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Universal and efficient electroporation protocol for genetic engineering of gastrointestinal organoids

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Corresponding Author:	Kristin Pape Technische Universitat Dresden Medizinische Fakultat Carl Gustav Carus Dresden, Saxony GERMANY
Corresponding Author's Institution:	Technische Universitat Dresden Medizinische Fakultat Carl Gustav Carus
Corresponding Author E-Mail:	kristin.pape@ukdd.de
Order of Authors:	Anne Marlen Gaebler, kand.med.
	Katharina Buczolich
	Alexander Hennig
	Jürgen Weitz
	Daniel E. Stange
	Kristin Pape
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TITLE:

Universal and Efficient Electroporation Protocol for Genetic Engineering of Gastrointestinal Organoids

AUTHORS AND AFFILIATIONS:

Anne-Marlen Gaebler[1], Katharina Buczolich[1], Alexander Hennig[1], Jürgen Weitz[1], Daniel E. Stange[1], Kristin Pape[1]

[1]Department of Visceral-, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

Email addresses of co-authors:

Anne-Marlen Gaebler (annemarlen.gaebler@uniklinikum-dresden.de)
Katharina Buczolich (Katharina.Buczolich@uniklinikum-dresden.de)
Alexander Hennig (Alexander.Hennig@uniklinikum-dresden.de)
Jürgen Weitz (Juergen.Weitz@uniklinikum-dresden.de)
Daniel E. Stange (Daniel.Stange@uniklinikum-dresden.de)

Corresponding author:

Kristin Pape (Kristin.Pape@uniklinikum-dresden.de)

KEYWORDS:

electroporation, transfection, CRISPR/Cas9, organoids, genetic engineering, FACS, PDAC, CRC, CCC, gastric cancer

SUMMARY:

This protocol describes an efficient electroporation method for the transfection of four different gastrointestinal organoid entities with larger plasmids (to the extent of 10 kB). It can be performed within one day and does not need extensive preparation or special, cost-intensive electroporation buffers.

ABSTRACT:

Electroporation is a common method for transfection with different kinds of molecules by electrical permeabilization of the plasma membrane. With the increasing use of organoids as a culturing method for primary patient material in the last years, efficient transfer methods of components for genetic engineering in this 3D culture system are in need. Especially for organoids, the efficiency of genetic manipulations depends on a successful transfection. Thus, this protocol was developed to facilitate the electroporation of organoids and to prove its universal functionality in different entities. Human colorectal, pancreatic, hepatic and gastric cancer organoids were successfully electroporated with small and large plasmids in comparison. Based on GFP encoding vectors, the transfection efficiency was determined by FACS. No extensive preparation of the cells or special, cost-intensive electroporation buffers are necessary, and the protocol can be performed within one day.

INTRODUCTION:

In recent years, a novel 3D cell culture system, termed organoids, was developed for various normal and cancerous tissues. Organoids are functionally and morphologically very close to their tissue of origin. They can be generated from different species, are easily expandable, genomically stable and genetically modifiable, which makes them an ideal model system for genetic investigations[1-3]. Genetic engineering techniques like the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 system enable diverse manipulations. The selection of clones can be realized by defined media conditions, for example, by WNT ligand withdrawal for *APC* (Adenomatosis Polyposis Coli) knockout clones[4,5]. Alternatively, selection markers have to be introduced by homologous directed repair of a targeting vector[6,7]. Due to the fact that often more than one plasmid needs to be introduced, an efficient transfection becomes a crucial parameter. Additionally, to reduce unspecific off-target effects, a transient expression of the Cas9 endonuclease is desirable[8].

Electroporation is a comparatively simple method to transfect cells with DNA, RNA, proteins or other macromolecules. By means of electric pulses, the cell membrane becomes more permeable and causes an increased uptake[9]. In a previously published electroporation protocol of colon organoids a 30 % efficiency with a piggy-bac GFP (green fluorescent protein) expressing vector (7.4 kB) was reached in a four days procedure[10]. The following protocol was developed to facilitate an efficient transfection of cancerous or healthy organoids with large plasmids encoding for the Cas9 endonuclease sequence and single guide RNA (sgRNA; e.g., px458 as vector with 9.3 kb). The whole electroporation process can be performed within one day, without special electroporation buffers, and with at least comparable efficiencies between different gastrointestinal organoids, namely pancreatic ductal adenocarcinoma (PDAC), colorectal cancer (CRC), cholangiocarcinoma (CCC) and gastric cancer (GC) organoids.

PROTOCOL:

Ethics approval was obtained from the ethical committee of the TU Dresden (#EK451122014).

1. Organoid culture and preparations before electroporation

1.1 Establish organoids by tissue digestion as described previously and expand them with their corresponding entity specific culture medium in a basement matrix (overview see **Table 1** and **Table of Materials**)[11-17].

NOTE: For human tissue samples informed consent and approval of the study by an ethical committee is necessary.

1.2 Prewarm 48-well plates at 37 °C for post-electroporation seeding.

1.3 Prepare basal medium w/o antibiotics as well as entity specific organoid culture medium w/o antibiotics (see **Table 1**) including 10 µM Y-27632 and 3 µM CHIR99021.

1.4 **Preparation of the organoids** (see **Figure 1**)

1.4.1 Cultivate 5 wells of organoids in a 48-well plate per electroporation sample in culture medium.

NOTE: Proliferative organoids should be used (around 2-3 days after last splitting).

1.4.2 Prepare 230 µL of dissociation reagent (see **Table of Materials**) including 10 µM Y-27632 per well.

1.4.3 Remove the culture medium from each well and dissociate the organoids mechanically in 230 µL of the prepared dissociation mixture. Pool 5 wells per electroporation sample into one 15 mL tube.

1.4.4 Mix by vortexing and incubate for 5-15 min at 37 °C until clusters of 10-15 cells occur. Therefore, check the dissociation microscopically. Stop the digestion by adding basal medium w/o antibiotics up to 10 mL.

NOTE: This step is very critical! The electroporation efficiency will be reduced, when incubation is too short, but long digestion will reduce survivability.

1.4.5 Centrifuge at 450 x *g* for 5 min at room temperature, discard the supernatant and wash twice with 4 mL of electroporation buffer (see **Table of Materials**).

2. Electroporation

NOTE: The following protocol is developed for electroporators capable of square waves and separated poring and transfer pulse sequences (see **Figure 2**). Optionally, impedance values as well as the voltages, currents and energies transferred into the sample can be measured as control for reproducible experiments.

2.1 Resuspend the organoid pellet in 100 µL of electroporation buffer (see **Table of Materials**) containing 30 µg of plasmid DNA.

NOTE: The concentration of the used plasmid DNA should exceed 5 µg/µL for an optimal salt concentration during the electroporation process. Therefore, endofree plasmid maxi kits (see **Table of Materials**) for the preparation of vectors are recommended. A total amount up to 45 µg of DNA can be used without cytotoxic effects.

