Methods in Molecular Biology 2050

Springer Protocols

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Electroporation Protocols

Microorganism, Mammalian System, and Nanodevice

Third Edition

Humana Press

METHODS IN MOLECULAR BIOLOGY

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-9739-8 ISBN 978-1-4939-9740-4 (eBook)
https://doi.org/10.1007/978-1-4939-9740-4

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Preface

Electroporation gene therapy, or gene electrotransfer, refers to the delivery of genetic materials into target tissues or cells via electric pulses for the treatment or prevention of disease. The genetic materials delivered by electroporation include oligoDNA, RNA, genes, and chromosomes. This term is a by-product of electrochemotherapy, in which chemical therapeutics or synthetic small molecules are delivered into targeted cells via electric pulses. Simultaneous delivery of both genetic materials and chemical therapeutics is referred to as electrochemogene therapy. The delivery of gentle/modest electric signal alone, without any chemical therapeutic or genetic material, which is used to message tissue for functional activation, is referred to as electrical (message) therapy, while the delivery of electric pulses with high intensity/frequency without any other chemicals/genetic materials, which can be used to remove bad tissue such as tumors, is referred to as irreversible electric therapy. The focus of this book is to provide in-depth knowledge and hands-on protocols for the delivery of naked DNA and small interfering RNA (siRNA) to the targeted cells, including fungus, tissues, and animals, for the treatment of disease and biological studies. Therefore, this book is primarily dedicated to electroporation gene transfer.

The first gene delivery via electroporation was carried out in 1982 by Professor Eberhard Neumann using an in vitro cell culture system. During the more than 30 years since then, this technology has evolved greatly, thanks to the remarkable progress in genetic sequencing, gene array analysis, gene cloning, gene synthesis, gene expression detection, DNA manufacture, and discovery and synthesis of siRNA. This third edition of *Electroporation Protocols: Microorganism, Mammalian System, and Nanodevice* builds on the success of the first two editions and on the progress made in the genetic delivery in single cells, nanodevice, and microorganism.

Because of the rapid development in electroporation gene therapy, 100% of the chapters in this third edition are completely new. Therefore, this book may be considered a continuation of the first two editions rather than a replacement in terms of the protocols. However, the style and format used for the third edition are similar to those of the first two editions and will be very familiar and accessible to investigators who have used the first two editions.

The major differences between this edition and the first two editions are the additions of a large section on microorganism and a section nanodevice. The other major differences are the substantial expansion of the section on applications in single cells because the electroporation of therapeutic genes has been used for preparing clinically used T cells for therapy. This type of application is extremely valuable. These additions and expansions in the third edition, unfortunately, required the sacrifice of chapters of the first two editions related to large animal applications. For details of those topics, the first edition should serve very well.

This editor would like to acknowledge the coeditors of this book, Drs. Lingqian Chang and Justin Teissie, who have done superb job in soliciting chapter authors, contributing their works, and finalizing the contents. I also am grateful to all the authors who worked so hard to get this book to press in a timely manner. Dr. Lingqian Chang also provided the art for the cover. Finally, I am very grateful to my department chair, Richard Gorlick, MD, for providing the time I needed to accomplish this work.

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Chapter 6

Nanofountain Probe Electroporation for Monoclonal Cell Line Generation

Horacio D. Espinosa, Prithvijit Mukherjee, and Cesar Patino

Abstract

In the field of genetic engineering, the modification of genes to produce stable cell lines has a variety of applications ranging from the development of novel therapeutics to patient specific treatments. To successfully generate a cell line, the gene of interest must be delivered into the cell and integrated into the genome. The efficiency of cell line generation systems therefore depends on the efficiency of delivery of genetically modifying molecules such as plasmids and CRISPR/CAS9 complexes. In this work, we describe a localized electroporation-based system to generate stable monoclonal cell lines. By employing the nanofountain probe electroporation (NFP-E) system, single cells in patterned cultures are selectively transfected with plasmids, grown, and harvested to obtain stably expressing cell lines. Methods for microcontact printing, cell culture, electroporation, and harvesting are detailed in this chapter.

