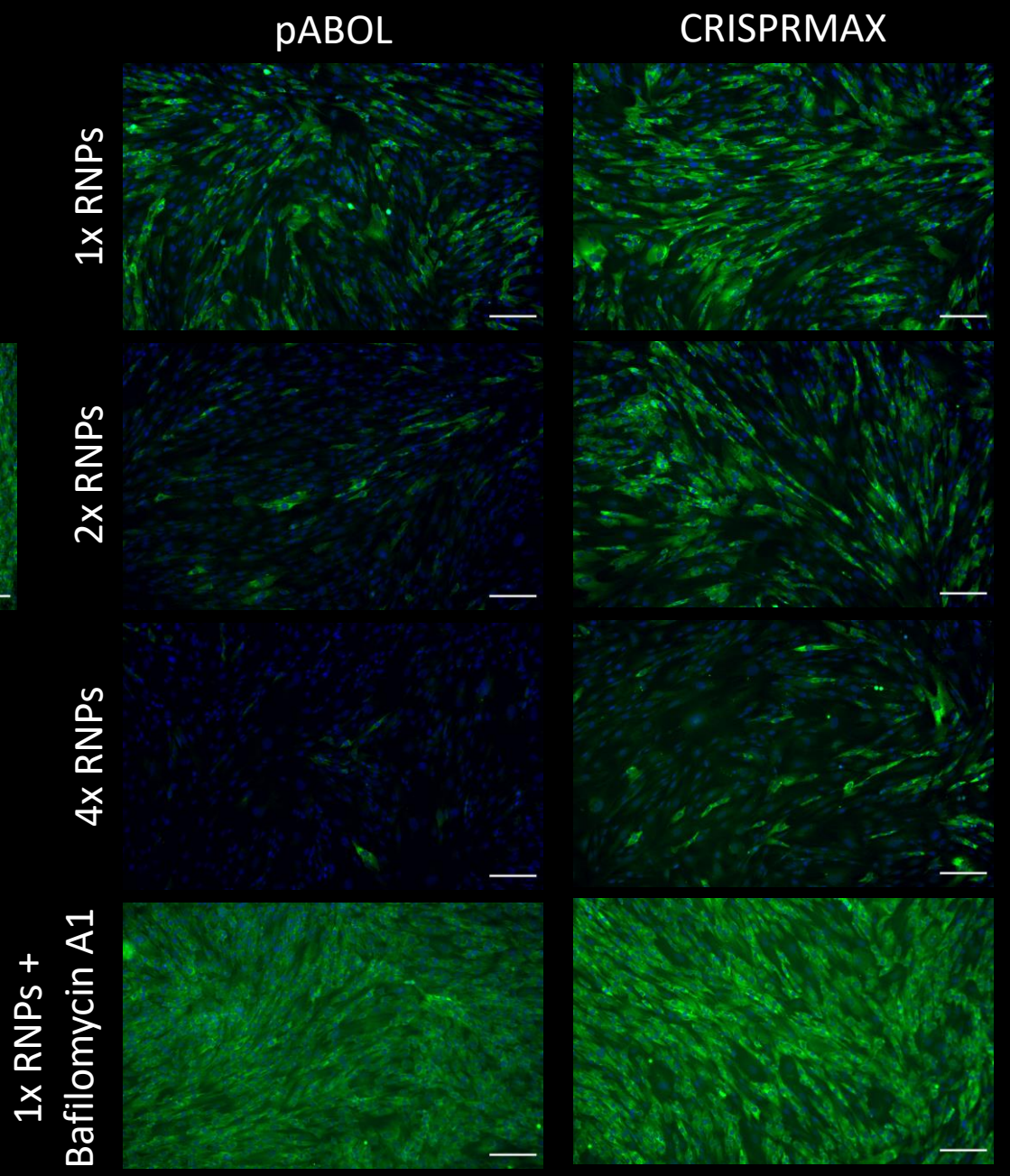




- Lysosome size altered by transfection with polymer nanoparticles
- Some Cy3-Cas9 appears in nuclear compartment
- SK-N-BE(2) cells too 3-dimensional for effective STORM imaging

hTERT-RPE1 Cells

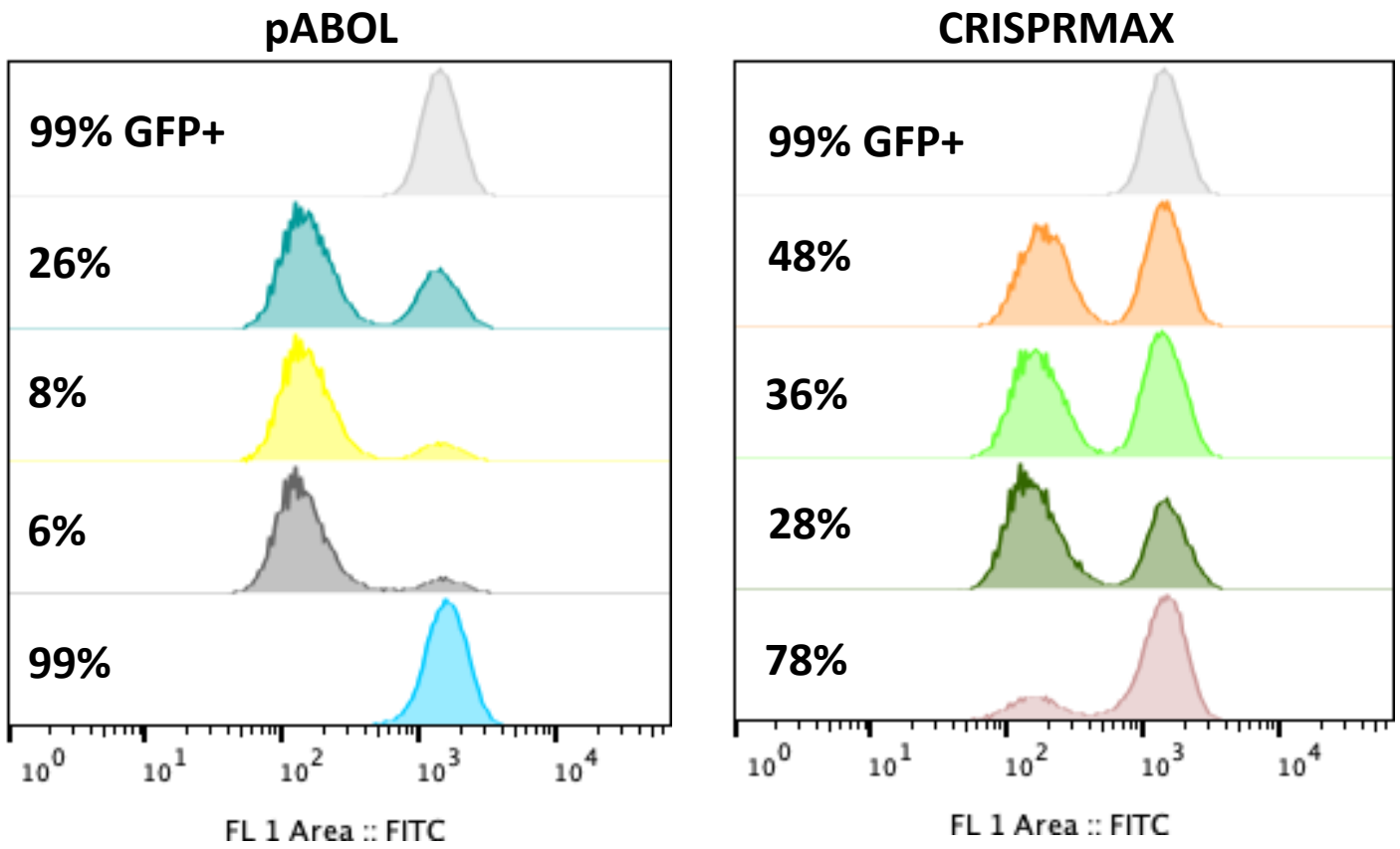


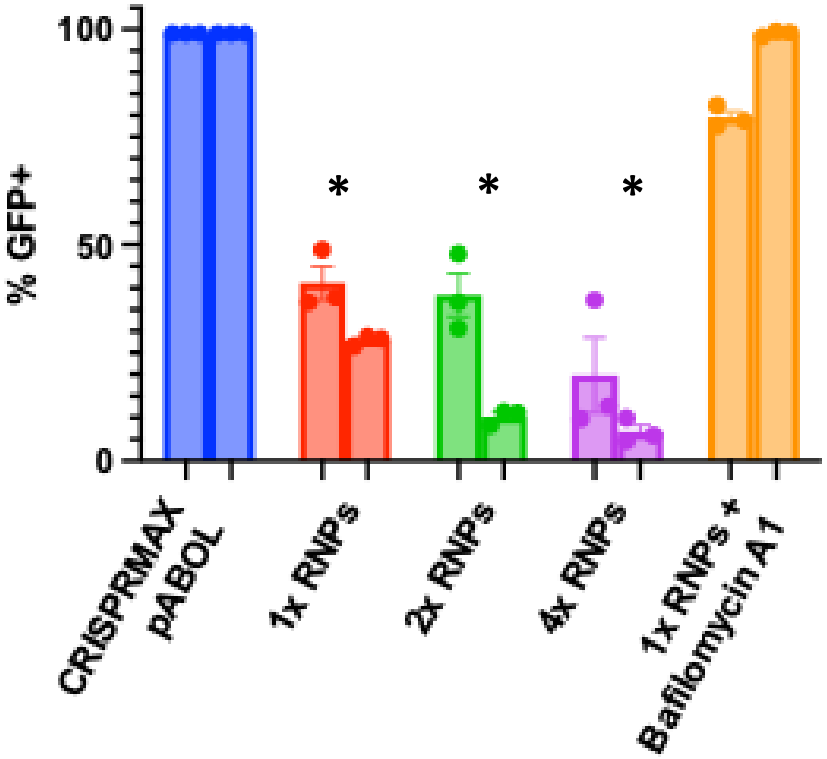


- hTERT-RPE1 cells with RPL24-GFP fusion transfected with pABOL-RNPs against GFP
- Imaged after immunostaining with anti-GFP antibody and DAPI
- Analyzed by flow cytometry