2.2 Dispense the complete DNA-organoid mixture into an electroporation cuvette with 2 mm gap width without producing air bubbles.

2.3 Set the electroporation parameters according to Fujii et al.[10] (see **Table 2**, **Figure 2**).

2.4 Mix the cells slightly without foaming by tapping the cuvette with a finger. Place the cuvette into the cuvette chamber.

2.5 Press the **Ω** button of the electroporator and make a note of the impedance value.

NOTE: An impedance between 30 – 40 Ω showed the best results. In general, it should be in the range between 30 – 55 Ω. If this is not the case, please control the following aspects: gap width of the cuvette used, cable connections of the electroporator, possible air bubbles, correct volume and salt concentration of the electroporation mixture.

2.6 Press the **Start** button to execute the electroporation program and control the values of currents, voltages and energies displayed.

NOTE: The values of measured voltages, currents and energies should correspond to the set electroporation parameters. For the comparison of repeated experiments, it can be helpful to note these data.

2.7 After electroporation, immediately add 500 µL of culture medium w/o antibiotics (with CHIR99021 and Y-27632; see step 1.3). Mix by pipetting up and down to dissociate the white foam.

NOTE: The white foam appears after the electroporation process and a significant number of cells is attached to it. So, dissociation of it is very important for not losing cells.

2.8 Transfer the sample completely from the cuvette into a new 15 mL tube using the pipette belonging to the electroporation cuvettes (see **Table of materials**). Rinsing the cuvette again with basal medium is recommended to obtain remaining cells.

2.9 For regeneration of the cells, incubate them for 40 min at room temperature.

3. Seeding of cells

3.1 Centrifuge the cells at 450 x *g* for 5 min at room temperature and discard the supernatant.

3.2 Resuspend the pellet in 100 µL of basement matrix and seed 20 µL drops in a prewarmed 48-well plate (see step 1.2). Incubate for 10 min at 37 °C for polymerization and add 250 µL of culture medium, which is supplemented with Y-27632 and CHIR99021 until the next splitting of the grown organoids (around 5-7 days).

4. Determination of transfection efficiency

NOTE: In general, it is recommended to electroporate a vector carrying a fluorescence marker

as additional transfection control. Dependent on the chosen marker and its chromophore maturation the fluorescence will be visible within around 24-48 h post transfection[18].

4.1 Check the fluorescence microscopically after 24-48 h in the transfection control (see **Figure 4B**).

4.2 Fluorescent activated cell scanning (FACS)

4.2.1 Harvest the cells analogously to step 1.4.2-1.4.4 and digest around 10-20 min until there are single cells. Add up to 10 mL of phosphate-buffered saline (PBS).

4.2.2 Centrifuge at 450 x *g* for 5 min at room temperature and discard the supernatant.

4.2.3 Optionally for discrimination of living cells: Resuspend the pellet in 1 mL of Dulbecco's phosphate-buffered saline (DPBS) and add a suitable antibody (see **Table of Materials**) or propidium iodide (PI). Mix very carefully only by tapping and incubate for 30 min at room temperature in the dark. Wash with 10 mL of DPBS, centrifuge and discard the supernatant.

4.2.3 Resuspend the cell pellet in 200 µL of PBS and optionally filter the suspension through a 100 µm cell strainer into a FACS tube.

4.2.4 Analyze the cells by a FACS machine using an appropriate gating strategy (see **Figure 3**; **Figure 4A**) and determine the transfection efficiency.

REPRESENTATIVE RESULTS:

Organoids of four different cancer entities (CRC, CCC, PDAC, GC) were electroporated at least 3 times using 30 µg of a small plasmid (pCMV-EGFP, 4.2 kb) or a large plasmid (px458, 9.3 kb). Both vectors carry a GFP cassette allowing the determination of transfection efficiency 48 h after electroporation by flow cytometry. To analyze only living cells, staining with a life-death antibody before scanning was performed. The gating strategy is shown in **Figure 3**.

In all four organoid entities the 4.2 kB sized plasmid was transfected with higher efficiency compared to the larger one (see **Figure 4**). The most efficient transfection of the small plasmid was reached in PDAC organoids with 92.1 ± 5.2 % GFP positive cells, whereas the large plasmid was transfected with an efficiency of 46.7 ± 3.7 % (mean ± standard deviation, n = 3). Compared to pancreatic cancer organoids, the larger plasmid was more efficiently transfected into CRC organoids with a mean efficiency of 53.4 ± 11.7 %, while the small plasmid was transfected with a mean efficiency of 84.3 ± 5.8 %. The most difficult entity to transfect were gastric cancer organoids: for both the large and the small plasmid, the lowest transfection efficiency was reached in this entity (32.3 ± 12.7 % and 74.1 ± 5.5 %, respectively). CCC organoids showed a mean transfection efficiency of 83.0 ± 13.1 % for the small plasmid and for the large plasmid 39.5 ± 10.4 % were obtained.

As proof of concept, human normal stomach organoids were electroporated with a

px458_Conc2 plasmid encoding for Cas9, GFP and two sgRNAs targeting *TP53*. The Cas9-induced double strand breaks on exon 8 were repaired by non-homologous end-joining (NHEJ), resulting in frameshifts and consequently a knockout of the gene (see **Supplementary Figure 1**).

FIGURE AND TABLE LEGENDS:

Table 1: Composition of basal media, digestion mixtures and cultivation media.

Table 2: Electroporation settings according to Fujii et al.[10].

Figure 1: Electroporation preparation workflow. First, organoids ought to be dissociated to clusters of 10-15 cells and antibiotics should get washed out. After electroporation the white foam need to be dissociated. Cells can be seeded after regenerating for 40 min at room temperature.

Figure 2: Two-step electroporation. Two poring pulses with higher voltage und short duration (175 V and 157.5 V, each for 5 ms, pause for 50 ms, voltage decay 10%) lead to the formation of pores in cell membranes. The following transfer pulses deliver the DNA into the cells: five positive transfer pulses (with 20 V, 12 V, 7.2 V, 4.32 V and 2,592 V, each for 50 ms, pause for 50 ms, voltage decay 40%), followed by five polarity exchanged transfer pulses (with -20 V, -12 V, -7.2 V, -4.32 V and -2,592 V, each for 50 ms, pause for 50 ms, voltage decay 40 %).

Figure 3: Representative gating strategy shown by CCC organoids. All electroporated organoids were analyzed by flow cytometry 48 h after electroporation. Cells electroporated without plasmid DNA were used as negative controls. The gates were set as following: **(A)** gating for cell shape, **(B, C)** gating for single cells (doublet discrimination), **(D)** gating for living cells (stained with an antibody for apoptotic cells) and **(E, F)** finally gating for eGFP expressing cells (FITC channel). FSC, forward scatter; SSC, side scatter.