Key words Genetic engineering, Localized electroporation, Nanofountain probe electroporation, Plasmid transfection, Monoclonal cell lines, Cell line generation

1 Introduction

Advances in gene editing and cell line generation technologies have profound implications in healthcare and fundamental biological research [1–4]. In cell line engineering, modification of the cell's genome is accomplished through a variety of methods such as; plasmid transfection, lentiviral transduction, recombinase-mediated cassette exchange (RMCE), and CRISPR/CAS9 gene editing [1, 5]. Successful gene editing involves the stable expression of the gene of interest over time. Furthermore, marker genes are introduced with the gene of interest to distinguish and isolate successfully edited cells from nonedited cells. For this purpose, fluorescent markers such as GFP as well as antibiotic resistant genes are often used [5]. Once the stably edited cells are isolated, they can be cultured and harvested to form monoclonal cell lines.

Shulin Li et al. (eds.), *Electroporation Protocols: Microorganism, Mammalian System, and Nanodevice*, Methods in Molecular Biology, vol. 2050, https://doi.org/10.1007/978-1-4939-9740-4_6,
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The overall efficiency of stable cell line generation depends on both the efficiency of delivery of gene editing molecules into the cell as well as the efficiency of integration of the DNA of interest in the genome. The focus of this protocol is to enhance the efficiency of delivery. Lipid-mediated delivery methods are inefficient, cell-dependent, and lack dosage control [6]. On the other hand, viral transduction protocols have higher delivery efficiencies but pose biohazard safety issues [5]. As an alternative, physical delivery systems like electroporation and mechanoporation can be employed to deliver precise doses of biomolecules into cells without incurring the biosafety hazards of viral vectors [7, 8]. In bulk electroporation systems, cells in suspension are subjected to an electric field that induces pore formation across the cell membrane. However, the viability of cells in bulk electroporation systems is low due to the strong electric fields the cells are subjected to. Localized-electroporation systems circumvent this issue by confining the electric field to small areas on the cell membrane, thus resulting in high cell viability [7, 9], uniform delivery [10], and high delivery efficiencies [11]. In these methods, the electric field is applied across nanostructures such as nanopores, nanoprobes, or nanopipettes [7, 9–13]**.** In this protocol the nanofountain probe electroporation system (NFP-E) is employed to transfect cells grown on a culture dish patterned with membrane proteins in an array format [10]. The transfection imparts green fluorescent protein (GFP) expression and antibiotic (zeocin) resistance to the cells. The stably transfected cells are then selected via antibiotic treatment and harvested.

2 Materials

2.1 Mold Fabrication

1. The mold fabrication for the PDMS stamps was performed in a clean-room facility having the following equipment: spin coater, hot plates, reactive ion etcher (Samco Inc., Kyoto, Japan), mask aligner (SUSS Microtec SE, Garching, Germany), and Parylene coater (SCS, Indianapolis, IN, USA).
2. Si wafers—Wafers are used for the lithographic patterning of photoresist features.
3. SU-82000 photoresist (MicroChem, Westborough, MA, USA)—Negative photoresist used for fabricating high aspect-ratio microscale features.
4. SU-8 Developer (MicroChem)—Developer is used to remove photoresist material not exposed to UV.

2.2 PDMS Stamp Fabrication

1. Polydimethylsiloxane (PDMS) Sylgard 184 (Dow Corning, Midland, MI, USA)—Mixed in a 10:1 elastomer to curing agent ratio and used for microcontact printing.

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2. Desiccator (Fisher Scientific, Hampton, NH, USA)—Used to remove air bubbles from the PDMS mixture.
3. Convection oven—Used for thermal curing of the PDMS mixture.

2.3 Stamp Coating

1. Fibronectin (Sigma-Aldrich, St. Louis, MO, USA)—A 25 μg/mL fibronectin in PBS solution is used to stamp the cell-culture dish to promote cell adhesion.
2. Pluronic F127 (Sigma-Aldrich)—A 0.2% w/v solution of Pluronic in DPBS is used to passivate surfaces and prevent cell adhesion.
3. DPBS (Thermo Fisher Scientific)—DPBS is used to prepare the fibronectin and Pluronic mixtures.
4. Ethanol—70% ethanol used to sterilize the PDMS stamp surfaces.
5. 60-mm culture dishes and 35-mm Polystyrene dish (Sigma-Aldrich)—The dishes are used as the substrate for protein fibronectin protein stamping and passivation with Pluronic.
6. Syringe filter (Millipore Sigma, Burlington, MA, USA)—A 0.22-μm syringe filter is used to filter the Pluronic solution prior to application.
7. Syringes (Becton Dickinson, Franklin Lakes, NJ, USA)—3 mL syringes used for the Pluronic passivation.

