Optimisation of electroporation of RNPs into DIPG IV cells

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Experiment Started:
Projects: **Characterisation**;**Exploratory**
Related Pages:
Referenced by:
Tags:

Aim

Optimise electroporation of ribonucleoprotein (RNP) 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 reported to produce greater frequencies of editing (better delivery, less stress on cells because they don't have to express the components) and lower off-target editing (lower levels of Cas9 and sgRNA exist in the cell and are present for less time).

Notes on methodology

How different papers optimised or measured RNP efficiency.docx
RNPs optimisation with fluorescent antibodies.docx

Title missing - double click to edit

This protocol adapted from the following references:

IDT protocol:
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 protocol:
http://tools.thermofisher.com/content/sfs/manuals/neon_device_man.pdf
Xu, 2018 protocol (use of AlexaFluor488 antibody in lieu of Cas9 for optimisation):
https://www.nature.com/articles/s41598-018-30227-w
Shalem, 2014 paper (EGFP sgRNA sequence):
https://science.sciencemag.org/content/343/6166/84.long

Pre-seeding of cells:

2 days before electroporation HSJD-DIPG-IV cells were seeded in TSM-C at a density of 2 million cells per T75 flask (2x) and 1 million cells per T75 flask (2x).

Processing of samples for electroporation:

Made up TSM-C (without antibiotics), added 500µL to each well of a 24 well plate, and set it to rewarm in the incubator. Cells from both 1 million and 2 million flasks were split as usual, counted, and resuspended in TSM-C (without antibiotics). All cells from the 2 million flasks were used and supplemented from the 1 million flask to have enough for 400k cells per electroporation.

Notes -

2 million flasks were at most 60% confluent and 1 million flask about 25% confluent, which is less than the ideal 70-90% confluent.

Mock RNPs (where Cas9 is replaced with AlexaFluor488 anti-mouse) were made as follows:
Anti-GFP sgRNA* was made up to 44µM (Neon protocol) and antibodies were made up to 10µM (for a final concentration of 0.5µM, as used in Xu, 2018) in TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0). sgRNA and antibody were combined in equal parts and allowed to combined for 10-20 min at room temperature. Meanwhile:

*sgRNA designed to target EGFP, used as a non-targeting control previously by (Shalem, 2014). Target sequence: GAAGTTCGAGGGCGACACCC. Ordered as a whole sgRNA from Sigma.

Cells were spun down, washed in PBS, and resuspended in enough buffer R (Neon 10µL electroporation kit) for 9µL per electroporation and 26 electroporations.

Each reaction consisted of 9µL cells, 1µL RNP complex, 2µL IDT electroporation enhancer (catalogue number 1075915) of which 10µL was taken for electroporation. (ALWAYS MAKE UP EXTRA TO AVOID BUBBLES).

Cells were electroporated according to the Neon 24-well optimisation protocol.

Notes -
* Well 7 threw up multiple errors before it worked, and later had a large clump of unidentified material in it
* Well 9 arced (expect reduced viability and efficiency)

Processing of samples for flow cytometry

Imaging of cells with the Celigo image cytometer was insufficient to detect AlexaFluor staining (the Xu, 2018 paper didn't specify their imaging technique, although it was likely to be confocal).

Following a 24h incubation to allow cell recovery: Growth medium was removed from plate and replaced with 400µL TrypLE. Plate was incubated at 37oC for 10 minutes. 800µL TSM-C (without antibiotics) was added to the plate to neutralise the TrypLE. The cell suspensions were each transferred to an FC tube and spun down at 500g, 4min. (Centrifuge in central room w fume cupboards). Supernatant was removed and cells were resuspended in 300µL ice cold PBS and kept on ice until FC was performed using a BD LSRFortessa. Analysis was performed using FlowJo.

Notes -
Cells need not be resuspended in PBS by hand - this can be done as they are vortexed before being read in the Fortessa
First row of samples went into 37oC PBS (before Ling told me not to).

Values for the mock electroporation were taken from the reported optimised values for U-87 cells (on website). Incidentally, these settings were in the top 5 most effective.

Notes on running electroporation

Fill electroporation chamber tube with 3mL buffer E (this is for 10µL kit)
Push the tube into the machine until it clicks in
This tube can be used for 10 different electroporations

To fit the tip to the pipette - press down on plunger until the metal holder sticks out. Use this to grip the metal spike down the centre of the tip and release the plunger whilst still pressing down to pull the spike into the pipette whilst pushing the outer plastic of the tip onto the pipette end.