Figure 4: Electroporation efficiency of four organoid entities. **(A)** FACS analysis (n = 3-4, mean ± standard deviation and each single value are shown) and **(B)** visual comparison by fluorescence microscope. The scale bar represents 1000 µm. BF, bright field; CCC, cholangiocarcinoma; CRC colorectal cancer; GC, gastric cancer; PDAC, pancreatic ductal adenocarcinoma.

Supplementary Figure 1: The px458_Conc2 vector (see Table of Materials) was cloned by combining the 2 gRNA concatemer vector, a generous gift from Bon-Kyoung Koo[19], with px458[20], resulting in a plasmid encoding for 2 sgRNAs, Cas9 and GFP. Two sgRNAs targeting *TP53* were introduced in px458_Conc2 vector by golden gate cloning (analogously to Andersson-Rolf et al.[19]). 10 µg of plasmid DNA were electroporated in human normal gastric organoids **(A)**. Clones were selected by Nutlin-3 administration **(B)** and the *TP53* knockout was confirmed by TOPO TA cloning and sequencing of the alleles, here exemplary shown for one clone **(C)**. The sgRNAs are underlined in the reference. The scale bar represents 200 µm. BF, bright field.

DISCUSSION:

This protocol gives detailed instructions for an efficient, quick and easy to perform electroporation of different organoid entities. Additional to the presented tumor organoids from PDAC, CRC, CCC and GC, it works successfully for organoids derived from healthy tissue as well. The protocol can be performed within one day. In published organoid transfection protocols the whole procedure lasted four days including two days of preparations with different types of cultivation media[10,21]. In our protocol no special pretreatment is required. By washing with electroporation buffer before electroporation the antibiotic components of the media were washed out and an adjustment of saline concentrations for optimal impedance values was achieved. Nevertheless, some critical aspects should be considered for a successful electroporation:

Cells

In the electroporation protocol by Fujii et al.[10] it is recommended to dissociate organoids to single cells and to filter them through a 20 µm cell-strainer. In our hands digestion to single cells strongly decreases the survivability of cells. As suggested in Merenda et al.[21], we also dissociated organoids to clusters of 10-15 cells and could not determine a decreased efficiency compared to single cell dissociation. After electroporation, it is a very important step to dissociate the white foam, so that no attached cells are getting lost.

For 2D cell culture, it has been shown that a regeneration time after electroporation of more than 10 min up to 40 min increases survivability and transfection efficiency especially of large plasmids[22]. In test experiments, the same could be documented for organoids, leading to an incubation step of 40 min after electroporation in this protocol. In order to increase recovery from the electroporation, we cultured them with Rho-associated protein kinase (ROCK) inhibitor Y-27632 for five to seven days[23]. Similarly, the additional supplementation of glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021 is meant to help single cells to recover[10].

Settings

One of the advantages of the used electroporator is that the impedance can be measured before electroporation for optimal conditions. According to the manufacturer, the impedance values should be 30-55 Ω. In our hands, impedance values of 30-40 Ω have shown optimal efficiencies. In a preliminary experiment, different voltages and pulse length values of the poring pulse were varied to find the optimal proportion of efficiency to survivability. In summary, we could confirm the described values of Fujii et al.[10] in the different entities described here.

DNA

The effect of different DNA amounts was tested in preliminary experiments up to 45 µg of DNA per sample. No cytotoxic effects could be detected. Transfection efficiency was increased in a dose dependent way with saturation > 30 µg. So, we used 30 µg per sample in the final protocol, but of course it can be increased (e.g., for the electroporation of more plasmids in parallel). Additionally, the purity and concentration of the DNA seems to be very important. A

concentration exceeding 5 µg/µL has shown optimal transfection efficiencies.

As expected, the 9.3 kB plasmid could be transfected with a lower efficiency than the smaller 4.2 kB plasmid (see **Figure 4**). The use of even larger plasmids than 10 kB is anticipated to further decrease the efficiency. For future applications, it might be interesting to test minicircle DNA as a vector, since these gene carriers lack the bacterial backbone of a plasmid which makes them smaller[24]. This should result in an enhanced transfection efficiency. Furthermore, for CRISPR-based manipulations of organoids a direct electroporation of sgRNAs bound to Cas9 as a ribonucleoprotein (RNP) complex could be an alternative or addition[25].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