2.4 Cell Culture

1. Human Embryonic Kidney Cells (HEK293)—HEK293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% penicillin–streptomycin (10,000 U/mL) (Thermo Fisher Scientific).
2. Trypsin—0.25% trypsin–EDTA solution (Thermo Fisher Scientific) is used for dissociating the cells.

2.5 Transfection Using NFP-E

1. Nanofountain probe electroporation (NFP-E) system (Infinitesimal, Skokie, IL, USA)—NFP-E is used to deliver molecules for transfection via localized electroporation.
2. Plasmid DNA—For transfection of HEK293 cells, a 4.2 kb plasmid pSELECT-GFPzeo-mcs (Invitrogen, Carlsbad, CA) is used to induce GFP expression and resistance to the antibiotic zeocin.
3. DPBS—The plasmid DNA stock solution is diluted in DPBS (Thermo Fisher Scientific). A typical final concentration of 50 ng/μL of plasmid DNA is used for the transfections.

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4. Zeocin—Zeocin antibiotic (InvivoGen, San Diego, CA) at a final concentration of 400 μg/mL is used in the culture media for selection of stably transfected cells.
5. Ethanol—70% ethanol is used for cleaning the electrodes.

2.6 Observation and Cell Line Generation

1. Fluorescence microscopy—The images are acquired using a CoolSnap HQ2 camera (Photometrics, Tucson, AZ) mounted on a Nikon Eclipse Ti microscope. Image acquisition is controlled using NIS Elements software (Nikon, Minato, Japan). Post processing of images is performed using Fiji image processing package.
2. Colony expansion—Stably transfected colonies are expanded in 60 mm tissue cultures dishes (Corning Inc., Corning, NY) and T-75 flasks (USA Scientific, Inc., Ocala, FL).
3. Flow cytometry—Characterization of GFP expression and sorting of GFP expressing cells of interest are done using a BD FACSAria SORP system at a speed of 1000 cells/s.

3 Methods

3.1 Mold Fabrication (Fig. 1a)

1. Si wafer (4 in) cleaning: oxygen plasma clean the Si wafer in RIE machine with the following settings: O_2: 100 sccm, 100 mT, 200 W, 5 min.
2. Place the clean Si wafer in the spin-coater machine, dispense 4 mL SU-8 photoresist on the wafer and set the following spin coater settings in two steps: (1) spread cycle; ramp to 500 rpm with100 rpm/s acceleration and hold for 10 s at 500 rpm, and (2) spin cycle; ramp from 500 rpm to 3000 rpm with 300 rpm/s acceleration and hold for 30 s at 3000 rpm.
3. Place the SU-8 coated wafer on a hotplate set at 65 °C for 3 min.

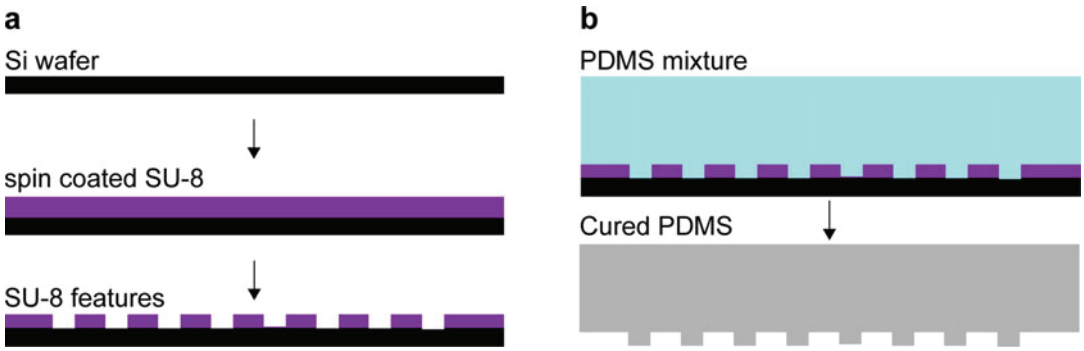

Fig. 1 PDMS stamp fabrication protocol: (**a**) Mold fabrication consisting of photolithographic patterning of SU-8 on Si wafer. (**b**) Thermal curing and peeling of PDMS mixture on mold to obtain stamps