(check that it is attached properly before trying with cells)

Aspirate cells - ensure there are no bubbles as this leads to arcing (sp?)
Push pipette and tip into machine until it clicks
Hit go on machine!

Notes on running flow cytometry

Before starting run make the following plots in the software:
(w machine on standby with a tube of water)

* FSC vs SSC to identify the live vs dead cell populations (dead are low on both axes)
* AlexaFluor488 vs count (i.e. a histogram)
* AlexaFluor488 vs PE - autofluorescent cells will lie on the diagonal + seeing populations in 2D helps separate them
* AlexaFluor488 vs FSC - again helps to separate populations

With the controls set up the following:
(Machine on RUN, and software on ACQUIRE)

* Get the correct voltage on FSC and SSC to collect all cells (do for all plots as you go)
* Gate loosely around the live cells
* Right click on next plot and gate it to live cells only
* Get the correct voltage on AlexaFluor
* Gate above all the negative cells (using negative control here)
* Gate in a triangle below/right of negative cells in AF vs PE
* Gate above negative cells in AF vs FSC

Once all that is set up can set machine to RUN and software to RECORD to collect data for 10k cells (set speed of run to LO, MED, HI as required, keep below 3000 cells/sec).

The software sorts everything in to an experiment>sample (i.e. cell type)>tube
Between each sample you need to hit next tube ! (it will number the next one)

NOTE that both RUN and STANDBY will slowly suck up your sample when you aren't looking

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Full FlowJo report

Analysis different layout.pdf

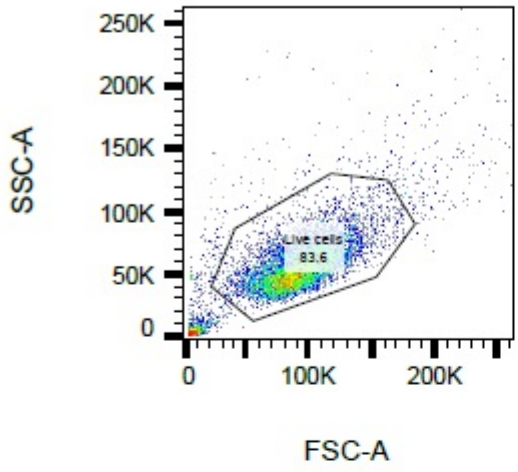
Quantification of each electroporation condition

sample	pulse voltage	pulse length	no. pulses	live cells	+ve cells (of live)	+ve cells - bg	+ve cells (of total)	notes
no EP no Ab				83.6	0			
no EP + Ab				81.3	1.44			(this is removed as background from otl
1	1300	30	1	84.8	0			mock EP without antibody

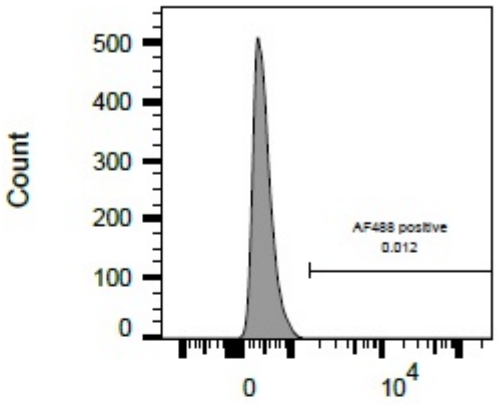
2	1400	20	1	82.9	99.3	97.9	81.1	
3	1500	20	1	81.7	99.7	98.3	80.3	
4	1600	20	1	81.5	99.7	98.3	80.1	
5	1700	20	1	77.8	99.7	98.3	76.4	
6	1100	30	1	84.2	93.2	91.8	77.3	
7	1200	30	1	84.9	92.8	91.4	77.6	had errors and something grew in it
8	1300	30	1	83.6	99.6	98.2	82.1	
9	1400	30	1	83.4	99.3	97.9	81.6	arced during electroporation
10	1000	40	1	84.9	88.1	86.7	73.6	
11	1100	40	1	84.3	99.1	97.7	82.3	
12	1200	40	1	82.7	99.7	98.3	81.3	
13	1100	20	2	84.5	92.4	91.0	76.9	
14	1200	20	2	84.3	99.4	98.0	82.6	
15	1300	20	2	82.1	99.6	98.2	80.6	
16	1400	20	2	75.8	99.5	98.1	74.3	
17	850	30	2	85.4	36.7	35.3	30.1	
18	950	30	2	84.5	83.7	82.3	69.5	
19	1050	30	2	84.5	99.4	98.0	82.8	
20	1150	30	2	82.7	99.7	98.3	81.3	
21	1300	10	3	84.3	96.2	94.8	79.9	
22	1400	10	3	82.6	99.3	97.9	80.8	
23	1500	10	3	83.6	99.7	98.3	82.1	
24	1600	10	3	80.4	99.7	98.3	79.0	

