Optimising delivery of ribonucleoprotein complexes into SU-DIPG-IV cells by electroporation

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Aim

Optimise the electroporation of ribonucleoprotein complexes into HSJD-DIPG-IV cells in preparation for later CRISPR editing.

RNP complexes will be used instead of plasmids as this has widely been adopted as a method that produces greater editing efficiency (better delivery into the cell, less stress on cells due to Cas9 expression) and lower off-target editing (lower concentrations of Cas9 and sgRNA in the cell, for shorter periods of time).

Methodology

Use of RNPs prevents the inclusion of a marker as would be present on a DNA plasmid. Instead to optimise electroporation settings Cas9 is substituted with an AlexaFluor488 antibody that can be detected with flow cytometry.

This protocol is adapted from the following references:

'IDT Genome Editing Protocol: Alt-R CRISPR-Cas9 System: Delivery of ribonucleoprotein complexes into Jurkat T cells using the Neon® Transfection System (Ver. 3.1)' accessed from: https://sfvideo.blob.core.windows.net/sitefinity/docs/default-source/protocol/alt-r-crispr-cas9-user-guide-ribonucleoprotein-electroporation-neon-transfection-system0601611532796e2eaa53ff00001c1b3c.pdf?sfvrsn=6c43407_26

Neon® Transfection System User Guide, publication number MAN0001557, accessed from: http://tools.thermofisher.com/content/sfs/manuals/neon_device_man.pdf

Xu, X., Gao, D., Wang, P. *et al.* Efficient homology-directed gene editing by CRISPR/Cas9 in human stem and primary cells using tube electroporation. *Sci Rep* 8, 11649 (2018). https://doi.org/10.1038/s41598-018-30227-w

Shalem O, Sanjana NE, Hartenian E, *et al.* Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 2014;343(6166):84-87. doi:10.1126/science.1247005

Method

Equipment/reagents used

- Neon electroporation system and Neon 10µL electroporation kit
- TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0)
- Anti-GFP sgRNA
  (sequence: GAAGTTCGAGGGCGACACCC, used previously by (Shalem, 2014), supplied as a synthetic RNA with a 2'-O-methyl/phosphorothioate linkage by Sigma)

Pre-seeding of cells

2 days before electroporation, HSJD-DIPG-IV cells (HIST1H3B K27M, ACVR1 G328V) were seeded in TSM-C (recipe) at densities of 1 million or 2 million cells per T75 flask. (Aiming to have 400,000 cells per electroporation reaction, harvested at 70-90% confluency).

Processing of samples for electroporation

TSM-B and then TSM-C were made up without the antibiotic/antimycotics that are usually included (TSM-C-NAB). A 24-well plate was made up with 500µL TSM-C-NAB, and pre-incubated at 37°C. Cells were split as usual [reference past protocol], counted, and re-suspended in TSM-C-NAB.

Note – When these cells were harvested they were at less than the ideal 70-90% confluent.

Mock RNPs formation:

Anti-GFP sgRNA (off-target control sgRNA) was made up to 44µM (according to Neon protocol) and antibodies were made up to 10µM (for a final concentration of 0.5µM) (Xu, 2018) in TE buffer. sgRNA and antibodies were combined in equal parts and incubated for 10-20min at room temperature.

Cells were pelleted, washed in PBS, and resuspended in enough buffer R for 9µL per electroporation, and 26 total electroporations. Each reaction consisted of 9µL of cells (400k total) and 1µL RNP complexes, 2µL IDT electroporation enhancer*, of which 10µL was used for each electroporation. (Excess must be made up to avoid bubbles, and arcing in the electroporation tip).

Electroporations were performed according to the Neon 24-well optimisation protocol.

Notes –
Well 7 threw up multiple errors, and later had a clump of unidentified material in it. Well 9 arced (expect reduced viability and efficiency).

Processing of samples for flow cytometry (FC)

AlexaFluor fluorescence could not be detected with the Celigo image cytometer so cells were prepared for flow cytometry. (Xu *et al.* didn't specify their imaging technique, although it was likely to be confocal).