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4. Increase the temperature of the hotplate to 95 °C and hold for 7 min.
5. Place the wafer and photomask in the mask aligner with the following settings: vacuum contact mode, power 9 mW/cm^2, exposure time 23 s.
6. Place the wafer on hot plate immediately after exposure at 65 °C for 3 min followed by an increase to 95 °C for 9 min.
7. Immerse the UV-exposed wafer in SU-8 developer for 10 min, while gently agitating the wafer with forceps.
8. Rinse with IPA. If white residue comes appears on the wafer, place back in the developer for additional time until no white residue appears.
9. Dry with nitrogen gas.
10. Hard-bake the mold on a hotplate set at 120 °C for 15 min.
11. Utilize the Parylene coater to deposit Parylene-C on the mold to facilitate the release of PDMS from the mold.

3.2 Fabrication of PDMS Stamps (Fig. 1b)

1. Prepare a 10:1 (w/w) elastomer base to curing agent solution of PDMS on a weighing dish.
2. For a 4″ wafer, mix 40 g of PDMS to obtain a thickness of ~5 mm.
3. Place the PDMS in a desiccator for 30 min to remove air bubbles from the mixture.
4. Place the mold on a flat aluminum foil sheet and wrap the foil around the edges of the mold to make an aluminum boat to contain the PDMS.
5. Pour the degassed PDMS on the mold and place back in the desiccator for 15 min to remove any remaining bubbles.
6. Place the aluminum boat containing the mold and uncured PDMS in a convection oven at 80 °C for 2 h to fully cure the PDMS. Ensure that the surface of the convection oven is level to achieve a uniform thickness of the PDMS stamps.
7. Once the PDMS is fully cured, cut the PDMS with a razorblade around the perimeter of the mold and remove the aluminum foil from the mold.
8. Slowly remove the PDMS from the mold and cut it into small 1 cm × 2 cm blocks; inspect pillars in microscope prior to stamping. Note that the Parylene coating on the mold facilitates this step and helps protect the mold for multiple uses.

3.3 Stamping Protocol (Fig. 2a)

1. Perform all the following procedures under a biological hood using sterile techniques.
2. Place the PDMS chip in a 60-mm dish filled with 70% ethanol (the whole chip should be covered) for 5 min.

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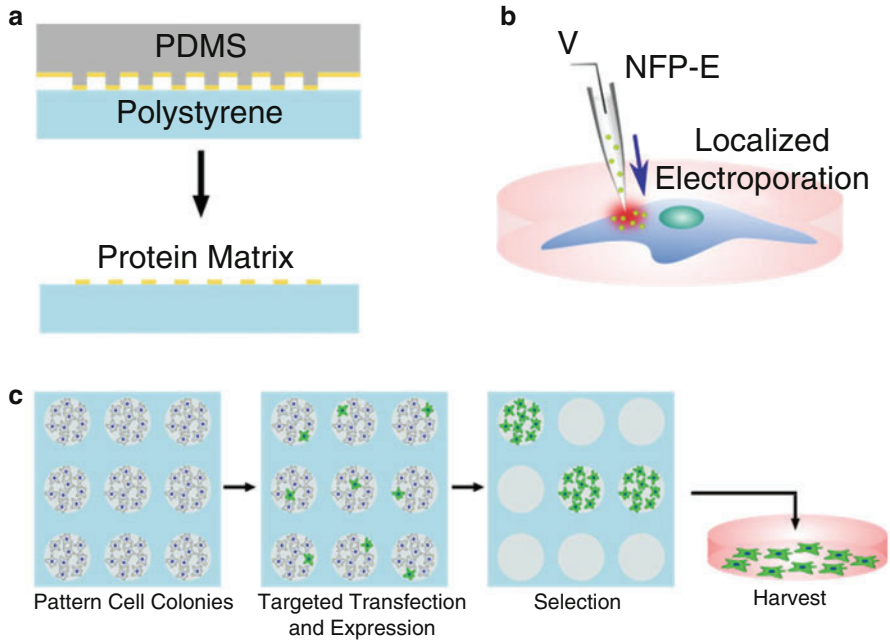

