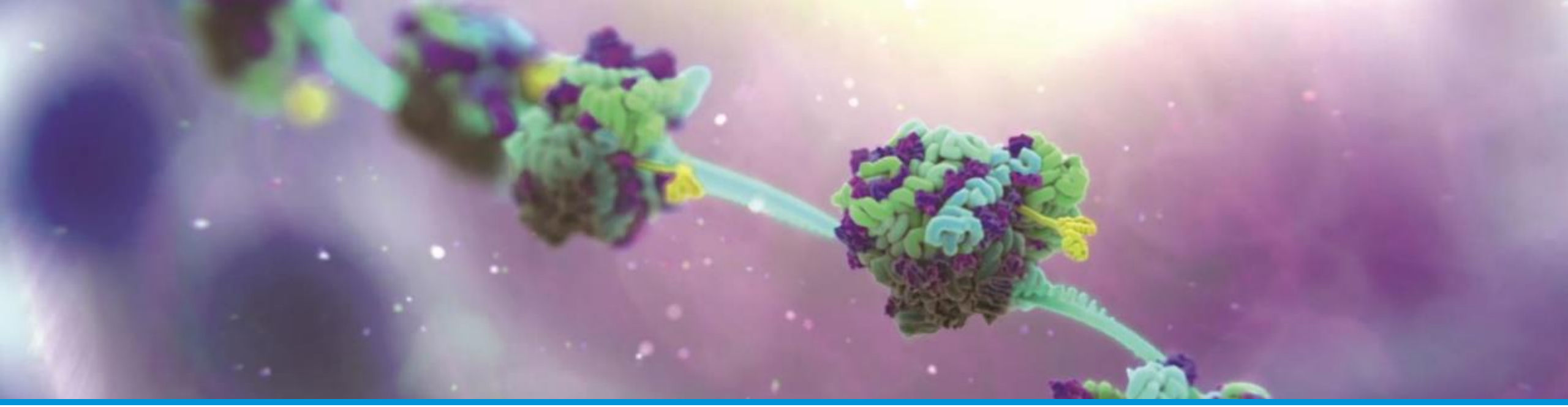


# Knock-In iPS Cell Line Generation Using End-Modified Linear DNA Donors

Aidan Simpson[1], Christopher B. Rohde[2], Mitchell R. Kopacz[2], Jasmine K. Harris[2], Franklin Kostas[2], I. Caglar Tanrikulu[3], Matthew Angel[2]

[1]Massachusetts Institute of Technology, [2]Factor Bioscience, Inc., [3]Novellus, Inc.

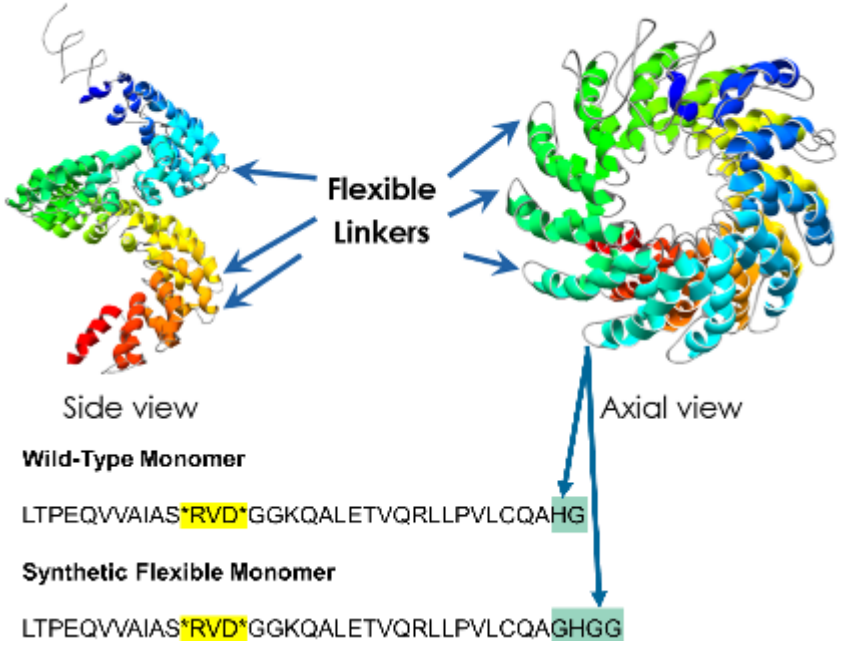
FACTOR BIOSCIENCE

ASGCT 2021

05.11.21

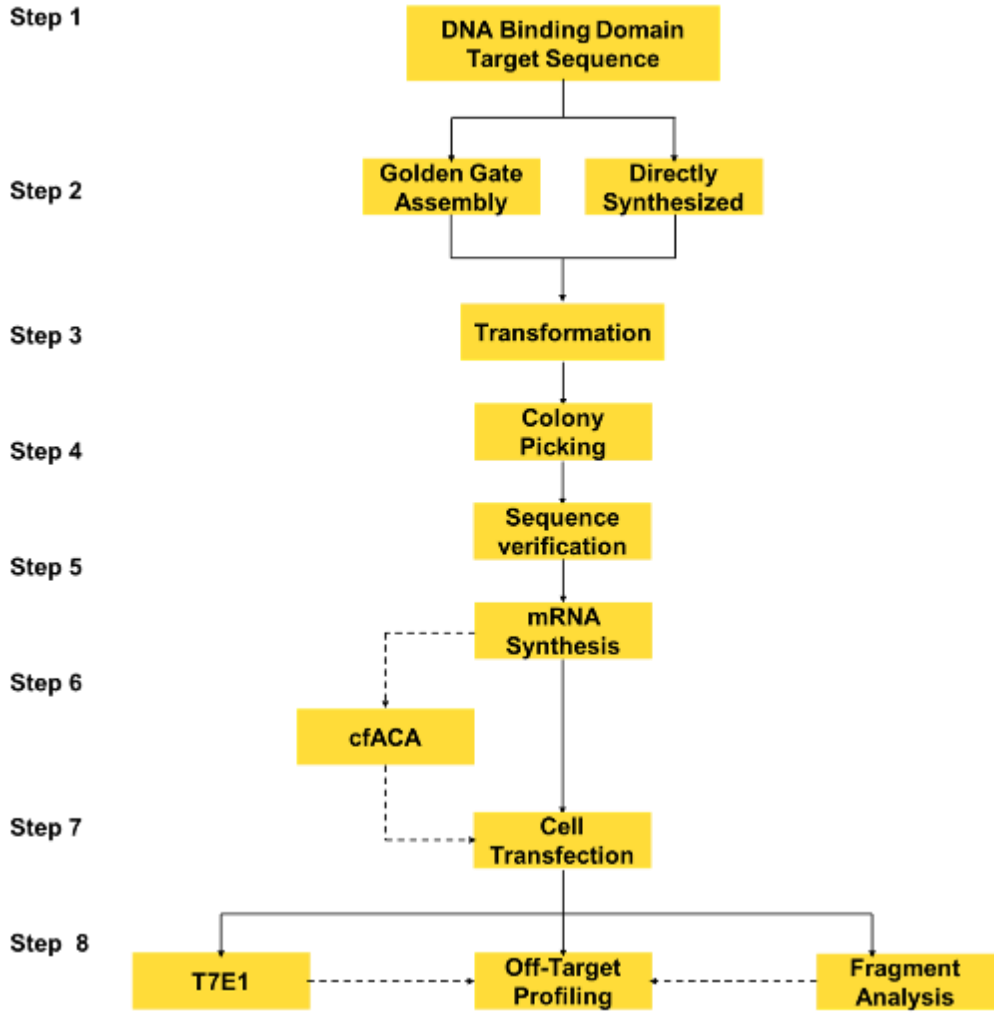
# NoveSlice Synthesis and Gene Editing

- NoveSlices are chromatin-context-sensitive gene-editing proteins which utilize synthetic flexible linkers.
- Once a target has been selected, NoveSlices are designed to cut at a selected target, and mRNA encoding for them is delivered to cells for editing.




Kopacz, M., et al. Mol Ther, Vol 28 No 4S1, 2020.

