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Monoclonal Cell Line Generation and CRISPR/Cas9 Manipulation via Single-Cell Electroporation

Dr. Ruiguo Yang[+],
Department of Mechanical Engineering, Northwestern University, Evanston, IL 60208, USA.
iNfinitesimal LLC, Skokie, IL 60077, USA

Dr. Vincent Lemaître,
iNfinitesimal LLC, Skokie, IL 60077, USA

Dr. Changjin Huang,
Department of Mechanical Engineering, Northwestern University, Evanston, IL 60208, USA.
iNfinitesimal LLC, Skokie, IL 60077, USA

Dr. Abbas Haddadi,
iNfinitesimal LLC, Skokie, IL 60077, USA. Department of Electrical Engineering and Computer Science Northwestern University Evanston, IL 60208, USA

Dr. Rebecca McNaughton, and
iNfinitesimal LLC, Skokie, IL 60077, USA

Prof. Horacio D. Espinosa
Department of Mechanical Engineering, Northwestern University, Evanston, IL 60208, USA.
iNfinitesimal LLC, Skokie, IL 60077, USA. Institute for Cellular Engineering Technologies Northwestern University Evanston, IL 60208, USA

Abstract

Stably transfected cell lines are widely used in drug discovery and biological research to produce recombinant proteins. Generation of these cell lines requires the isolation of multiple clones, using time-consuming dilution methods, to evaluate the expression levels of the gene of interest. A new and efficient method is described for the generation of monoclonal cell lines, without the need for dilution cloning. In this new method, arrays of patterned cell colonies and single cell transfection are employed to deliver a plasmid coding for a reporter gene and conferring resistance to an antibiotic. Using a nanofountain probe electroporation system, probe positioning is achieved through a micromanipulator with sub-micron resolution and resistance-based feedback control. The array of patterned cell colonies allows for rapid selection of numerous stably transfected clonal cell lines located on the same culture well, conferring a significant advantage over slower and labor-intensive traditional methods. In addition to plasmid integration, this methodology can

Correspondence to: Horacio D. Espinosa.
[+]Present address: Department of Mechanical and Materials Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588, USA

Conflict of Interest

The authors declare the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: iNfinitesimal LLC, which commercializes the NFP system, was founded and is partially owned by Horacio D. Espinosa.

be seamlessly combined with CRISPR/Cas9 gene editing, paving the way for advanced cell engineering.

Keywords

cell line generation; electroporation; nanofountain probe; single cell

1. Introduction

Production of therapeutic proteins and monoclonal antibodies often relies on the development of mammalian cell lines. The generation of a stable cell line begins with the cytoplasmic delivery of a DNA vector containing the gene of interest and a cassette for antibiotic selection. The vector subsequently enters the nucleus and, in a rare event, integrates into the genome of the cell. Selection of stably transfected cells is performed by adding the antibiotic to the culture, such that cells that did not integrate the DNA, and therefore are not resistant to the antibiotic, will not survive the treatment.

Random integration and variable copy number of the transfected gene of interest into the genome result in a high degree of heterogeneity among individual cells.[1] Consequently, investigators must isolate and characterize many clones to select the stably transfected cell lines that express enough recombinant protein.[2] Lengthy and tedious limiting dilution procedures are traditionally used to isolate individual cells that will grow and form monoclonal lines.[3] In particular, minimizing cellular heterogeneity through limiting dilutions has been shown to be a key to the success of therapeutic monoclonal antibody production.[4] Hence, new methods for monoclonal cell line generation and isolation that do not require limiting dilutions would potentially confer a significant advantage compared to regular techniques, in terms of time, cost, and labor.

The need for limiting-dilution procedures in cell line development processes stems from the limited options in gene delivery methods, which are often cell-specific, population oriented, and low in efficiency and viability.[5] Typical carrier-mediated delivery methods include viruses, lipids, and nanoparticles, which are limited by the sizes and types of transfected molecules, and are often cell specific.[6] Importantly, these methods exhibit cytotoxicity[7] and, in the case of viral transfection, can introduce undesirable mutations. As an alternative, a commonly employed method is bulk electroporation, which uses high electric fields to open nanopores in the cell membrane.[8] Similarly to carrier-mediated delivery, it is only applicable to a population of cells and lacks transfection uniformity and dosage control.[9] Furthermore, the high voltages used for bulk electroporation induce a significant stress on cells, leading to lower efficiency and viability.[10]

In contrast to bulk electroporation, single-cell electroporation using the nanofountain probe (NFP) system[11–13] is capable of delivering a precise amount of biomolecules, such as proteins,[13] plasmids,[13] and RNA/DNA molecular beacons,[14] into different cell types with high efficiency and cell viability. The NFP uses a probe, with an embedded microchannel and electrode, which results in a localized and well-controlled electric field upon voltage application. In this study, we have combined the capabilities of the NFP system

with an original model of extracellular matrix patterning to generate and isolate stably transfected cell lines without the need for limiting dilutions.