Fig. 2 NFP-E cell line generation workflow: (**a**) Microcontact printing of fibronectin on culture dish to promote adhesion of cells in isolated colonies. (**b**) Single-cell electroporation and delivery of plasmid encoding gene of interest using the NFP-E system. (**c**) Stably transfected single cells in stamped arrays are selected by antibiotic treatment, picked, and harvested to form monoclonal cell lines

3. Transfer the chip (using forceps) into a dish containing sterile water for 5 min, and then place the chip in an empty dish.
4. Let the chip dry for 5 min (If necessary, remove remaining water from the sides using a low-lint wipe).
5. Prepare fibronectin: Dilute the stock solution (1 mg/mL) 40 times in sterile D-PBS (25 μg/mL final) (e.g., for two chips, add 25 μL of stock fibronectin to 975 μL of DPBS).
6. Add 400–500 μL of the diluted fibronectin solution on top of the chip, making sure its covers the whole surface, and wait for 1 h to ensure the fibronectin coats the surface of the pillars.
7. In the meantime, prepare Pluronic at 0.2% w/v in D-PBS (e.g., 40 mg in 20 mL)—and incubate at 4 °C for 15 min.
8. After **step 6** is complete, remove all of the fibronectin solution from the chip by placing a wipe on the side of the chip and letting it dry for 10 min.
9. Carefully place the coated side (top) of the chip onto a 35-mm polystyrene dish (do not use a regular cell-culture treated 35-mm dish); press chip gently with forceps (once the chip is in contact with the well, its position should not be changed).

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10. Wait for 15 min to allow the transfer of the fibronectin from the pillars onto the well.
11. In the meantime, take the pluronic solution prepared in **step 7** and filter 1 mL per stamp using a 22 μm syringe filter.
12. Carefully remove the chip from the well with forceps and add 1 mL of the filtered pluronic solution (this solution will passivate the parts of the dish that do not contain fibronectin). Let pluronic sit for 45 min.
13. Remove the pluronic solution with a pipette.
14. Add 25,000 cells per well in a total of 2 mL of culture media (cell number can vary based on experimental needs and cell type).
15. Place dish in incubator and transfect the cells that form colonies over the micropatterned regions the next day (*see* **Note 1**).

3.4 Cell Culture on Stamped Substrates

1. Harvest HEK293 cells by adding trypsin and collecting the detached cells. Dissociate the clusters by gentle pipetting and then count the cells.
2. Plate the cells on the micropatterned petri dishes by putting 5×10^4 cells in 2 mL DMEM per dish and put them in the incubator (37 °C and 5% CO_2) overnight to allow them to adhere.

3.5 Transfection Using NFP-E (Fig. 2b)

1. Observe the cells on the micropatterned petri dish to confirm that they have adhered on the fibronectin stamped regions to form isolated circular colonies. Wash the petri dish with PBS to remove debris and unattached cells and put fresh media.
2. Fill the NFP tip slowly with 20 μL of the plasmid solution using an Eppendorf Microloader. Ensure that no bubbles are trapped by visual inspection or under the microscope.
3. Clean the electrodes with ethanol.
4. Load the NFP tip on the piezo-actuated arm of the system, which is mounted on the microscope column.
5. Place a petri dish with the patterned cells on the microscope stage. Switch to phase contrast and bring the colonies of interest in focus (10× or 20× objective).
6. Lower the NFP tip using the controller until it touches the top surface of the media. Bring the tip to the center of the field of view and slowly lower the tip again until it is slightly above the cells (~100 μm).
7. Calibrate the position of the tip using the NFP-E software.
8. Make sure that the resistance reading is stable (~30–50 MΩ) (*see* **Note 2**).

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9. Use the Point Click Transfect System to select one cell per colony and automatically transfect them. A bilevel pulse is typically applied for the transfection ($V_1 = 15$ V, $t_1 = 0.5$ ms, $V_2 = 10$ V, $t_2 = 2.5$ ms, $f = 200$ Hz, $n = 100$ pulses). The amplitude, duration or the number of pulses can be adjusted to improve the performance (*see* **Note 3**).
10. Move to a different region and transfect cells in other colonies if desired.
11. Disengage the tip and remove the petri dish from the stage. Replace the media in the petri dish and put it in the incubator.
12. Monitor the cells regularly and add zeocin after 2 days in culture to select the stably transfected cells.