## NoveSlice creation and testing

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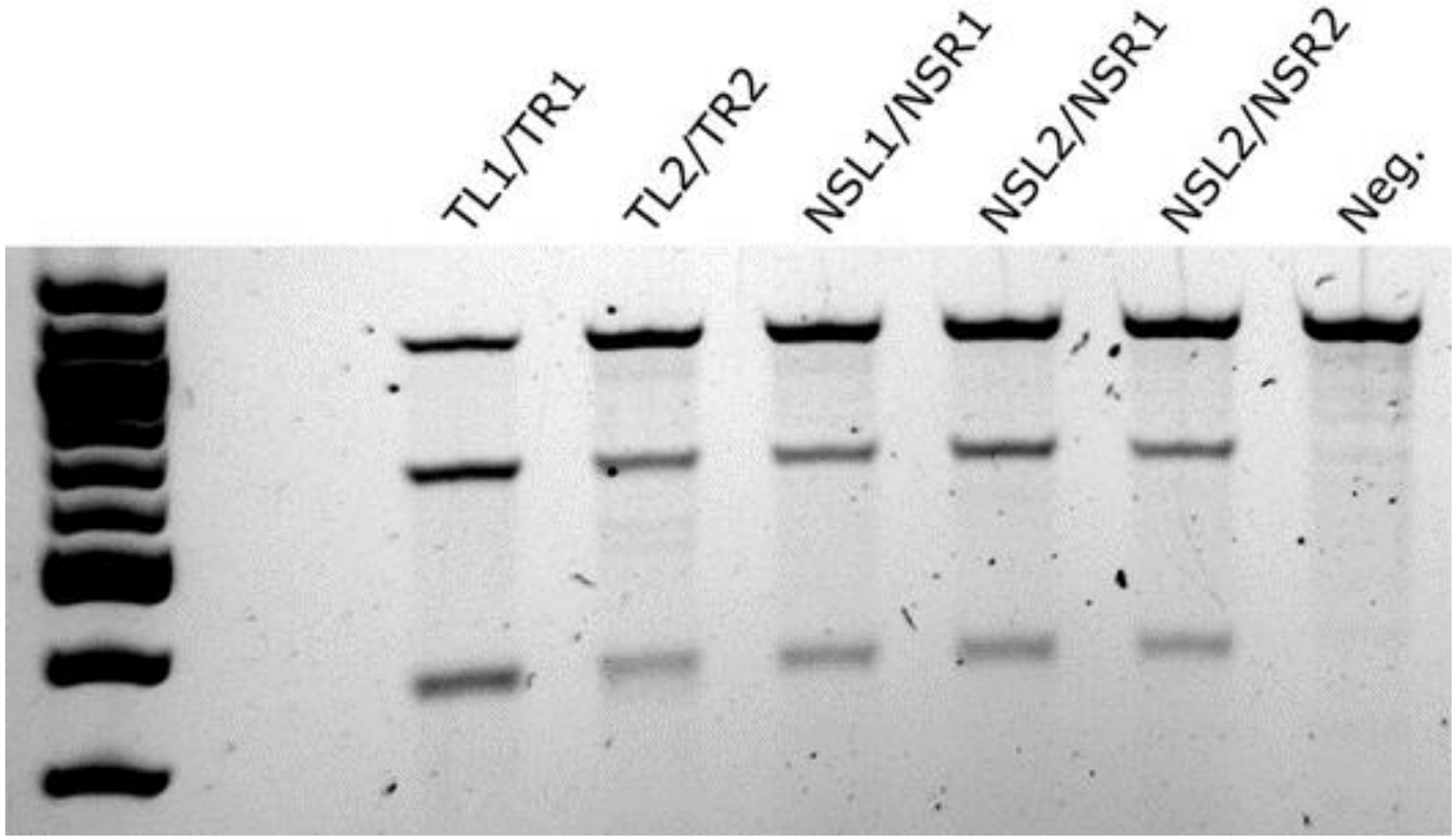
# NoveSlice Synthesis and Gene Editing

- mRNA encoding the NoveSlice protein was electroporated into iPS Cells.
- Gene editing efficiency was determined via a T7E1 Mismatch assay after electroporation with mRNA encoding either TALENs or NoveSlice.

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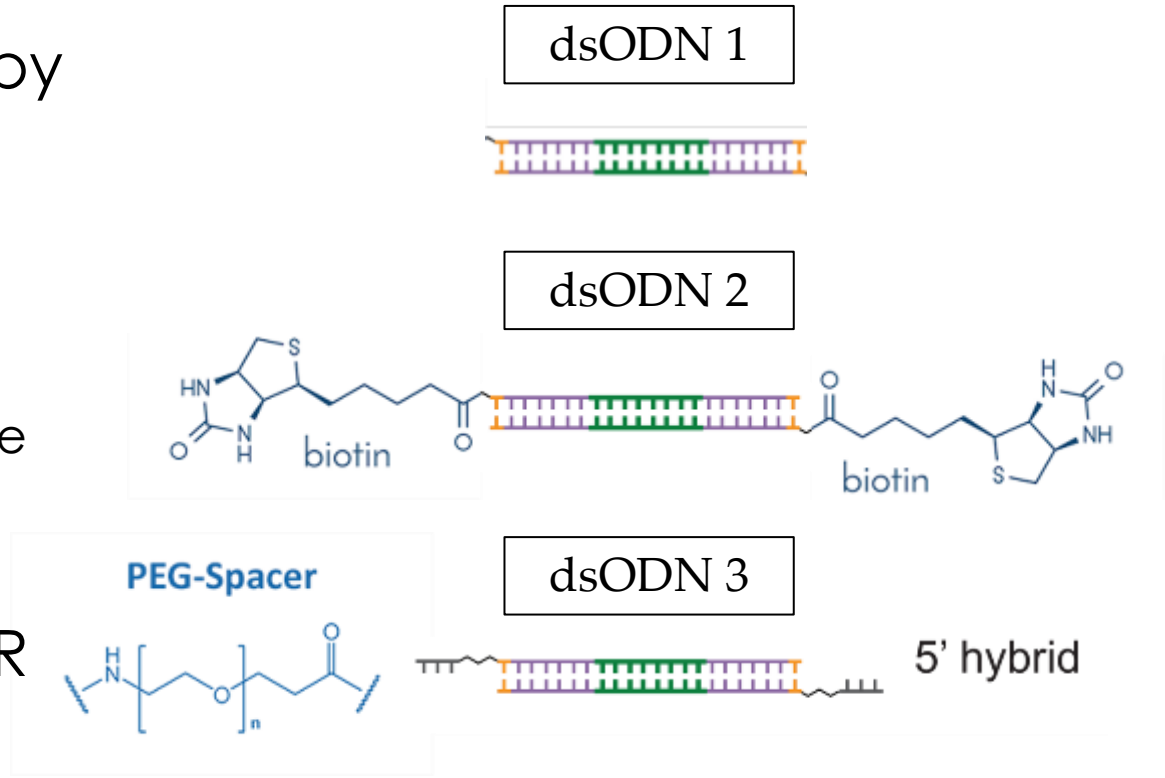
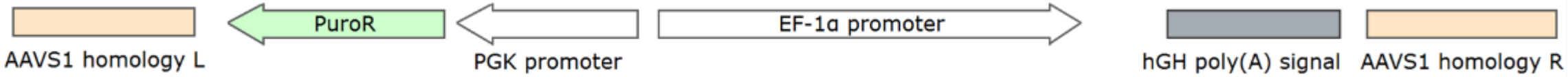
# HDR Donor Templates

- We focused on 3 linear dsODNs with different end modifications enabled by PCR amplification using modified primers
  - dsODN 1 (blunt): standard primers
  - dsODN 2 (biotin): biotinylated primers
  - dSODN 3 (ssDNA-PEG): random DNA sequence followed by polyethylene glycol (PEG) spacer
- Each donor encodes a bidirectional EF1a/PGK promoter driving GFP/PuroR expression flanked homology to the AAVS1 cut site.