No EP, no Ab control

Specimen_001_no EP no Ab_001.fcs
Ungated
10000



Specimen_001_no EP no Ab_001.fcs
Live cells
8357

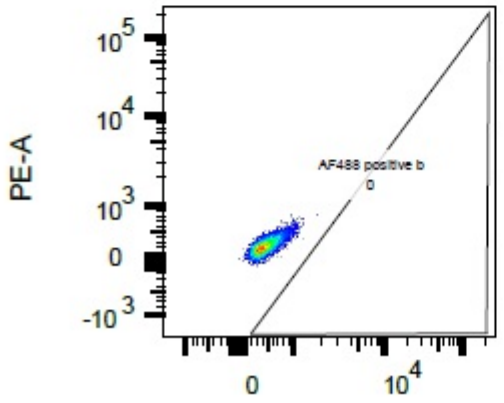


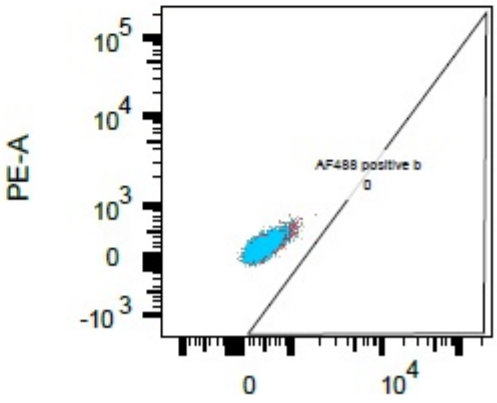
Live cells : Freq. of Total : 83.6

Specimen_001_no EP no Ab_001.fcs
Live cells
8357

	Sample Name	Subset Name	Count
	Specimen_001_sample_001_003.fcs	Live cells	8479
	Specimen_001_no EP no Ab_001.fcs	Live cells	8357

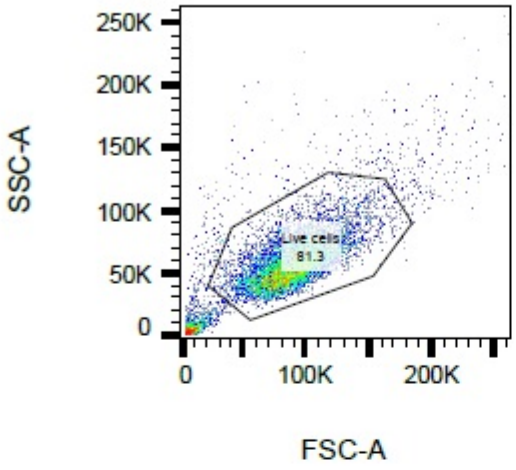
AF488 positive b : Freq. of Parent : 0



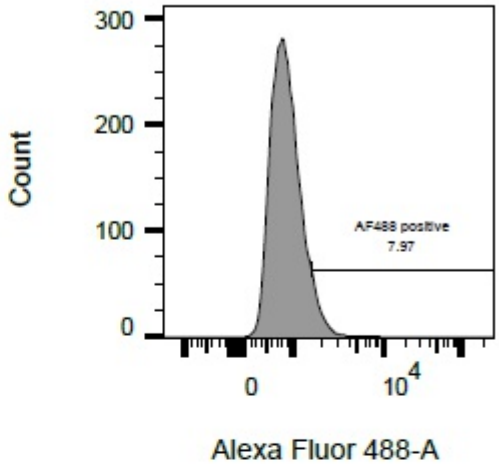


No EP + Ab control (taken as background level of SURFACE rather than internal staining)