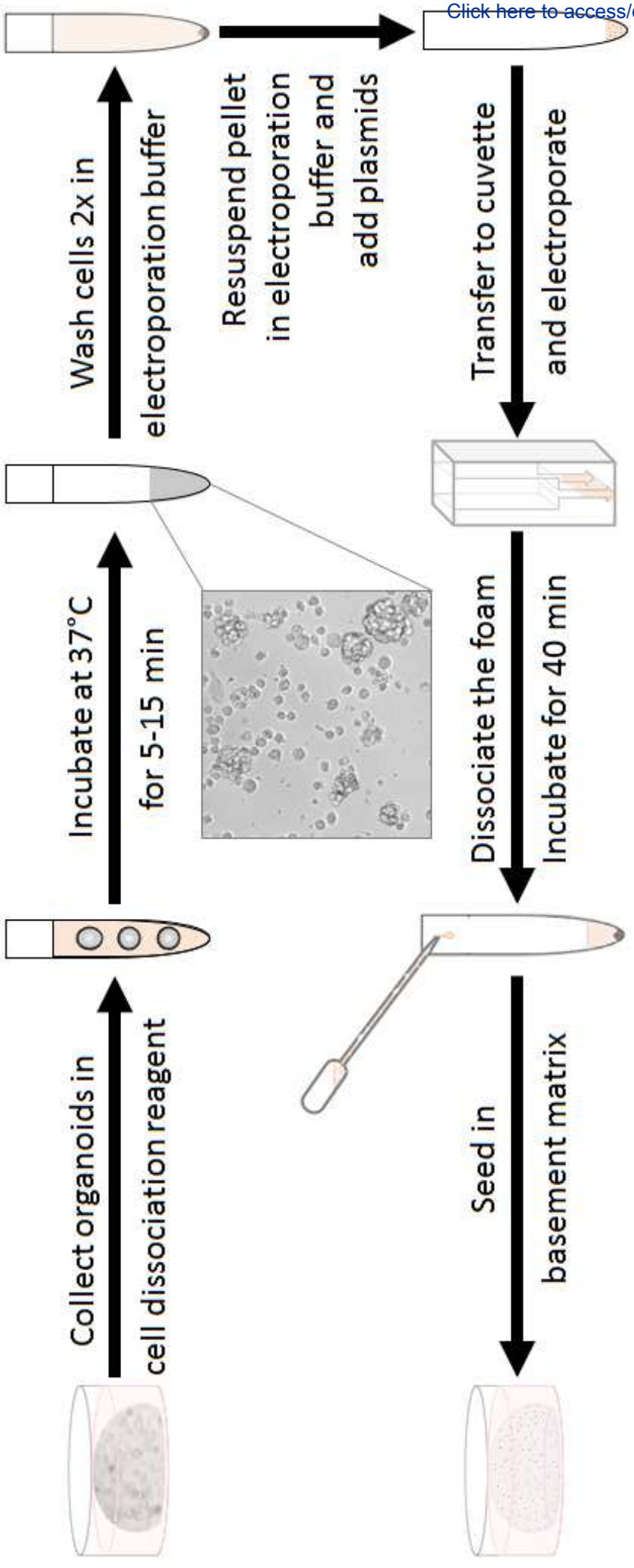
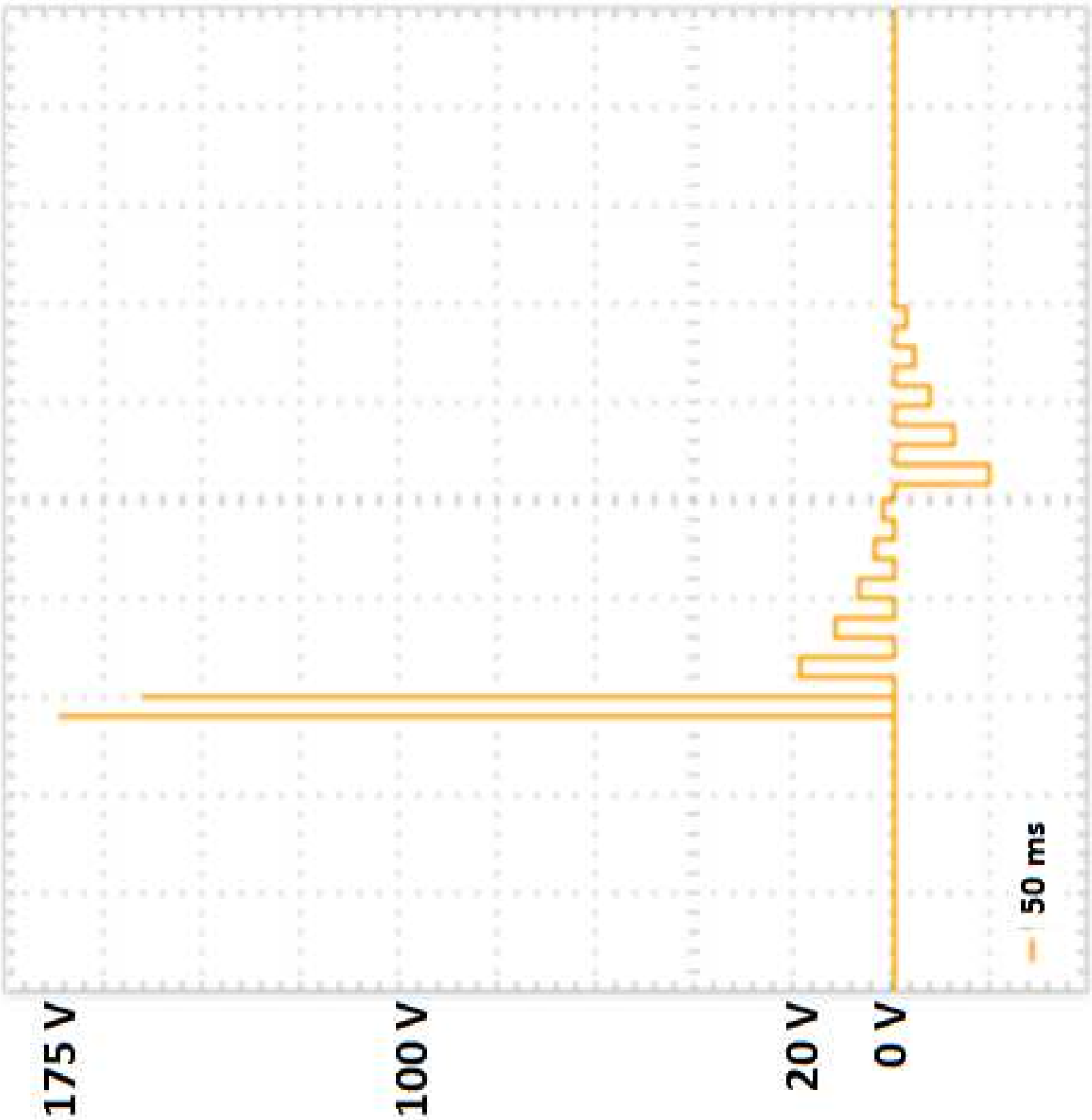