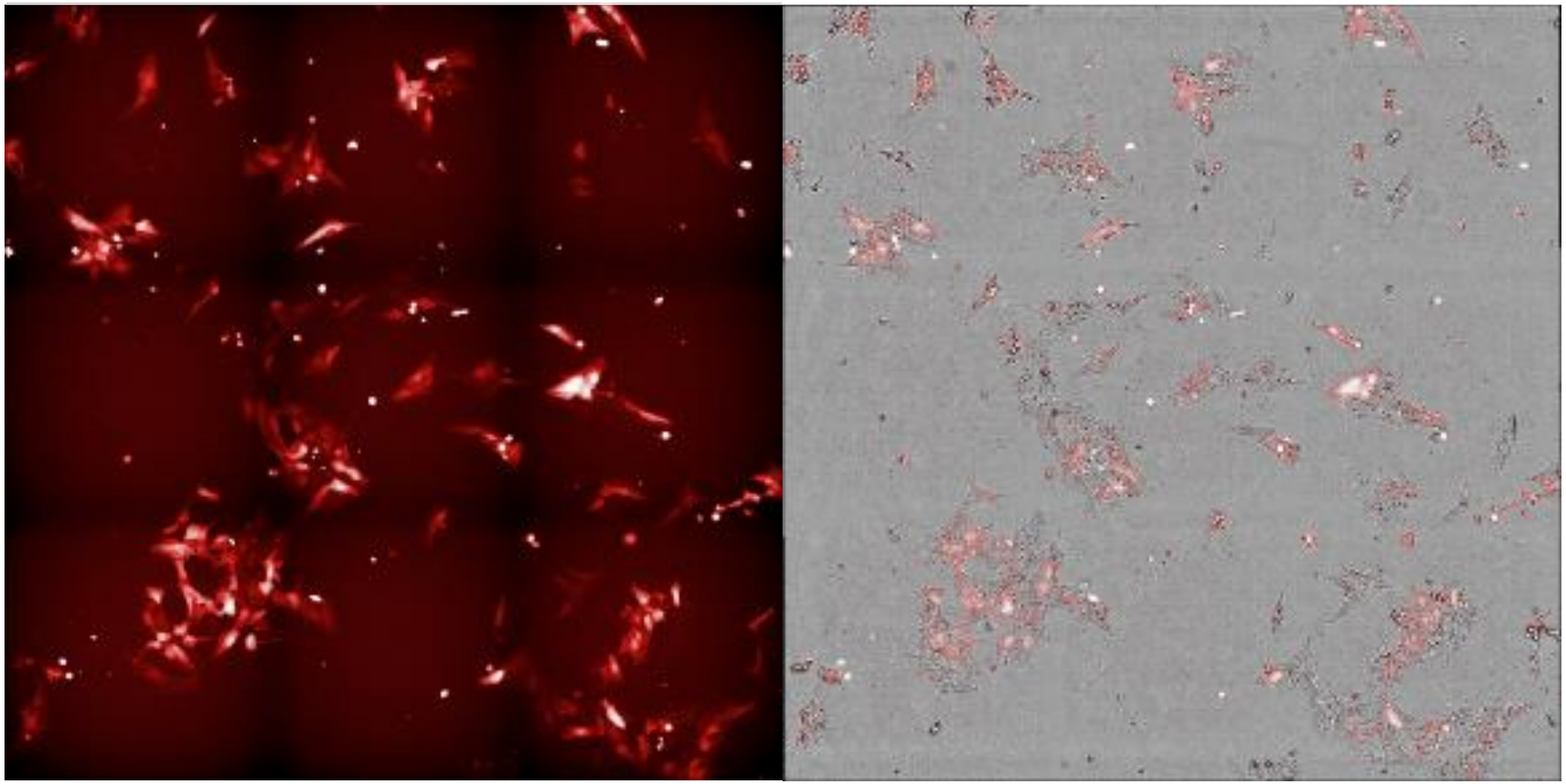




FACTOR BIOSCIENCE

# mRNA Electroporation Conditions

mCherry

mCherry + Brightfield



- The electroporation conditions used to deliver mRNA to iPSCs:
  - Voltage- 950v
  - Width- 30ms
  - # Pulses- 1 pulse
- These conditions have been used to successfully deliver mRNA for gene editing and protein expression as seen with this mRNA encoding RFP.

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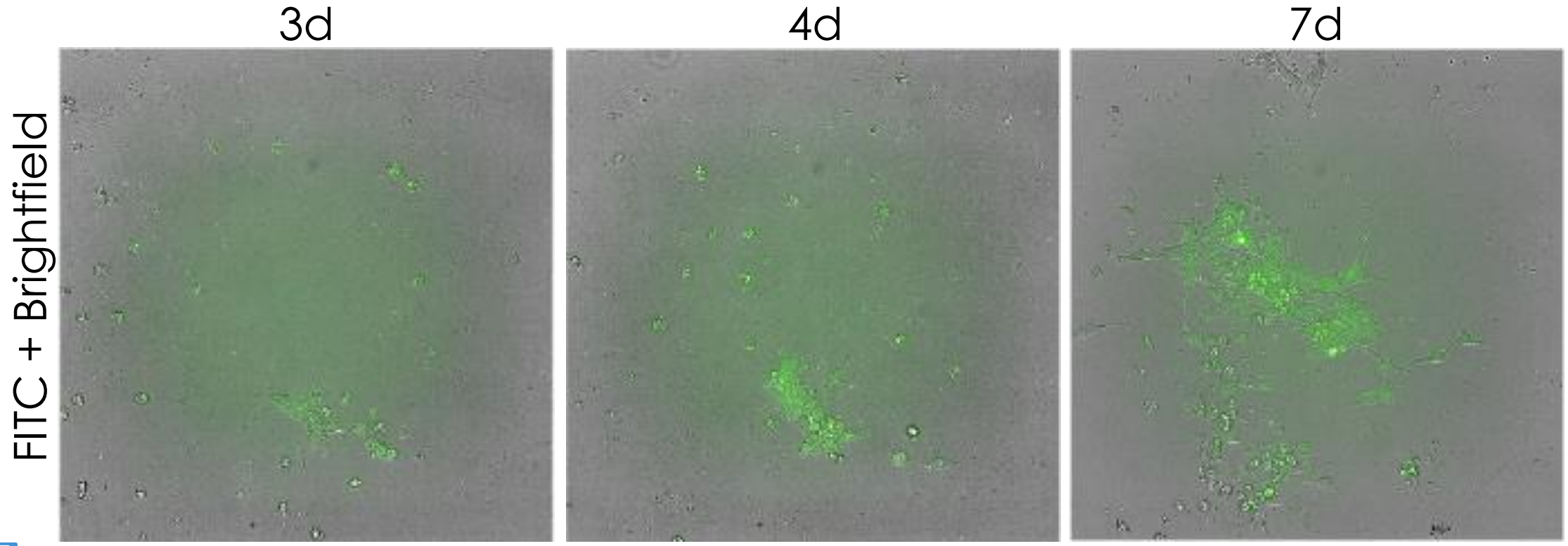
# mRNA Electroporation Condition Not Optimized for DNA + mRNA Co-Delivery

- Over numerous experiments, low numbers of iPSCs had presented GFP as indicate by green cells. However when co-transfected with mRNA encoding RFP, cells showed strong levels of RFP expression.
- This indicated that the current electroporation conditions failed to effectively deliver HDR Donor DNA to the cells.

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# Electroporation Optimization for DNA + mRNA co-delivery

- In order to determine the best electroporation for optimal gene editing and gene insertion, we would co-delivery of mRNA encoding RFP and Donor DNA encoding GFP.
- We would also co-deliver the same amount Donor DNA encoding GFP but swap out the mRNA encoding RFP for mRNA encoding NoveSlice.

| | 1 | 2 | 3 | 4 | |
|---|---|---|---|---|---|
| A | 400ng dsODN 1 | 400ng dsODN 1 + 500ng RFP mRNA | 400ng dsODN 1 + 1µg GE mRNA | Electro. Only | 1400V/2x20ms (Best DNA + RNA Condition) |
| B | 400ng dsODN 1 | 400ng dsODN 1 + 500ng RFP mRNA | 400ng dsODN 1 + 1µg GE mRNAmRNA | Electro. Only | 1100V/2x20ms (Best Protein Complex Condition) |
| C | 400ng dsODN 1 | 400ng dsODN 1 + 500ng RFP mRNA | 400ng dsODN 1 + 1µg GE mRNA | Electro. Only | 1400V/3x10ms (Best 3-Pulse Condition) |
| D | 400ng dsODN 1 | 400ng dsODN 1 + 500ng RFP mRNA | 400ng dsODN 1 + 1µg GE mRNA | Electro. Only | 1200V/1x30ms (Best Single-Pulse Condition) |