Specimen_001_no EP + Ab_002.fcs
Ungated
10000



Specimen_001_no EP + Ab_002.fcs
Live cells
8128

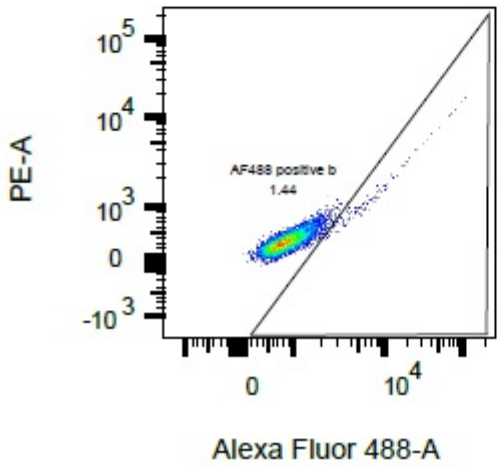


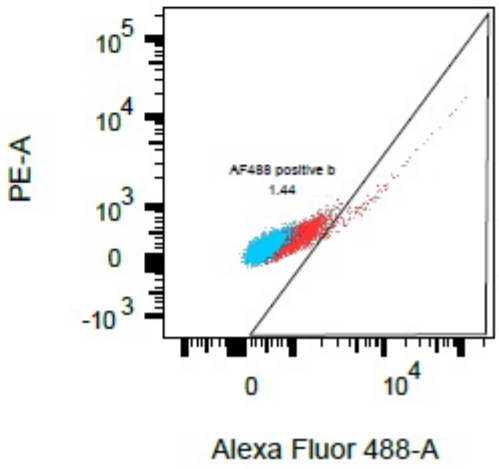
Live cells : Freq. of Total : 81.3

Specimen_001_no EP + Ab_002.fcs
Live cells
8128

	Sample Name	Subset Name	Count
	Specimen_001_sample_001_003.fcs	Live cells	8479
	Specimen_001_no EP + Ab_002.fcs	Live cells	8128

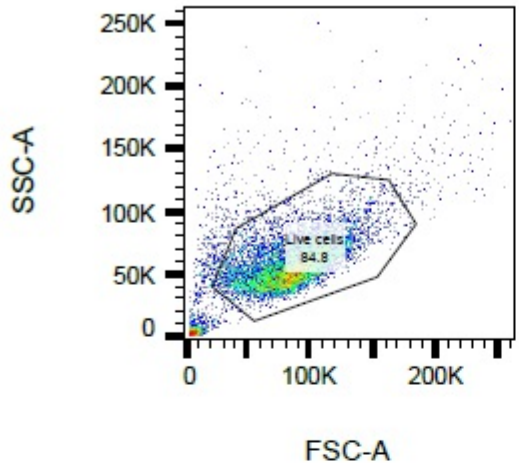
AF488 positive b : Freq. of Parent : 1.44