2. Results

2.1. Optimization of Single-Cell Electroporation Parameters for Cell Line Generation

In single-cell electroporation, a microfluidic probe or a glass micropipette filled with electrolyte is positioned in close proximity of a cell using a closed-loop micromanipulator. Then, a short electric pulse is applied across the cell membrane, (Figure 1a–c), leading to the formation of nanopores. The amplitude and duration of the pulse are tuned to be long enough for molecular transport, but short enough for the nanopores to reseal. The aperture of the probe (500 nm) covers only a small area of the cell membrane, focusing the electric field and limiting cell stress.[13]

To ensure a uniform molecular delivery among individual cells, the voltage across the cell membrane or transmembrane potential (TMP) must be precisely controlled. TMP is the dominant factor in membrane poration as well as in the electrophoretic forces applied to the charged molecules traveling across the membrane.[15,16] To this end, a position control system with resistance as feedback was designed and implemented. As the probe approaches the cell membrane, the resistance of the entire circuit increases as the access resistance is inversely proportional to the distance (d) between the probe and the membrane (Figure 1a).[13,17] Considering the total resistance in the circuit, TMP increases as the access resistance rises (Figure 1b,c). Multiphysics modeling revealed that TMP can be regulated by controlling the position of the probe with respect to the cell membrane through resistance change measurements (Figure 1b).[13]

Labeled protein markers, enabling fluorescence analysis immediately after electroporation, were used to determine the effect of contact resistance change (ΔR), as well as applied voltage (amplitude and duration) on delivery uniformity and cell viability. HEK293 cells transfected with FITC-labeled bovine serum albumin (BSA), at a constant ΔR of 5%, exhibited a uniform level of fluorescence intensity, cells 1 through 5 in Figure 2a,b, and a reduced intensity at a lower ΔR of 4.0% for cell 6 and 2.0% for cell 7 (Figure 2a,b). As shown in Figure 2a, a very reproducible curve was obtained on all probe-cell approaches when the same media and transfectant were used (baseline resistance and resistance increase would change under other conditions). Before each transfection, the resistance value was obtained as a baseline (generally 20 to 40 MΩ), and the percentage of resistance increase was used to control the relative dosage. For different conditions, changes to the applied far-field voltage are needed to maintain the same TMP. Multiphysics analysis shows that with a baseline resistance of 20 MΩ, a 5% resistance increase corresponds to a TMP of 0.6 V under a 50 V far-field voltage. A significant variation of fluorescence intensity was achieved by regulating ΔR from 10% to 2% across different concentrations of transfected molecules (Figure 2c). On the other hand, a higher ΔR led to decreased cell viability (Figure 2d), due to higher contact forces during probe–cell interaction and also, possibly, to a cytotoxic overdosed transfection.[18] Interestingly, up to 5% resistance increase, the reduction in cell viability was lower than 10%.

Alternatively, relative dosage can also be tuned by the applied electrical field. A TMP of approximately 0.1 V is required for membrane poration through the electrical field-induced stress on the lipid bilayer.[5,19] At a certain access resistance, the strength of the applied electrical field and pulse duration have a great impact on delivery and cell viability.[20] The combination of a short high voltage pulse followed by a prolonged low voltage one has been shown to be more effective compared to a single pulse, both in vivo and in vitro.[21] It was found that the magnitude of the high voltage pulse (V_1) and the duration of the prolonged low voltage pulse (t_2) have largest impact on the number of molecules delivered into HEK293 cells. Indeed, transfection of fluorescence-labeled BSA resulted in an almost proportional increase of relative fluorescence intensity with V_1 (Figure 2e). A similar effect was observed with the increase of t_2 (Figure 2f). However, the dose was also dependent on concentration. At a high concentration of transfected proteins (200 ng μL^{-1}), the effect was significant and, as the concentration was reduced (50 to 10 ng μL^{-1}, Figure 2f), it became less pronounced, likely an indication of the electrophoresis-driven molecular transport.