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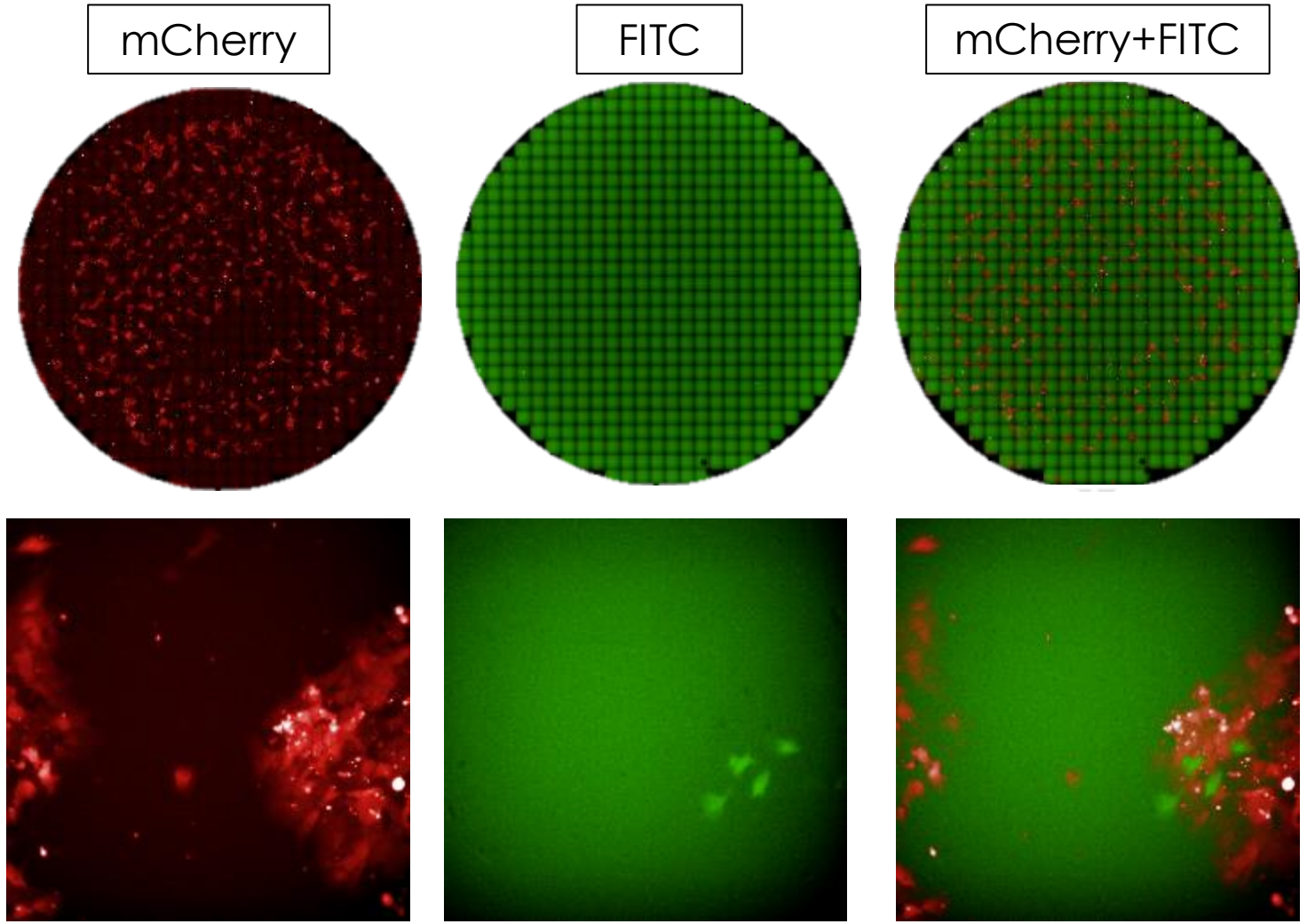
Electroporation Conditions from Xiquan Liang et al., 2015. Rapid and Highly Efficient Mammalian Cell Engineering via Cas9 Protein Transfection. *Journal of Biotechnology* 208, 44-53.

# Electroporation Optimization for DNA + mRNA co-delivery

- Here, we tested the co-delivery of mRNA encoding RFP and donor DNA encoding GFP.
- It was observed that the GFP encoded by the dsODN are expressed independent of integration due to the presence of a Strong Promoter, EF1a.
- Expression of unintegrated dsODNs decreases over time.

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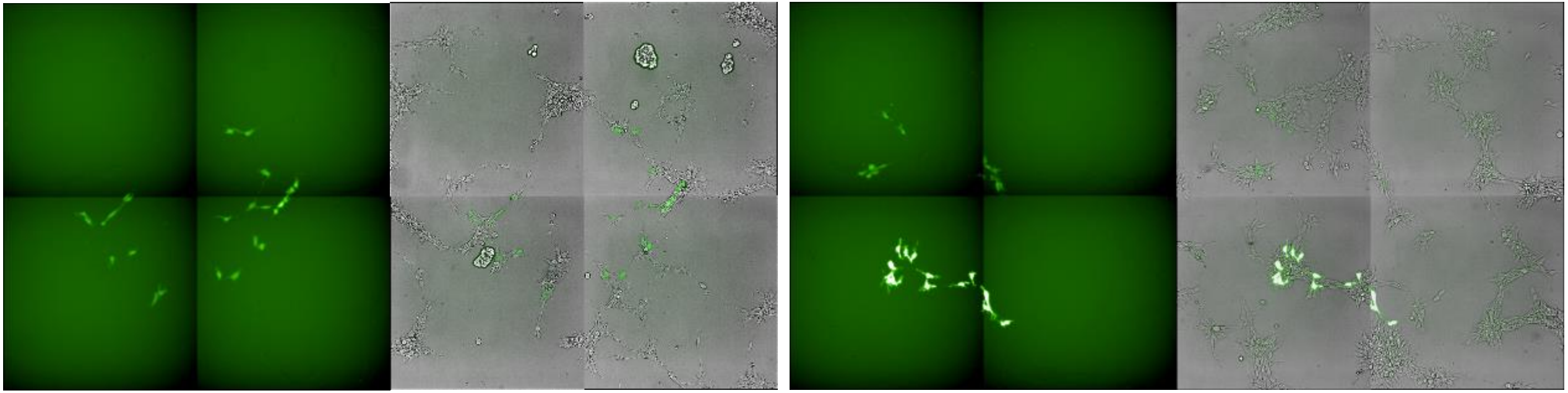
# Electroporation of dsODN + NoveSlice mRNA co-delivery

- Utilizing the same electroporation conditions as the previous co-delivery of mRNA encoding RFP and donor DNA encoding GFP. We co-delivered the mRNA encoding NoveSlice gene editing protein and dsODN 1 to iPSCs.
- We observed and increased number of GFP fluorescence cells in the samples co-delivered with dsODN and mRNA encoding NoveSlice.