Cells were cultured for 24h in the 24-well plate to allow cell recovery. TSM-C-NAB was removed from plate and replaced with 400µL TrypLE. Plate was incubated at 37°C for 10 minutes. 800µL TSM-C-NAB was added to neutralise the TrypLE. The cell suspensions were each transferred to an FC tube and spun down at 500g, 4min. Supernatant was removed and cells were resuspended in 300µL ice cold PBS and kept on ice until FC was performed using a BD LSRFortessa, recording the FSC, SSC, AlexaFluor488, and PE amplitudes. Analysis was performed using FlowJo V10.

Notes -
Cells need not be resuspended in PBS by pipetting - this can be done as they are vortexed before being read in the Fortessa.
The first row of samples were mistakenly resuspended in 37oC PBS and only later put on ice.

Values for the mock electroporation were taken from the reported optimised values for U-87 cells (https://www.thermofisher.com/content/dam/LifeTech/migration/en/filelibrary/cell-culture/neon-protocols.par.80909.file.dat/u-87%20mg-brain.pdf).

Analysis

Live cells were gated using FSC-A and SSC-A as illustrated in (Figure 1a). Within live cells AF488 was gated into positive (according to the antibody negative control, and the first

electroporation), and hyper-positive (around the peak of a high amplitude AF488 peak) (Figure 1b, c).