Scale 200 µm

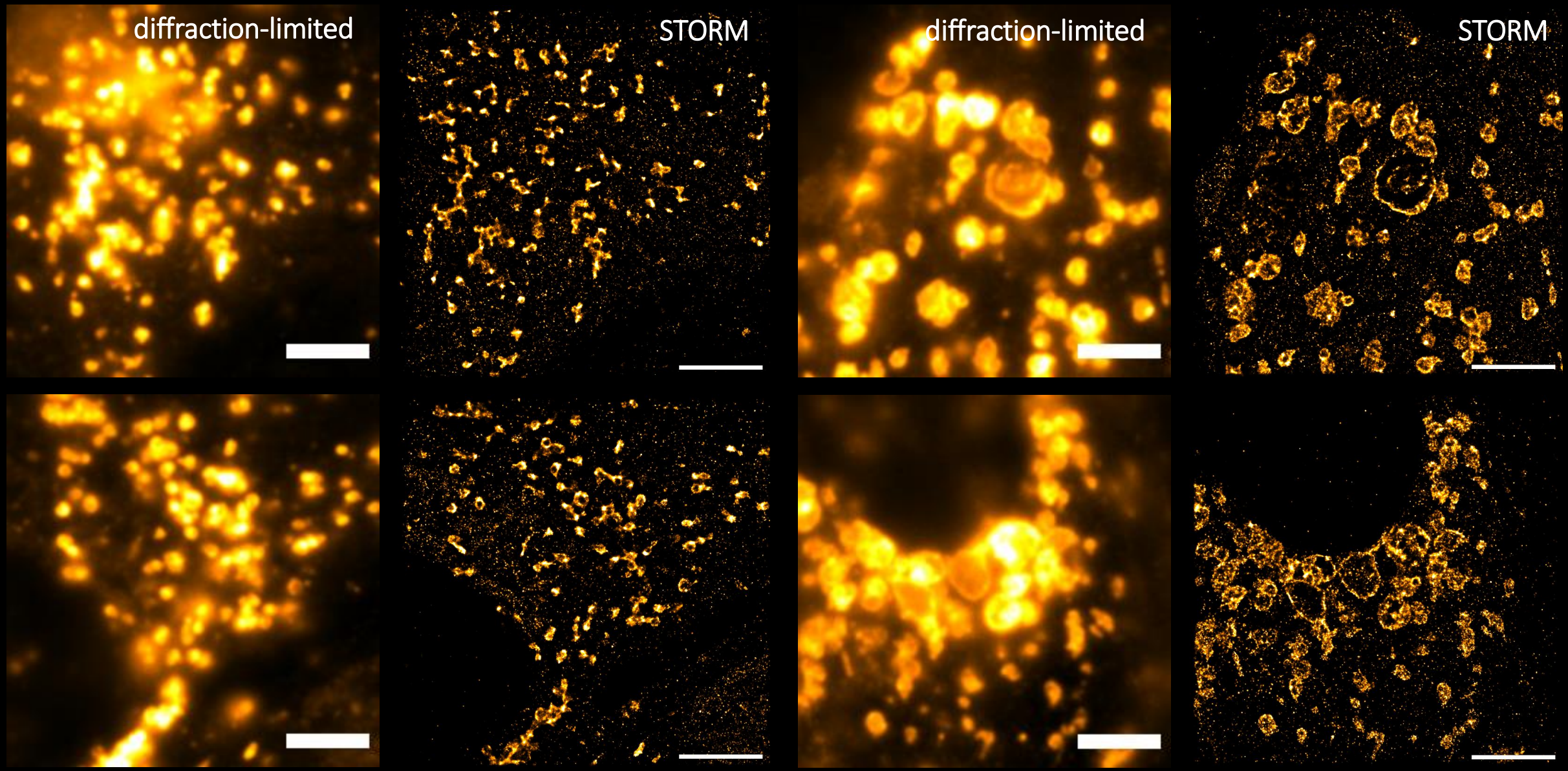
Flow Cytometry



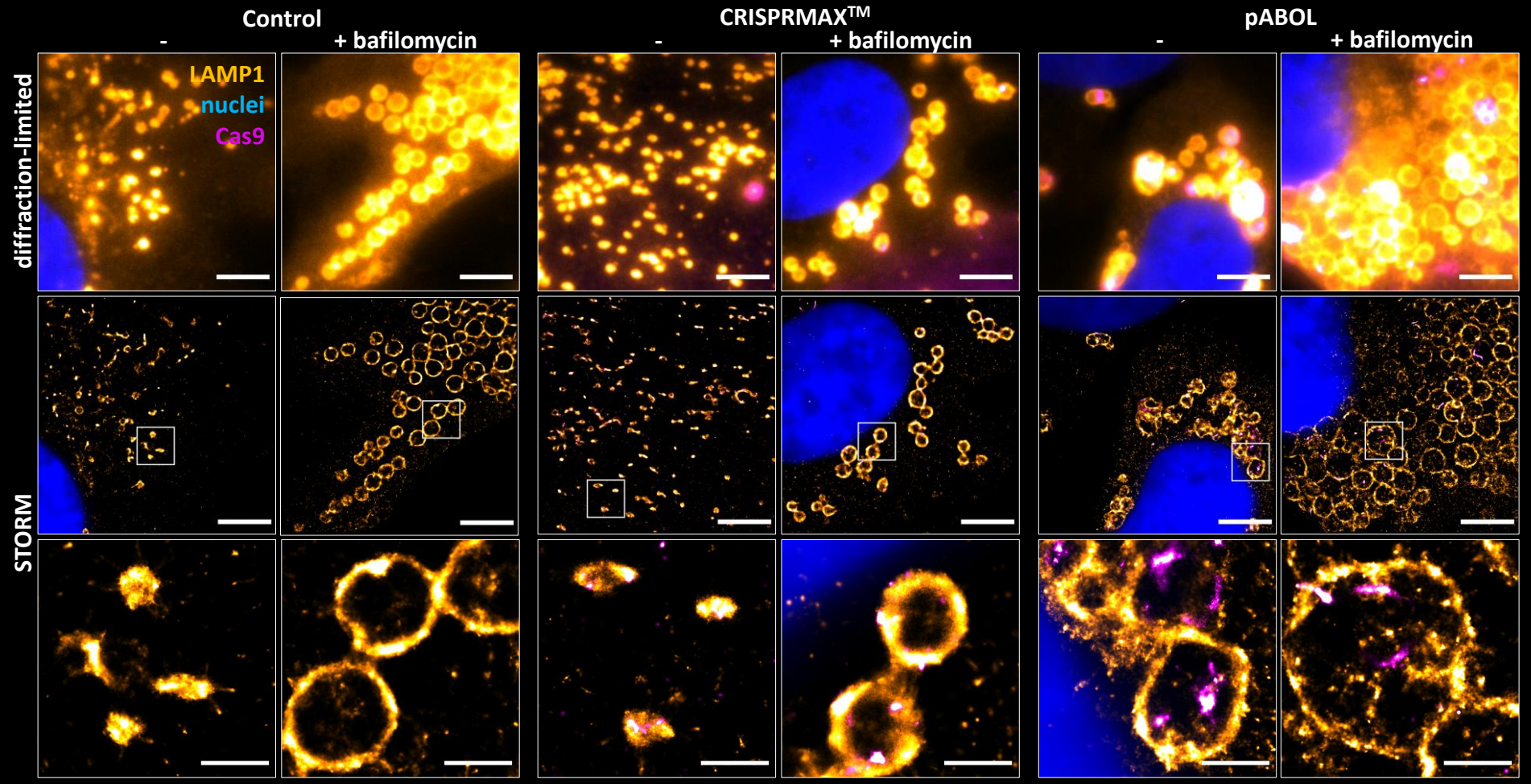


- pABOL outperforms lipoplex CRISPRMAX at each dose with increasing efficacy
- Proton pump inhibitor Bafilomycin A1 inhibits delivery/editing

STORM Microscopy



STORM Microscopy



Scale bars: diffraction-limited and full view STORM 5 µm, STORM zoom-in 1 µm.

- Hypertrophic perinuclear lysosomes observed with nanoparticle delivery
- Delivery via lipoplexes seems to occur via another mechanism

STORM Image Analysis

Google Collab

Stardist neural network (Schmidt, Uwe, et al. "Cell detection with star-convex polygons." *International Conference on Medical Image Computing and Computer-Assisted Intervention*. Springer, Cham, 2018.)
(a deep-learning method that can be used to segment cell nuclei from bioimages)

ZeroCostDL4Mic: exploiting Google Collab to develop a free and open-source toolbox for Deep-Learning in microscopy (von Chamier et al. Democratising deep learning for microscopy with ZeroCostDL4Mic. Nature Communications, 2021. DOI: https://doi.org/10.1038/s41467-021-22518-0)

Free of charge cloud computing platform (virtual computers)

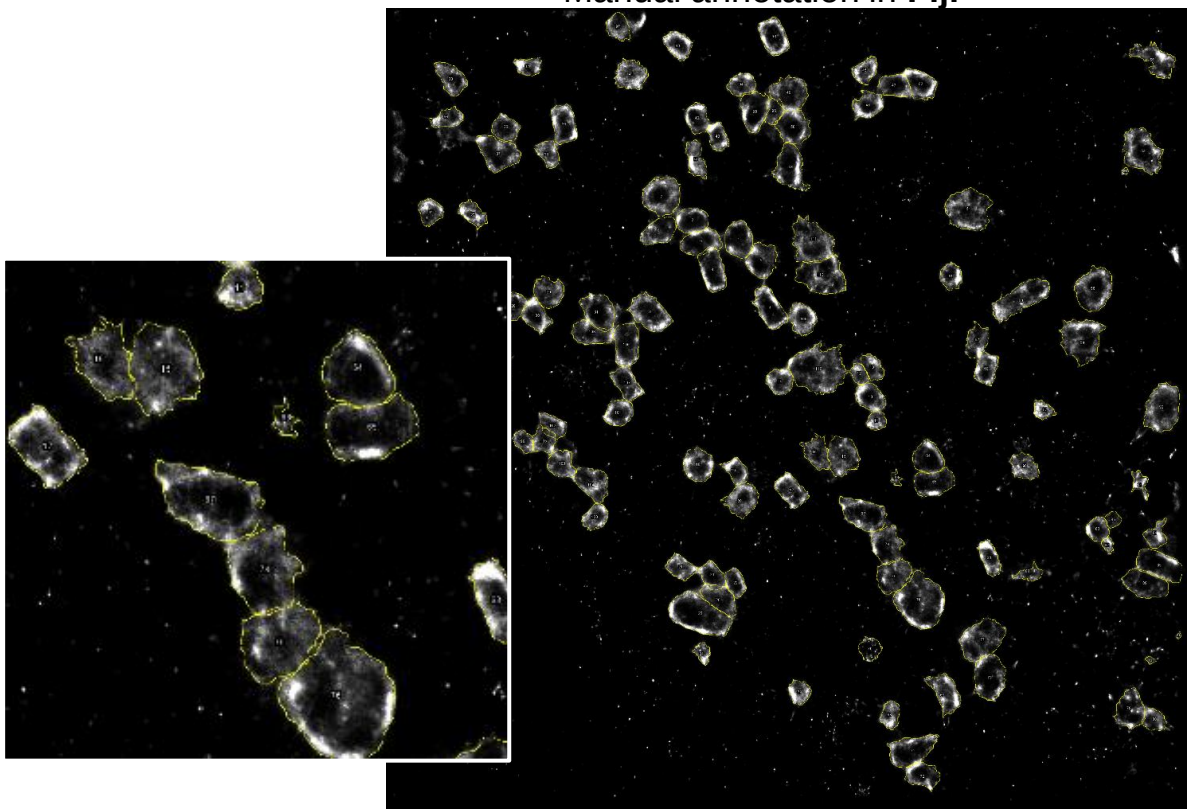
Jupyter = interactive web-based Python code