Figure 2

Click here to access/download;Figure;Figure2.eps



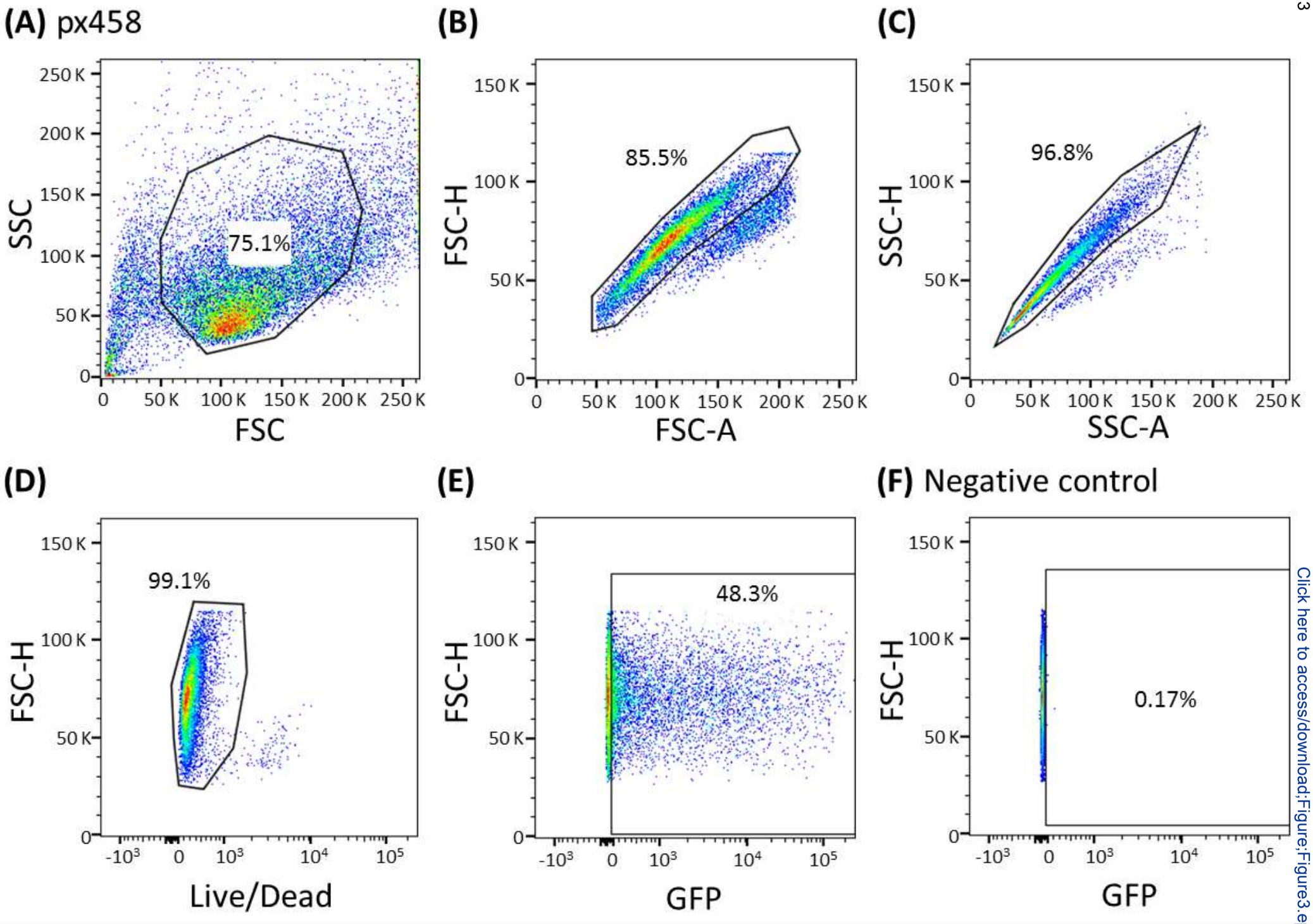


Figure 4

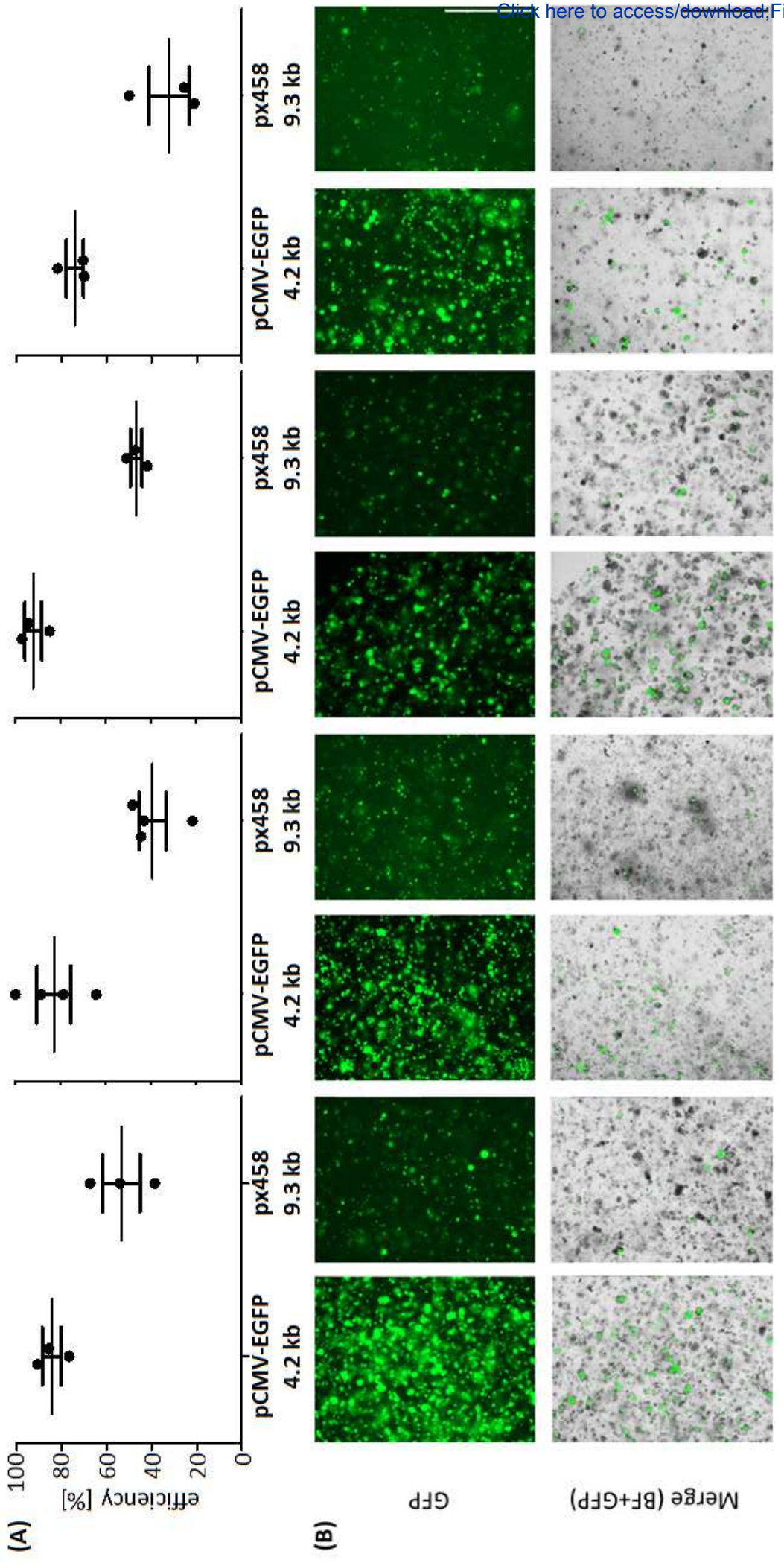


Table 1

Basal medium components:	**I**	**II**
Advanced DMEM/F-12		
Glutamax	1x	1x
Hepes	10 mM	10 mM
Penicillin Streptomycin	-	1x
Primocin	1x	

Establishment by digestion								
	Colon		**Stomach**		**Liver**		**Pancreas**	
Digestion mix:	**normal**	**tumorous**	**normal**	**tumorous**	**normal**	**tumorous**	**normal**	**tumorous**
Collagenase II	-	-	-	-	-		0,625 mg/ml	
Collagenase XI	-	0,1 mg/ml	-	0,1 mg/ml	-		-	
Collagenase D	-	-	-	-	2,5 mg/ml		-	
Dispase II	-	1 mg/ml	-	1 mg/ml	-		2,5 mg/ml	
DNase I	-	-	-	-	0,1 mg/ml		-	
EDTA	2mM	-	10mM	-	-		-	
Diluted in	Chelating buffer*	Basal medium I	Chelating buffer*	Basal medium I	in EBSS		Basal medium I	
Incubation for:	0,5 h on ice	1h at 37°C	0,25-0,5 h at RT	1h at 37°C	0,5-1 h at 37°C	1,5 h at 37°C	0,5-1 at 37°C	2-3 h at 37°C
Adapted from reference:	Sato et al. 2011		Bartfeld et al. 2015		Broutier et al. 2016		Hennig et al. 2019	