To further assess dosage control, HEK293 cells were transfected with a green fluorescent protein (GFP)-expressing plasmid using either NFP (50 μg μL^{-1}) with the same ΔR (5.0%) (a typical NFP transfection image is shown in Figure 3a) or lipofectamine (Figure 3b), a commonly used transfection method. Twenty-four hours after transfection, quantification of the relative fluorescence intensities showed a significantly higher variation (increased standard deviation) when using lipofectamine as compared to cells transfected with the NFP (NFP: 80.0 ± 11.9, n = 7 cells; Lipofection: 57.6 ± 37.7, n = 32 cells) (Figure 3c). This is consistent with the better dosage control capability the NFP system.

2.2. Monoclonal Cell Line Generation using Single Cell Transfection and a Patterned Substrate

Cells grown on patterned substrates were used for clonal cell line generation. The process is illustrated in Figure 4a. Cellular confinement necessary to form individual colonies was achieved by microstamping, a thin layer of fibronectin onto a polystyrene substrate, followed by passivation of the untreated surface with pluronic acid (Figure 4b). The size and spacing of the microstamp were optimized to maintain a healthy growth and proliferation of transfected cells (Figure 4c). Each colony had 10 to 20 cells (other cell lines may require a different number of neighboring cells to grow appropriately). An individual cell in each colony was specifically targeted and transfected with a plasmid encoding GFP and resistance to the antibiotic Zeocin. A selection process was subsequently performed using Zeocin until a colony of GFP-positive cells proliferated from the initial transfected cell. GFP-expressing colonies were then harvested and expanded as clonal cell lines with stable transfection.

As expected, cellular expression of GFP depended on transfection parameters. Based on the parametric study described earlier, a transfection parameter of ΔR = 5.0% with a bi-level electrical pulse (V_1 = 20 V, t_1 = 0.5 ms, V_2 = 10 V, t_2 = 2.5 ms) was selected for the experiments. The GFP expression level was significantly lower when ΔR = 2.5% (Figure 4d). Note that different parameter sets can be chosen to modulate GFP expression and to generate cell lines with distinct properties. High GFP expression level was detected in 77.8 ± 16.7% of the transfected cells 24 h after transfection. No difference in GFP expression was

observed between electroporation targeting the cytoplasm or the nucleus (data not shown). A representative image is shown in Figure 5a, in which all but one colony expressed GFP. Surrounding cells in the colony appeared to provide good growth conditions for the transfected cells as shown in the zoom-in image in Figure 5b. Cells were then selected with Zeocin 2 days after transfection and 7 days after treatment, 61% of all transfected cells (22 cells out of 36) survived (representative image is given in Figure 5c and zoom-in image in Figure 5d). After 15 days, 6.9% of them formed a colony of entirely GFP-positive cells (average of 2.5 out of 36 cells; Figure 5e). A representative image of a GFP-positive cell colony is shown in Figure 6a. Stably transfected cell colonies were detached from the well using trypsin and transferred by means of micropipette aspiration onto a 60 mm cell culture dish for further expansion.

After 2 weeks of culture, confluent cells were subsequently characterized by fluorescence assisted cell sorting (FACS) analysis of GFP expression. As shown in Figure 6b, GFP fluorescence signal spanned over an order of magnitude, with a normal distribution and a coefficient of variation of 0.719 (Figure 6c). This spread of fluorescence signal was consistent with monoclonal cells exhibiting intraclonal variation of EGFP expression levels.[22] The observed variation in intraclonal GFP expression may be due to a range of factors, including cell cycle and cell size.[22] This experiment indicated that the analyzed cells originated from one single transfected cell in the colony. Overall, the process of stable monoclonal cell line generation, using the NFP system for single-cell electroporation, took less than 5 weeks. This time is dictated by the cell biology. No repetition of any of the processes used in the described protocol was needed. When this is combined with the ability to transfect many individual cells in a 2D array, with dosage control and high viability, a much-improved throughput, compared to other methods, is achieved.

In order to illustrate the suitability of the here presented monoclonal cell line generation to other applications of interest, NFP-based gene editing was carried out. For this purpose we employed the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, a popular method to generate monoclonal cell lines with specific gene knockout and editing.[23] A HEK-293 cell line containing a single copy of the EGFP gene as a target for Cas9 cleavage was used for single-cell transfection with the NFP system. These cells grown in arrayed colonies were transfected with Cas9 nuclease linked to a guide RNA (gRNA) specifically targeting EGFP (Figure 7a).[24] Two days after transfection, a loss of green fluorescence signal was observed in the progeny of the transfected cells, consistent with EGFP gene cleavage by Cas9 and knockout (Figure 7b). In nontransfected control colonies, all cells exhibited the expected green fluorescence signal (Figure 7c). These experiments indicated that, in addition to using plasmid integration, CRISPR/Cas9 gene editing can also be employed to generate monoclonal cell lines using patterned cell colonies and the NFP device.