400ng dsODN 1

400ng dsODN 1 + 1µg NoveSlice mRNA



**FITC** **FITC+Brightfield** **FITC** **FITC+Brightfield**

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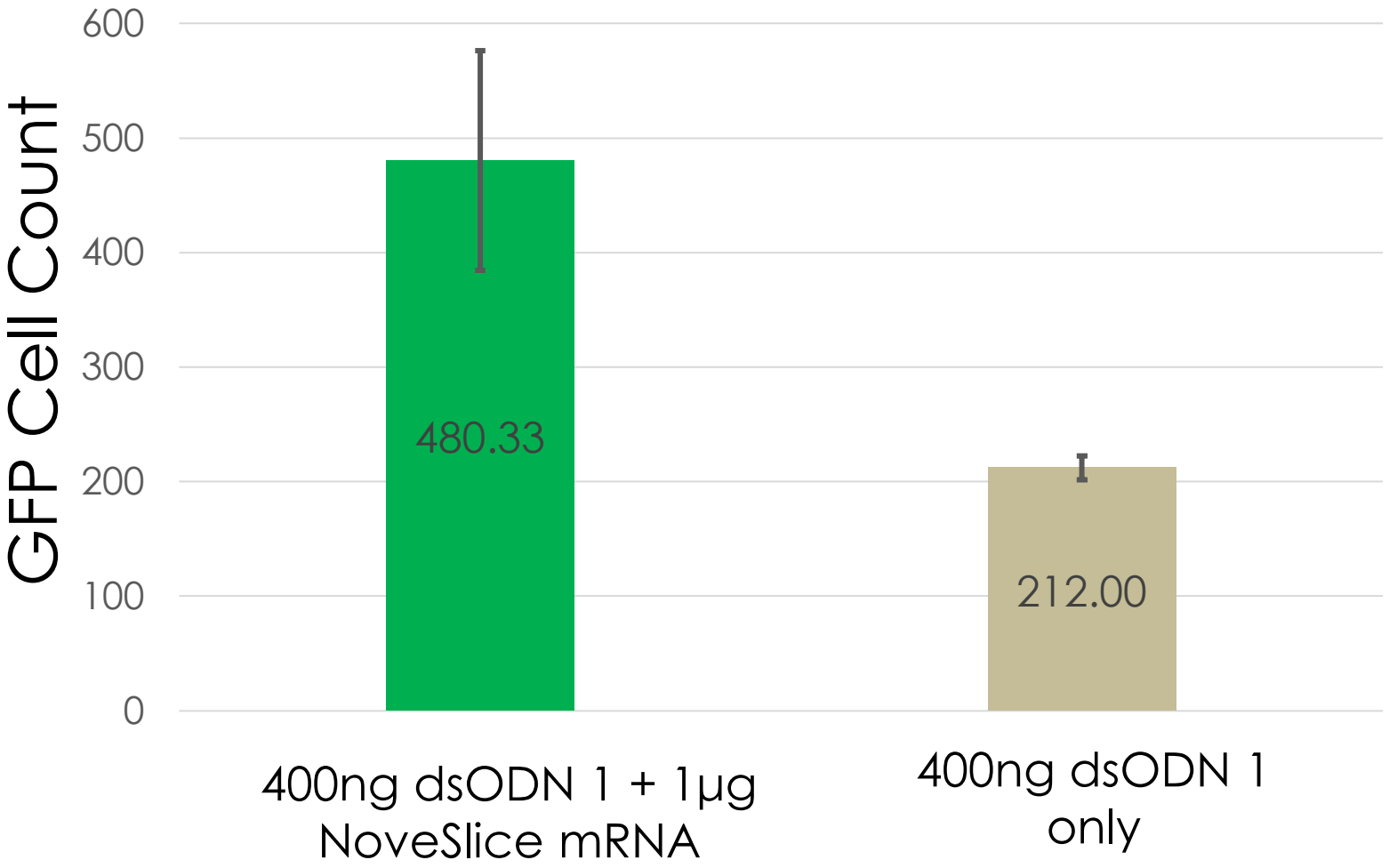
# Gene-edited Cells Show Higher GFP Expression on Day 9




- Whole-well tiled images were captured and assembled using the PerkinElmer Operetta High-content Imaging System for three electroporation replicates of each condition in separate wells of a 24-well plate.
- Cells expressing GFP in each image were counted manually.

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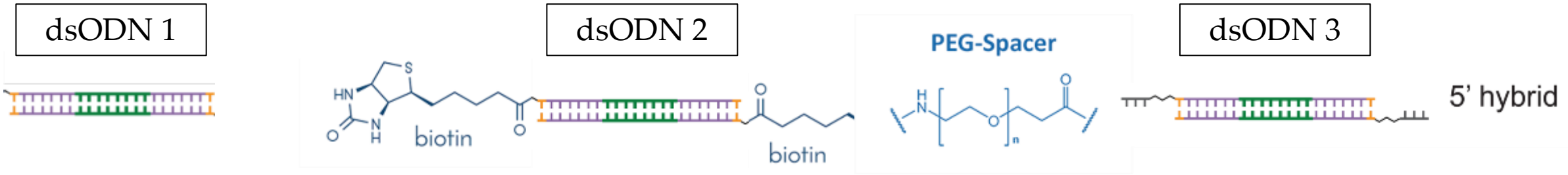
# Comparing dsODN Templates for Optimal Gene Expression

- To create the dsODN templates we used the following primers:
  - dsODN 1 (blunt): standard primers
  - dsODN 2 (biotin): biotinylated primers
  - dSODN 3 (ssDNA-PEG): random DNA sequence followed by polyethylene glycol (PEG) spacer

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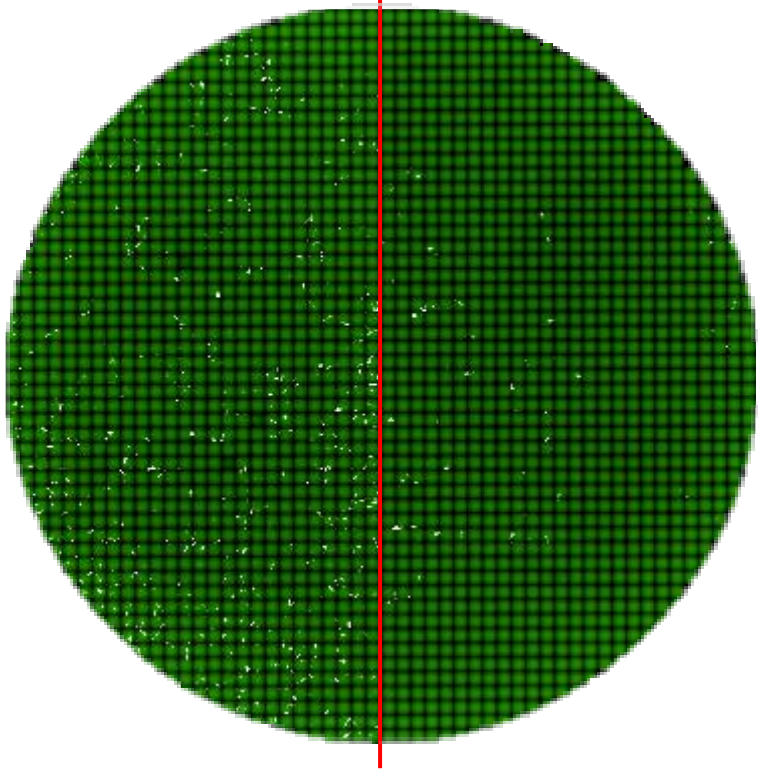
# dsODN Comparison – 7 Days Post-electroporation