Miina Ojansivu

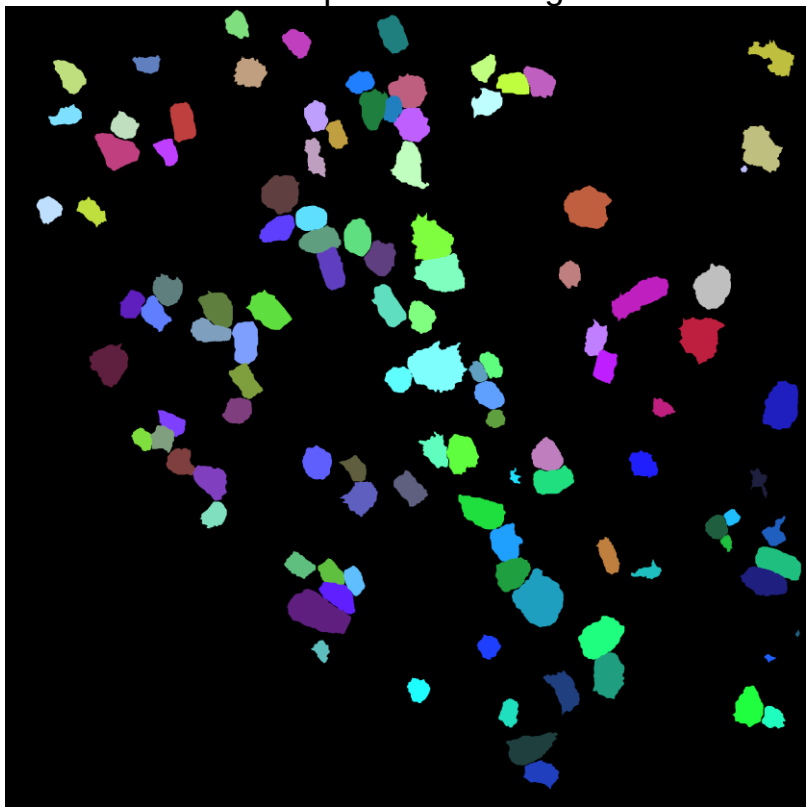
STORM Image Analysis

STORM quantification: training data for the neural network

Manual annotation in **Fiji**

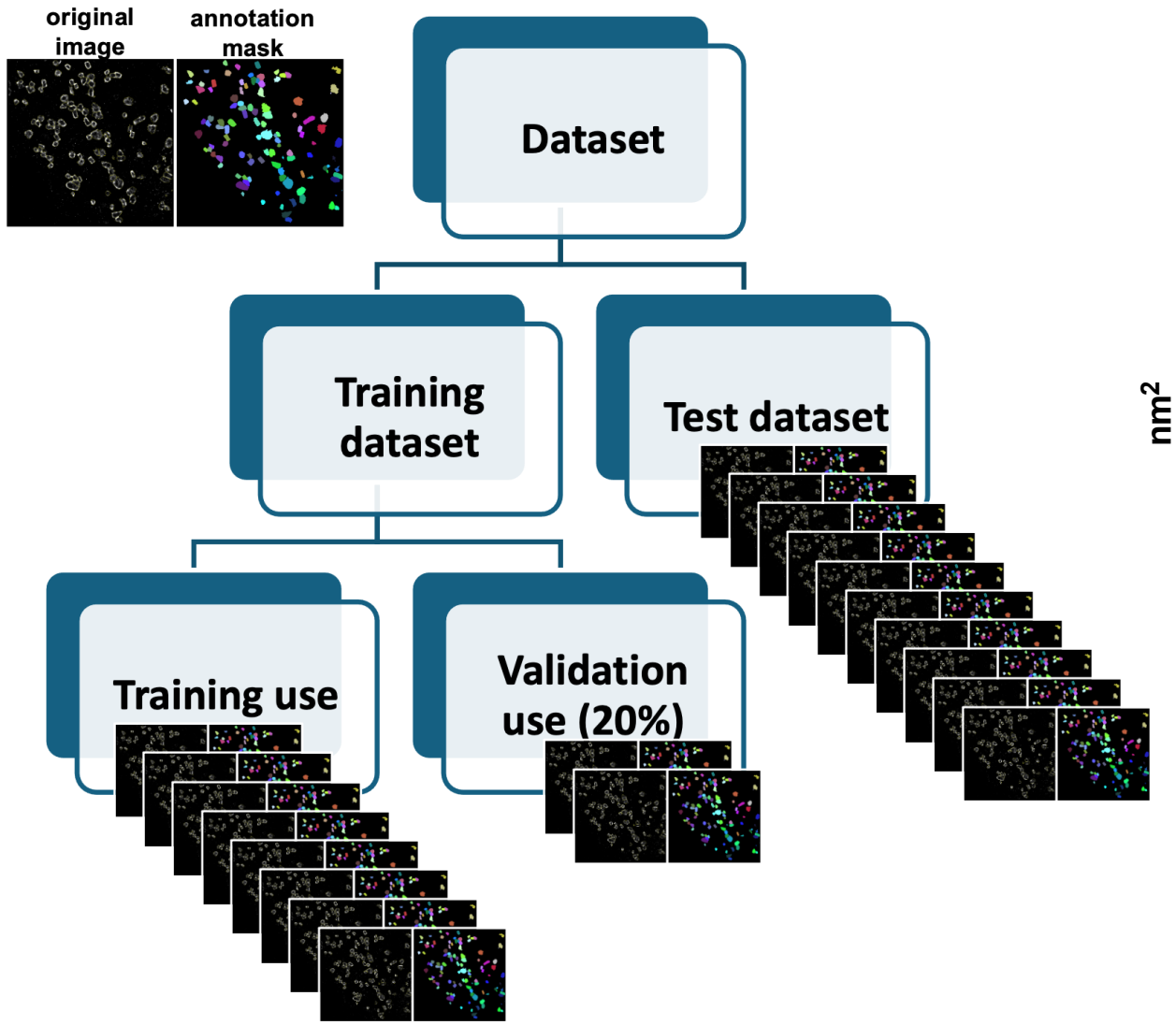


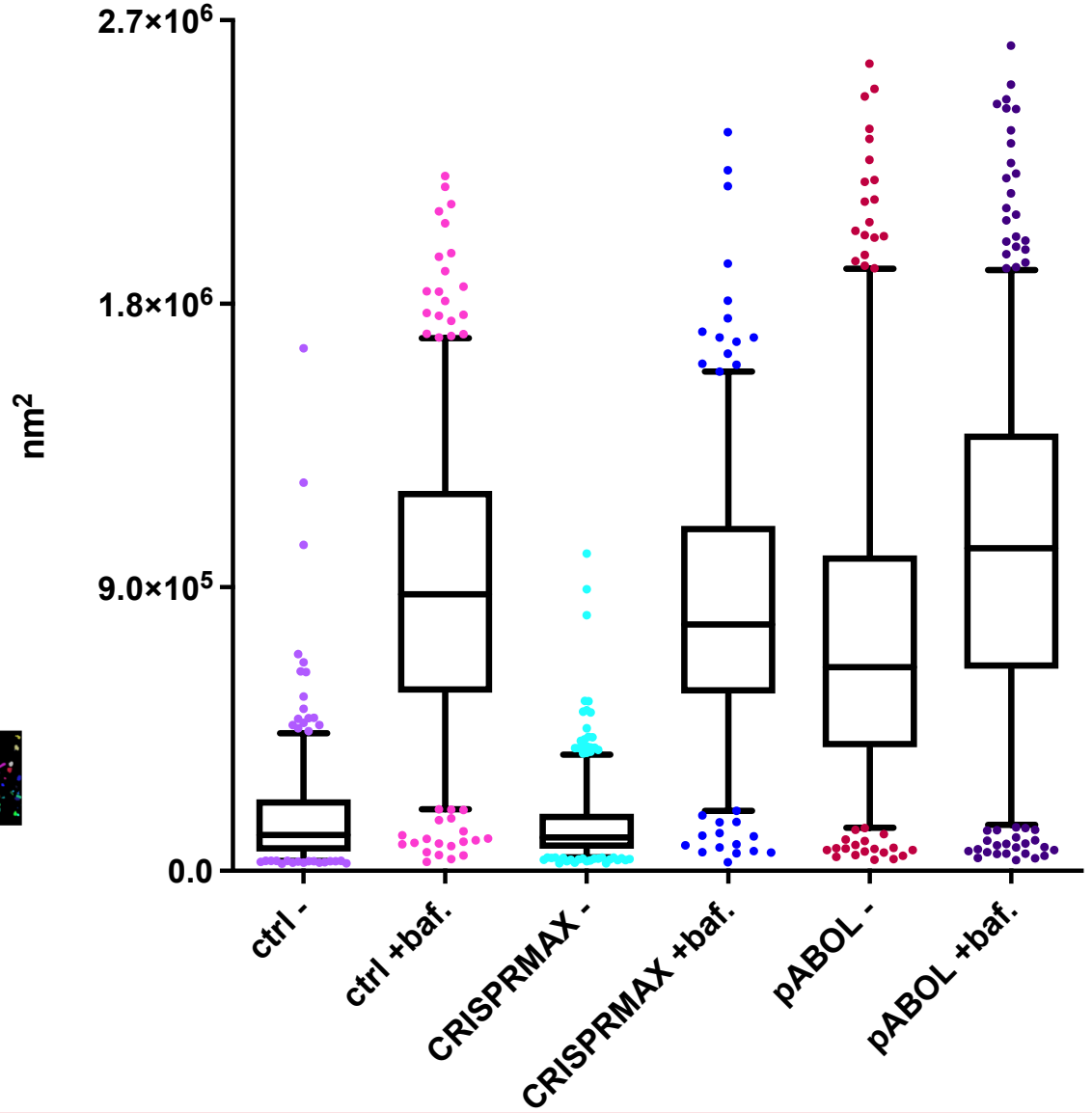
LOCI plugin (Fiji) →
ROI map = **mask** image



Done for one image of each treatment condition (10 conditions → 10 images in the training). Cell lines treated separately.