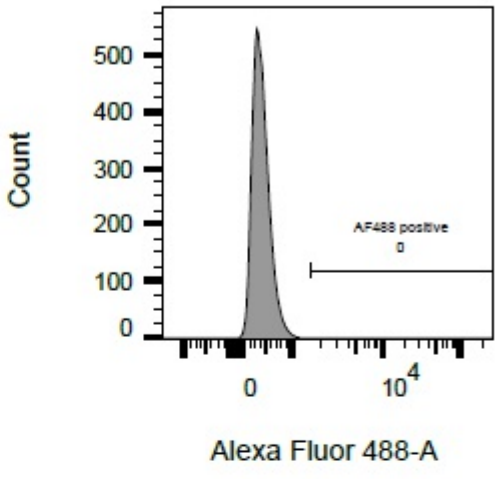


EP, no Ab control

Specimen_001_sample_001_003.fcs
Ungated
10000



Specimen_001_sample_001_003.fcs
Live cells
8479



Live cells : Freq. of Total : 84.8

Specimen_001_sample_001_003.fcs
Live cells
8479

	Sample Name	Subset Name	Count
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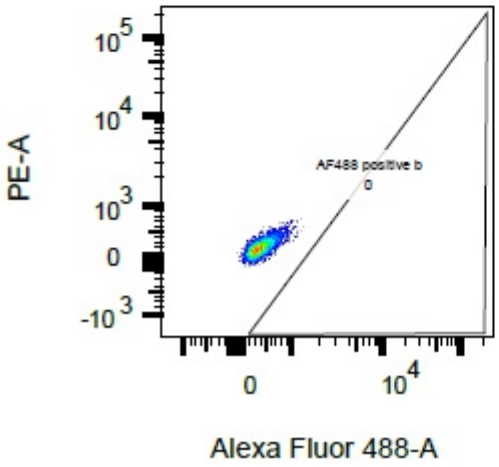
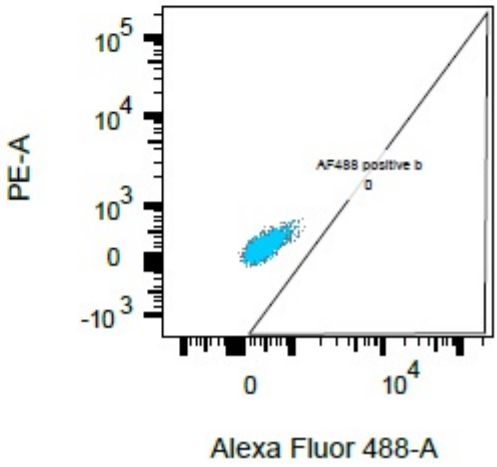
	Specimen_001_sample_001_003.fcs	Live cells	8479

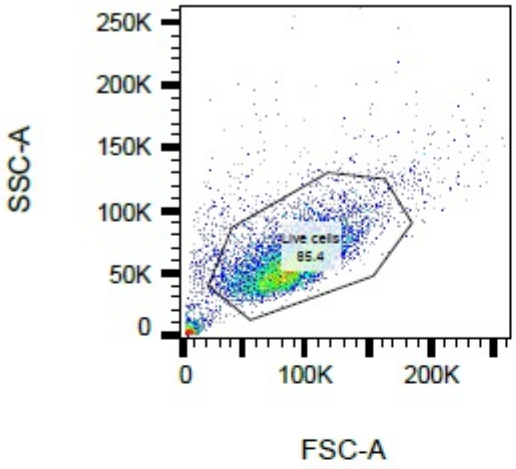
AF488 positive b : Freq. of Parent : 0



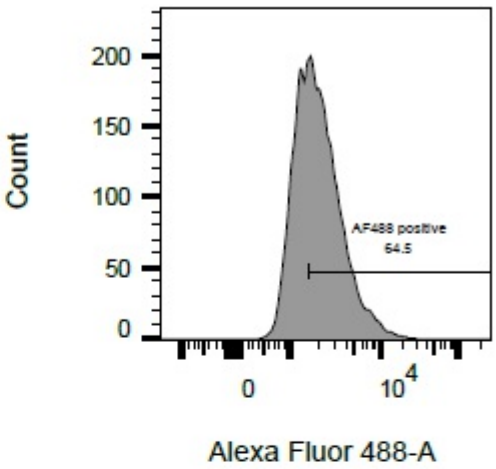


Least successful treatment

Specimen_001_sample_017_019.fcs
Ungated
10000



Specimen_001_sample_017_019.fcs
Live cells
8543

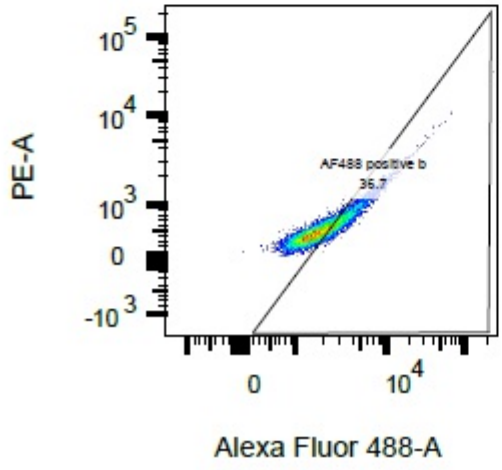


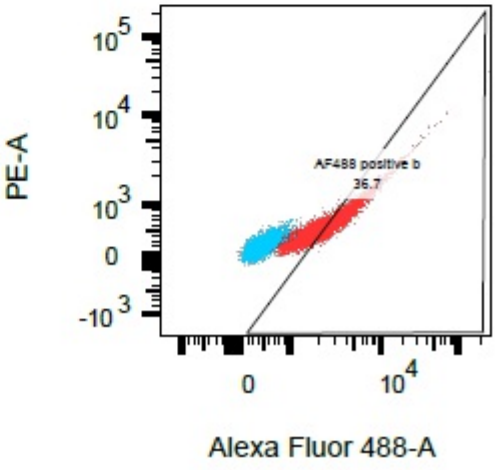
Live cells : Freq. of Total : 85.4

Specimen_001_sample_017_019.fcs
Live cells
8543

	Sample Name	Subset Name	Count
	Specimen_001_sample_001_003.fcs	Live cells	8479
	Specimen_001_sample_017_019.fcs	Live cells	8543