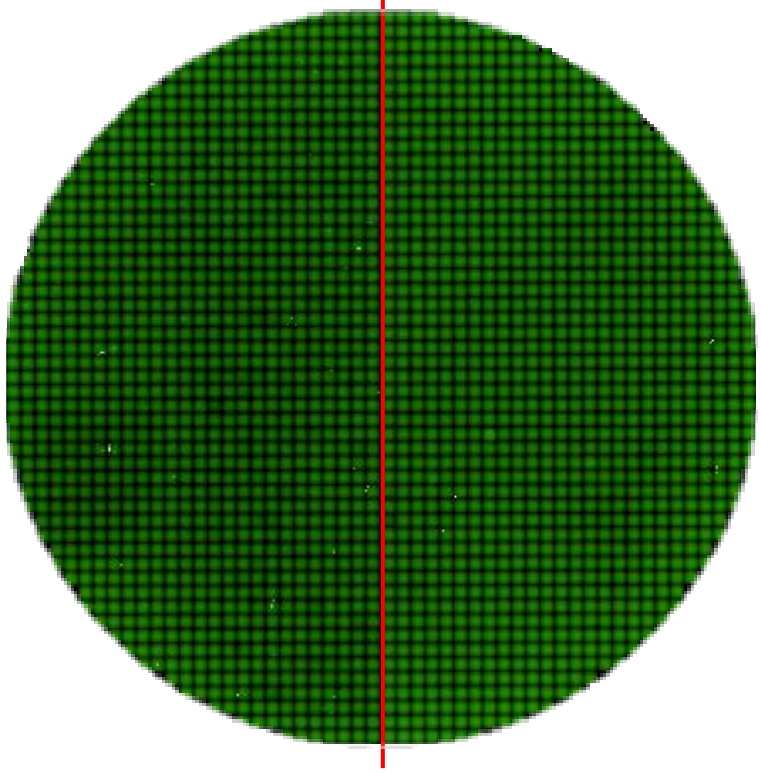
dsODN 1 – Blunt end

+NoveSlice | - NoveSlice



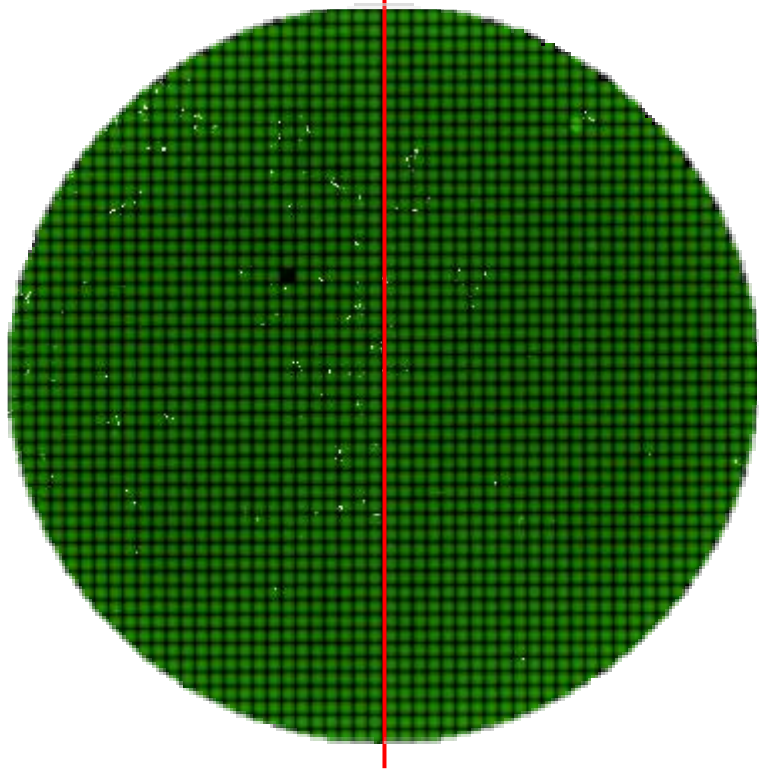
dsODN 2 – Biotin

+ NoveSlice | - NoveSlice



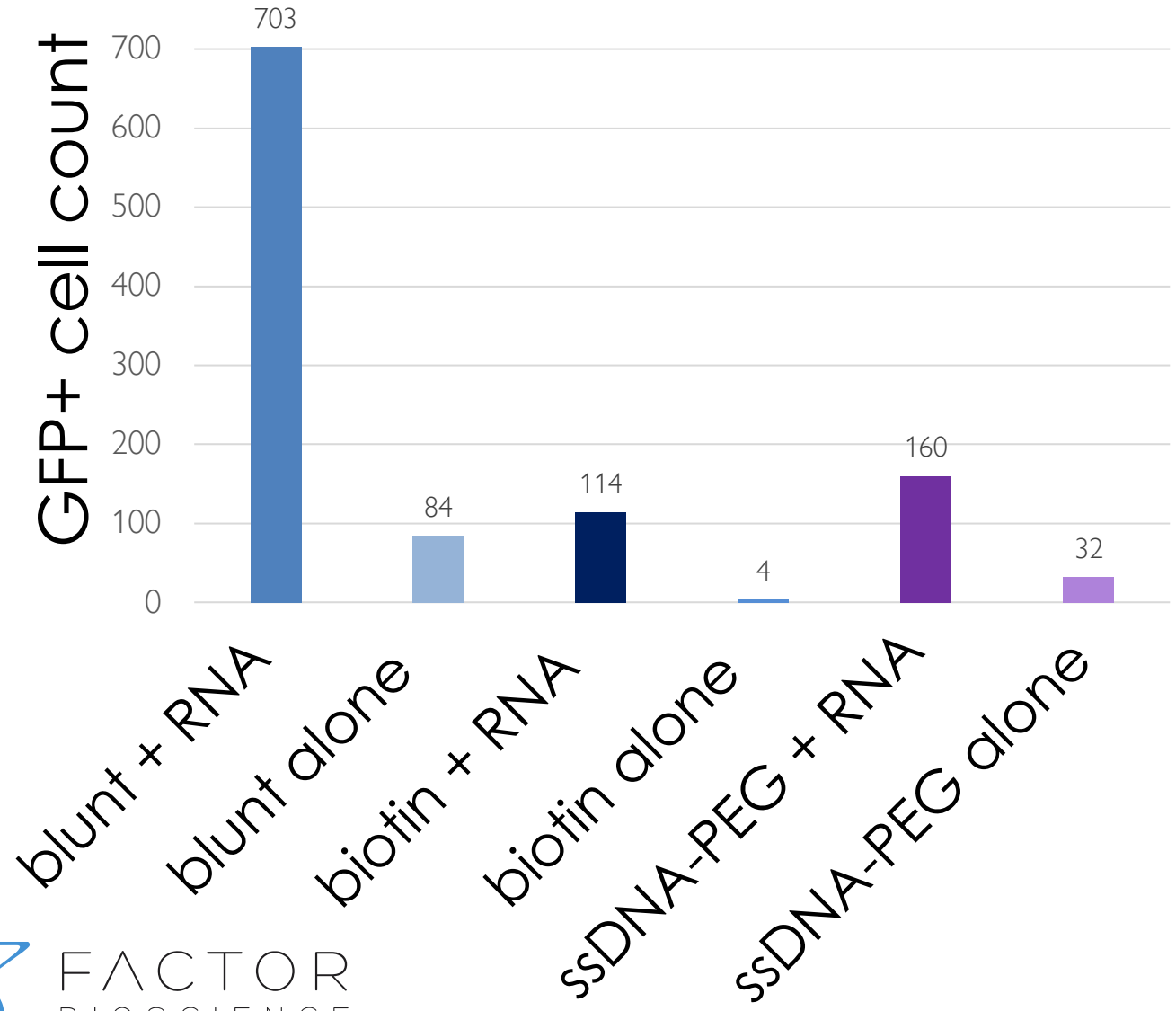
dsODN 3 – PEG

+ NoveSlice | - NoveSlice



FACTOR BIOSCIENCE

# End-Terminal Modifications Lead to Variation in dsODN Expression




- Whole-well tiled images were captured and assembled using the PerkinElmer Operetta High-content Imaging system for wells containing three electroporation replicates of each condition in a 6-well plate.
- Cells expressing GFP in each image were counted manually.

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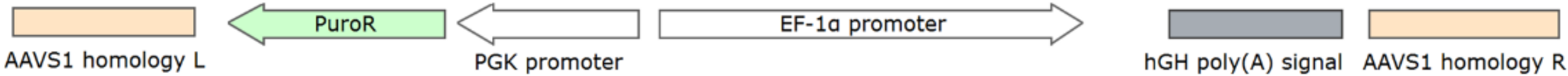
# Puromycin Selection of dsODN Integration

- Each HDR donor DNA template (dsODN ) contains a Puromycin resistence gene under the control of the Human Phosphoglycerate kinase (PGK) promoter.

- With Puromycin gene under the control of the PGK promoter, cells with stable integration of the dsODN into the genome will produce sustained levels of Puromycin resistance.

- Proper puromycin dosing conditions needed to be optimized. Initial testing conditions were selected as 0, 0.1, 0.5, 1, 5, and 10 µg/mL.

- We observed that the optimum Puromycin dose was 0.1 µg/mL.