3. Discussion

Stably transfected cell lines are widely used in drug discovery and biological research. To avoid genetically mixed cell populations, investigators use dilution techniques to select single cells that will thereafter generate clonal lines. However, limiting dilutions require

several cycles of cell detachment, centrifugation, and manual pipetting of small volumes,[25] thus rendering this process tedious and time consuming. By transfecting individual cells cultured in arrayed colonies using the NFP electroporation system, we present a new method for efficient generation of clonal eukaryotic cell lines, in less than 5 weeks, without dilution cloning. The method is not only less labor intensive, but also employs very small volumes of expensive reagents (nucleic acids, proteins) and is considerably quicker when compared to traditional cell line generation workflows that generally require 8 to 10 weeks.[26] Moreover, the entire protocol is amenable of full automation. Whereas conventional methods of transfection often result in high cytotoxicity and subsequent cell death, our technique exhibited excellent cell survival, with more than 90% of viable cells after transfection, due in part to the precise positioning of the probe resulting in minimal cellular stress and localization of the electric field that porates the cell membrane.

Single-cell electroporation with micromanipulator-controlled lateral movement of the microfluidic probe enables a convenient selection of target cells. Indeed, in this study, target cells were randomly selected within a colony, but because of the precision and resolution of the micromanipulator, our system also allows the targeting of specific cells present on the same culture well. The NFP system would therefore be useful for investigators using multicellular systems, such as a neuron-muscle cell coculture[27] where only the neuron cells need to be electroporated.

It is worth mentioning that clonality could be affected if a cell detaches from its colony and adheres to another adjacent one. To examine this possibility, cells would have to be transfected with plasmids coding for different reporters and tracked overtime.

Our stamping method offers a practical solution to transfection of single cells that grow slowly or do not survive when isolated.[28] In effect, the size of the microstamp can be optimized to get a sufficient number of neighboring cells for normal growth and functioning. Moreover, it provides flexibility in tuning electroporation parameters by comparing their effect side by side across the array of colonies. Another significant advantage of our protocol of patterned colonies compared to current techniques is the ability to rapidly select numerous stably transfected clonal cells grown on the same culture well. Moreover, it is worth noting that one can transfect different nucleic acids on each row of the array, with each row containing multiple replicas of the same biological process. This is particularly important when generating multiple cells lines for drug screening or toxicity studies performed on cells containing targeted mutations, such as specific single nucleotide polymorphism generation using the popular CRISPR/Cas9 system.[29,30]

The CRISPR/Cas9 system consists of a guide RNA (gRNA) bound to the bacterial nuclease Cas9.[31] A specific 20-nucleotide region of the gRNA targets the genomic sequence to be modified, immediately upstream of a protospacer adjacent motif (PAM). After cleavage, the resulting double strand break is repaired through the non-homologous end joining (NHEJ) pathway. This pathway often introduces undesired nucleotide insertion or deletion, leading to the knockout of the target gene. The CRISPR/Cas9 system can also precisely introduce single nucleotide mutations or larger insertions, using the high-fidelity homology directed repair (HDR) pathway.[31] In this case, a DNA repair template containing the desired edit

located in between sequences homologous to the target gene is used together with Cas9 and the gRNA. Experiments performed on a HEK293 cell line expressing the green fluorescence protein demonstrated the ability of the NFP system to knockout the GFP gene after transfecting these cells with the Cas9 nuclease and a specific gRNA. These data indicate that the generation of monoclonal cell lines using our model of micro-stamping in conjunction with NFP single cell electroporation may also involve the simple and potent CRISPR/Cas9 system for gene knockout and editing, in addition to transfecting plasmids for chromosomal integration. In particular, the array can be used to perform parallel experiments with each row transfected with a different gRNA.

Contrary to conventional methods of transfection, our results demonstrate that dosage control can be achieved by monitoring the probe–cell membrane proximity through resistance change, and by modulating the applied electrical field. For absolute dosage control, gene profiling can serve as a basis by examining the copy number of inserted genes. Our future work will focus on evaluating the effect of dosage on the number of genes inserted in the chromosomes of monoclonal cells.

4. Conclusion

We have demonstrated a novel process for generating monoclonal cell lines from individually transfected cells using NFP-based single-cell electroporation and arrays of cell colonies. The method eliminates the limiting dilution process and has the potential to be integrated with state-of-the-art gene editing techniques, which promise to revolutionize cell engineering and therapeutics.