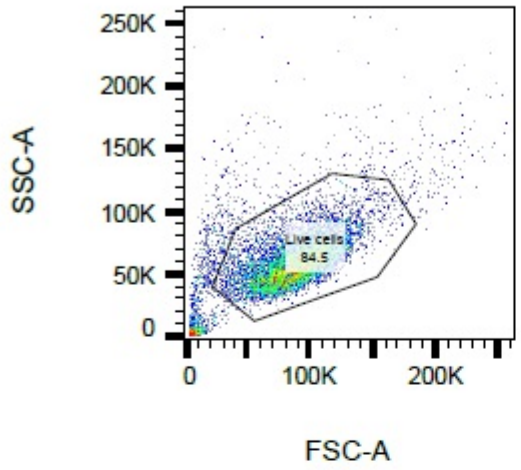
AF488 positive b : Freq. of Parent : 36.7



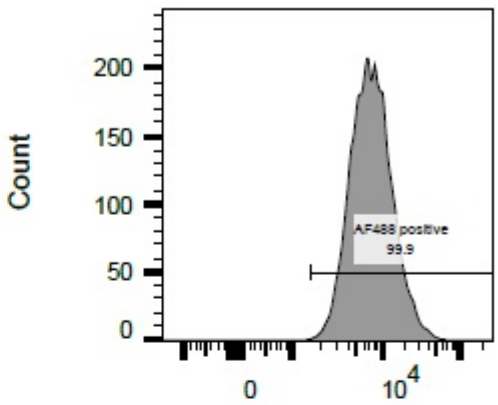


Most successful treatment

Specimen_001_sample_019_021.fcs
Ungated
10000



Specimen_001_sample_019_021.fcs
Live cells
8447

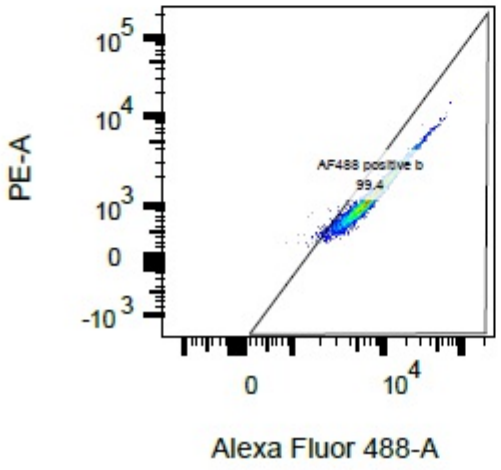


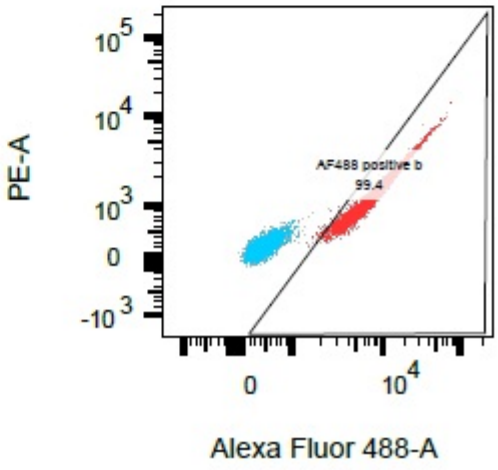
Live cells : Freq. of Total : 84.5

Specimen_001_sample_019_021.fcs
Live cells
8447

	Sample Name	Subset Name	Count
	Specimen_001_sample_001_003.fcs	Live cells	8479
	Specimen_001_sample_019_021.fcs	Live cells	8447

AF488 positive b : Freq. of Parent : 99.4





Conclusions

All treatments were highly effective. The best 3-5 conditions will be taken forwards to the next optimisation step.