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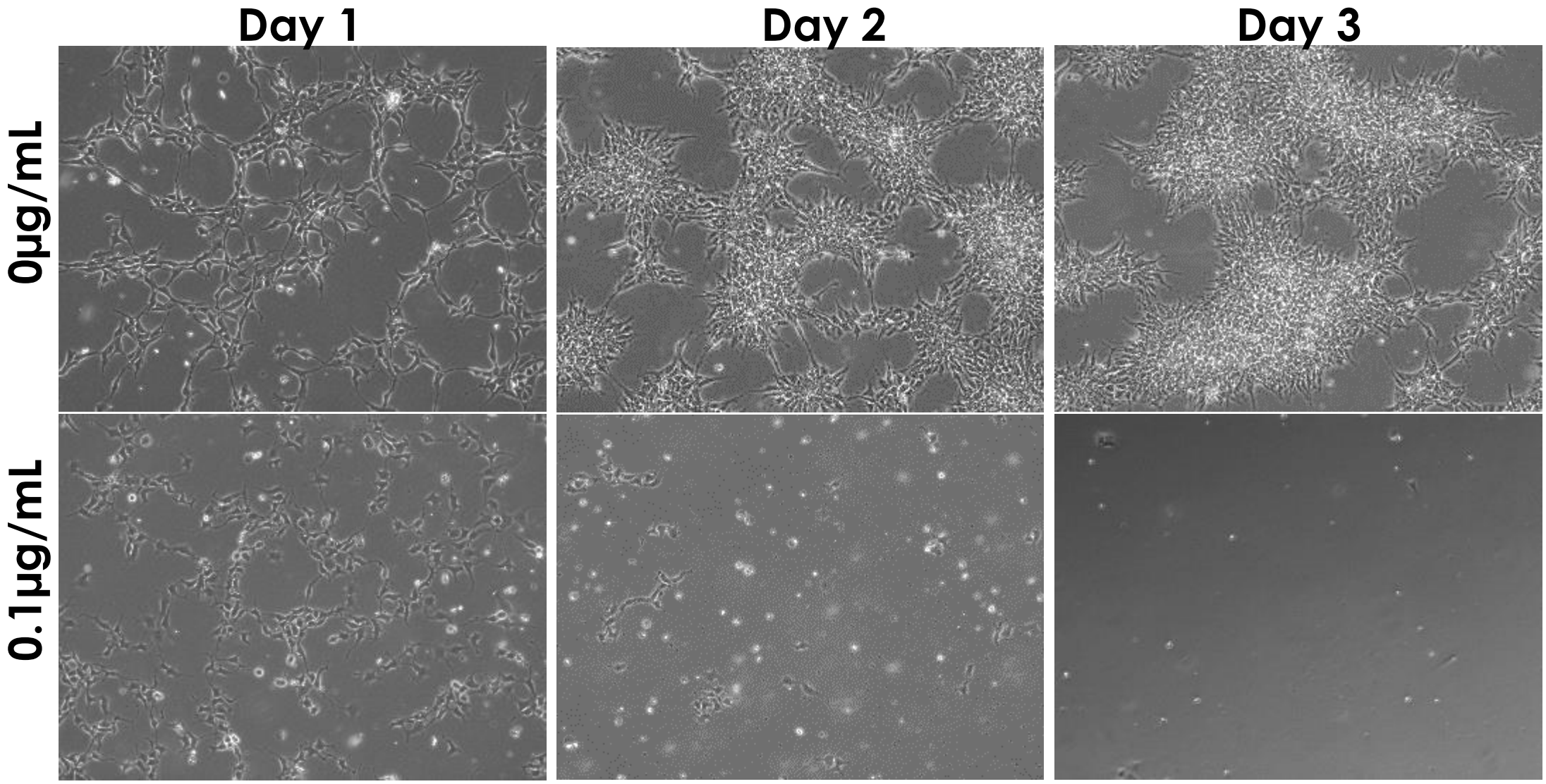
# Optimization of Puromycin Dosing on iPSCs

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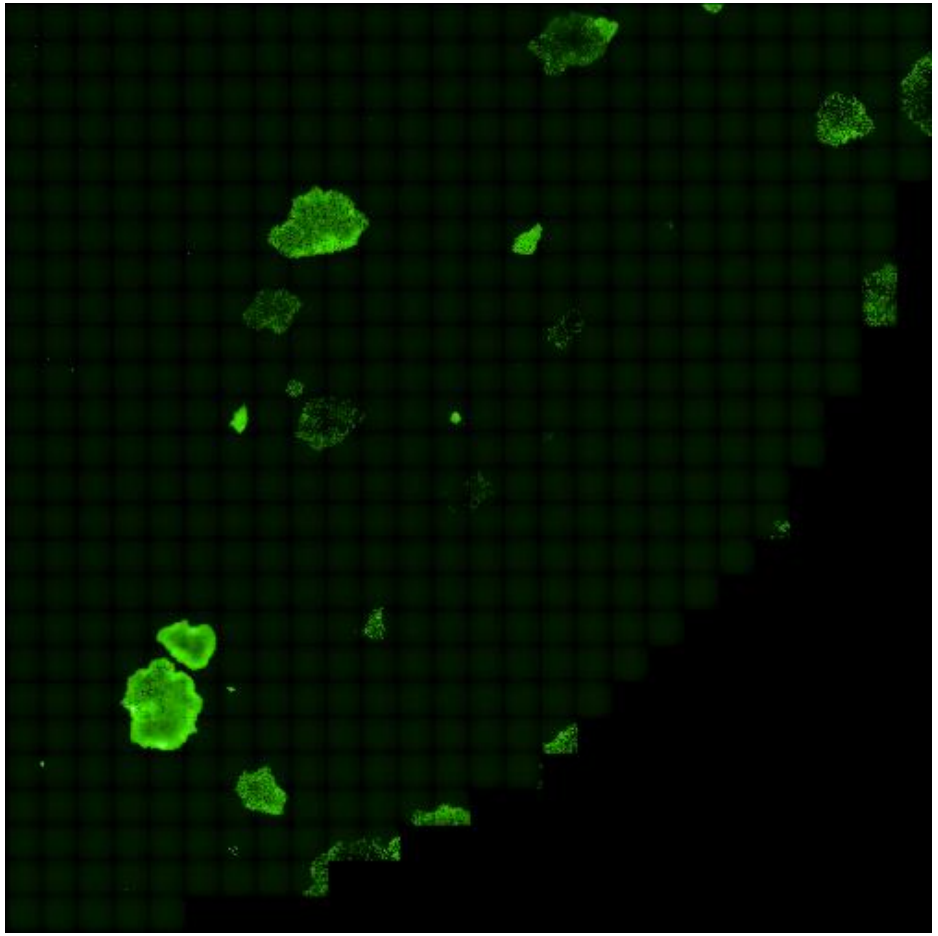
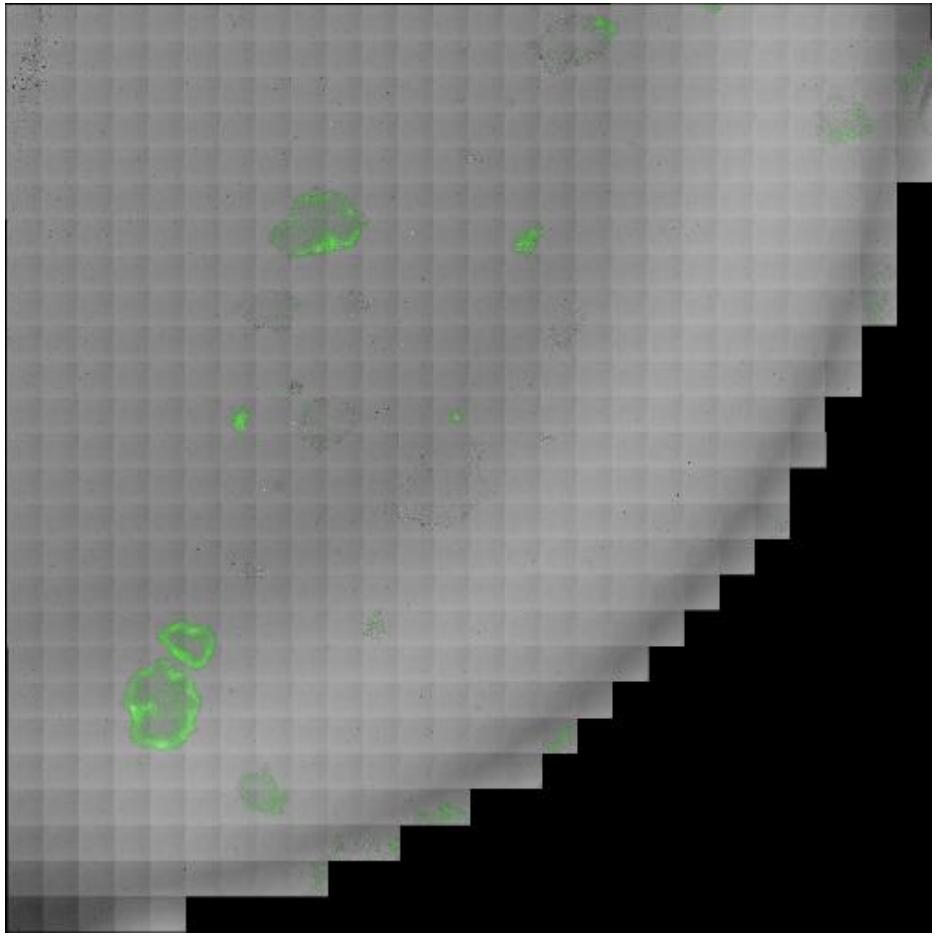
# Stable GFP Integration in iPSCs Post-Puromycin Selection