Cultivation medium (Basic: Basal medium II)				
A83-01	0.5 µM	2 µM	5 µM	0.5 µM
B27	1x	1x	1x	1x
B27 without Vitamin A	-	-	1x	-
Forskolin	-	-	10 µM	-
hFGF-10	-	200 ng/ml	100 ng/ml	100 ng/ml
hHGF	-	-	25 ng/ml	-
[Leu15] Gastrin	10 nM	1 nM	10 nM	10 nM
mEGF	50 ng/ml	50 ng/ml	50 ng/ml	50 ng/ml
N2	-	-	1x	- | 1x
N-Acetyl-L-Cystein	1.25 mM	1 mM	1 mM	1 mM
Nicotinamid**	10 mM	10 mM	10 mM	10 mM
Noggin	10 %	10 %	-	10 %
Primocin	1x (100 µg/ml)	1x (100 µg/ml)	1x (100 µg/ml)	1x (100 µg/ml)
Prostaglandin E2	10 nM	-	-	- | 1 µM
Rspondin	20 %	10 %	10 %	10 %
SB202190	3 µM	-	-	-
Wnt3A	50 %	50 %	-	50 %

*Chelating buffer: 5.6 mM Na_2HPO_4, 8.0 mM KH_2PO_4, 96.2 mM NaCl, 1.6 mM KCl, 43.4 mM sucrose, 54.9 mM d-sorbitol, 0.5 mM dl-dithiothreitol in distilled water

** Nicotinamid only for the first passages, reduces lifespan (Bartfeld et al. 2015)

Table2

	Voltage	Pulse length	Pulse interval	Number of pulses	Decay rate	Polarity
Poring pulse	175 V	5,0 ms	50,0 ms	2	10%	+
Transfer pulse	20 V	50,0 ms	50,0 ms	5	40%	+/-

Material	Company	Catalog Number
For establishment and culture medium		
[Leu15] Gastrin	Sigma-Aldrich	G9145
A83-01	Tocris Bioscience	2939
Advanced DMEM/F-12	Invitrogen	12634010
B27	Invitrogen	17504044
B27 Supplement, minus vitamin A	Thermo Fisher Scientific	12587010
CHIR99021	Stemgent	04-0004
Collagenase II	Life tech	17101-015
Collagenase XI	Sigma-Aldrich	C9407-100MG
Collagenase D	Roche	11088866001
Dispase II	Roche	4942078001
Dnase I	Sigma Aldrich	D5319
D-sorbitol	Roth	6213.1
Dithiothreitol	Thermo Scientific	1859330
EDTA	Roth	8040
Forskolin	Tocris Bioscience	1099
Glutamax	Life Technologies	35050061
Hepes	Thermo Fisher Scientific	15630106
hFGF-10	Preprotech	100-26
KCl	Sigma Aldrich	P9541
KH_2PO_4	Roth	3904.2
Matrigel	Corning	356231
mEGF	Invitrogen	PMG8043
N2	Invitrogen	17502048
NaCl	Roth	3957.1

Na_2HPO_4	Roth	K300.2
N-Acetyl-L-Cystein	Sigma-Aldrich	A9165
Nicotinamid	Sigma-Aldrich	N0636
Noggin	n.a.	n.a.
PBS	Gibco	14190169
Penicillin Streptomycin	Life Technologies	15140122
Primocin	InvivoGen	ant-pm-1
Prostaglandin E2	Tocris Bioscience	2296
Recombinant Human HGF	Preprotech	100-39H
Rspondin	n.a.	n.a.
SB202190	Sigma-Aldrich	S7067
Sucrose	VWR	27,480,294
TrypLE Express	Gibco	12604021
Wnt3A	n.a.	n.a.
Y-27632	Sigma-Aldrich	Y0503
Consumables		
Cell Strainer 100 µm	Falcon	352360
48-well plate	Corning	3548
Nepa Electroporation Cuvettes 2mm gap w/pipettes	Nepa Gene Co., Ltd.	EC-002S
Tubes 15 ml	Greiner	188271
Tubes 15 ml low binding	Eppendorf	30122208
Equipment		
Electroporator Nepa21	Nepa Gene Co., Ltd.	n.a.
EVOS FL Auto	Invitrogen	AMAFD1000
LSRFortessa	BD Bioscience	647800E6
Reagents and plasmids		

Live/dead fixable blue dead cell stain kit	Invitrogen	L34962
EndoFree Plasmid Maxi Kit	Qiagen	12362
Nutlin-3	Selleckchem	S1061/07
Opti-MEM	Gibco	31985047
2 gRNA concatemer vector	AddGene	84879
pCMV-EGFP	Nepa Gene Co., Ltd.	n.a.
px458 plasmid	AddGene	48138
px458_Conc2 plasmid	AddGene	134449
sgRNA_hTP53_1a	Eurofins Genomics	n.a.
sgRNA_hTP53_1b	Eurofins Genomics	n.a.
sgRNA_hTP53_2a	Eurofins Genomics	n.a.
sgRNA_hTP53_2b	Eurofins Genomics	n.a.

Comments/Description
basement matrix

Conditioned medium produced from HEK293 cells (Hek293-mNoggin-Fc)

Conditioned medium produced from HEK293 cells (HA-Rspo1-Fc-293T)

Dissociation reagent

Conditioned medium produced from L-Wnt3a cells (from Sylvia Boj)

Tubes for FACS preparing

Fluorescence microscope

FACS

Includes antibody for live/dead staining for FACS analysis

Electroporation buffer

coding for sgRNA and Cas9

px458 plasmid containing 2x U6 promotors for two different sgRNAs

5´-CACCGG**GTAGTGGTAATCTACTGGGA**GT-3´

5´-TAAAA**CTCCCAGTAGATTACCACTAC**CC-3´

5´-ACCGG**TGGGAGAGACCGGCGCACAG**G-3´

5´-AAAAC**CTGTGCGCCGGTCTCTCCCA**C-3´

Dear editor(s) and reviewers,

Please find enclosed our revised manuscript “Universal and efficient electroporation protocol for genetic engineering of gastrointestinal organoids” for publication in JoVE. We appreciate the interest and time that the editors and reviewers have taken to help us with constructive suggestions.

Following this letter are the editor and reviewer comments. Our responses and changes within the revised manuscript are marked in red.

Thank you again for consideration.

Sincerely,
Kristin Pape, Dr. rer. nat.
Universitätsklinikum Carl Gustav Carus
Fetscherstr. 74, 01309 Dresden
Germany

Editorial comments:
The manuscript has been modified and the updated manuscript, 60704_R0.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Answer: Done.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Answer: No figures from previous publications were used.