Figure 1

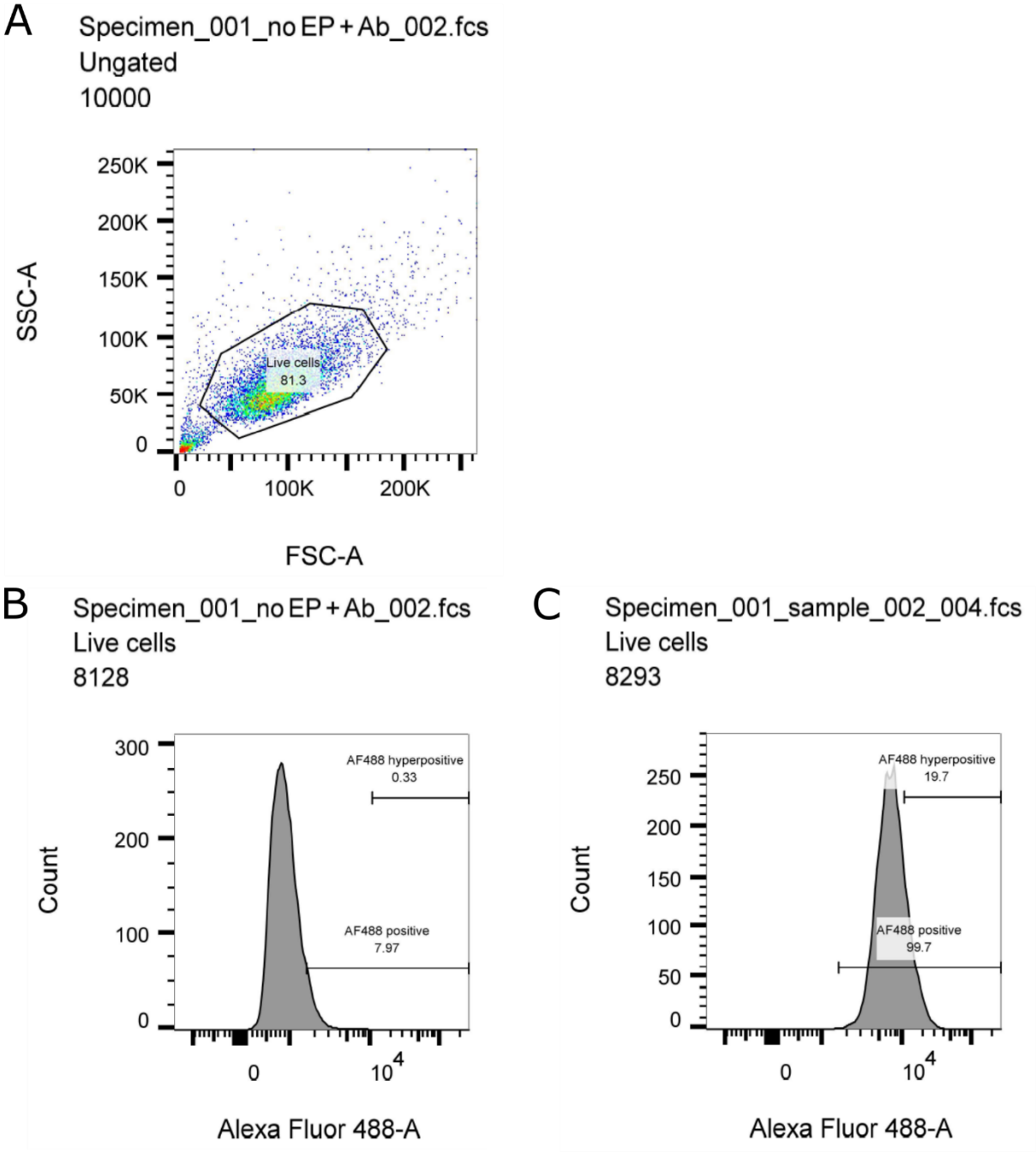

Figure 1 - analysis setting for flow cytometry of SU-DIPG-IV electroporated with AlexaFluor488 antibodies

A, A scatter plot showing the forward scatter and side scatter through SU-DIPG-IV cells, an a gate used to select the live cells. **B, C,** Histograms showing the amplitude and number of cells stained with AlexaFluor488 following electroporation with sgRNA:AlexaFluor488 RNPs (**B**) or mock electroporation with the same RNPs (**C**). The plots include gates for positive and hyper-positive cells, showing a background staining of 7.97% and 0.33% respectively.

The hyperpositive group was added because all electroporation presets produced a high percentage of positive cells. Including a 'hyper-positive' groups identified electroporation presets that produce cells with a greater amplitude of AF488 signal, suggesting a greater level of protein delivery.

Background/external staining, which can be seen in the cells that weren't electroporated but were incubated with the antibody was 1.44% positive cells, and 0.33% hyper-positive cells (Figure 1b).

The percentage of live cells, percentage of live cells that are AF488 positive and hyperpositive, estimated percentage of total cells that are alive and AF488 hyperpositive are tabulated below:

Sample	Pulse voltage	Pulse length	No. of pulses	% live cells	Positive cells (% of live cells, background removed)	Hyper-positive cells (% of live cells)	Hyper-positive cells (% of total cells)
no EP no Ab				83.6	0	0.33	
no EP + Ab				81.3	1.44	0	
1				84.8	0	0	
2	1400	20	1	82.9	99.3	19.7	16.3
3	1500	20	1	81.7	99.7	44.2	36.1
4	1600	20	1	81.5	99.7	68.7	56.0
5	1700	20	1	77.8	99.7	92.6	72.0
6	1100	30	1	84.2	93.2	6.58	5.5
7	1200	30	1	84.9	92.8	7.67	6.5
8	1300	30	1	83.6	99.6	52.2	43.6
9	1400	30	1	83.4	99.3	19.7	16.4
10	1000	40	1	84.9	88.1	6.87	5.8
11	1100	40	1	84.3	99.1	21.1	17.8
12	1200	40	1	82.7	99.7	57.1	47.2
13	1100	20	2	84.5	92.4	6.55	5.5
14	1200	20	2	84.3	99.4	24.6	20.7
15	1300	20	2	82.1	99.6	58.2	47.8
16	1400	20	2	75.8	99.5	85.1	64.5
17	850	30	2	85.4	36.7	1.38	1.2
18	950	30	2	84.5	83.7	5.17	4.4
19	1050	30	2	84.5	99.4	27	22.8
20	1150	30	2	82.7	99.7	71.4	59.0
21	1300	10	3	84.3	96.2	5.93	5.0
22	1400	10	3	82.6	99.3	18.9	15.6
23	1500	10	3	83.6	99.7	35.9	30.0
24	1600	10	3	80.4	99.7	62.6	50.3

This analysis highlights that the harshest presets (those that produce the most cell death) also provide the best protein delivery, but that the best preset still produces a final estimated 72% alive and hyper-positive cells.

Analysis of all controls and electroporations is attached separately as a FlowJo V10 PDF report.