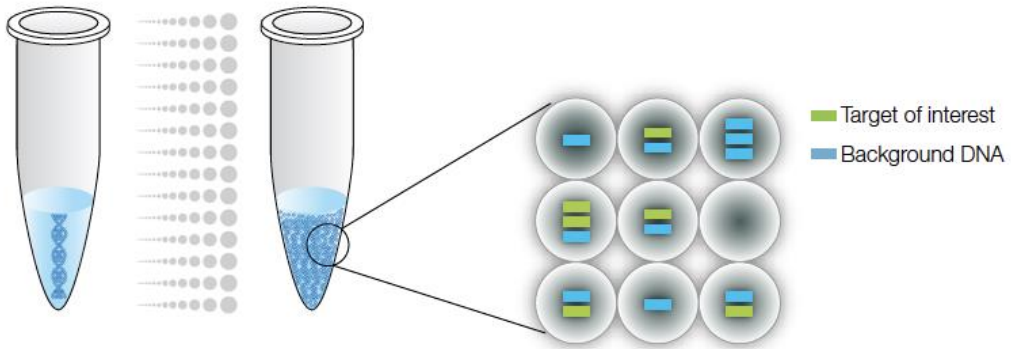
STORM Image Analysis

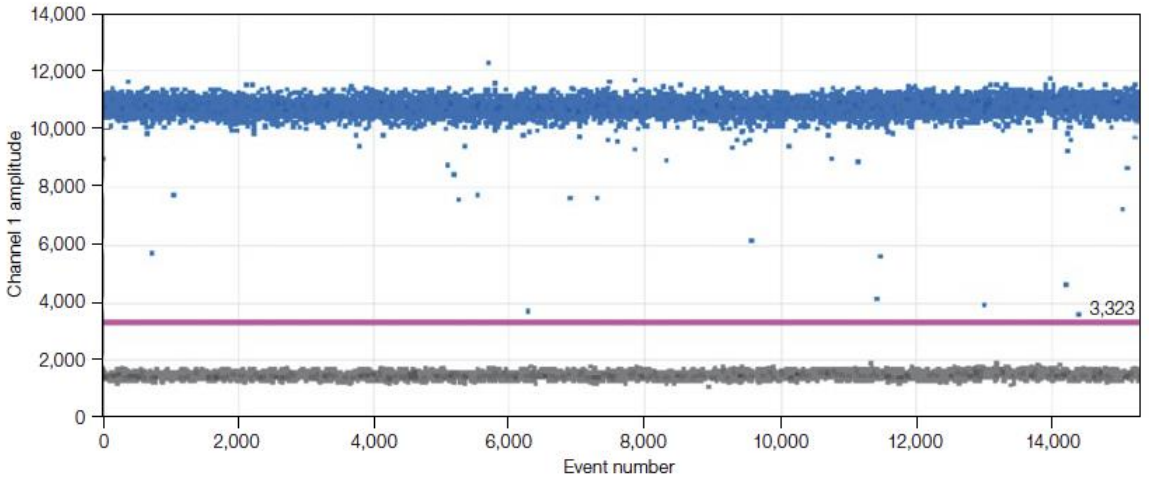


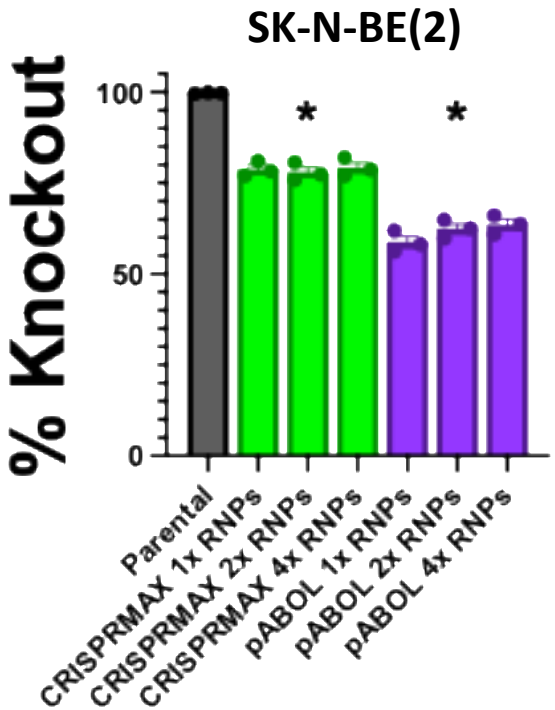


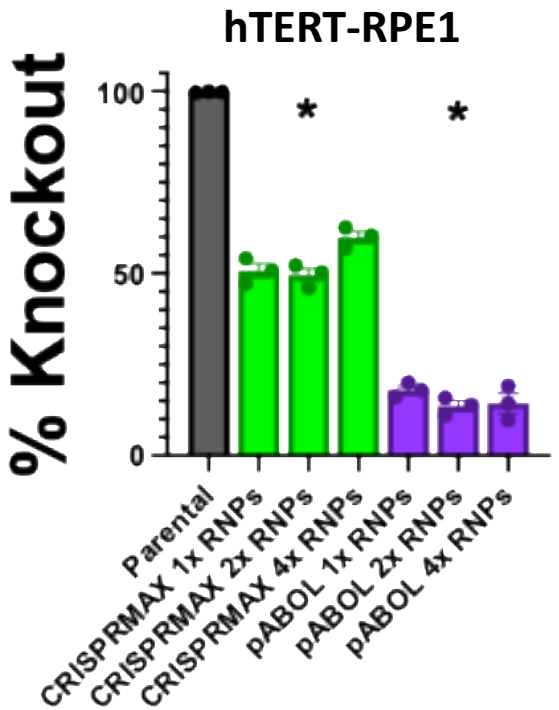
Endogenous Targets – *SOX9*

Digital Droplet PCR (ddPCR)



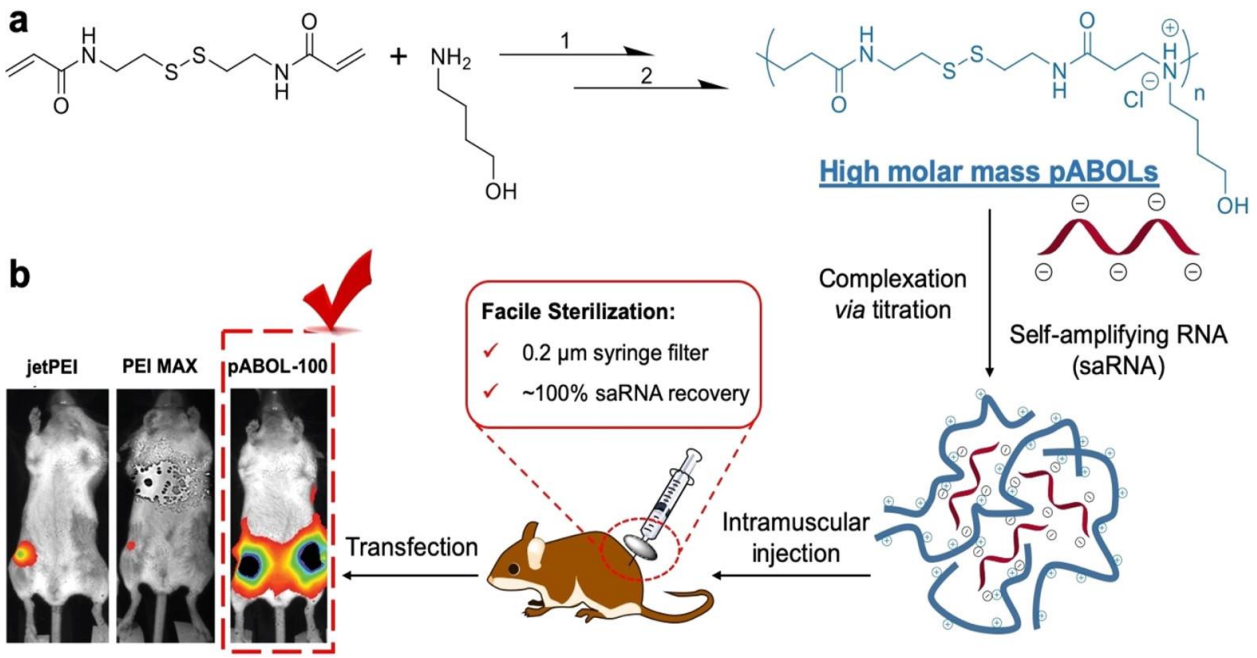


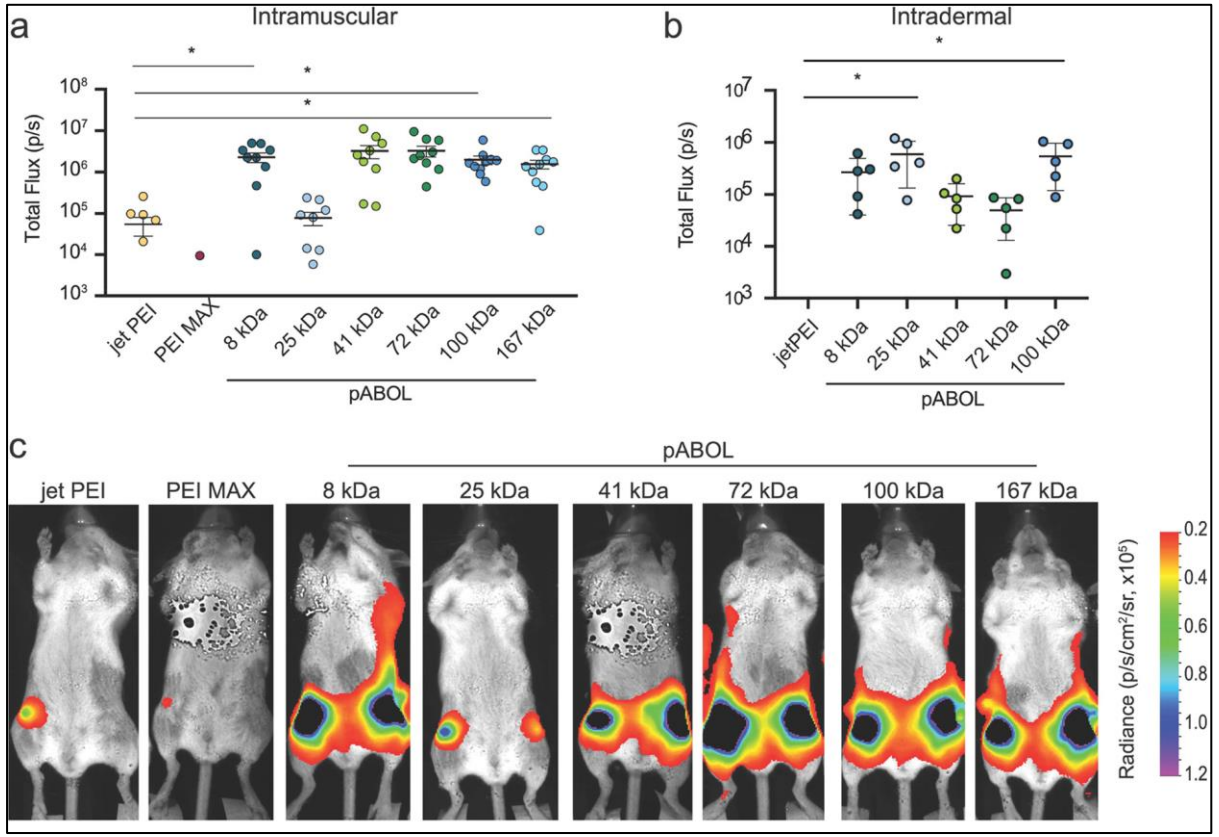




* One-way ANOVA with Tukey's multiple comparisons test

Delivery in vivo

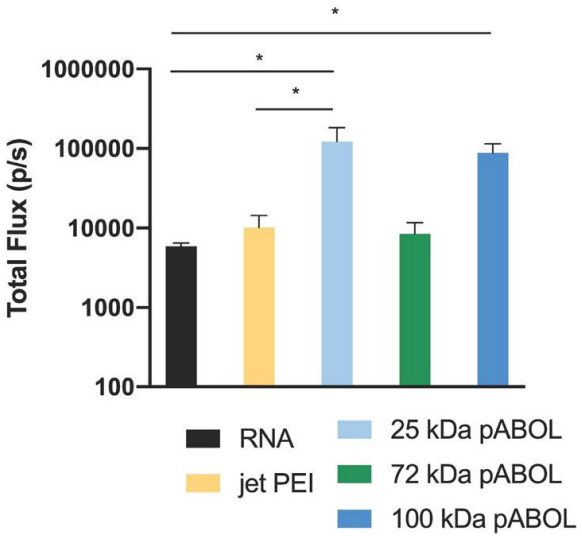




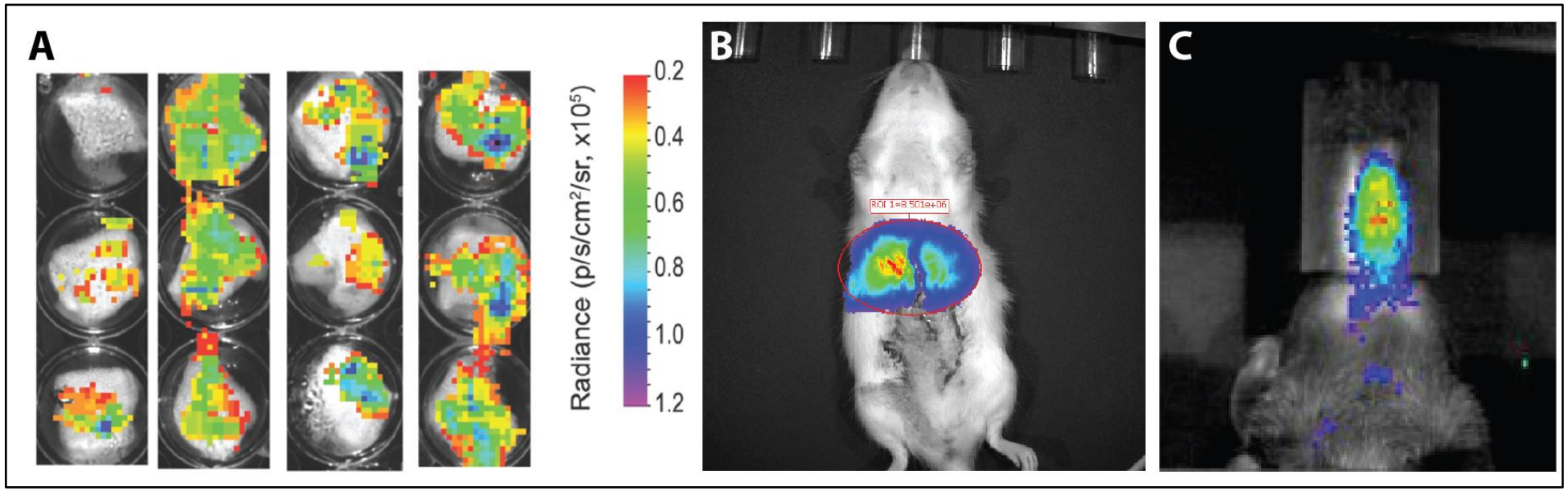
Blakney, Zhu, et al. *ACS Nano* (2020)

- Optimized synthetic conditions can increase molecular weight of pABOL polymers
- pABOL nanoparticles can deliver saRNA in vivo by intramuscular administration

Delivery in vivo



Blakney, Zhu, et al. *ACS Nano* (2020)



Macromolecular payloads and targets tested for delivery in vivo:

A) Self-amplifying RNA delivery to human skin explants

B) Minicircle plasmid delivery to liver in rats

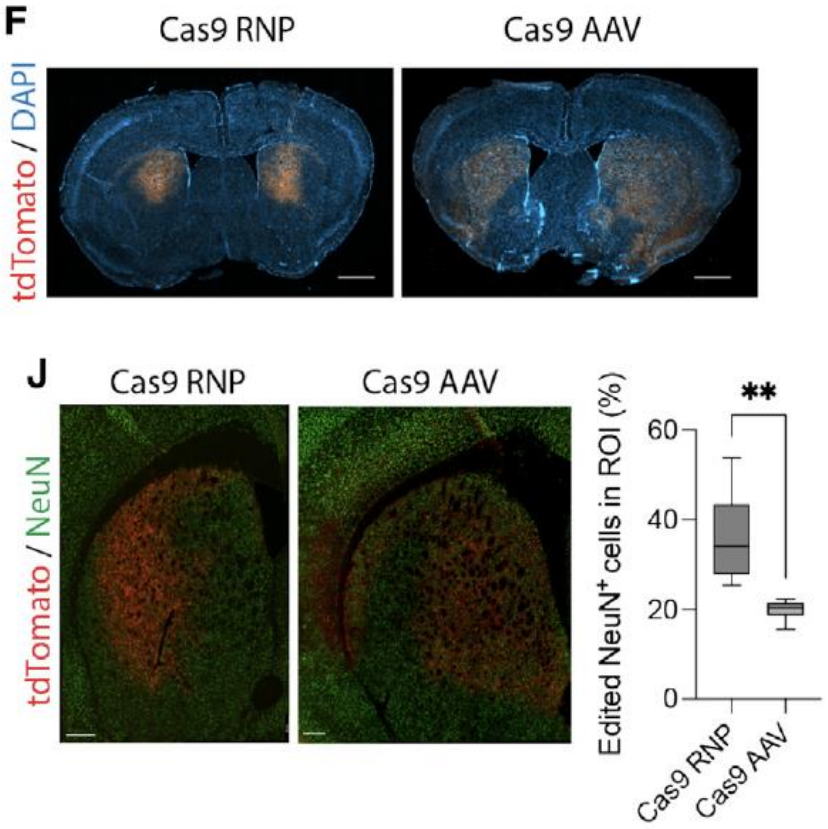
C) mRNA intranasal infusion in mice

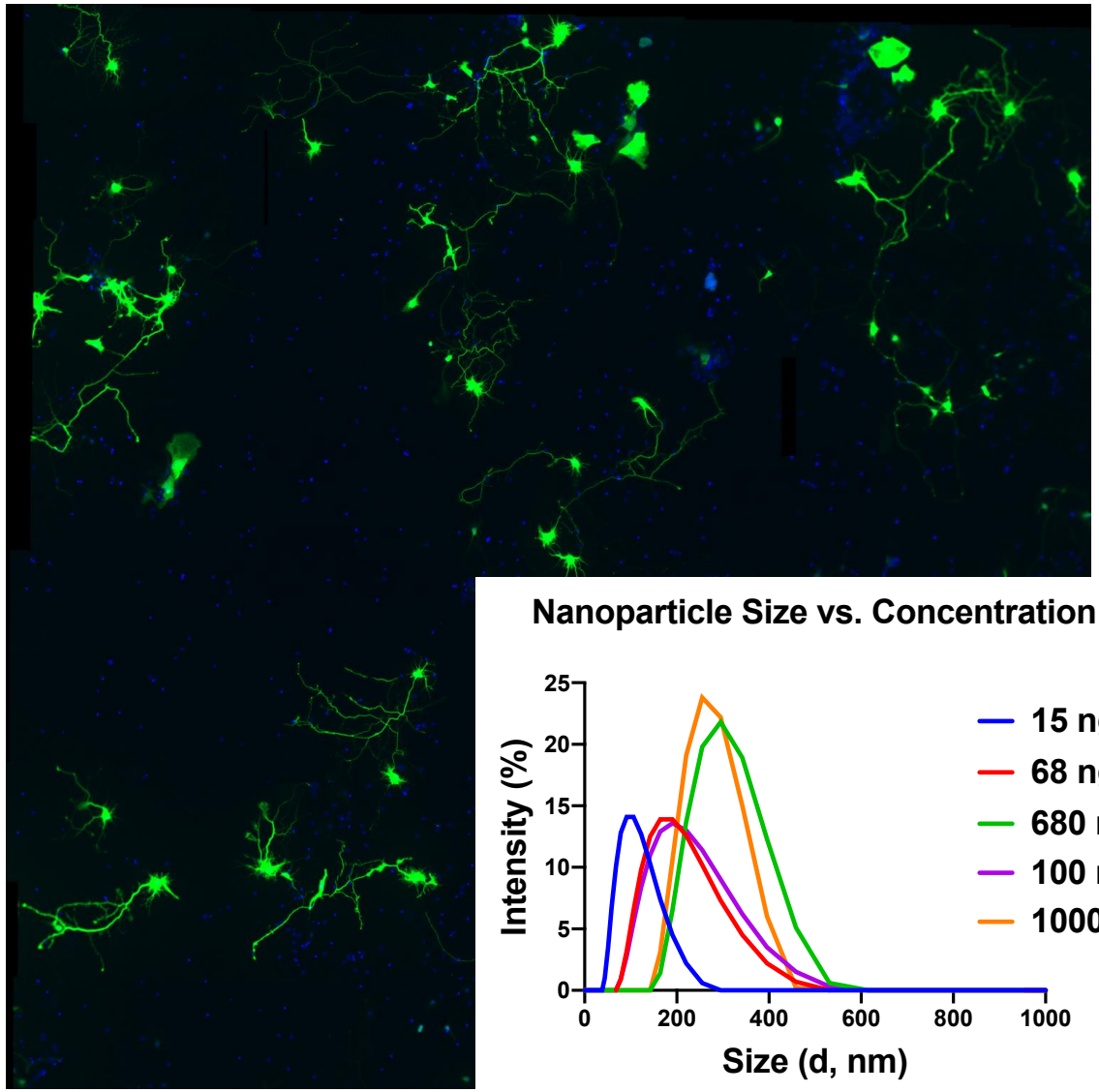
Delivery to the Brain

Molecular Therapy
Original Article

Genome editing in the mouse brain with minimally immunogenic Cas9 RNPs

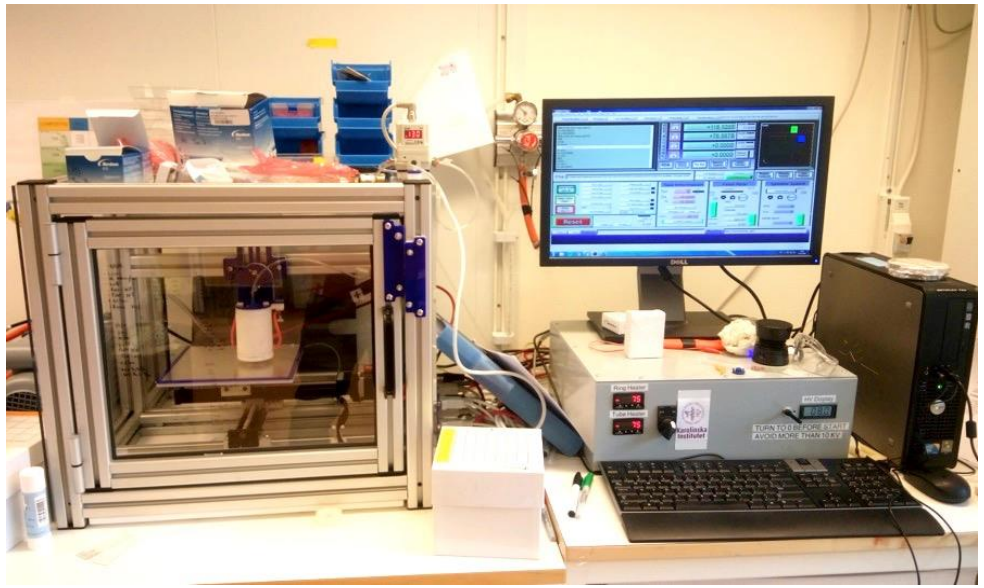
Elizabeth C. Stahl,[1,2,3] Jennifer K. Sabo,[1] Min Hyung Kang,[1,5] Ryan Allen,[1] Elizabeth Applegate,[1] Shin Eui Kim,[1] Yoonjin Kwon,[1,3] Anmol Seth,[1,3] Nicholas Lemus,[1,3] Viviana Salinas-Rios,[1] Katarzyna M. Soczek,[1,2,3] Marena Trinidad,[1] Linda T. Vo,[1,9] Chris Jeans,[2] Anna Wozniak,[4] Timothy Morris,[4] Athen Kimberlin,[4] Thomas Foti,[4] David F. Savage,[1,3,5] and Jennifer A. Doudna[1,2,3,5,6,7,8,9]