400ng dsODN 1 + 1µg NoveSlice mRNA

**FITC**



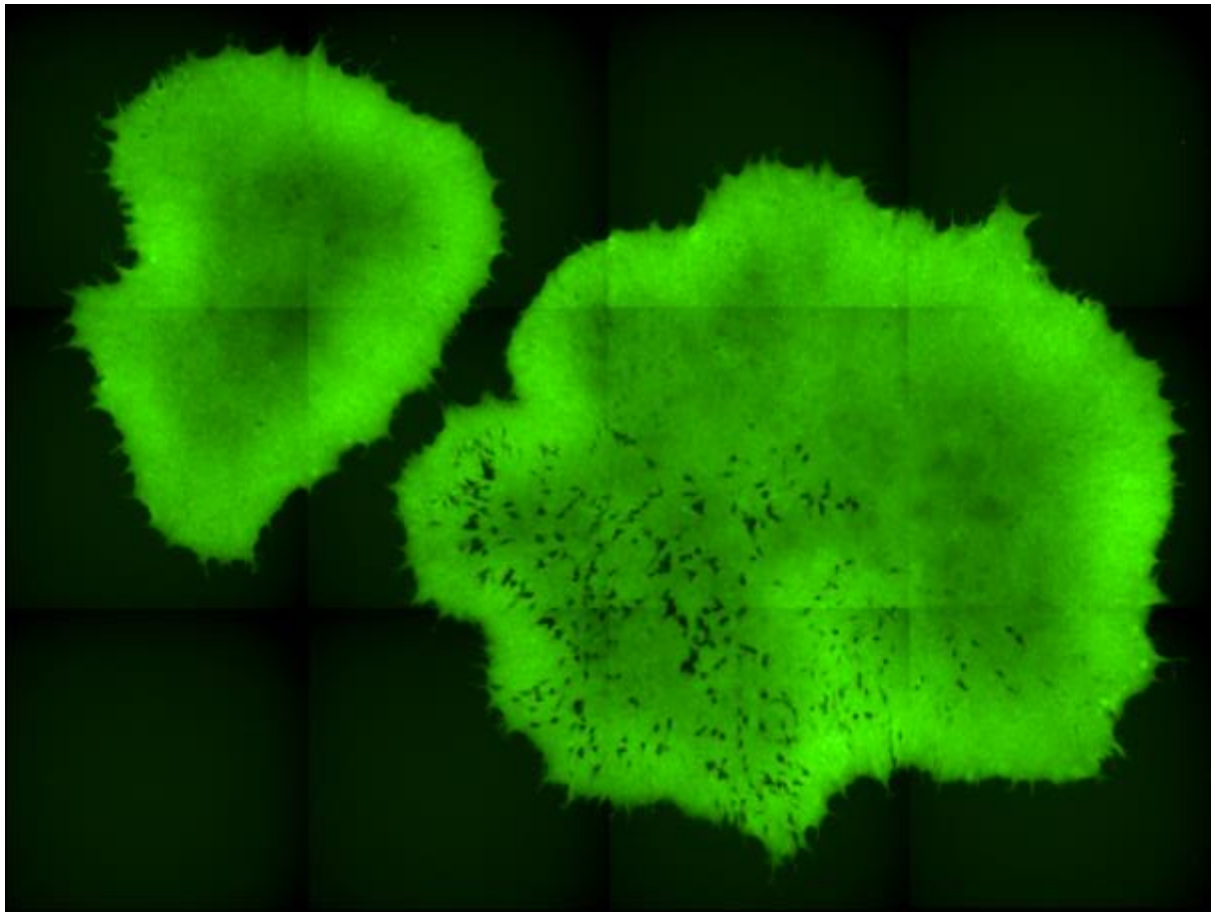
**FITC+Brightfield**



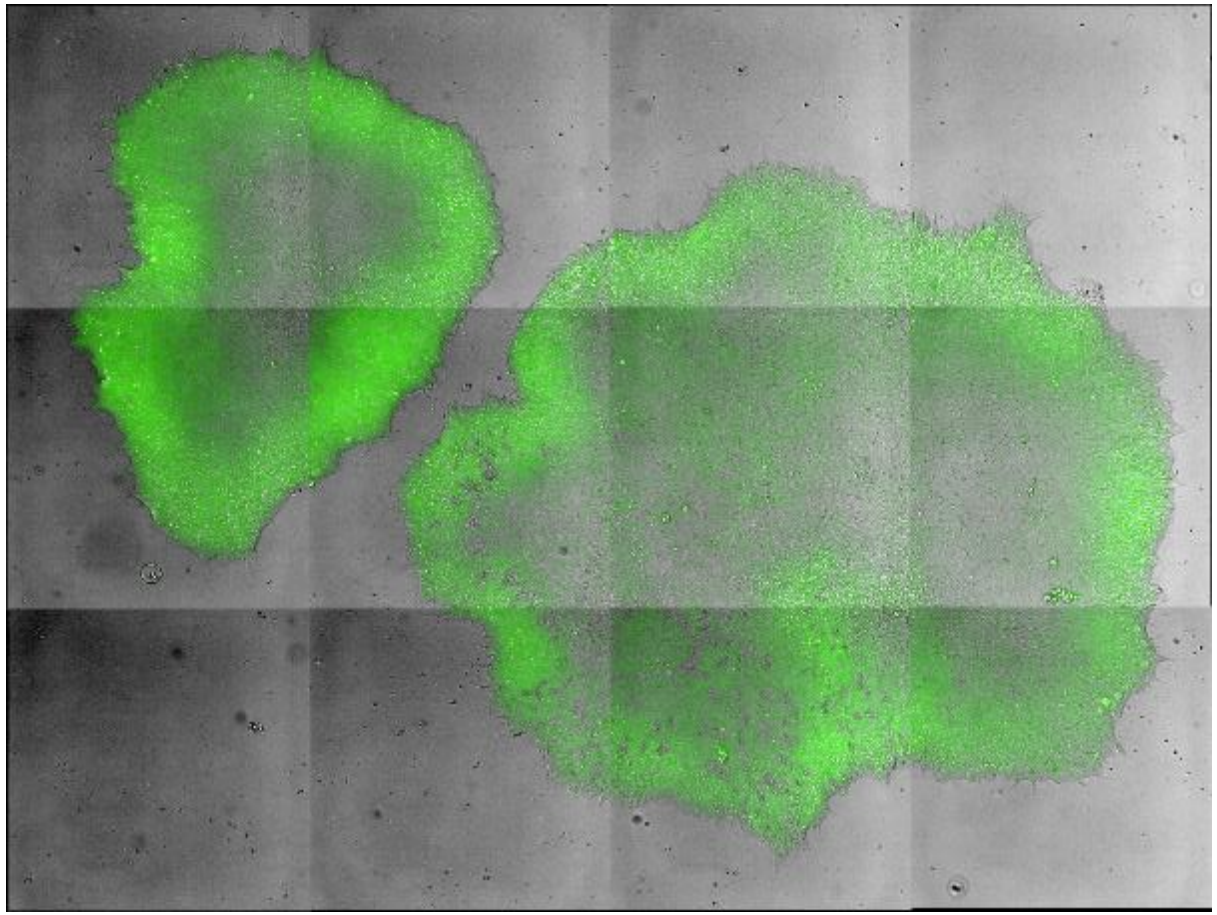
FACTOR BIOSCIENCE

# Stable GFP Integration in iPSCs Post-Puromycin Selection

**FITC**



**FITC+Brightfield**



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# Discussion

- Results indicate successful genomic integration of the GFP and Puromycin Resistance genes
- The blunt-ended dsODN emerged as the most successful construct, but its rate of on-target integration relative to other constructs remains to be determined
- The most important factors for successful gene editing of human iPSCs are optimal cell culture conditions including cell culture media and cell coating material to prevent overgrowth and differentiation.
- A high dose of puromycin delivered in a short time frame is sufficient to isolate edited cells from the background unintegrated cells.
- Different nucleic acids and their combinations require specific electroporation conditions in terms of voltage, pulse duration, and pulse number. Linear dsODNs containing genes and promoters are robustly expressed when successfully electroporated

# Acknowledgements


- I would like to thank everyone at Factor Bioscience Inc, for their help and support.


*CR and MA are authors on several gene editing patents held by Factor Bioscience Limited

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