5. Experimental Section

Cell Culture and Microstamping for Cell Colony Control

Human embryonic kidney cells (HEK293) were grown in Dulbecco's modified Eagle media (DMEM) (Gibco) containing 10% fetal bovine serum and antibiotics. The polydimethylsiloxane (PDMS) micropillar arrays, containing 100 μm pillars, were fabricated from a silicon mold. A fibronectin solution (Sigma-Aldrich) at 50 μg mL^{-1} in PBS (Gibco) was deposited onto a PDMS chip and, after one hour at room temperature, the solution was gently removed, and the chip was dried for 5 min. The chip was then placed upside down onto a 35-mm polystyrene substrate for 10 min to allow the transfer of the fibronectin onto the substrate. After removal of the stamp, 1 mL of pluronic acid solution (2 mg mL^{-1} in PBS) (Sigma-Aldrich) was added for 45 min to passivate parts of the substrate devoid of fibronectin, preventing nonspecific cellular adherence. After removal of the pluronic acid solution, HEK293 cells were added. The substrate was placed in an incubator for 24 h allowing cells to adhere on the fibronectin and form small colonies.

Fluorescence Microscopy

Fluorescence images were acquired with a CoolSnap HQ2 (Photometrix) camera mounted on a Nikon Eclipse Ti microscope. AF-488 and GFP fluorescence was analyzed using a B-2E/C filter (Nikon), and image acquisition was controlled by the NIS Elements software (Nikon).

Single Cell Transfection by NFP

The NFP was positioned on top of cells using a micromanipulator (Eppendorf Injectman 2). The probe–cell membrane contact was detected by monitoring resistance change with an approach velocity of 1 μm s^{-1}. When a specified threshold resistance change was measured, the NFP motion was stopped and bi-level pulses were applied. The HEK293 cells were transfected with BSA conjugated with Alexa Fluor 488 (BSA-AF488, ThermoFisher) at 2.5 mg mL^{-1} in PBS (Gibco) or with the plasmid pSELECT-GFPzeo-mcs (Invivogen) at a concentration of 52 ng μL^{-1} of PBS (Gibco).

Transfection by Lipofectamine

HEK293 cells were transfected using Lipofectamine 2000 (LifeTechnologies) following the manufacturer's instructions. Briefly, HEK293 cells were plated on a 24-well plate and at 80% confluence, a mixture containing 4 μL of lipofectamine and 1 μg of pSELECT-GFPzeo-mcs plasmid (InvivoGen) in a final volume of 50 μL of DMEM was added per well. Twenty-four hours post-transfection, pictures were taken at 50 ms exposure and the relative fluorescence intensities were measured using the ImageJ software.

Generation of Recombinant Cell Lines Expressing GFP

The plasmid pSELECT-GFPzeo-mcs (Invitrogen) of 4.2 kb was used to express GFP and confer resistance to the antibiotic Zeocin. One individual HEK293 cell, randomly selected in each colony, was transfected using the NFP system with pSELECT-GFPzeo-mcs. The process was repeated for all the colonies in the array. Two days after transfection, Zeocin (Invivogen) was added at a final concentration of 400 μg mL^{-1} in the culture media in order to select the stably transfected cells. The selection process was performed for 2 weeks to eliminate all nonstably transfected cells.

Characterization of Recombinant Cell Lines Using Fluorescence-Activated Cell Sorting

Stably transfected cell colonies were transferred to a 60 mm cell culture dish and grew to confluence. The cells were subsequently trypsinized and grown in a T-75 flask till confluence. Prior to the FACS sorting, the cells were suspended in DMEM medium at 1 million cells per mL. The FACS characterization was performed on a BD FACSAria SORP system with a speed of 1000 cells s^{-1}.

CRISPR/Cas9 Gene Knockout of GFP in HEK293 Cells

A transgenic HEK293 cell line containing a single copy of the EGFP gene in the genome (a gift from Dr. W. Miller, Northwestern University) was used in these experiments. These cells were grown on patterned substrates as described above in "Cell culture and microstamping for cell colony control." Colonies of 10 to 20 cells were transfected using the NFP-E with a solution containing Cas9 nuclease (NEB) and a guide RNA (targeting sequence: GGGCGAGGAGCUGUUCACCG; Synthego) both at 1×10^{-6} M final in DPBS. This gRNA has been shown to efficiently knock-out the green fluorescent protein gene.[24] A bi-level electrical pulse (V_1 = 15 V, t_1 = 0.5 ms, V_2 = 10 V, t_2 = 2.5 ms) was used for electroporation. After transfection, cells were observed every day under fluorescence microscopy for GFP signal analysis.