3. Please use 12 pt font and single-spaced text throughout the manuscript.
Answer: Done.

4. Please add a one-line space between each of your protocol steps.
Answer: Done.

5. Please define all abbreviations before use.
Answer: Done.

6. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol:
Answer: This part was mentioned in line 231-232: "After electroporation, it is a very important step to dissociate the white foam, so that no attached cells are getting lost. "
b) Any modifications and troubleshooting of the technique
Answer: In preliminary experiments the troubleshooting led to a high purity and concentration of the deployed DNA, that is necessary to reach high efficiencies. This is explained in lines 251-252 now.
c) Any limitations of the technique
Answer: As shown in Figure 4, the transfection efficiency decreases with a larger plasmid size. This was described in lines 254-255.
d) The significance with respect to existing methods
Answer: The significance concerning other existing methods was described in lines 219-225.
e) Any future applications of the technique
Answer: Future applications are explained in lines 255-259 (e.g. minicircle DNA vector).
Please do not abbreviate journal titles for references.
Answer: Done.

7. Figure 2, Figure 3A: Please use . instead of , for decimal point.
Answer: Done. We also replaced it in the "representative results" section.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The patient-derived organoid culture system that mimic the structural and functional characters of an organ is widely used in any fields in not only basic biology, but also applied research fields such as medical biology and pharmacology. Here, Pape et al. describe the method of electroporation in tumor organoids established from different organs: colon, pancreas, bile duct, and stomach. The author actually tested the efficiency of transfection using different size of GFP-expressing plasmids and, consequently many GFP expression derived from the transgenes could be observed in their system. The another also developed a cost-intensive electroplating buffer.

Overall, the manuscript represents a very useful protocol for the organoid-gene editing by using CRISPR/Cas9 and I strongly believe this protocol improves the efficiency to establish gene-engineering organoid. Therefore, the protocol by Pape et al. might be expected to be applies in a wide range of fields. However, a major drawback of the manuscript is that some of the represented methods are preliminary and less explanation. Several issues need to be addressed in a revised manuscript, as detailed below:

Major Concerns:

1. Compared with the similar reports in JoVE previously (DOI: 10.3791/55916, DOI: 10.3791/57374, and DOI: 10.3791/55159), The authors just referred previous reports at both line-71 and line-106, their explanation in each step were very poor. As the journal policy of JoVE is a scientific methods journal, the author should write methods in detail by themselves that every reader can recapitulate their protocol by reading those manuscript and movie.

Answer: We extended Table 1 as detailed overview for the establishment of organoid cultures and their cultivation media. The detailed description for normal gastric, liver, pancreas and colon organoids as well as CRC, GC, CCC and PDAC organoids would go beyond the scope of this manuscript. Regarding line 106 we referred to Fujii et al. to make clear that this setting is not our finding. We tried different settings, but this one worked best. In the published protocol the details will be clearly visible by the belonging Table 2 including the settings. We also added a picture illustrating the two-step electroporation (see Figure 2)

2. The author claimed "genetic engineering" in the title, but this protocol does NOT contain any results of gene-engineering. As they just showed the result of transfection efficiency after the electroporation, the authors should consider removing "genetic engineering" from the title or showing any experimental results of generation of knockout or knocked-in organoids if they want to include the part of "genetic engineering".

Answer: Detailed genetic engineering techniques are already published for organoids (e.g. DOI: 10.3791/55916, DOI: 10.1038/nm.3802, DOI: 10.1126/science.aao3130, DOI: 10.1016/j.stem.2013.11.002). As we want to publish our detailed results elsewhere, we focused in this methodology paper on optimizing the electroporation of DNA. Nevertheless, we added a representative result as Supplemental Figure 1 showing a knockout of *TP53* in normal human stomach organoids.

3. As the authors mentioned at the line-90, the one of important tips in their protocol is that tumor organoid need to be dissociated into 10-15 cell aggregation before electroporation, NOT into single cells. This might be their original point to improve survivability of organoid in electroporation. However, in my experience, it is more difficult to control the number of aggregated cells than generating single cells in dissociation step of organoid, especially in the case of tumor organoids. Therefore, the authors need to explain in detail of this part how the authors prepare 10-15 cells aggregation entirely (which splitting enzyme they used, trypsin? collagenase? others?), and show the exact picture of cells after digestion in the Figure or in the movie of JoVE. Definitively, it helps readers to image this step.

Answer: We rephrased 1.4.4 (line 92-94) and hopefully we could point out that it is only a time-dependent enzymatic digestion. Due to the policy of JoVE it is not allowed to mention TrypLE as brand name directly. For a better understanding we referred to the Table of Materials (line 87). A picture of the digestion is included in Figure 1 now and we will show this step in a detailed way within the video.

Minor Concerns:

1. Another major finding of their author is about electroporation buffer because they emphasized "electroporation buffer, which is not expensive but very efficiency" in human tumor orgnaoids in the part of SUMMARY, ABSTRACT, and INTRODUCTION, respectively. There is no information about this buffer which authors used. They just mentioned at Line#100 like 100 uL electroporation buffer. The authors need to describe contents of the buffer if this is your original finding.

Answer: Due to the policy of JoVE it is not allowed to mention Opti-Mem as electroporation buffer directly, because it is a brand name. For a better understanding we referred to the Table of Materials (line 105). There it is described as electroporation buffer.

2. The authors make clear tables. The part of culture medium, regents, and equipment should be separated.

Answer: Done.

3. Mistype of CHIR99021 at line-117 and -130. Forget "1" at the last.

Answer: We changed it.

Reviewer #2:

Manuscript Summary:

Gaebler et al. described a method for electroporation of human gastrointestinal organoids. This method is potentially useful for readers to genetically engineer intestinal organoids. This protocol is mostly well-written, although there are some concerns:

Major Concerns:

none.

Minor Concerns:

1. Since this is a protocol paper, it would be helpful if the authors would include a Table with medium components they have used to culture the specific organoid lines. They do include components in the materials list, but only refer to references when it comes to composition of the expansion media.

Answer: We extended Table 1 as an overview about the basal media, digestion mixtures for establishing and cultivation media.

2. At 4.2 include "FACS" as abbreviation of the full term.

Answer: Done.

3. In figure 2, indicate in legends which type of organoids were used for the example.

Answer: These were CCC organoids. We added it.

4. In figure 3. Please indicate abbreviations in the legends. In B) include "by fluorescent microscope" and abbreviation for BF in the legends.

Answer: Done.

5. In discussion Settings: please rephrase "In a pre-experiment…". Suggestion: "We assessed different voltages (…) pulse to find (…). In summary, we confirm (…)"

Answer: We rephrased this part.

6. In materials list, please indicate that noggin, Rspondin and Wnt3A are conditioned media.
Answer: We extended the descriptions.