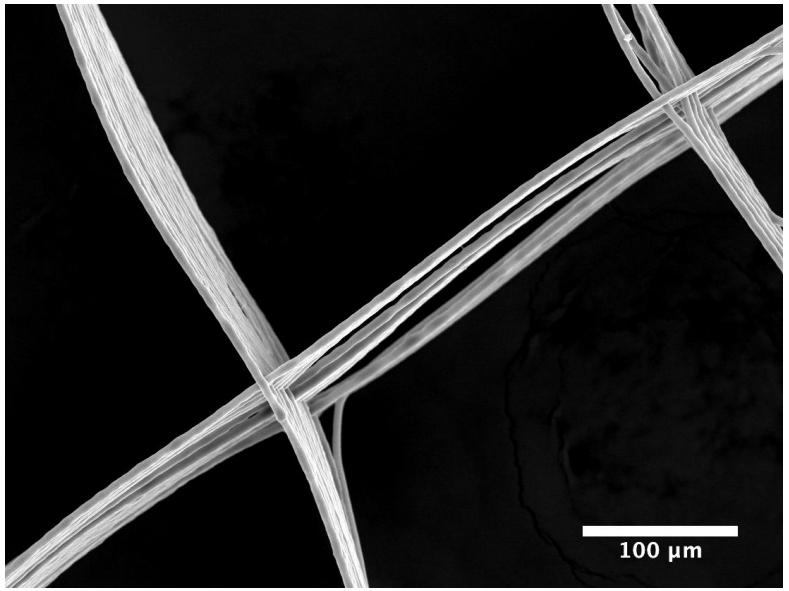


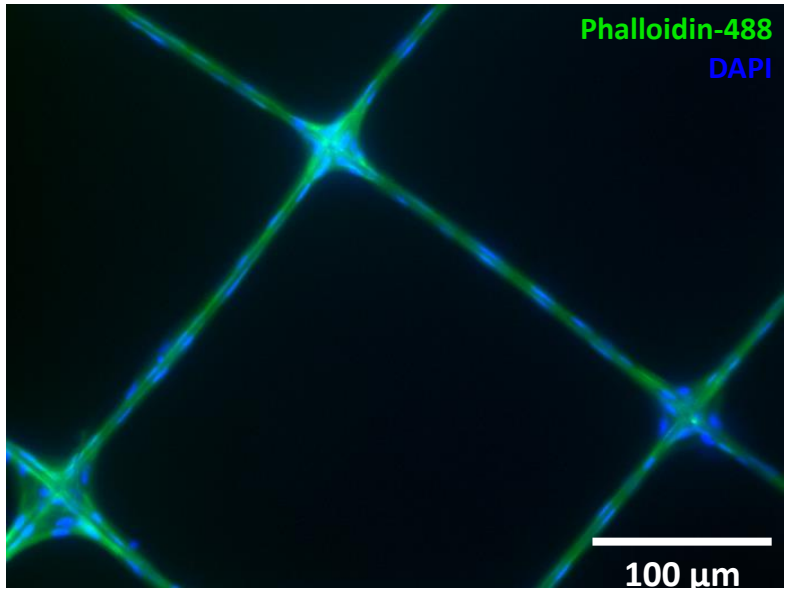


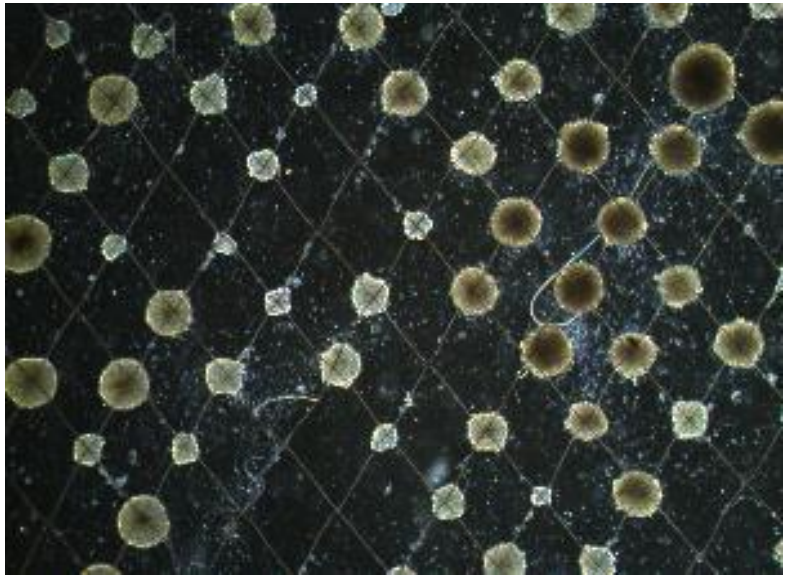
Nanoparticle Size vs. Concentration

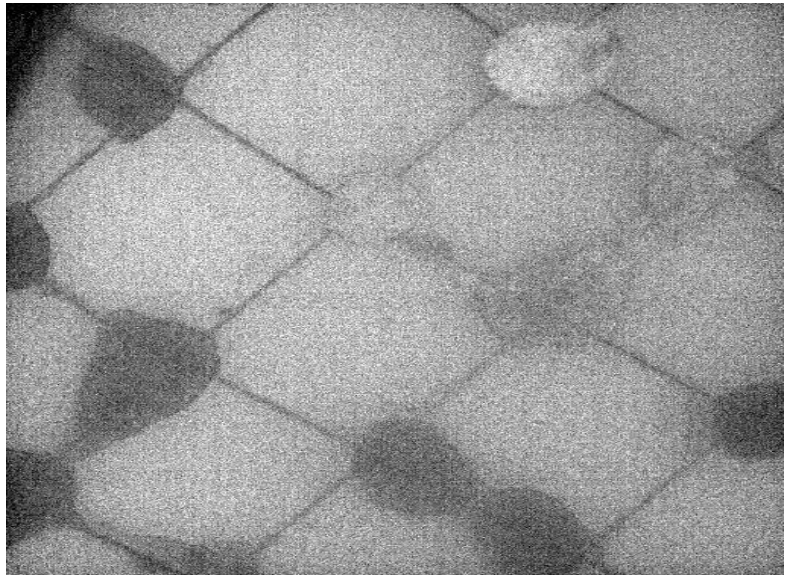
Delivery to the Brain



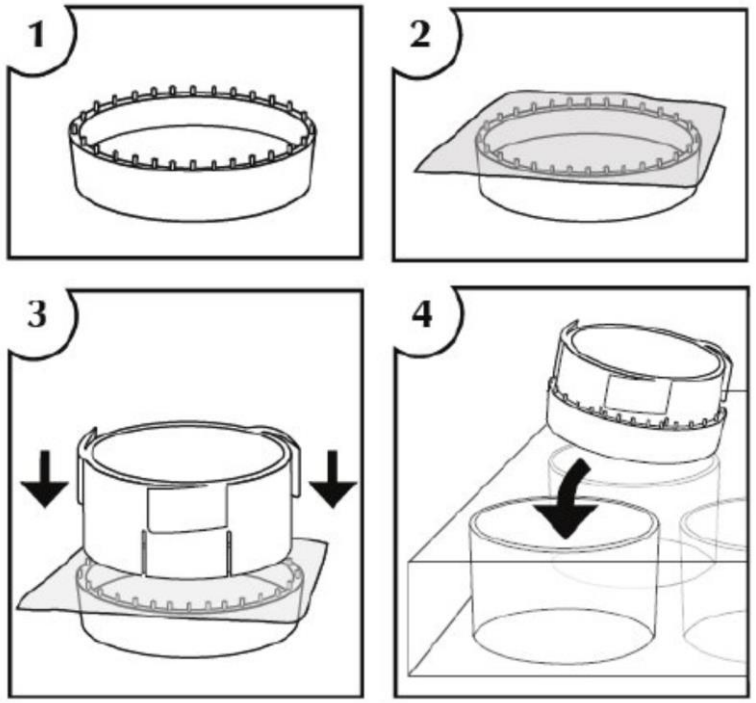


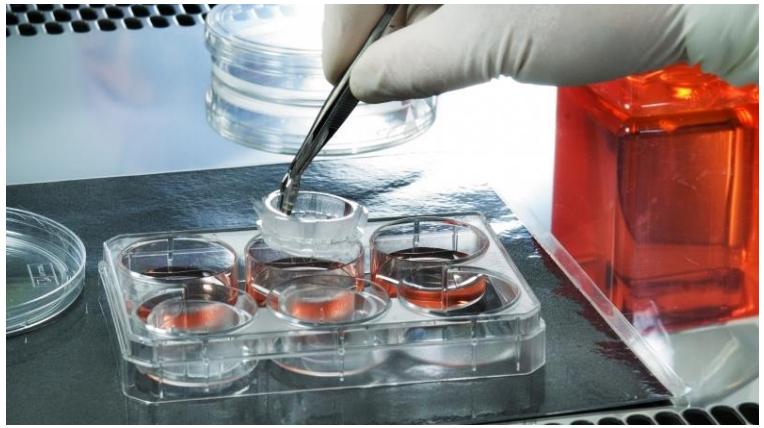


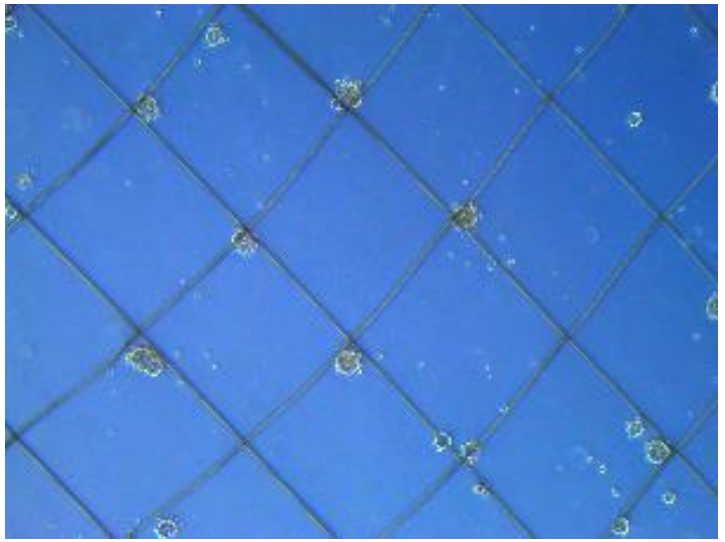


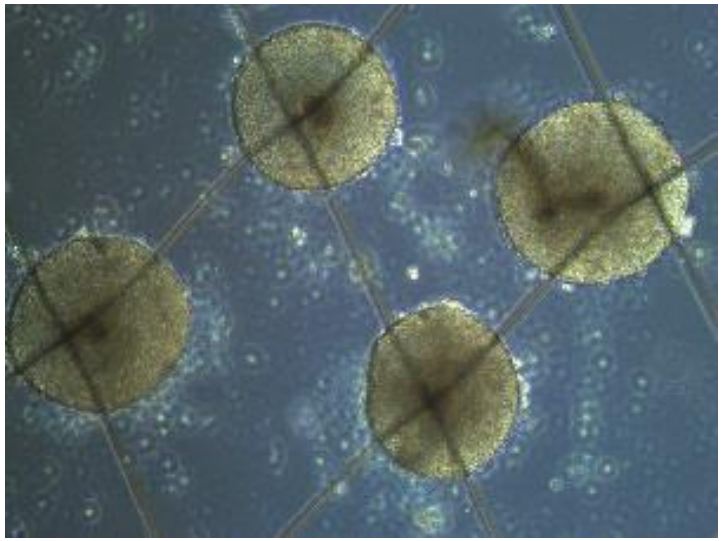


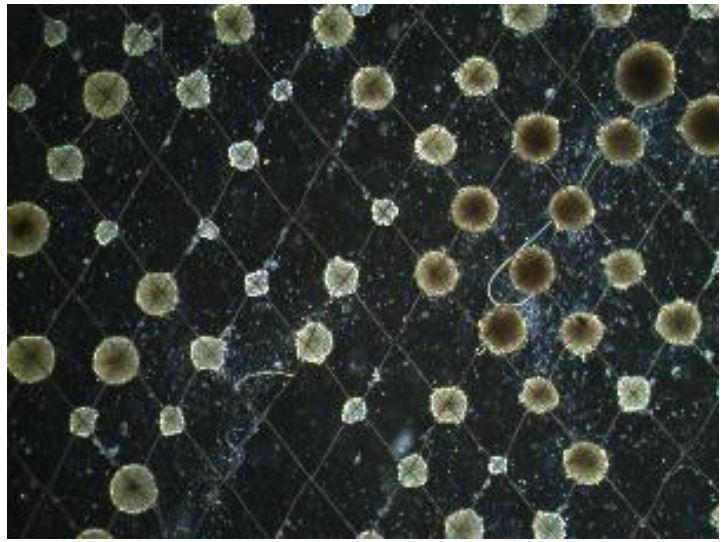
Scaffold Seeding

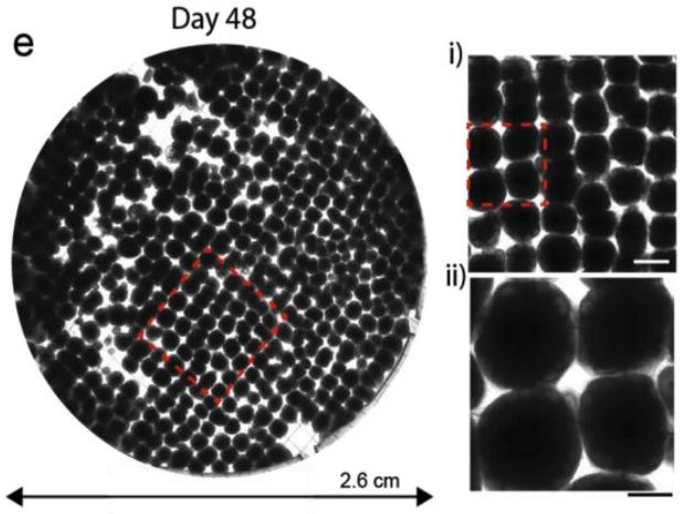






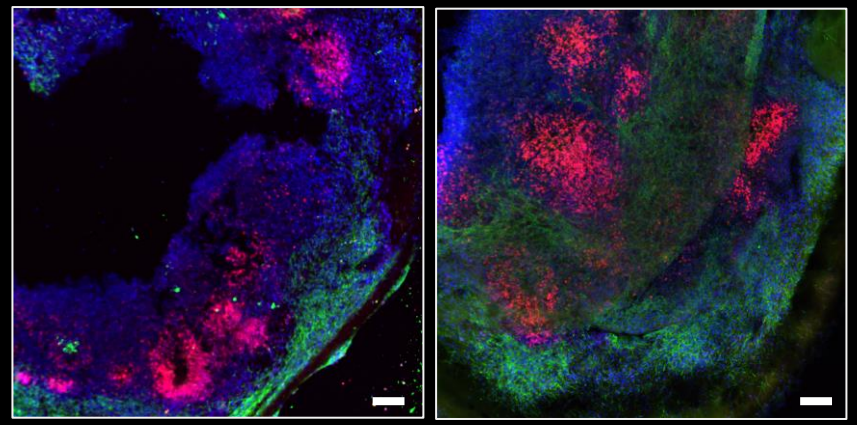




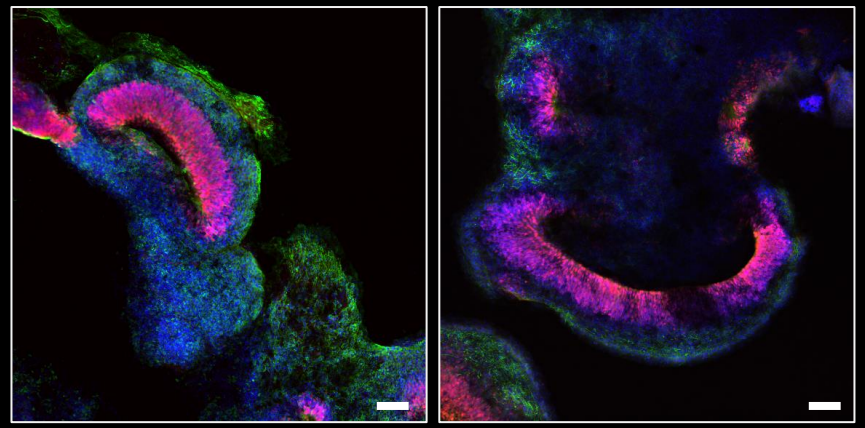


Brain Organoids on Scaffolds

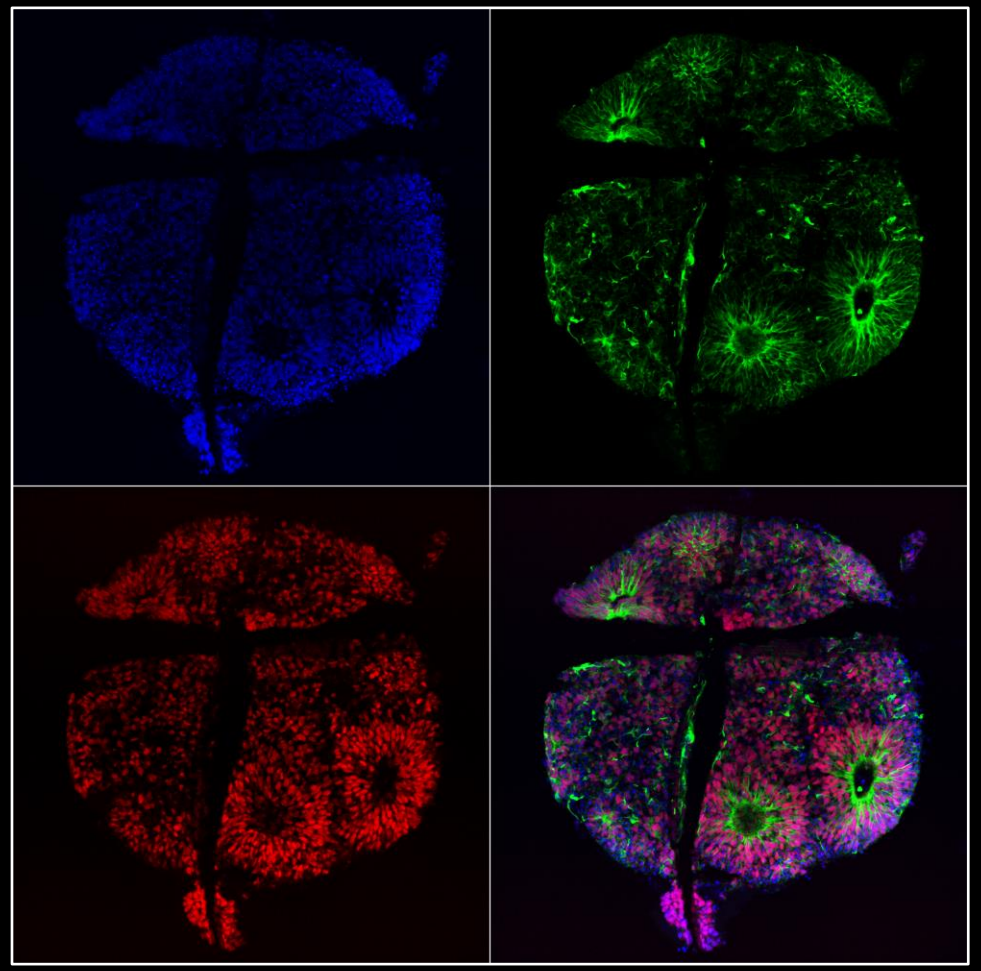
Round organoids

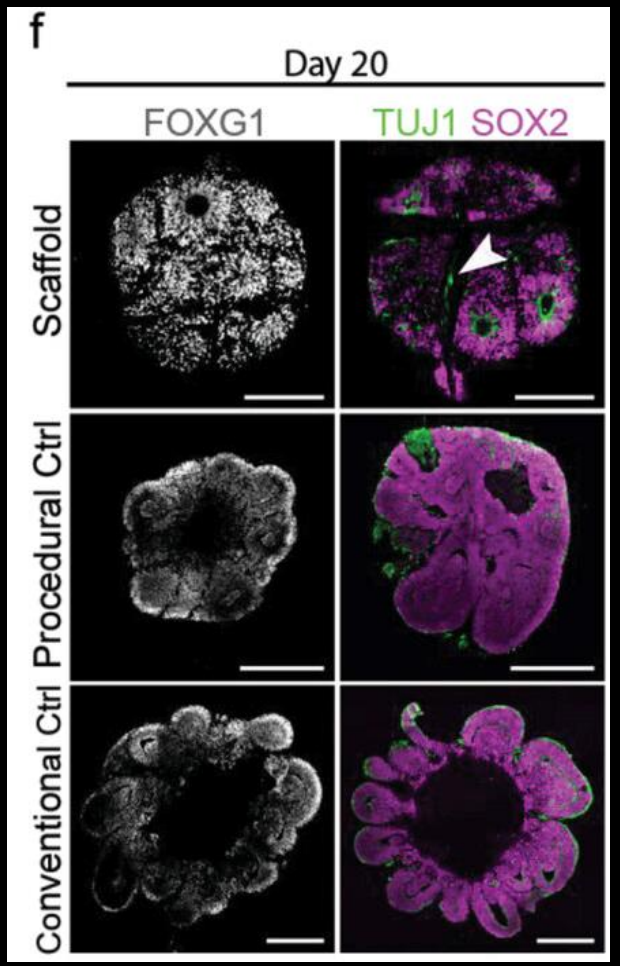