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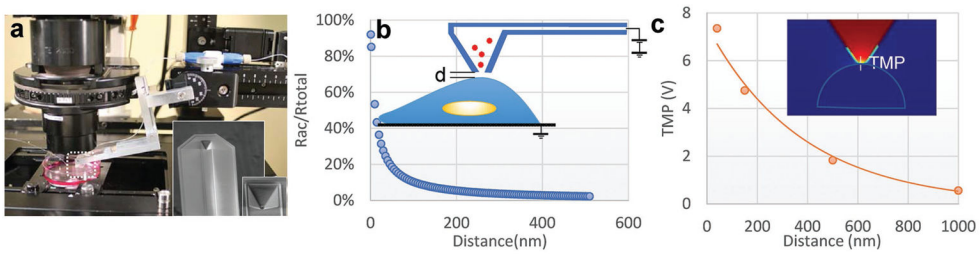

Figure 1.

Experimental setup and resistance-based control of transmembrane potential (TMP). a) The experimental setup includes an NFP microchip containing molecules to be transfected; the NFP chip consists of a microchannel-embedded cantilever fabricated out of silicon dioxide and a pyramidal tip with an opening of 500 nm (SEM image shown in inset); the chip assembly is mounted on a 3 degree-of-freedom micromanipulator, and the entire setup is mounted on top of an inverted microscope. b) Normalized access resistance–distance curve obtained from a lumped model that considers the serial connection of resistances including contact resistance between the electrode and the fluid. As the resistance increases, the voltage across the interface increases accordingly. c) The COMSOL simulation[13] shows that the TMP increases as the probe draws near to the cell membrane; a 50 V potential is applied to the entire circuit. A representative simulation result is shown in the inset. The property parameters for the simulation are: membrane conductivity, 5×10^{-7} S m^{-1}, intracellular conductivity 0.4 S m^{-1}, and fluid conductivity, 1 S m^{-1}.

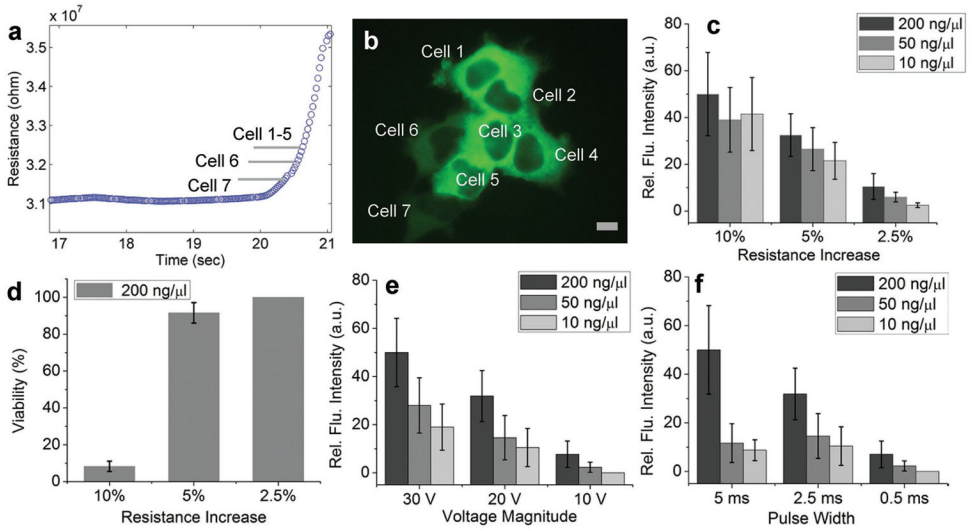

Figure 2.

Optimization of NFP transfection parameters. a) Resistance measurements during probe approach. b) Intensity level of BSA-AF488 after transfection at different resistance increases ΔR (cell 1–5, ΔR = 5.0%; cell 6, 4.0%; cell 7, ΔR = 2.0%). c) Comparison of the relative fluorescence intensity in HEK293 cells after transfection of BSA-AF488 at 200, 50 and 10 ng μL^{-1}, respectively, under the same applied electrical voltage but at varied ΔR. d) Viability of transfected cells at varied ΔR. e) Fluorescence intensity level at different voltages together with data comparison for different concentrations of transfected BSA. f) Fluorescence intensity level at different pulse durations together with data comparison for different concentrations of transfected BSA. For each data point, the sample size is 15 cells. For the data reported in (e), V_2 = 10 V, t_1 = 0.5 ms and t_2 = 2.5 ms was used. For the data reported in (f), V_1 = 20, V_2 = 10, and t_1 = 0.5 ms was used. Error bar represent the standard deviation for at least 25 cells. Scale bar = 10 μm.