Reviewer #3:

Manuscript Summary:

As clearly stated by the authors, organoids have become a wiedely used tool in biomedical research. And tools and techniques to manipulate organoids has become an important need. The authors describe an interesting and as seen from outside easy to handle methods to transfect different types of organoids. The transfection efficiency appear to be very high making it it potentially very useful and probably also very economical method to manipulate organoids. I have few comments and questions:

Major Concerns:

The authors propose the use of a very specific electroporator. Have the authors used electroporators from different brands? Are the same settings adjustable in these different electroporators?
The nulecofection device from Lonza (Amaxa) is frequently used to transfect cells. Given the wide distribution of this device, have authors tested whetehr it can also be used to transfect organoids?
Answer: This protocol with the chosen settings can be performed with all electroporators capable of square waves and a two-step electroporation setting (see Note line 101-104).
To our knowledge it is not possible to define the settings with the Nucleofactor from Lonza exactly. You can choose between different programmed settings, but without an exact knowledge of the voltage, time etc. So, it is not possible to compare both electroporators directly with the same settings. Because Fujii et al. achieved a significantly better transfection efficiency with the Nepa21 compared to the Nucleofactor from Lonza (DOI: 10.1038/nprot.2015.088) and the group from Bon-Kyoung Koo (IMBA Vienna) reported similar results, we decided to buy the NEPA21 and focused on optimizing electroporation with this machine.

The authors should provide some information on how to best concentrate plasmids as most plasmid siolation kits will not give such a high concentration but rather in the range of 1 ug/ml
Answer: We added the advice to use Maxi kits for the preparation and recommended one in the Table of Materials.

Since most GFP expression plasmids contain a neomycin casette, have the authors tried to select organoids by antibiotics?
Answer: Of course. Using our electroporation technique for concrete application examples we also selected with antibiotics successfully afterwards. In our representative results we focused on a good (optical) presentation of the transfection efficiency and both plasmids did not encode for any eukaryotic resistance cassette.

Minor Concerns:

Line 147: should say 100 um, not 100 ul
Answer: Done.

Reviewer #4:

Manuscript Summary:

The authors proposed an optimized electroporation method for GI organoids in this protocol. They tested the protocol in 4 different cancer organoids, pancreatic ductal adenocarcinoma (PDAC), colorectal cancer (CRC), cholangiocarcinoma (CCC) and gastric cancer (GC) organoids, and they showed highly efficient transfection results from all conditions (around 40% and 80% efficiency on average for the transfection of 9.3kb and 4.2kb plasmids, respectively). Even though their protocol adapted previous electroporation protocols in organoids (Fujii et al., 2015 and Merenda et al., 2017), this protocol could still be very useful and versatile since they tested it in several different gastrointestinal organoids from different tissue origin and focused on higher electroporation

efficiency. They also tried to make the whole procedure less-expensive, easier and simpler than the previous ones. I only suggest some minor points to improve the manuscript.

1. Line 54-55: What is the logic behind the claim "an efficient transfection becomes a crucial parameter" "(due to the fact that) transient expression of the Cas9 endonuclease is desirable to reduce off-target effects"? It is difficult to understand the link between these two phrases. Any better explanation?
Answer: We rephrased this part of the text. Here, we wanted to address the fact that it is also possible to stably integrate vectors encoding for Cas9 (by viral transduction), if you want to circumvent low transfection efficiency. But permanent expression of Cas9 will increase not wanted off-target effects.

2. Line 60-61, 190-191: The authors mentioned that their protocol works well with healthy organoids, but they did not show any data.
Answer: An exemplary electroporation-mediated knockout of *TP53* in healthy human gastric organoids was added as representative result and is shown in Suppl. Figure 1.

3. Line 84, Step 1.4.2: Please add "(see Table of Materials)" after "dissociation reagent".
Answer: Done.

4. Line 87, Step 1.4.4: I wonder whether vortexing has any drawback in terms of cell viability. How harsh should it be? Is it specific for cancerous organoids or for healthy ones too? Please specify.
Answer: We could not detect any drawbacks and no differences in handling healthy or cancerous organoids.

5. Line 93, Step 1.4.5: How can authors wash the organoids after dissociation in order to remove antibiotics? By two times of centrifugation after resuspension? Please add "(see Table of Materials)" after "electroporation buffer" as well.
Answer: Yes, two washing steps with electroporation buffer (Optimem) were enough to wash out the medium components including antibiotics.

6. Line 102-103: How can the authors keep their DNA stocks with such a high concentration (5ug/ul)? It is not clear whether "the use of less DNA (total amount?)" reduces the efficiency or "the use of less DNA concentration" reduces it or both. Please clarify it.
Answer: We rephrased it, added the advice to use Maxi kits for the preparation of vectors and recommended a kit in the Table of Materials.

7. Line 110-111: It would be great if the authors provide some infomation on how to increase or decrease the impedance to match 30~40 Ω when it does not match.
Answer: We added some troubleshooting guidelines (line 118-120).

8. Line 128-130, Step 3.2: How many wells would the authors recommend after the electroporation using 5 wells of organoids? Should the CHIR9902 (3uM) be added on top of the complete media even if the complete media contains Wnt3a? Please describe how many days (7d) in general the media should be supplemented with Y-27632 and CHIR9902 here.
Answer: As mentioned (“Resuspend the pellet in 100 µl basement matrix and seed 20 µl drops”) 5 wells were originally used and also seeded after electroporation. Of course this can be adapted dependent on how dense the individual culture likes to grow.
Yes, we added CHIR99021 additionally, because it seems to increase the recovery rate (10.1038/nprot.2015.088, Figure 2). In a preliminary experiment we could confirm this for our cultures. We rephrased 3.2. regarding the supplementation of around 5-7d.

9. Even though the authors mentioned that "often more than one plasmid needs to be introduced" by electroporation, there is no guideline for using more than one DNA plasmid. I guess one can just try the same ratio of each plasmid up to 45ug in total amount, but it would be helpful if they provide a guideline when using several plasmids in one electroporation.

Answer: We are sorry, that we cannot give a general guideline. The ratio for “using more than one plasmid” is completely dependent on the aim of the experiment (overexpression, knockout, knock-in, CRISPR-based etc.) and the used plasmids/ DNA strands. For example, for generating a NHEJ-based CRISPR knockout, which will be selectable by medium composition like shown in Suppl.Figure 1, it is possible to use:

- vector 1 encoding for Cas9 + vector 2 encoding for sgRNA1 (+ vector n encoding for sgRNAn)
- vector 1 encoding for Cas9 + vector 2 encoding for sgRNA1+2 (e.g. 2 gRNA concatemer vector, Addgene # 84879)
- 2-n x vector(s) encoding for Cas9 & sgRNA (e.g. px458, Addgene #48138)
- vector encoding for Cas9 and two sgRNAs (e.g. our new px458_Conc2 vector, available on Addgene after publication; which also can be combined with a repair template for HDR-based approach etc.).

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);SupplFigure1.eps