Scaffold organoids



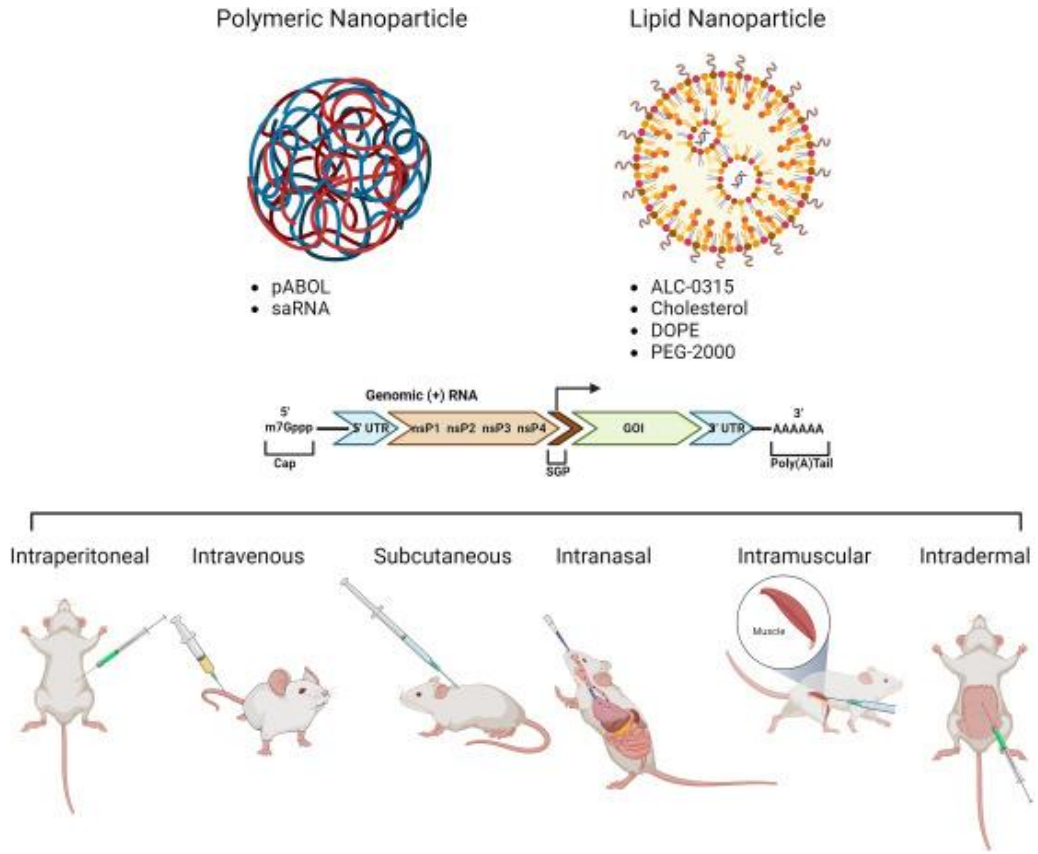
Scale bars, 100µm

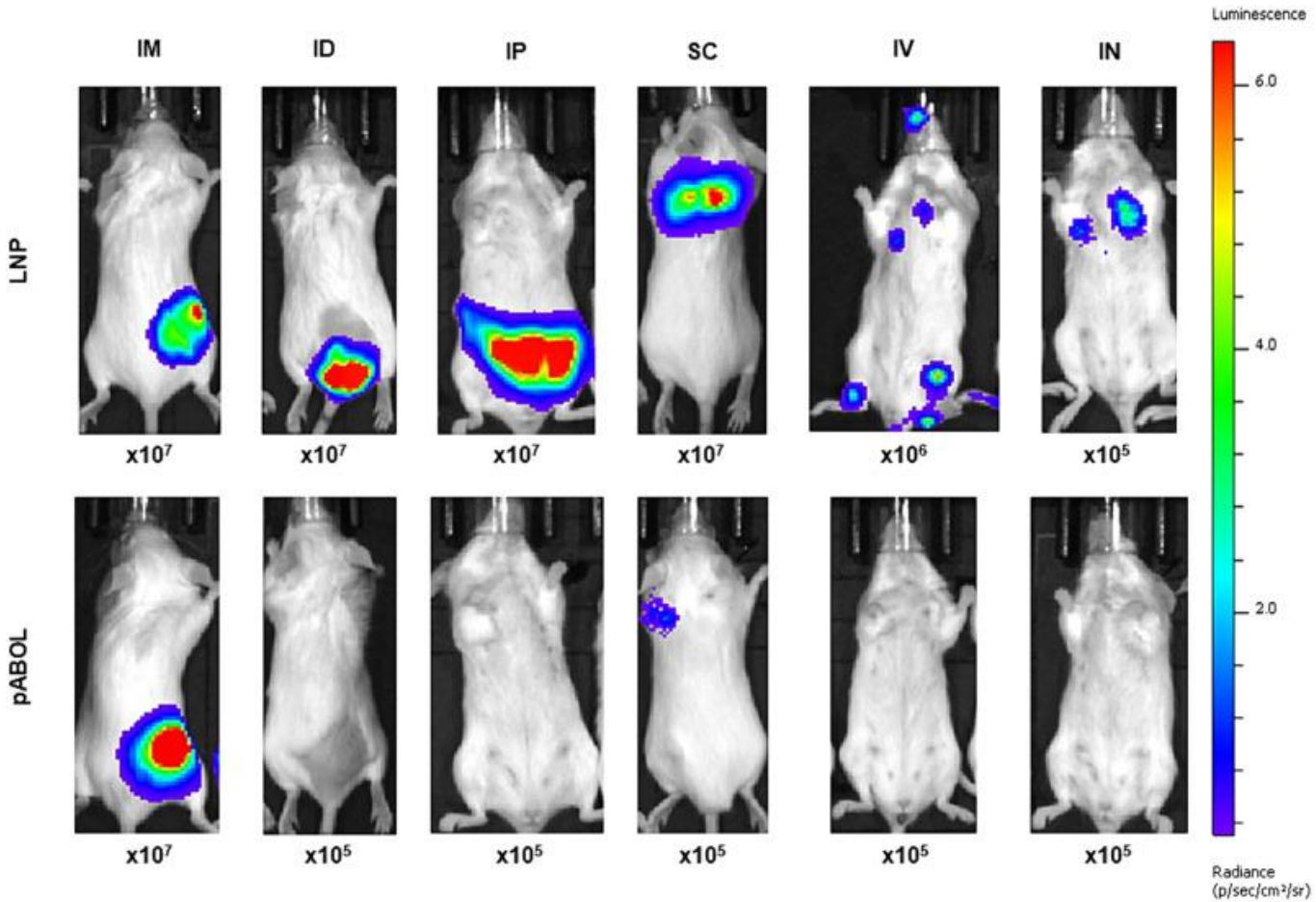




MAP2 late neuronal marker
SOX2 Neural stem cell marker

Tissue Targets





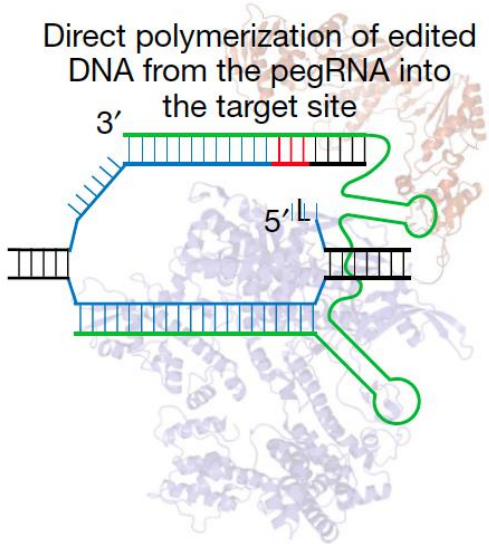
- pABOL nanoparticles outperform LNPs when administered to muscle
- Protein expression levels 10-fold higher observed with IM pABOL

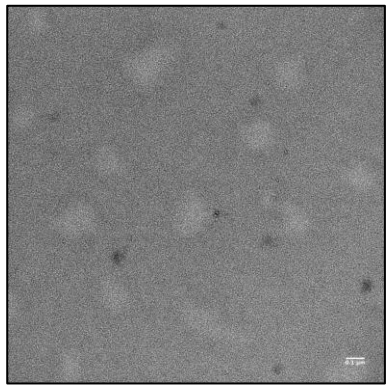
Bathula et al. *J Controlled Release* (2024)

Prime Editing with RNPs

Anzalone *et al.*, Nature, Dec 5 2019

A precision 'search and replace' technology



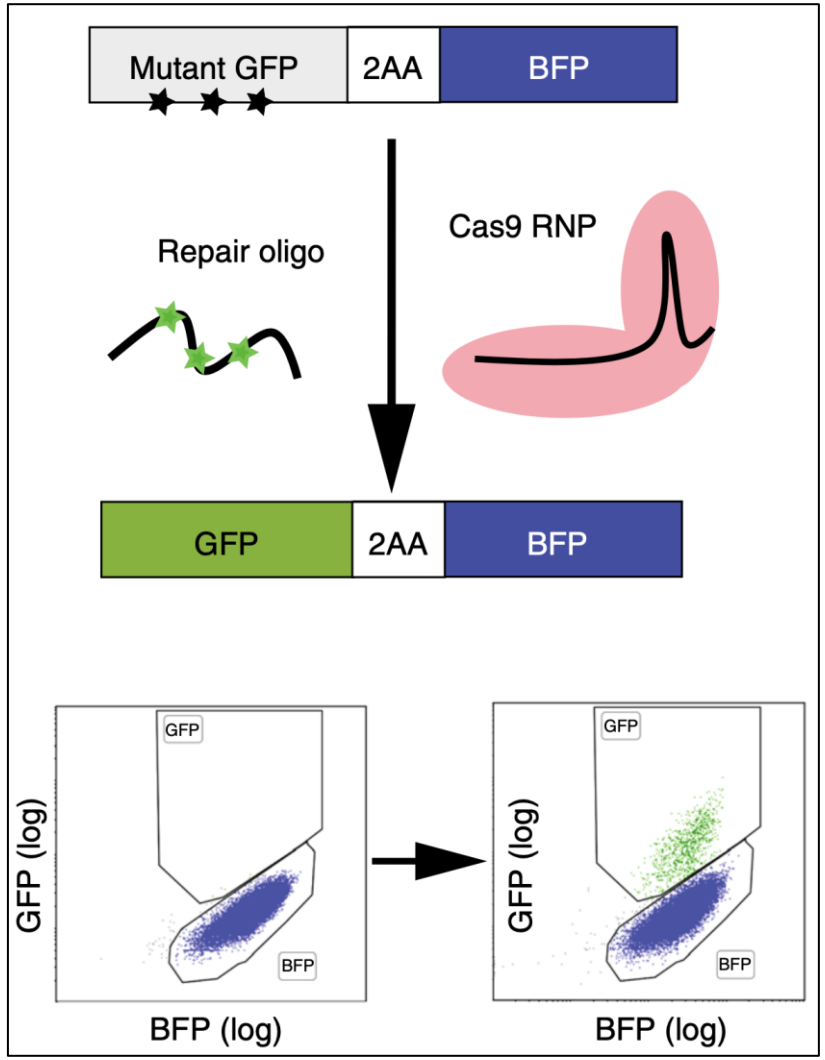


nCas9-MMLV-RT produced by KI Protein Science Facility

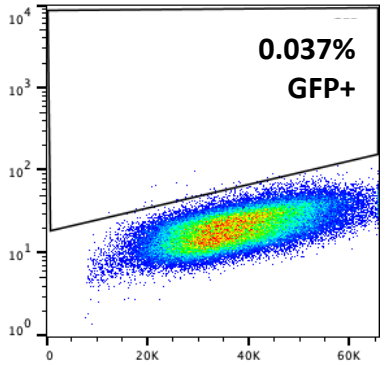
Lentiviral Reporter:

- Stable expression of deadGFP-2A-BFP

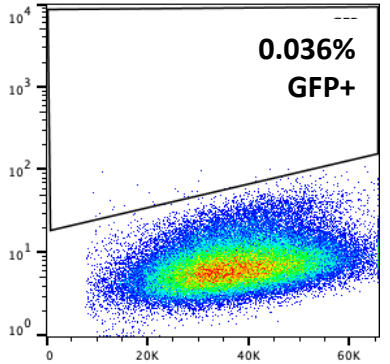
Thr-Tyr-Gly → Ala Ala Ala



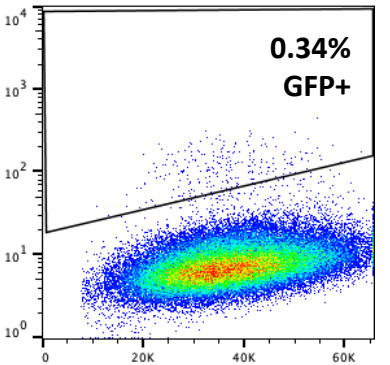
Haapaniemi et al. *Nat Med* (2018)



Control

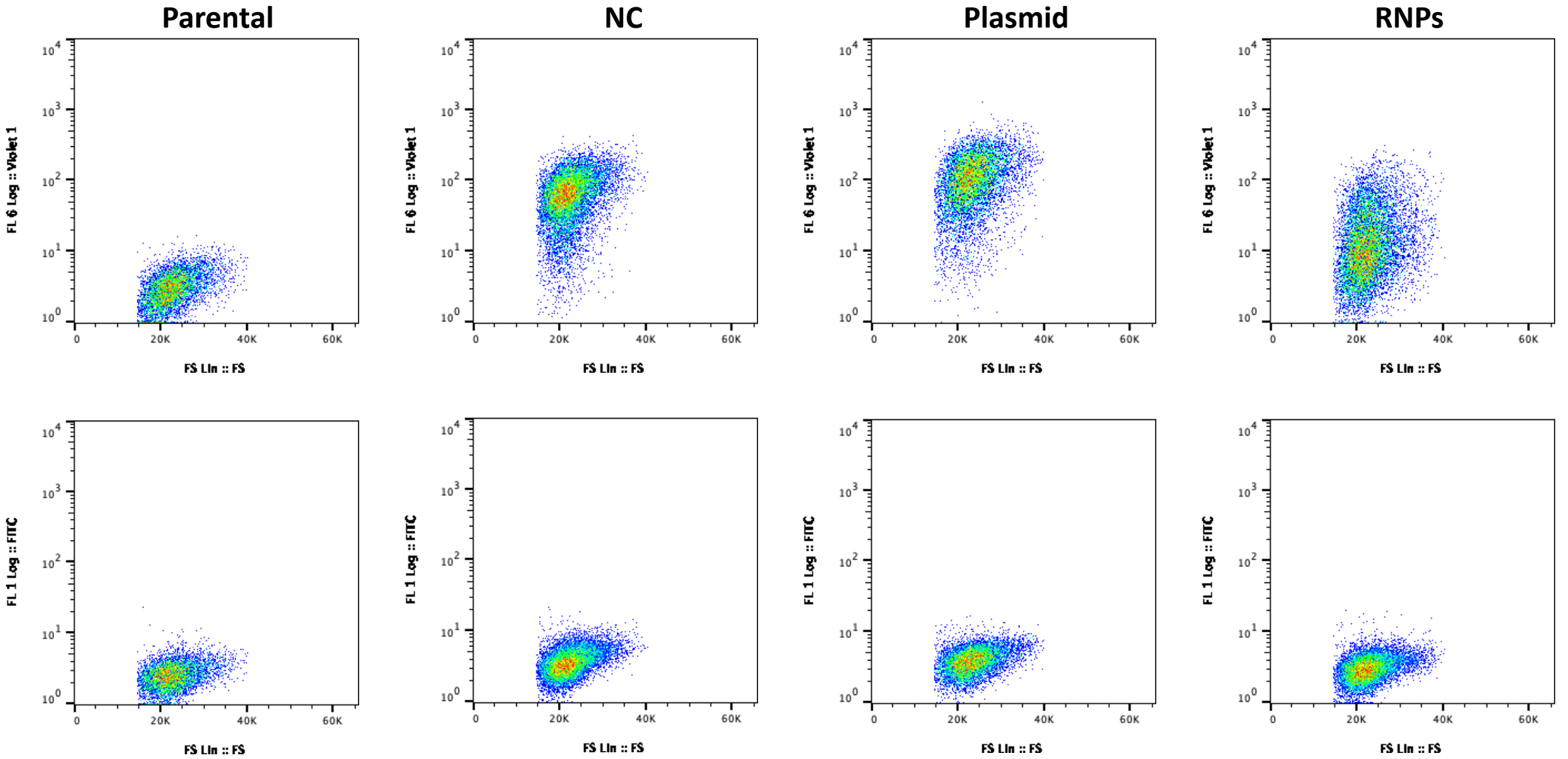


CRISPRMAX™



pABOL

Prime Editing with RNPs



Conclusions

- The bioreducible polycation p(CBA-ABOL) enables the complexation and delivery of Cas-derived RNPs capable of more efficient genome editing in cells
- STORM microscopy reveals distinct changes in endolysosomal morphometrics in cells treated with p(CBA-ABOL) nanoparticles
- p(CBA-ABOL) nanoparticles may require careful choice of tissue targets for in vivo applications

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