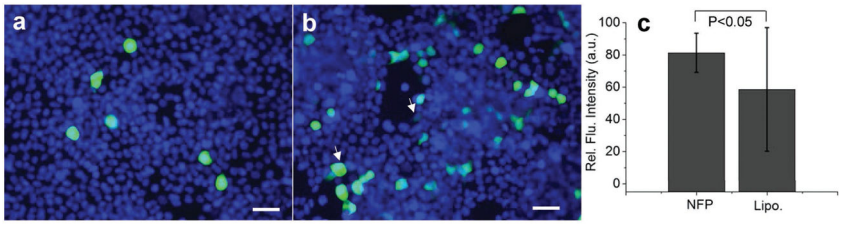

Figure 3.

Comparison of HEK293 cells transfected with an EGFP-expressing plasmid using NFP or lipofectamine. a) Image of NFP transfected cells, same ΔR of 5%, twenty-four hours posttransfection. b) Image of lipofectamine transfected cells. All images were taken at the same exposure time of 50 ms. Arrows show two cells with large variations in expression levels. c) Green fluorescent signals (nuclei appear in blue after Hoechst stain) measured using the ImageJ software show more fluorescence variability when using lipofectamine. Scale bar = 200 μm.

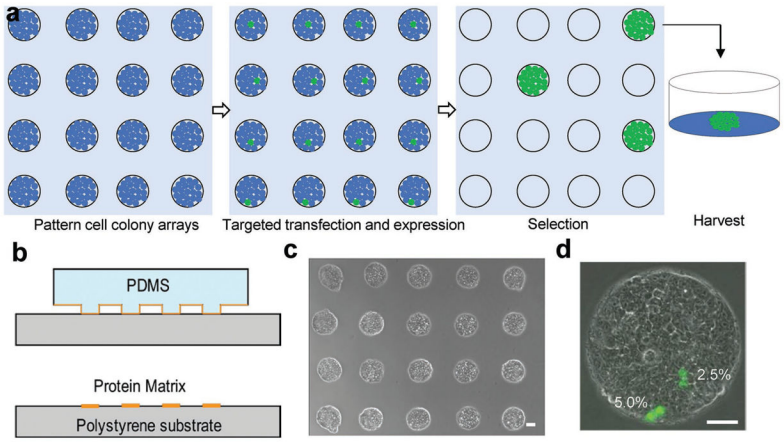

Figure 4.

Monoclonal cell line generation process using NFP-based single cell transfection and micro-stamping. a) Diagram of the workflow used to generate a monoclonal cell line. b) Transfer of an extracellular matrix protein (fibronectin) onto a polystyrene substrate by microstamping. c) Image showing array of cell colonies on protein stamped substrates. d) Targeted transfection and expression of the GFP-Zeocin plasmid with different dosages, which demonstrates control of expression level via ΔR selection. Scale bar = 50 μm.

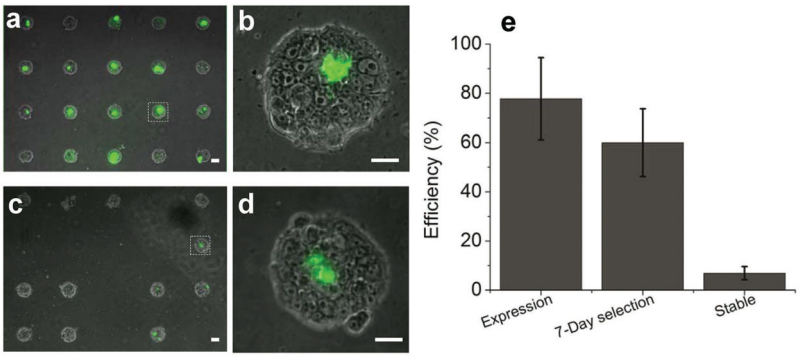

Figure 5.

Monoclonal cell line generation process by NFP-based single-cell transfection. a) GFP-expressing plasmid transfection of HEK293 cells after 24 h. b) Zoom-in image of individual colony shows healthy cells surrounding the transfected cell. c) Image showing selection, after Zeocin treatment 72 h posttransfection, with zoom-in image in (d). e) Efficiency of transfection, selection, and stable integration. Scale bar = 50 μm. The images are representative of three independent experiments.