3.6 Observation, Colony Isolation and Cell Line Generation (Fig. 2c)

1. Observe the cells under the microscope (green channel) to monitor GFP expression in each colony (*see* **Note 3**).
2. Observe the colonies over 2 weeks. Due to zeocin selection only the stably transfected cells will survive (*see* **Note 4**) and proliferate to form individual colonies at the micropatterned regions (Fig. 3).
3. Use a cell scraper or pipette to extract and transfer the colonies to 60 mm petri dishes. Once confluent, transfer the cell to T-75 flasks and expand.
4. The GFP expression can be analyzed using flow cytometry.

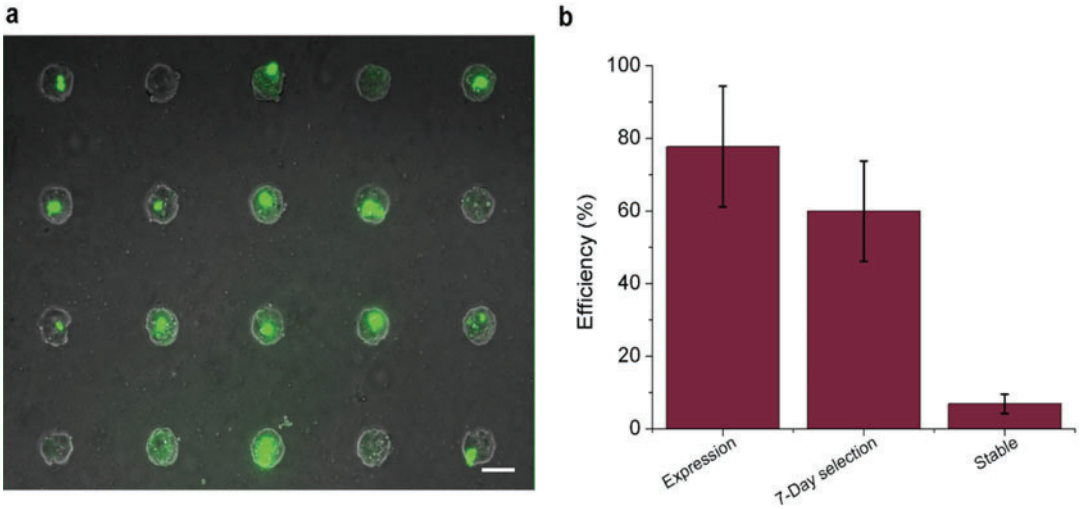

Fig. 3 Transfection and stable cell line generation results (**a**) Transfection of GFP-expressing plasmid in HEK293 cells cultured on fibronectin-stamped array (100 μm scale bar). (**b**) Transfection efficiencies of plasmid transfection, antibiotic selection, and stable generation of monoclonal cell lines

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4 Notes

1. The microcontact printing protocol may need to be optimized if different matrix proteins and substrates are used than those discussed here. The various substrates, proteins and stamp polymers used in microcontact printing are discussed in the following reference [14].
2. In some cases, the contact detection based on the resistance change might not work due to the presence of air bubbles or clogging of the probe tip by the transfectant. In such cases a new pipette with fresh transfectant should be used. The biomolecule concentration can also be lowered to prevent clogging.
3. If other cells are used, the transfection efficiency may not be optimal. The pulse parameters and the biomolecule concentration may need to be adjusted to obtain maximum transfection efficiency. Typically, the voltage amplitude (between 10 V and 25 V) and number of pulses (between 50 and 500) should be changed to improve transfection.
4. The cell viability may be low in some experiments. This may be because the pulse profile is too harsh for the cell type. The voltage amplitude and number of pulses should be reduced in this case. Keeping the cells for a long time outside the incubator and DNA toxicity due to high plasmid concentrations are other causes of low viability. Typically, cells should not be kept outside the incubator for more than 20 min, and lower plasmid concentrations (20 ng/μL to 200 ng/μL) should be used.

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