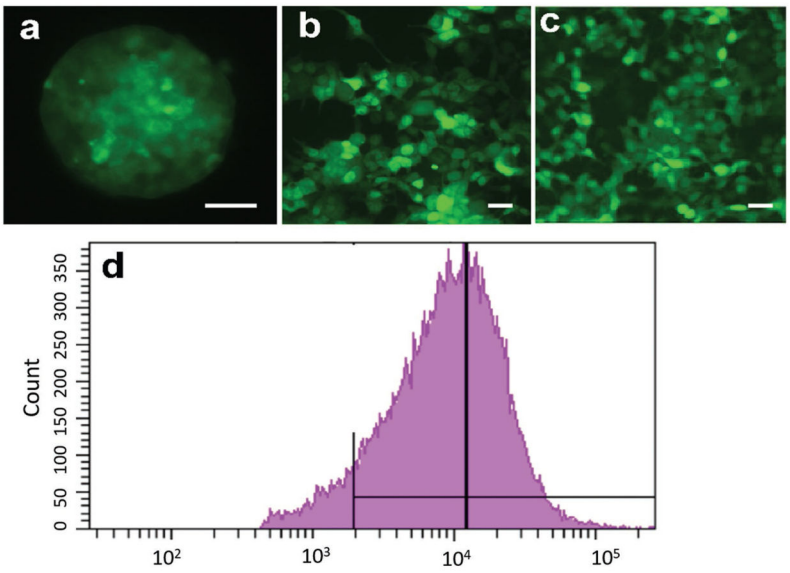

Figure 6.

Flow cytometry analysis of GFP expression in a stably-transfected monoclonal HEK293 cell line. a) All cells stably expressing GFP in a single colony are derived from a single transfected cell within the colony. b) Fluorescence microscopy image of the monoclonal cell line generated by single-cell electroporation. c) Image of sorted subpopulation from the higher half of GFP expressing cells (GFP + brt). d) Plot showing the distribution of fluorescence intensity (Fluorescence in arbitrary units, A.U.). There is an inherent intraclonal heterogeneity of GFP expression mostly due to the cell cycle and cell size.[22] This heterogeneity is evident in presorted cell and postsorted cell populations as shown in b and c. The overall spread of the fluorescence intensity falls within the intraclonal differences, with approximately two orders of magnitude and a coefficient of variance of 0.719.[22]

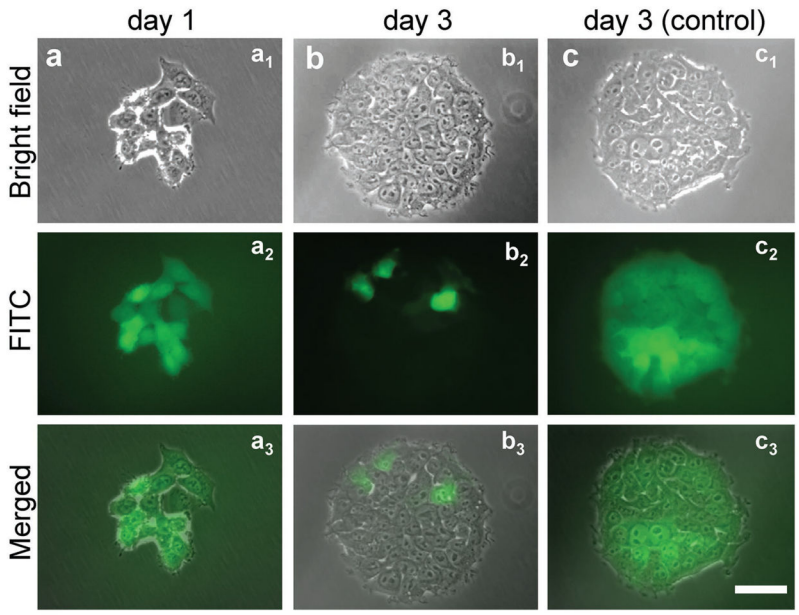

Figure 7.

CRISP/Cas9 gene knockout using single-cell electroporation. a) Patterned EGFP-HEK293 cells colonies transfected using the NFP-E with Cas9 nuclease and a specific guide RNA targeting EGFP. All cells of a colony of 14 cells were transfected on day 1 with Cas9/gRNA. b) On day 3, transfected cells multiplied but only three cells in the colony remained positive for GFP fluorescence signal, consistent with knockout of EGFP. c) A nontransfected control colony exhibiting EGFP signal in all cells.